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Hyper IgD syndrome
and
type AA amyloidosis

Hyper IgD syndrome and type AA amyloidosis

Een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

Proefschrift
ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S. C. J. J. Kortmann,
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door

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Voor mijn ouders

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Outline of thesis

Some 25 years ago van der Meer *et al*/described a new syndrome resembling familial Mediterranean fever in six patients of Dutch ancestry. All patients had a long history of recurrent fever episodes. Because the patients all had a high serum IgD level the authors named it the HyperImmunoglobulinemia D and periodic fever Syndrome (HIDS). Ten years after the first description of HIDS, the International HIDS Study Group was established to collect data of patients with this rare disease. And although it is almost a decade ago that mutations in the mevalonate kinase gene was identified to cause HIDS, there are still many questions that remained to be elucidated. This included questions about basic mechanisms (how does mevalonate kinase deficiency leads to inflammation) as well as clinical questions: how does HIDS evolve during life, what are the effects on quality of life, what is the best therapeutic approach, what is a rational strategy for making a diagnosis. These questions are explored in the **first part** of this thesis.

Chapter 1 gives a clinical overview of the autoinflammatory syndromes. In **chapter 2**, we have investigated the question regarding the natural course of 103 HIDS patients and the influence of HIDS on quality of life. In **chapter 3** we addressed the question regarding the pathogenesis of HIDS. We hypothesized that one of the reasons that patients develop attacks after a trivial stimulus is a reduced apoptosis of lymphocytes. In **chapter 4**, an important clinical problem is approached. In daily practice physicians are confronted with the question which patients with fever attacks should have genetic testing for HIDS. A decision rule is presented that can be used in clinical practice to reduce the number of unnecessary genetic tests of MVK mutations. In **chapter 5** we wanted to know what the effect of the biological agents Etanercept and Anakinra on a vaccination provocation model is.

Type AA amyloidosis, also known as reactive amyloidosis, is caused by the accumulation of proteolytically cleaved acute phase protein Serum Amyloid A (SAA). Since production of SAA is strongly upregulated during inflammation, all patients that have a chronic inflammatory disease are at risk of developing amyloidosis. Based on the experience from our international HIDS registry, we concluded that the incidence of AA amyloidosis is much lower in HIDS than in the other auto-inflammatory syndromes despite a similar or even more severe inflammatory response during and in between attacks. Possible explanations for this observation together with additional studies on the pathogenesis of AA amyloidosis are the focus of the **second part** of this thesis.

Chapter 6 provides an overview of the current knowledge on type AA amyloidosis in the autoinflammatory syndromes. Next, in **chapter 7** we asked the question what could be an explanation for the low incidence of amyloidosis in HIDS. In **chapter 8**, we present a study that describes an *in vitro* model of human amyloidosis, using human mononuclear cells.

The effect of inhibition of the isoprenoid pathway on amyloidogenesis is then studied in this cell culture model.

Although each of the different types of amyloidosis are very rare, dual expression of different types of amyloidosis has been described repeatedly. In **Chapter 9** an explanation is explored using a cell culture model of monocytic cells from a patient with AL amyloidosis. Single nucleotide polymorphisms in the gene coding for SAA have been shown to influence the risk of developing amyloidosis. In **chapter 10**, a study is presented that explores the effect of the different SAA isotypes on degradation by MMP-1, as a possible explanation for the observed difference in risk.

Amyloid fibrils contain SAA that is cleaved at the C-terminus. The process of SAA proteolysis is an essential step in amyloidogenesis and it is thought to be carried out by the cathepsin family of proteases. In **chapter 11** the contributing role of different cathepsins in the process of human SAA degradation is investigated.

This thesis ends with a summary of the research described and a discussion of future perspectives



Autoinflammatory fever syndromes

J. C. H. van der Hilst, J. W.M. van der Meer, J. P. H. Drenth

Chapter 1

Chapter 60 "Autoinflammatory Fever Syndromes," in *Clinical Immunology: Principles and Practice 3rd ed.*, edited by Rich R.R. et al (Mosby, St Louis, 2008)

Autoinflammatory syndromes, also known as hereditary periodic fever syndromes, encompass a group of genetic disorders characterized by lifelong recurrent febrile attacks of noninfectious origin. Each syndrome is characterized by a typical mix of symptoms that may include abdominal symptoms, arthralgias, arthritis, lymphadenopathy, and skin manifestations. Attacks of fever are always accompanied by an intense acute-phase response with elevated C-reactive protein (CRP), serum amyloid A (SAA), and leukocytosis.¹ Autoinflammatory syndromes can be distinguished from autoimmune diseases on several grounds. First, periodic fever is not a typical feature of autoimmune disorders. Moreover, several serological and cellular parameters such as the presence of autoantibodies or antigen-specific T cells do not play a role in the pathogenesis of autoinflammatory syndromes. In recent years, important steps have been made in understanding the pathogenesis of the autoinflammatory syndromes.

Without exception, autoinflammatory syndromes are disorders with a clear mendelian inheritance pattern. The progress in molecular genetics over the last decade has allowed the discovery of the genes implicated in a number of autoinflammatory disorders. The discovery of these genes and the corresponding proteins led to the identification of a new family of inflammatory proteins and their scaffold (named the inflammasome) that have a role in innate immunity. Recent research efforts have broadened the understanding of the mechanism of inflammation. Collectively this group of syndromes can be regarded as gain-of-function disorders in which the causative mutations lead to inappropriate and increased secretion of inflammatory cytokines, such as interleukin (IL)-1 β . In turn, this has led to the development of effective treatment strategies for these rare syndromes. This chapter describes the clinical pathology of six autoinflammatory disorders (Table 1).

The cornerstone of the diagnosis of the autoinflammatory diseases is clinical assessment. This includes a detailed medical and family history and clinical observation of an attack. Based on age of onset of symptoms, family history, ethnic background, accompanying symptoms, and duration of fever, most autoinflammatory syndromes can be differentiated on clinical grounds (Table 2).

Table 1: The autoinflammatory syndromes: names and acronyms

Name	Acronym
Familial Mediterranean fever	FMF
Hyperimmunoglobulin D and periodic fever syndrome	HIDS
Tumor necrosis factor (TNF) receptor associated periodic syndrome	TRAPS
Familial cold autoinflammatory syndrome	FCAS
Muckle-Wells syndrome	MWS
Neonatal onset multisystemic inflammatory disease. (also known in the European literature as chronic infantile neurologic cutaneous and articular syndrome)	NOMID / CINCA

Table 2: The autoinflammatory fever syndromes

	FMF	TRAPS	MWS	FCAS	NOMID	HIDS
Mode of Inheritance	Autosomal recessive	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal recessive
Age of Onset (yrs)	<20	Variable most <10	<20	<1	<1	<1
Main ethnic distribution	Turks, Arabs, Jews, Armenians	All races	European	European	European	Dutch, French
Gene involved	<i>MEFV</i>	<i>TNFRSF1A</i>	<i>CIAS1</i>	<i>CIAS1</i>	<i>CIAS1</i>	<i>MVK</i>
Duration of typical attack	12 hours-3 day	3 day-weeks	1-2 days	<24 hours	?	4-6 days
Distinguishing symptoms	Pleural effusion erysipelas-like skin lesion	Severe myalgia, peri-orbital edema	Sensorineural deafness	Induction by cold-exposure	Epiphysion bone lesions, dysmorphic features	Cervical lymphadenopathy
Risk of amyloidosis	Up to 75%	25%	33%	Unknown	Unknown	<2%
Treatment	Colchicine	Etanercept, anakinra	Anakinra	Anakinra	Anakinra, corticosteroids	Simvastatin, anakinra

FCAS, familial cold autoinflammatory syndrome; FMF, familial Mediterranean fever; HIDS, hyperimmunoglobulin D and periodic fever syndrome; MWS, Muckle–Wells syndrome; NOMID, neonatal-onset multisystemic inflammatory disease; TRAPS, tumor necrosis factor (TNF) receptor-associated periodic syndrome.

Despite the fact that clinical assessment allows the identification of a number of syndromes, we fail to make a classifiable diagnosis in the majority of patients who consult us with periodic fever. It is therefore to be expected that study of the pathogenesis of the various autoinflammatory syndromes will identify new proteins and pathways implicated in hitherto unrecognized syndromes.

Epidemiology

It is important to realize that autoinflammatory diseases are rare and that the prevalence of the different autoinflammatory syndromes depends on the background population as the gene distribution depends to some extent on ethnicity. Without doubt, familial Mediterranean fever (FMF) is the most prevalent of these diseases, with more than 10 000 persons affected worldwide. It is found in persons originating from the Mediterranean basin, including Turks, Jews (primarily non-Ashkenazi), Armenians, and Arabs. However, sporadic cases in other ethnic groups have been described. With the discovery of the incriminated gene, the Mediterranean Fever gene (*MEFV*), it was appreciated that FMF is also relatively common in Italians and Ashkenazi Jews, although often with a less severe phenotype than in the high-prevalence populations. In selected populations, the carrier frequency can be as high as 1 in 3 to 1 in 5.² The high carrier rate suggests that carriers of the *MEFV* gene in the founder population may have had an evolutionary selection advantage, possibly due to protection against an infectious agent by an exaggerated

inflammatory response. The first patients with hyperimmunoglobulin D and periodic fever syndrome (HIDS) were described in 1984 in the Netherlands.³ So far, over 200 patients have been reported in a central registry (www.HIDS.net). The majority of patients come from western Europe, particularly the Netherlands and France. This observation is partly explained by the increased awareness among pediatricians in these countries, but also a founder effect has been described for the most prevalent mutation.⁴ Two mutations (V377I and I268T) account for almost 90% of the patients described to date.^{5,6} Almost all patients are of Caucasian origin, though cases from India and Japan have been described. Men and women are equally affected. Tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) was originally named familial Hibernian fever after the first family described, a Scottish–Irish family with periodic fever. Though TRAPS has been reported in families from Central America, Australia, and Central Europe, most families originate from north-western Europe. So far, a few dozen families and over 200 sporadic cases have been reported.^{7,8} The cryopyrin-associated syndromes are all very rare. The exact prevalence is unknown: this can be explained by a lack of a central registry. In addition, the genotype–phenotype relation is not always clear, which may leave many patients undiagnosed. In each of the three syndromes fewer than 200 cases have been reported in the literature, most often from European families.

Pathogenesis

Familial Mediterranean fever

The MEFV gene responsible for FMF was discovered in 1997 by two international collaborative groups and is located on the short arm of chromosome 16. So far, more than 70 mutations have been reported in a central registry (www.fmf.igh.cnrs.fr/infevers), most of which are clustered in exon 10 of the gene. The six most prevalent mutations (M694V, V726A, M680I, V694I, E148Q) represent some 80% of all cases. The gene encodes a 781-amino-acid protein termed pyrin that is primarily expressed in neutrophils, macrophages, and eosinophils, but not in lymphocytes. Pyrin is a cytosolic protein and its expression is upregulated during myeloid differentiation. Several proinflammatory cytokines upregulate transcription of the MEFV gene.⁹ Pyrin is the first of a new class of proteins discovered that play an important role in the regulation of inflammation and apoptosis.¹⁰ These PYrin Domain (PYD)-containing proteins are a subfamily of the Death Domain (DD) superfamily. More than 20 different PYD-containing proteins have been described. The PYD domain of pyrin can bind to the PYD domain of other proteins like the apoptosis-associated speck-like protein (ASC), forming complexes that activate caspase-1 to produce IL-1 β . Possibly, pyrin can also bind to other PYD proteins that can initiate apoptosis or activate NF- κ B (Fig. 1).

Cryopyrin-associated periodic syndrome

The incriminated gene in cryopyrin-associated periodic syndrome (CAPS) encodes another member of the PYD protein family alternatively called cryopyrin, NALP3 and PYPAF1.¹¹

Cryopyrin is predominantly expressed in monocytes and granulocytes. Cryopyrin contains three domains: a pyrin domain (PYD), a nucleoside oligomerization domain (NOD), and a domain of leucine-rich repeats (LRR).¹⁰ Cryopyrin is able to associate with other proteins to form a complex termed the inflammasome. The central component of the inflammasome is a member of the NALP family (in case of the cryopyrin inflammasome: cryopyrin). It associates with the adaptor protein ASC, which in turn recruits proinflammatory caspase precursors (such as procaspase-1) (Fig. 2). The conversion of procaspase-1 into caspase-1 leads to the conversion of pro-IL-1 β into IL-1 β .¹² IL-1 β is a key mediator of inflammation, with a wide variety of effects ranging from induction of fever and extravasation of leukocytes to enhanced expression of adhesion molecules on endothelial cells and induction of bone resorption.

Tumor necrosis factor receptor-associated periodic syndrome

TRAPS is caused by mutations in *TNFRSF1A*, located on chromosome 12p13. The gene encodes TNF receptor superfamily 1A (TNFRSF1A), the main surface receptor for TNF.¹³ TNFRSF1A has three important domains: an extracellular ligand-binding domain, a transmembrane portion, and an intracellular effector domain (DD) belonging to the DD superfamily. More than 50 different *TNFRSF1A* mutations have been described that cause TRAPS. The TRAPS-associated mutations are all located within the extracellular domain of the molecule. The TNF receptor forms trimers after binding to its ligand. Upon binding of TNF, the intracellular DD recruits TRADD. TRADD is able to recruit other molecules to initiate a downstream signalling cascade leading to NF- κ B activation and caspase-induced apoptosis (Fig. 3). Furthermore, upon receptor activation through TNF, the extracellular domain of the TNF receptor is shed from the

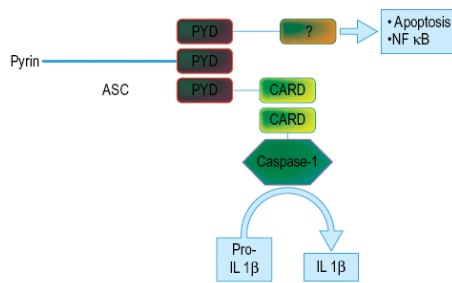


Figure 1. Inflammatory mechanism of pyrin. Pyrin contains a PYD domain that can bind to apoptosis-associated speck-like protein (ASC). ASC in turn, can recruit caspase-1 via the CARD domain. Caspase-1 is capable of cleaving inactive proIL-1 β into active IL-1 β .

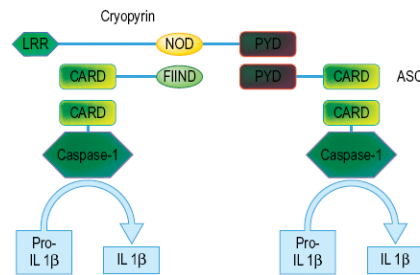


Figure 2. The cryopyrin inflammasome. Cryopyrin is the central component of the cryopyrin inflammasome. Cryopyrin contains three domains: a pyrin domain (PYD), a nucleoside oligomerization domain (NOD), and a domain of leucine-rich repeats (LRR). Cryopyrin binds ASC through its PYD domain and through the NOD domain. The association of these proteins ultimately leads to the release of active caspase-1, which in turn activates IL-1 β through the cleavage of proIL-1 β . Upon its secretion into the extracellular milieu, IL-1 β sets off a series of events that result in inflammation.

membrane. This shedding of receptors leads to a pool of soluble TNF receptors that is thought to mitigate the immune response. Two hypotheses are offered to explain the inflammatory phenotype of TRAPS. The *shedding hypothesis* was first coined after the initial observation that patients with TRAPS have reduced serum levels of soluble TNF receptors. Furthermore, *in vitro* experiments showed that some mutated cell lines exhibit impaired shedding of TNF receptors.¹³ The defective shedding would lead to a deficiency of soluble TNF receptors that can scavenge excess TNF and diminish the immune response. Furthermore, since receptors remain present on the membrane, there is ongoing stimulation of the cells. However, the shedding hypothesis fails to explain the complete phenotype, as not all TRAPS patients have defective shedding. Recently an alternative explanation, the so-called *misfolding hypothesis*, was proposed.^{14, 15} Several mutations in the extracellular domain of TNFRSF1A lead to misfolding of the molecule. The misfolded receptors are not expressed at the surface, but are retained intracellularly. *In vitro* results show that the aggregated TNFRSF1A retain signal function and can induce ligand-independent NF- κ B activation and apoptosis.

Hyperimmunoglobulin D and periodic fever syndrome

HIDS is caused by mutations in the mevalonate kinase gene (*MVK*), located on the long arm of chromosome 12 (12q24). Mevalonate kinase is a key enzyme in the isoprenoid pathway and follows 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG coA reductase). In patients with HIDS, the activity of mevalonate kinase is reduced to 5–15% of normal levels.⁵ In the isoprenoid pathway cholesterol is produced in addition to a number of nonsterol isoprenoids. Isoprenoids are essential compounds in diverse cellular function and include ubiquinone, heme A, farnesyl, and geranyl. How a reduced activity of mevalonate kinase leads to an autoinflammatory condition is not known. It can be caused

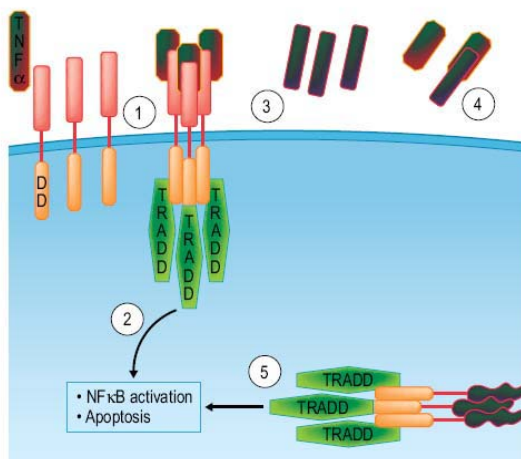


Figure 3. Proposed pathophysiology of tumor necrosis factor receptor-associated periodic syndrome (TRAPS). (1) Tumor necrosis factor (TNF) binds to the TNF receptor on the surface of inflammatory cells (2). After receptor triggering, TRADD is recruited and a signal leads to apoptosis and cytokine production (3). In healthy individuals the receptors are shed from the surface, leading to a pool of receptors that dampen immune response (4). In patients with TRAPS there is diminished shedding, which may lead to a proinflammatory state by ongoing stimulation of retained cell surface receptors. Alternatively, the deficiency of soluble TNF receptors reduces scavenging of excess TNF. (5) Other research has shown that mutated TNF receptors form aggregates and are retained intracellularly. These aggregated receptors are capable of binding TRADD and stimulate ligand-independent cytokine production

by a lack of isoprenoids or by an excess of the substrate of mevalonate-kinase: mevalonic acid. Proinflammatory cytokine production by mononuclear cells of patients with HIDS is strongly enhanced.¹⁶ Furthermore, a defect in apoptosis of HIDS lymphocytes has been detected.¹⁷ It has been hypothesized that this leads to an inability to curtail an excessive cytokine response after a trivial stimulus.

Familial Mediterranean fever

Clinical features

FMF is an autosomal recessive disease. Over 90% of patients become symptomatic within the first two decades of life. Typically an attack has an abrupt onset with high fever, reaching a peak soon after onset, lasting from 12 hours to 3 days and then rapidly subside.¹⁸ There are no consistent triggers, but in some patients vigorous exercise, emotional stress, or menstruation precedes an attack. The frequency of attacks varies greatly between patients, from once every week to only once every few years. Even in a given patient the frequency can vary greatly. Signs of painful serositis accompanying the fever are the hallmark of the disease. Abdominal pain of 1 or 2 days' duration occurs in > 95% of patients. The abdominal pain may originally be focal, and then progresses to become more generalized. It is caused by a sterile peritonitis with a major influx of neutrophils. Many patients have undergone exploratory abdominal surgery because of an acute abdomen, with the resection of an uninflamed appendix. Sometimes adhesions are seen, presumably the result of recurrent peritoneal inflammation. Pelvic adhesions can reduce fertility in female patients. Other serosal and synovial membranes are often involved. Pleural inflammation presenting as unilateral pleural pain is experienced by ~40% of patients. Synovitis presenting as monoarthritis with effusion of the knee, ankle, or wrist occurs in half to three-quarters of patients. An arthritic attack may have a more protracted course, with fever lasting up to a week. The synovitis usually resolves completely without joint destruction. Pericarditis is rare (< 1%). Erysipelas-like skin lesions are reported in 7–40 % of patients.¹⁹ The lesions mimic acute infectious cellulitis occurring on the lower extremities. Other less frequently encountered symptoms include vasculitis, such as polyarteritis nodosa and Henoch–Schönlein purpura, orchitis, aseptic meningitis, and myalgia. The literature is replete with genotype–phenotype studies, and the most consistent finding is that the M694V/M694V genotype carriers have earlier onset of symptoms and higher frequency of arthritis. The life expectancy of patients depends on receiving appropriate treatment to prevent amyloidosis (see related paragraphs).

Laboratory investigation

There is no specific biological marker for FMF. During attacks patients show a strong acute-phase response with high CRP concentrations and leukocytosis. During remission laboratory analysis shows signs of persistent inflammation despite the fact that patients

are clinically well.²⁰ Proteinuria (more than 0.5 g of protein per 24 hours) in patients with FMF is highly suggestive of amyloidosis.

Diagnosis

Table 3: Criteria for the diagnosis of familial Mediterranean fever

Major Criteria

- Typical attack* with abdominal symptoms
- Typical attack with pleural symptoms
- Typical attack with monoarthritis
- Typical attack with only fever
- Incomplete attack† with abdominal symptoms

Minor criteria

- Favourable response to colchicine
 - Incomplete attack with monoarthritis
 - Exertional leg pain
-

*Typical attacks are defined as at least three attacks with fever >38 °C.

†Incomplete attacks are painful and recurrent attacks not meeting the criteria for typical attack. The diagnosis of FMF requires 1 major or 2 minor criteria. The sensitivity and specificity of these 2 criteria sets were >95% and >97%²¹

FMF is a clinical diagnosis (Table 3). A set of validated diagnostic criteria has been designed at the Sheba Medical Center at Tel Hashomer in Tel Aviv, Israel.²¹ When applied in a population with high pre-test probability, it has a high positive and negative predictive value. It is unknown whether these criteria can be applied to other populations with lower disease frequency. In general, when a patient has a typical medical history and stems from a high-prevalence ethnic group, the diagnosis is not difficult to make. FMF has been described in a wide variety of ethnic groups, so ancestry should not be used to rule out the diagnosis. In atypical cases and in patient from low-prevalence ethnic groups, genetic testing can be useful in the diagnostic process.

Cryopyrin-associated periodic syndrome

Originally, CAPS was identified as three different syndromes with distinct clinical features. All types of CAPS are autosomal dominantly inherited. With the discovery of the incriminated gene there is increasing awareness that there are many patients with overlapping symptoms.²² Furthermore, a particular genetic mutation can result in different phenotypes, suggesting the role of other disease-modifying genes. Though each of the cryopyrin-associated syndromes has distinctive clinical features, the common genetic basis and the many overlapping clinical manifestations support the notion of a continuous spectrum of diseases with different severities.

Familial cold autoinflammatory syndrome

Clinical features

Familial cold autoinflammatory syndrome (FCAS) has an almost complete penetrance. The disease becomes clinically manifest in early childhood. In 60% of patients, the first symptoms develop within the first days of life. Symptoms typically occur after a few minutes to a maximum of 3 hours after being exposed to temperatures lower than 22°C. A recurrent characteristic rash is always present. The rash usually starts on the extremities and can extend to other parts of the body. It is described by patients as itchy or burning. Most attacks are accompanied by fever and chills, recurrent arthralgia, and conjunctivitis. Other commonly reported symptoms are profuse sweating, headache, extreme thirst, and nausea.²³ Attacks generally resolve spontaneously after a few hours to a day. Localized exposure to cold does not provoke an attack, which distinguishes it from acquired cold urticaria. Many patients with FCAS also show evidence of chronic inflammation between attacks, particularly a daily pattern of rash developing in the afternoon that can be associated with headaches, myalgia, and fatigue. The symptoms tend to become less severe with advancing age.

Laboratory investigation

The most consistent finding during attacks is a polymorphonuclear leukocytosis, with white blood cell counts up to 36 000/mm³. Erythrocyte sedimentation rate can be moderately elevated, as are other acute-phase reactants. No cold agglutinins or cryoglobulins are present. Skin biopsies from the urticarial rash show neutrophil efflux.

Diagnosis

FCAS is diagnosed based on the typical clinical features in combination with a positive family history.²⁴ Genetic testing is available.

Muckle–Wells syndrome

Clinical features

This autosomal dominant disorder was originally described by Muckle and Wells as a triad of urticaria, deafness, and amyloidosis in a large family. Patients with Muckle–Wells syndrome (MWS) have short bouts of inflammation (12–48 hours); sometimes the attacks are triggered by cold exposure, minor trauma, or emotional stress. The age of onset is variable and ranges from neonatal onset to adolescence. Inflammatory attacks are preceded or accompanied by an urticarial skin rash. The trunk and extremities are most frequently involved, more rarely the face. Arthralgias are a common feature of attacks and can be disabling. Sometimes they are accompanied by large-joint effusion. Arthralgias tend to subside when skeletal growth ceases. Other symptoms may include conjunctivitis, uveitis, severe fatigue, and aphthous ulcers.²⁵ A distinctive feature of MWS is sensorineural deafness. It has been reported to occur in about 70% of patients. The progressive loss of

hearing usually starts in early childhood, but late onset of perceptive deafness is not uncommon.

Laboratory investigation

Leukocytosis and elevated acute-phase reactants are invariably present. Skin biopsies of the lesions show a polymorphonuclear infiltrate.

Diagnosis

Most patients have a positive family history, but isolated cases have been reported. The diagnosis is made on clinical grounds in combination with genetic testing. Genetic testing of rare disorders, such as MWS, is commercially available (www.genedx.com).

Neonatal -Onset Multisystem Inflammatory Disease

Clinical features

Neonatal-onset multisystem inflammatory disease (NOMID) is an autosomal dominant inherited condition characterized by a triad of cutaneous, articular, and neurological symptoms. Patients have chronic inflammation with episodic bouts of fever and worsening of symptoms. NOMID has a much more severe clinical course than the other two cryopyrin-associated syndromes. Typically, patients are born prematurely and have a nonpruritic urticarial-like rash. Central nervous system manifestations include chronic aseptic meningitis, increased intracranial pressure, hydrocephalus, seizures, and sensorineural deafness.¹¹ Chronic papilledema with optic nerve atrophy can result in loss of vision. Headache caused by aseptic meningitis is a prominent feature. Magnetic resonance studies from the brain show ventriculomegaly and mild-to-moderate cerebral atrophy. High-resolution images show arachnoid adhesions and cochlear enhancement. The central nervous system involvement often leads to mental retardation. Articular symptoms are a prominent feature of NOMID.²⁶ A highly characteristic arthropathy, with distinct radiographic findings of premature patellar and epiphyseal long bone ossification and resultant osseous overgrowth, develops early in life. It leads to short stature, severe contractures, and disability. It is unclear whether the bone manifestations are caused by IL-1 β -driven inflammation or result from impaired apoptosis of chondrocytes. The prognosis is grave, with a reported mortality rate of 20% before adulthood due to infection, vasculitis, and amyloidosis.

Laboratory investigations

Patients with NOMID have a continuous inflammation, with elevated SAA and CRP and elevated leukocyte counts. There is meningeal inflammation with increased protein concentrations and elevated white cell counts in the cerebral spinal fluid.

Diagnosis

NOMID can be diagnosed based on characteristic clinical manifestations. Mutational analysis is available (www.genedx.com), but in some patients no mutations are found.

Tumor necrosis factor receptor associated periodic syndrome

Clinical features

TRAPS is inherited in an autosomal dominant manner. The age of onset varies widely, but most patients become symptomatic within the first few years of life. The median age of onset is 3 years. The usual duration of episodes in TRAPS is considerably longer than in the other autoinflammatory syndromes. An attack persists for a minimum of 3 days, but can last for weeks. The interval between attacks can vary substantially within a single patient. Localized myalgia affecting a limb associated with fever is found in virtually all patients.²⁷ Patients describe it as a deep cramping muscle pain, often severely disabling. The symptoms are probably due to a monocytic fasciitis. Cutaneous manifestations are present in the large majority of patients during attacks. They consist of localized erythematous macules and patches that tend to migrate to the distal part of the extremities.²⁸ Abdominal pain, often accompanied by vomiting, constipation, and bowel obstruction, occurs in almost all patients. Arthralgia and monoarticular arthritis involving hips, knees, and ankles are present in a quarter of patients at some point in the natural history of their disease. Chest pain is a frequent feature and may be either pleural in origin or reflect musculoskeletal involvement.

Characteristic ocular symptoms in TRAPS range from conjunctivitis and periorbital pain to severe uveitis and iritis. Periorbital edema with conjunctival injection is another distinctive but infrequent feature of TRAPS. Other less frequently observed symptoms are pericarditis, and inflammation of the tunica vaginalis, leading to scrotal pain. Lymphadenopathy is rare.

Laboratory investigations

During an attack of TRAPS laboratory investigations indicate an acute-phase response, with elevated erythrocyte sedimentation rate, CRP, haptoglobin, fibrinogen, and ferritin. A large proportion of patients also demonstrate an elevated acute-phase response between clinically symptomatic attacks. Complete blood count may demonstrate neutrophilia, thrombocytosis, and anemia of chronic disease. Polyclonal gammopathy as well as small monoclonal gammopathy occur.



Figure 4. Migrating erythematous macular rash during an inflammatory attack in a tumor necrosis factor receptor-associated periodic syndrome (TRAPS) patient.

Autoantibodies, including antinuclear antibodies, anti-cardiolipin antibodies and rheumatoid factor are negative or are found at low titers.

Diagnosis (Table 4)

Hull et al. have proposed a set of diagnostic criteria for TRAPS. However, these criteria have not been validated, and information about sensitivity and specificity of this diagnostic test is lacking. Genetic testing is the mainstay in the diagnosis of TRAPS.

Table 4: Diagnostic indicators for TRAPS

-
- Recurrent episodes of inflammatory symptoms spanning a period of more than 6 months duration. Inflammatory symptoms include fever, abdominal pain, myalgia, rash, conjunctivitis/periorbital oedema, chest pain and arthralgia or monoarticular synovitis
 - Episodes lasting more than 5 days
 - Responsiveness to glucocorticosteroids but not colchicine
 - Affected family members (not always present)
 - Any ethnicity may be affected
-

Hyperimmunoglobulin D and periodic fever syndrome

Clinical features

HIDS is an autosomal recessively inherited disease that was first recognized as a separate disease entity in 1984. Patients with HIDS have recurrent fever attacks of 4–6 days' duration that start in early childhood.¹⁶ The inflammatory attacks occur on average every 4–6 weeks, although there is considerable variation within a single patient and between patients. An attack begins with chills followed by a sharp rise in temperature. Factors known to provoke an attack are infection, trauma, vaccination, and both physical and emotional stress, although often a trigger is not obvious. Typically, parents recall the first attack of their child occurring after the first childhood vaccination. The febrile episodes are accompanied by cervical lymphadenopathy and abdominal pain with vomiting and diarrhea. Cutaneous manifestations of HIDS present primarily as erythematous macules and papules, urticaria, and different forms of rash and exanthema. A minority of patients have painful oral and genital aphthous ulcers. Arthralgias and arthritis accompany attacks in nearly 70% of patients, involving the large joints in a polyarticular fashion. Synovial aspirates show a leukocyte-rich fluid negative for bacterial growth. Other findings in HIDS are splenomegaly, hepatomegaly, and tendinitis. After 4–6 days there is gradual defervescence, leaving patients completely asymptomatic in between attacks. Patients with HIDS have a normal life expectancy. The frequency and severity of attacks tend to decrease later in life. It should be noted that the genetic abnormality in HIDS – mevalonate kinase deficiency – is also present in a more severe disease: mevalonic aciduria. In this inborn error of metabolism there is also periodic fever, but the clinical picture is dominated by

psychomotor retardation, ataxia, failure to thrive, cataract, and dysmorphic facies. Most patients die in early childhood.

Laboratory investigation

Laboratory evaluation at the time of attack reveals a vigorous acute-phase response with leukocytosis, raised serum concentrations of CRP, and proinflammatory cytokines such as IL-6, TNF- α , and interferon- γ . Even in between attacks, when patients are asymptomatic, half the patients have laboratory evidence of continuing inflammation. The principal laboratory finding is a persistent elevation of polyclonal immunoglobulin D (IgD). In 80% of patients this is accompanied by an elevation of IgA as well. IgD concentrations do not correlate with disease activity. The mechanism of elevation of IgD is unknown. During attacks traces of mevalonic acid in the plasma can be found, but the increase is only slight.

Diagnosis

The diagnosis of HIDS can be established by characteristic clinical findings in combination with persistent elevated IgD (> 100 IU/ml). Elevation of IgD is not pathognomonic as it also occurs in other inflammatory conditions, including FMF. Furthermore, in the very young, IgD levels can be normal, and in a small number of patients IgD levels remain low. The diagnosis can be confirmed by DNA analysis; however, in almost half the patients with a periodic fever, elevated acute-phase response, and elevated IgD, no mutations in *MVK* are found. The latter group, which has been described as variant HIDS,⁹ suffers from less severe disease, usually with a later onset and IgD concentrations that are not as high as in classic HIDS.

Amyloidosis

Reactive or type AA amyloidosis is a serious complication of all autoinflammatory syndromes.²⁹ It is caused by the deposition of insoluble fibrils in the extracellular matrix of organs and tissues, most notable the kidneys, spleen, and liver. The fibrils are composed of a degradation product of SAA. Since SAA is an acute-phase reactant there is a close relationship between the continuous elevation of SAA and the development of amyloidosis. Before the recognition of colchicine as an effective treatment for FMF, amyloidosis occurred in up to 75% of patients. Even before the advent of effective treatment, not all FMF patients developed amyloidosis, suggesting that other factors contribute to the risk of developing amyloidosis. In FMF there is a strong correlation between ethnicity and risk of amyloidosis, with the highest risk for Sephardic Jews. Another identified risk factor for developing amyloidosis is single nucleotide polymorphisms (SNPs) in the *SAA* gene. Two SNPs define three different SAA proteins: SAA 1.1, 1.3, and 1.5. Patients with the *SAA* 1.1/1.1 genotype have a three- to sevenfold increased risk of developing amyloidosis.³⁰ Up to a quarter of TRAPS patients develop amyloidosis. There seems to be a strong family predilection. In some families almost all adults are affected whereas in other families no

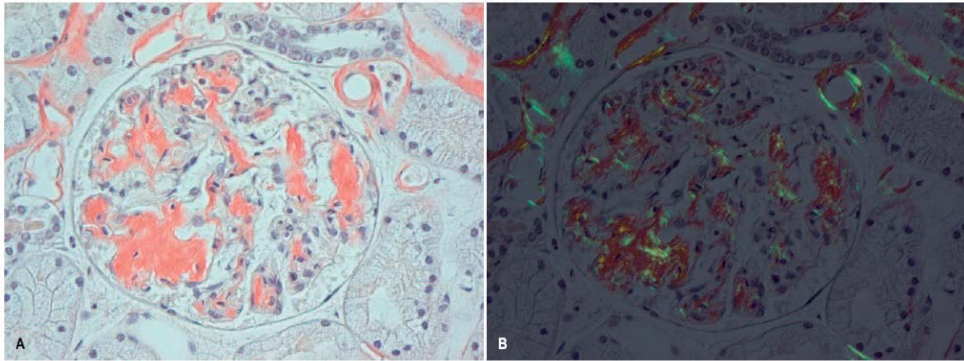


Figure 5. Tissue section of a renal biopsy from a patient with AA amyloidosis. Amyloid deposits are visualized by staining with Congo red (A). Under polarized light microscopy amyloid deposits shows typical apple-green birefringence (B).

cases of amyloidosis are found. Hopefully, with the recent progression in treatment, the number of new cases of amyloidosis will decrease in the near future.

Although MWS was originally described as a triad of deafness, urticaria, and amyloidosis, not all patients with MWS develop amyloidosis. Approximately one-third of patients develop amyloidosis, and there is familial clustering. Several cases have been described in FCAS and NOMID, but numbers are too small to make an accurate estimate of the prevalence.

Patients with HIDS have a relatively small risk of developing amyloidosis. In fact, only recently the first cases of amyloidosis were described. Still there is a remarkably lower incidence of amyloidosis in HIDS compared to the other periodic fever syndromes, despite a similar acute-phase response.

A diagnosis of amyloidosis is confirmed by Congo red staining of affected tissues, showing a typical apple-green birefringence under polarized light microscopy (Fig. 5). Biopsy of subcutaneous fat or rectal tissue can be used to detect amyloid fibrils. If these are negative and there is a high index of suspicion, a direct biopsy from an affected organ can be considered.

The prognosis of patients with established amyloidosis is grave, with a median survival of 24–53 months. The natural history of amyloidosis is progression to renal failure. If inflammation cannot be controlled, amyloid deposits in a variety of organs (liver, spleen, gastrointestinal tract, heart) occur. As a consequence malabsorption with severe diarrhea may ensue. Cardiac failure and rhythm disturbances are typical manifestations of cardiac involvement. The progression of amyloidosis is strongly dependent on the ability to control the underlying inflammatory process. If the SAA concentration can be kept under 10 mg/l, progression of amyloidosis can be halted in many cases, and in some the amyloid mass even slowly regresses.³¹

Treatment

Colchicine

Colchicine is the treatment of choice in FMF.³² It is highly effective in preventing attacks. In fact, it is so effective in preventing attacks that response to colchicine has been used as a clinical criterion for diagnosing FMF. The mechanism of action is unknown. Colchicine therapy is also very efficient in preventing amyloidosis. Therefore, all patients with FMF should receive colchicine, regardless of the severity and frequency of attacks. In patients who already have amyloidosis, intensive treatment can sometimes arrest progression or even partially reverse the process. The average daily dose is 1 mg/day. In cases in which this is not sufficient to prevent attacks the dose can be increased to up to 3 mg. There is a small subset of patients who do not respond to colchicine. The most encountered side effect, gastrointestinal discomfort with diarrhea, usually resolves with dose reduction. Myopathy, neuropathy, and leukopenia are rare, but serious side effects that primarily occur in patients with renal or liver impairment. In animal studies teratogenic effects are only seen at extremely high dosages. The potential teratogenic role of colchicine arises from its effect on microtubules, and there has been some concern that colchicine could therefore increase inborn errors, especially trisomy 21. However, recent data suggest that colchicine is safe to use during pregnancy. It can also be used while breastfeeding. In therapeutic dosages colchicine does not interfere with sperm quantity or quality. Furthermore, a clinical series of male patients on colchicine did not detect a negative effect on fertility. There is no place for colchicine in the treatment of autoinflammatory syndromes other than FMF.

Soluble TNF receptor

The initial observation of reduced concentrations of soluble TNF receptors in the serum of TRAPS patients raised the possibility of etanercept as a therapeutic agent. Etanercept is a fusion protein consisting of two chains of the recombinant TNF- α receptor p75 monomere fused with the Fc domain of human IgG1. There are several reports of successful treatment with etanercept, although most patients do not have complete remission of symptoms. In TRAPS patients with established amyloidosis, etanercept can significantly reduce proteinuria. Etanercept has also been tried in HIDS with promising results in a small number of patients. The arthropathy in chronic infantile neurologic cutaneous and articular syndrome (CINCA) may respond to etanercept therapy.

Anakinra

Anakinra is a recombinant form of human IL-1Ra that competitively inhibits binding of IL-1 α and IL-1 β to the IL-1 receptor type 1. With the advances in the understanding of the genetic and molecular basis of the autoinflammatory syndromes, the concept emerged that IL-1 β plays a key role in inflammation and the successful intervention with anakinra in these syndromes provides the best evidence for such a role of IL-1. Recently there has

been a focus on the effects of anakinra in CAPS, TRAPS, and HIDS, and although preliminary, the results are very promising. In 2003, Hawkins and Lachmann described a patient with MWS who had been unsuccessfully treated with an array of immunosuppressive drugs, but who had a virtual instant and complete response to anakinra.²⁵ These beneficial effects were confirmed in a larger study of 22 patients. Anakinra is well tolerated and gives a complete resolution of fever, rash, conjunctivitis, and joint symptoms. Furthermore, because of the suppression of acute-phase reactants it can probably prevent amyloidosis.

These favourable results triggered research in other cryopyrin-associated syndromes. In NOMID, a similar dramatic response was found in a clinical study of 18 patients.¹¹ The skin rash disappears within 24 hours of start of treatment. Also, neurological symptoms resolve and in many patients hearing improves. Further, steroids can be tapered in the majority of patients. The follow-up has been too short to evaluate the effects of anakinra on prevention of bone deformities. FCAS also responds to anakinra treatment. In a well-designed provocation model, Hoffman et al. showed that anakinra effectively prevented attacks when given prior to cold stimulation.²³ In TRAPS no controlled trials are available, but there are reports in the literature indicating a similar improvement in symptoms. Evidence is accumulating that blocking IL-1 β is effective in treatment in HIDS.³³ Anakinra can prevent attacks when given before a trigger, and it has been shown effective in reducing attacks in severe cases. On theoretical grounds, anakinra may be effective in FMF, but since colchicine is so effective, this option has not been studied to a great extent. In a patient with colchicine-resistant FMF we observed a clinical response to anakinra. An unsettled question is in which way anakinra should be given. One option is chronic suppression of the inflammatory response by daily injections, and this is probably the best regimen in the cryopyrin-associated syndromes, where there is continuous inflammation. In HIDS and TRAPS it is less clear. Here, one may consider patient-initiated treatment at the first sign of an attack. It is unclear as yet how long treatment should be given under these circumstances. With continuous treatment with anakinra in TRAPS we have observed breakthrough attacks, of which the mechanisms are unclear. Under such circumstances we have been able to induce a remission for several weeks with a couple of intravenous injections of 300 mg of anakinra. The latter observation suggests that in patients who do not readily respond to anakinra, a higher dose (intravenously) should be considered.

Corticosteroids

A course of steroids (30 mg daily for 1 week) can be used to treat attacks of TRAPS. Severe attacks may respond to high doses of corticosteroids, such as 1 g methylprednisolone infusion. Corticosteroids, however, while reducing the severity of symptoms, do not alter the frequency of attacks, and often escalating dosages are needed over time. In NOMID, corticosteroids can ameliorate skin manifestations and joint symptoms, but remission cannot usually be induced. Corticosteroid treatment is ineffective in FMF and HIDS.

Simvastatin

The possibility that the inflammatory phenotype in HIDS is caused by an excess of mevalonic acid would make an inhibitor of the preceding enzyme, i.e., HMG-coA reductase inhibitors, a therapeutic option. A small controlled trial has demonstrated some advantage of simvastatin over placebo in terms of reduction of the number of days of illness, but the overall efficacy is limited.³⁴

Other immunosuppressive drugs

Testament to the problems in treating the autoinflammatory diseases are the multiple therapeutic agents that have been tried. The often disabling features and the lack of therapeutic agents might lead to a trial-and-error practice exposing these patients to empirical treatment with an array of immunomodulating drugs, such as ciclosporin, thalidomide, dapsone, azathioprine, mycophenol, and infliximab. Results are disappointing and there is no evidence to support the use of these agents.

Conclusion

The autoinflammatory syndromes are a still expanding group of disorders, characterized by incapacitating attacks of inflammation. In recent years, the genetic background, the molecular pathophysiology, the focus point of the inflammasome, and a key role for IL-1 have surfaced. These insights have provided us with tools for diagnosis and counseling and also for effective treatment. An important challenge is to find other – preferably orally effective – drugs that interfere with IL-1 action.

Although the autoinflammatory syndromes are rare, the study of these diseases has contributed to the insight into important mechanisms of inflammation in general. In the future, with advancing knowledge, we expect new syndromes to be discovered that can contribute to further insight into basic mechanisms of inflammation.

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Long-term follow-up, clinical features, and quality of life in a series of 103 patients with Hyper-Immunoglobulinemia D Syndrome

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Chapter 2

Abstract

The Hyper Immunoglobulinemia D and periodic fever syndrome (HIDS), one of the auto-inflammatory syndromes, is caused by mutations in the gene coding for Mevalonate Kinase (MVK). The aim of our study was to assess the genetic, laboratory and clinical features as well as complications and course of disease in patients with genetically confirmed HIDS. In addition, we studied the quality of life and course of life in a selection of patients. Follow-up data were obtained by a questionnaire that was sent to all physicians of patients in the database. In addition the course of life and quality of life (QoL) was assessed in Dutch patients >16 years using validated QoL instruments.

Data was obtained from 103 patients from 17 different countries. The median age of first attack was 6 months (range 0-120). There was a median period of 9.9 years from onset of disease to diagnosis. Most frequent symptoms that accompanied attacks of fever include lymphadenopathy, abdominal pain, arthralgia, diarrhoea, vomiting, skin lesions, and aphthous ulcers. Amyloidosis is a severe, but infrequent complication (2.9%). The median serum IgD level was 400 U/ml. IgD levels were normal in 22% of patients. The four most prevalent mutations (V377I, I268T, H20P/N, P167L) account for 71.5% of mutations found. During life there is a decrease in the frequency of attacks, although 50% of patients over the age of 20 years still have 6 or more attacks per year. Many drugs have been tried in HIDS. Some patients respond to high dose prednisone (24.4% good response). Anakinra and etanercept can also be effective (good response 33.3%). Quality of life was determined in a subgroup of patients (n=28). Social functioning, general health perception, and vitality are significantly lower in HIDS patients than controls, as are autonomy and social development. In addition, HIDS had an adverse impact on educational achievements and employment status. In conclusion, HIDS is an early-onset disease that is accompanied by an array of inflammatory symptoms. Although the frequency of attacks decreases during life, many patients continue to have frequent attacks. HIDS impairs several aspects of quality of life.

Introduction

The hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) is one of the auto-inflammatory fever syndromes. The auto-inflammatory syndromes form a group of hereditary disorders characterized by lifelong recurrent inflammatory attacks, with fever as its most prominent symptom, usually accompanied by other phenomena such as arthritis, abdominal pain, diarrhoea, lymphadenopathy, and skin lesions (14). Inflammatory attacks are invariably associated with a vigorous acute-phase response and strongly elevated C-reactive protein (CRP) and Serum Amyloid A (SAA) (37). The autoinflammatory syndromes consist of at least 6 different inherited disorders. They can be distinguished on basis of mode of inheritance and on genetic basis. Familial Mediterranean Fever (FMF), and hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) are autosomal recessively inherited. On the other hand, TNF receptor 1-associated periodic fever syndrome (TRAPS), and the Cryopyrin associated periodic syndromes (CAPS) (38) are dominantly inherited. Research into the auto-inflammatory disorders has been boosted by the elucidation of the molecular origins. HIDS is caused by mutations in the gene that encodes mevalonate kinase (MVK) (13) (19). Mevalonate kinase is an enzyme in the isoprenoid pathway in which cholesterol is produced in addition to a number of non-sterol isoprenoids. Isoprenoids are essential compounds in diverse cellular functions and include farnesyl, geranyl, and ubiquinone (20). Mutations in MVK lead to two distinct syndromes: HIDS as a mild and mevalonic aciduria (MVA) as a more severe phenotype, and collectively they are sometimes designated as Mevalonate Kinase Deficiency (or MKD). Although the incriminating gene was discovered in 1999, the exact mechanism of inflammation in HIDS remains to be elucidated. An increased production of interleukin 1β by mononuclear cells has been suggested as a central mechanism in the inflammatory phenotype of HIDS (12,16). Recently, it has been shown that a deficiency in geranylgeranyl, one of the isoprenoids, leads to increased production of interleukin- 1β by human mononuclear cells, providing further evidence for a central role of IL- 1β in the pathogenesis of HIDS (22,24). Also, it has been shown that lymphocytes from HIDS patients show a defective apoptosis. This may lead to an unbridled inflammatory response after a minor stimulus, as an explanation for the inflammatory phenotype of HIDS (4).

This year it is exactly 25 years ago that the first patients with HIDS were described. (39) In 1994, the International HIDS Study Group was established to collect data of patients with this rare disease. In the first publication 14 years ago, prior the discovery of the causative gene mutations, we presented preliminary data on 50 patients who, on clinical grounds, were classified as HIDS (11).

Apart from the fact that that study necessarily included patients diagnosed only based upon clinical criteria, it also left a number of questions unanswered. The prevalence of amyloidosis or the development of other complications was unknown, as well as a possible effect on mortality. Further, general clinical impression was that the frequency of attacks

abates with time, but that has never been substantiated. Also, the effect of this syndrome on several dimensions of the quality of life (QoL) has not been studied previously. The aim of the present study was to characterize a large international cohort of patients with mutation positive HIDS in detail, including assessment of the genetic, laboratory, and clinical features, but most especially the prevalence of complications, survival, and progression of disease. In addition, we studied the quality of life and course of life in a selection of patients.

Patients and methods

In the International HIDS database (www.hids.net) we collect data about patients with suspected and confirmed HIDS. Data is submitted by the patient's physician. With the submission of a new patient to the registry, physicians are asked about clinical features, inflammatory markers, serum immunoglobulin concentrations, and results of mutational analysis. This database does not contain data on patients with the more severe phenotype of mevalonic aciduria (MVA). Although somewhat arbitrary, we considered patients with any of the following as having MVA: severe psychomotor retardation, progressive cerebellar ataxia, typical dysmorphic features, and progressive visual impairment (18). A total of 244 patients have been submitted until January 2007. In 47 patients genetic testing did not reveal mutations in MVK. In 71 patients genetic testing was not performed or data about testing was unavailable. Some genetic laboratories only tested for the most common mutations. A total of 126 patients with mutation positive HIDS, defined as recurrent attacks of fever and at least one mutation detected in the *MVK* gene, were eligible for this study. All registered physicians of these patients received an additional questionnaire about clinical features, complications, course of disease, response to therapy, and, where applicable, were asked to supply missing information. Patients were included when follow-up data was available up until January 2007 or until their death.

Quality of life (QoL) and course of life (CoL) assessment.

For the assessment of QoL and CoL we included only Dutch patients >16 years of age, who had the ability to understand the standardized questionnaires in the Dutch language. The patients were approached by their treating physician, and after giving informed consent, received a letter with explanation of the study and a questionnaire booklet. After one month a reminder letter and a new booklet were sent to those who did not respond. The questionnaire included three items:

The *Course of Life questionnaire*, a Dutch questionnaire, was used to assess the achievement of developmental milestones retrospectively in persons aged 16-30 years. This questionnaire was developed to investigate the course of life in persons who have grown up with a chronic or life threatening disease compared to peers without a history of disease (17). The validity of the course of life scales and the test-retest reliability are good (17). For this study we used two scales: development of autonomy and social development. In addition, the questionnaire measures sociodemographic outcomes in young adulthood,

such as living situation, education and employment. Data derived from a group of 508 Dutch controls was available for comparison (32).

The *RAND-36 Health Survey* was used to assess the quality of life and is almost identical to the MOS SF-36. The RAND-36 is the most commonly used health status measurement for assessing quality of life in the world. It measures eight different health domains: physical functioning, role limitations due to physical health, social functioning, role limitations due to emotional problems, bodily pain, vitality, general health perception, and mental health. The validity and reliability of the RAND scales are satisfactory (40). Norm data were available from a sample of 1036 persons (41).

Since the RAND-36 does not measure cognitive functioning satisfactorily, the cognitive function scale of the *TNOAZL Adult Quality of Life (TAAQoL)* questionnaire was added to this study. The TAAQoL is a validated, generic health-related quality of life questionnaire developed by researchers from TNO and the Leiden University Medical Center. The cognition scale consists of four items; a higher score indicates better cognitive functioning. (15) We compared the TAAQoL cognition score of the HIDS patients with the Dutch norms provided by Fekkes and colleagues (15).

The local ethical committee approved the study design.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) Windows version 14.02 was used for all the analyses. We used chi-square test to evaluate categorical data, and Student's t-test to compare the mean between groups.

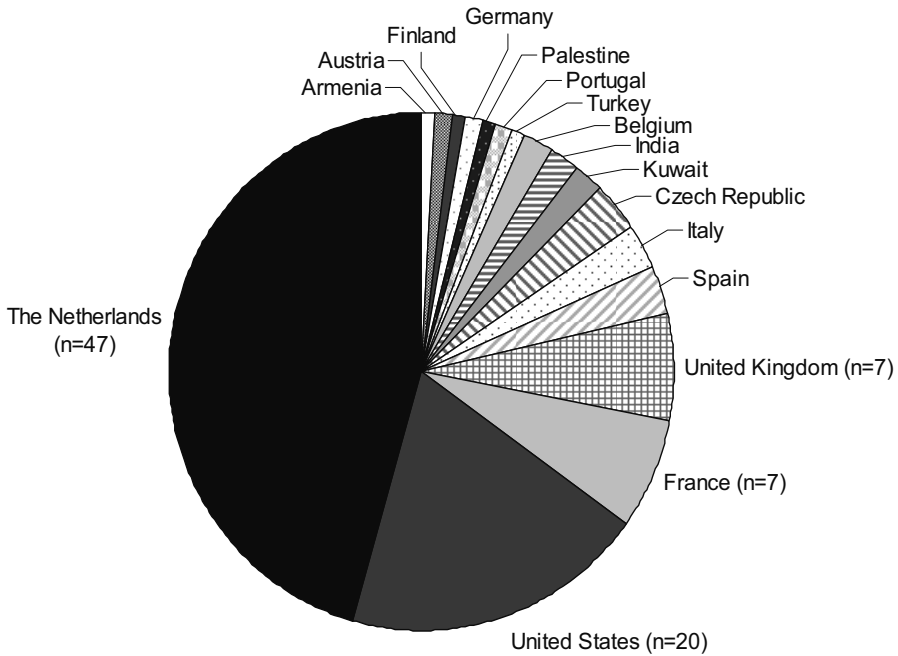
We performed nonparametric Mann–Whitney *U*-tests to test group differences in the number of attacks per year and Friedman-test to test for change of number of attacks during life. To adjust for multiple testing, a significance level of $p < 0.01$ was used for all tests of quality of life.

Results

Demographic features

Out of the 126 patients with mutation-positive HIDS, 103 patients could be included in this study. Reasons for exclusion of the other patients included no response from registered physician ($n=17$) and loss to follow-up ($n=6$). Excluded patients did not differ from included patients in current age, age of onset, or IgD concentrations (data not shown). The patients originate from 17 different countries on 3 different continents, although the majority of patients were European, or from European ancestry (figure 1).

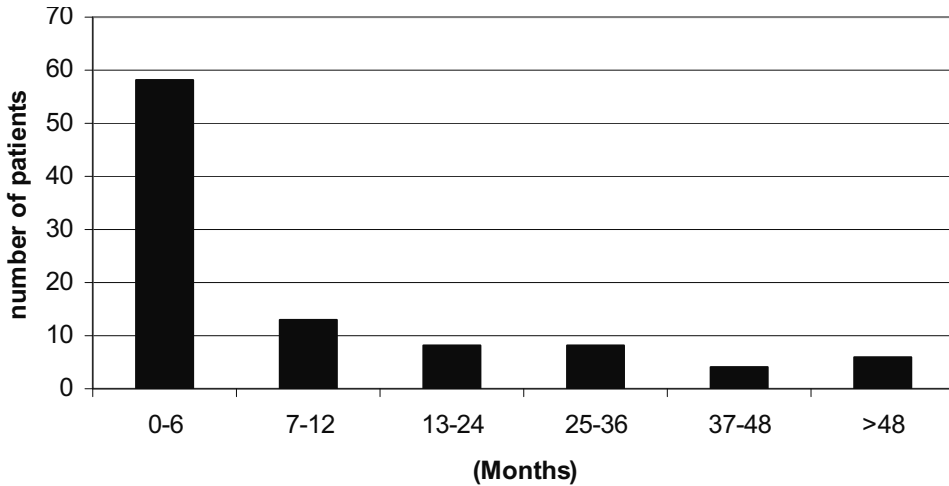
Figure 1: Country of origin of 103 HIDS patients.



The median current age of patients was 19.0 years (mean 24.5) with a range of 2-74 years (table 1). The median age of onset of symptoms was 6 months ranging from the first week of life to 10 years. The majority of patients had their first attack within the first year of life (78,1 %), 6 patients had the first symptoms after the age of 4 years (figure 2). The diagnosis of HIDS was made at a mean age of 15.7 years (median 10.0, range <3 month-52 years) .There was a mean delay in diagnosis of 13.9 years (median 9.9) .

Table 1. Demographic features

		Data available (N)
Age (yrs, median(range))	19.0 (2-74)	103
Gender (male:female)	52:51	103
Age at onset (mnths, median((range))	6 (0-120)	96
Follow-up from onset (yrs, median(range))	17.6 (2-74)	96

Figure 2. The distribution of the age of onset of symptoms.

In recent years the delay in time to diagnosis has not changed significantly. In patients who have been diagnosed after the year 2000 (n=50) there is still a mean delay in diagnosis of 11.7 years (median 8.4). In 33 patients an alternative diagnosis was made before patients were diagnosed as HIDS (table 2).

Table 2 Incorrect diagnoses made prior to HIDS diagnosis.

Diagnosis	Number of patients
FMF	13
Adult onset Still's disease	6
JCA	5
Rheumatic fever	3
Chronic infection	3
Behçet's disease	3

JCA = juvenile chronic arthritis

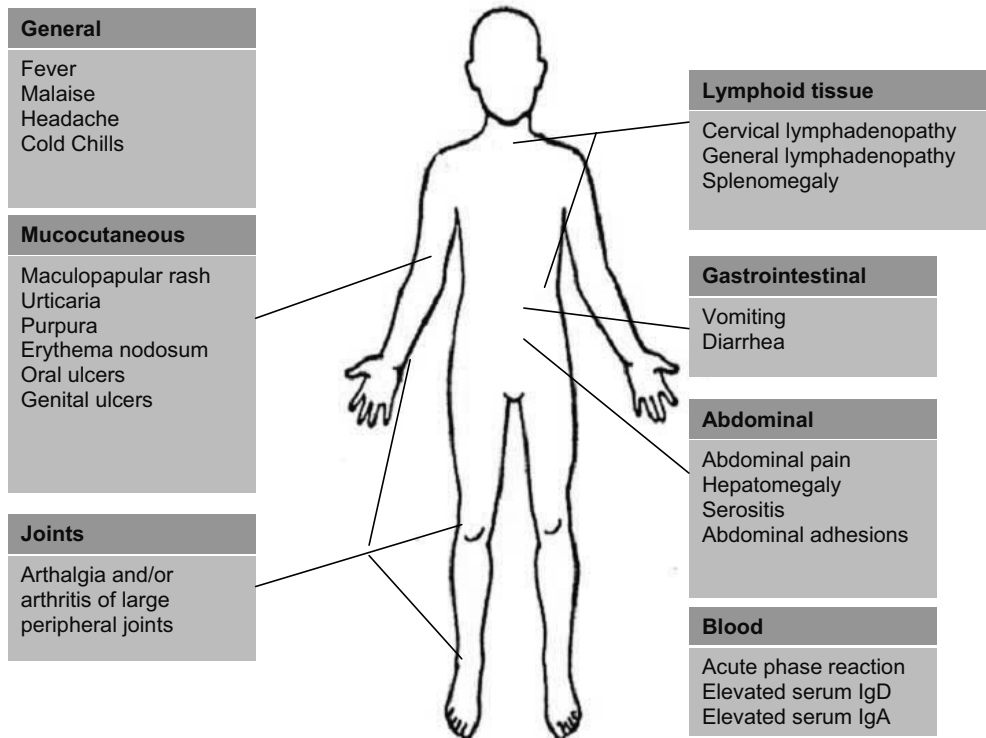
Clinical features:

Attacks of fever are accompanied by an array of signs and symptoms (Figure 3). A typical attack of HIDS starts with prodromal symptoms such as malaise and headache, followed by a rapid rise in temperature, often $>40^{\circ}\text{C}$. Cold chills accompany fever in two third of patients. Physical and emotional stress is recognized by many patients as a precipitating factor. Childhood vaccinations often induce the first attack; this was reported to occur in 63 % of patients.

Table 3

Symptoms during attacks	% of patients
Lymphadenopathy	87.4%
Abdominal pain	85.4%
Arthralgia	83.5%
Diarrhea	71.6%
Vomiting	70.9%
Skin lesions	68.9%
Cold chills	62.7%
Headache	63.3%
Arthritis	55.3%
Aphthous ulcers	48.5%
Splenomegaly	32.4%
Hepatomegaly	21.6%
Serositis	18.6%

Figure 3. Clinical features of HIDS



Lymphadenopathy and splenomegaly. Lymphadenopathy accompanies attacks in almost 90% of patients. The enlarged lymph nodes are generally painful on palpation and primarily located in the cervical region. Axillary and inguinal localization occur less frequently. Splenomegaly is found in 32.4% of patients and all patients that had splenomegaly also have accompanying lymphadenopathy (table 3).

Gastrointestinal symptoms. Abdominal pain, vomiting and/or diarrhea accompanied attacks in all but 5 patients. Abdominal pain can be severe and resemble acute abdomen tempting the attending physician to order explorative surgery. At least 7 patients had surgery for suspected appendicitis. In 6 patients abdominal adhesions were found on explorative surgery, suggesting repeated sterile peritonitis as the cause of abdominal pain.

Articular symptoms. Arthralgia is a prominent feature of HIDS, experienced by 83.5% of

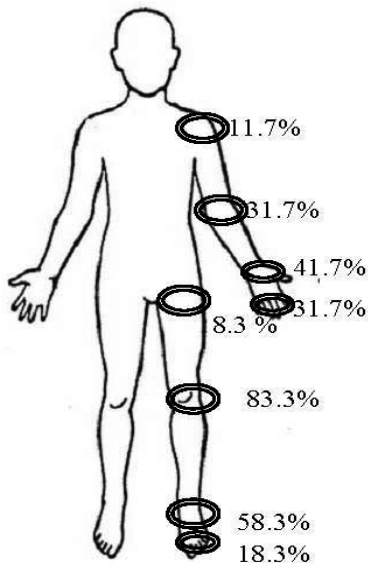


Figure 4. The location of articular symptoms in HIDS. This includes arthralgia and arthritis. Note that more than one joint localization is often involved in a single patient

patients, making it the third most frequent symptom of HIDS after lymphadenopathy and abdominal pain. Fifty percent of the patients have arthritis, defined as swollen, tender joints. In 60 of 86 patients with arthritis and/or arthralgia data of location of joint involvement was available (figure 4). Arthralgia and arthritis are mainly restricted to large peripheral joints. In the hands, both MCP and PIP joints can be affected.

Cutaneous and muco-cutaneous manifestations. Skin manifestations accompany attacks of fever in more than two-thirds of patients¹. Usually this is a maculopapular rash, but urticarial rash, purpura, and erythema nodosum have also been reported. Oral aphthous ulcers with or without accompanying genital ulcerations are reported in 48.5% of patients. That aphthous ulcers can be a very prominent symptom is illustrated by the fact that in 3 patients a diagnosis of Behcet's disease was made prior to the diagnosis of HIDS.

Laboratory results

All patients have a vigorous acute phase response during attacks, with elevated ESR (median 76 mm/h), leukocytosis, and CRP (median 163 mg/l, range 36-404). Between

attacks, some patients have continuous elevation of inflammation markers, although much lower than during attacks. An elevation of serum polyclonal IgD is considered a hallmark of the disease. The median IgD concentration of the highest IgD measured in the patients was 400 U/ml (range <0.8-5300 IU/ml). However, an elevated IgD concentration is not universally present in HIDS patients. In 22% of patients, highest concentration of IgD measured was below the upper limit of normal (<100 IU/ml).

Elevation of serum IgD is frequently accompanied by elevation of serum IgA. We had data about serum IgA concentration available of 86 patients. In 55 patients (64%) the IgA concentration was above the upper limit of normal of 2.6 g/l (Median 4.05 g/L) .

Genetics

Table 4 shows the most prevalent mutations. The four most prevalent mutations account for 71.5% of mutations found. Only 4 patients did not have any of these four prevalent mutations. In 12 patients only one mutation was identified. Seventy-seven patients were compound heterozygote, while 14 patients were homozygote. We compared compound heterozygotes for V377I , I268T, H20P, P167L, and V377I homozygotes (n=11) with patients who did not carry these mutations. There was no association between these genotypes and age of onset, symptoms during attacks, and number of attacks per year (data not shown).

Table 4 Allele frequencies of MVK mutations

Mutations	Allele frequencies
V377I	50%
I268T	14.7%
H20P/N	4.4%
P167L	2.4%
H380R	1.5%
R215Q	1.5%
W188X	1.5%
25 other mutations and deletions	All <1%

Follow-up: course of disease and complications

Frequency of attacks during life. We asked physicians to indicate the number of attacks of their patients. During life there is a significant decrease in the frequency of attacks (figure 5), although no patients had a remission. In the first decade of life 44.1% of patients have more than 12 attacks per years. This decreases to 23.9% in the second decade of life. After the age of twenty, 17.8% of patients continue to have attacks more than 12 times per year, while 50% of patients still have more than 6 attacks per year (p=0.001). Thirty-three of a total of 45 patients above the current age of 20 years, had fewer attacks after the age of 20 years than in the first decade of life.

Amyloidosis. Type AA amyloidosis, a frequent complication in the other auto-inflammatory diseases, was reported in 3 patients (2.9%) that have been described elsewhere (27). All three patients had recurrent fevers for more than 20 years before the manifestation of amyloidosis. No additional cases of amyloidosis in HIDS were discovered in the present follow-up study.

Abdominal adhesions. Repeated peritonitis resulting in abdominal adhesions found during laparotomy was reported in 10 patients.

Joint contractures. Joint contractures as a result of arthritis was reported in 2 siblings of 13 and 14 years and in two brothers, aged 17 and 27 years. None of the other patients manifested any signs of erosive arthritis.

Mortality. Three patients in the cohort died during this follow-up. The cause of death did not seem to be associated with HIDS symptoms or complications. The causes of death were suicide (age 50), cerebral haemorrhage (age 61), and pneumococcal sepsis (age 31).

Therapeutic interventions

A wide variety of immunomodulatory drugs has been tried to treat and prevent attacks in this cohort of patients (table 5). Apart from the drugs listed in the table, the following medication has been tried with limited, if any, success: methotrexate, azathioprine, salazopyrine, tacrolimus, dapson, intravenous immunoglobulins, montelukast, cimetidine, and ranitidine. When prednisone is given in high dosage at the onset of an attack, a considerable number of patients experience a reduction in severity and duration of attacks (24.4% good response, 37.8% some response). Colchicine is ineffective in preventing or treating attacks in HIDS, in contrast to FMF. In 80% of patients in whom it has been tried (n=20) the biological agents anakinra and etanercept have at least some response. When anakinra is ineffective patients can respond to etanercept and vice versa.

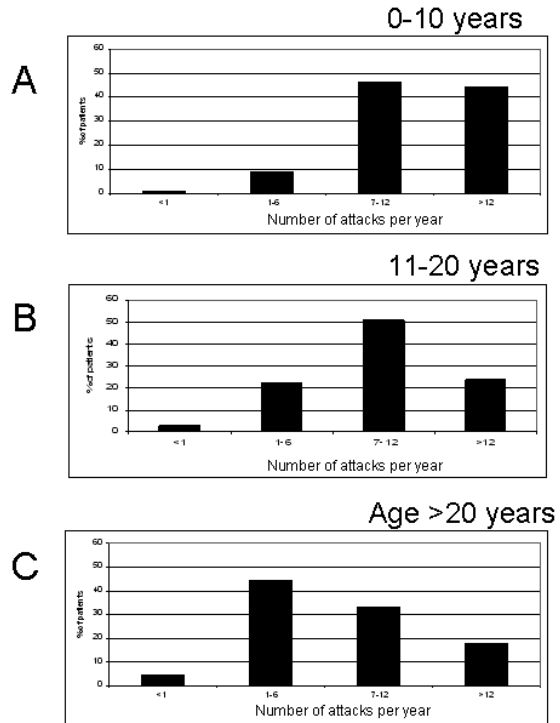


Figure 5. Number of attacks per year in the first decade of life (A), in the second decade of life (B), and after the age of 20 years (C), as retrospectively reported by their physician.

Table 5 Medications that have been tried and response to therapy in HIDS patients. Patients may have used multiple medications.

	No response (n)	Some response (n)	Good response (n)
Prednisone (n=45)	17	17	11
Colchicine (n=44)	37	7	0
Statin (n=18)	12	4	2
Antibiotics (n=13)	11	2	0
Etanercept (n=13)	4	5	4
Anakinra (n=11)	4	3	4
Thalidomide (n=8)	7	1	0
Cyclosporine (n=7)	6	1	0

Quality of life and Course of life

Thirty-eight patients were eligible for this study. Twenty-eight patients returned the QoL questionnaire (73.7 %). Patient characteristics are listed in table 6. There was no significant difference between the study population and other patients from the cohort >16 years of age concerning prevalence of symptoms, age of onset, and number of attacks.

Health related quality of life. The RAND 36 dimensions role physical functioning, social functioning, general health perception, and vitality were significantly lower ($p < 0.01$) in patients than in the general population (figure 6). Patients who experienced more than 6 attacks last year scored significantly lower on the scales for pain (53.1 ± 11.6 vs 87.3 ± 6.5 , $p < 0.01$), physical role functioning (18.8 ± 12.3 vs 80.8 ± 10.3 , $p = 0.01$), and general health perception (27.5 ± 5.1 vs 47.8 ± 5.0 , $p = 0.01$) than patients with 6 or less attacks last year.

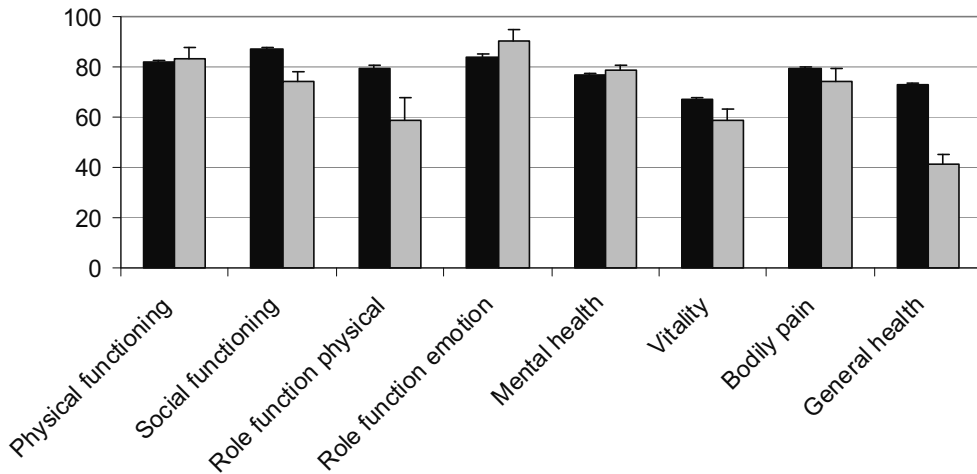
There was no significant difference on the TAAQoL cognition scale between HIDS patients and comparison group (data not shown).

Course of life. Autonomy development and social development during childhood were significantly lower in patients with HIDS compared to controls. In addition, 45.8 % of patients indicated that their disease delayed their education and in 17% this made high-school graduation unachievable. During primary education, 44% had to repeat a year, while 41% of patients repeated a year during high school. Furthermore, 34.8% of patients reported that their disease contributed to discharge from their jobs. Currently, 26.6% of the patients were unemployed, a much higher percentage than in the general Dutch population (4.0%) (7).

Table 6 Characteristics of patients included the quality of life assessment:

Mean age (range)	35,7 (16,5-67,5)
Age of onset months (median)	6
Disease duration yr mean (range)	31,9 (16.3-65.1)
Male: female (n:n)	15:11
Number of attacks last year (n)	
• <1	1
• 1-6	16
• 7-12	6
• >12	3

Figure 6. Comparison of health related quality of life (RAND-36) in HIDS patients and Dutch control group. Errors bars represent SEM. * $p < 0.01$.



patients were unemployed, a much higher percentage than in the general Dutch population (4.0%) (7).

Table 7 Mean scores and SD between HIDS and comparison group on two scales of the Course of Life questionnaire

	HIDS (N=14)	Comparison group (N=501)	
Social development	19.17	20.96	$p=0.01$
Mean	3.07	2.48	
SD			
Autonomy development	8.31	9.87	$p < 0.01$
Mean	1.18	1.48	
SD			

Discussion:

This study is the largest series of HIDS patients reported so far and the first to investigate progression of disease and the effects of HIDS on quality of life. Although patients were entered into the database over a period of 14 years and patients originated from 18 different countries, we were able to obtain follow-up data from 81,7% of patients.

One part of the present study was the collection of new data on clinical features of HIDS. The frequency of symptoms we found in this study differs to some extent from our earlier report of 50 patients that had been diagnosed with HIDS purely based on clinical grounds

i.e. recurrent fever episodes and persistent elevation of serum IgD (11). In the current study we find more abdominal pain (85.4 % vs 72%), vomiting (70.9% vs 56%), and headache (63.3% vs 52%) while skin lesions (68.9% vs 82%) and splenomegaly (32.4% vs 48%) are reported less often. Furthermore, an onset of disease after childhood was not found in this study. An explanation for this difference is that of the 50 patients in our initial 1994 study, only 31 patients later proved to have mutations in the MVK gene (22 of whom were also included in the present study). Two patients were later diagnosed as TRAPS, 5 patients had a normal MVK gene upon testing, while in the remaining 12 patients, the clinical diagnosis HIDS could not be confirmed at the molecular level because of lack of biological material.

Our data indicate that HIDS is a difficult diagnosis to make. There was a median delay in diagnosis of 9.9 years after onset of symptoms. Although genetic testing has been available since the end of the last millennium, there continued to be a large delay in diagnosis in patients who were diagnosed after the year 2000. Furthermore, in many patients an alternative diagnosis was offered prior to the correct diagnosis of HIDS. Aphthous ulcers can be a prominent feature in HIDS patients. It can be restricted to the oral cavity but it can also include genital ulcers. Recently, in a genetic analysis of 96 patients that had a clinical diagnosis of Behçet's disease, mutations in the MVK gene were found in two patients. Both patients had clinical characteristics of HIDS including recurrent fever attacks and arthralgia (21). In our cohort, a diagnosis of Behçet's disease was made in 3 patients that ultimately proved to have HIDS. Twelve patients had previously been diagnosed with FMF. HIDS can be distinguished from FMF on several grounds. First, FMF is very rare in patients from other than Mediterranean or Jewish ancestry (35). Furthermore, lymphadenopathy and aphthous ulcers, both important features of HIDS, do not accompany attacks in FMF (36). In addition, HIDS does not respond to treatment with colchicine. Although raised IgD concentrations have been described in FMF patients, it is infrequent and IgD concentrations rarely exceed 200 IU/l (23). Elevation of IgD may provide a clue to the diagnosis, but it is not diagnostic. Although the majority of patients have an elevated IgD concentration, in 22% of patients IgD values were normal. This is in line with other observations (1,9,19,29). Furthermore, an elevation in serum IgD has been described in other auto-inflammatory diseases (10,26). In addition, we have previously found that IgD levels do not correlate with inflammatory symptoms (30). This suggests that IgD should not be a requirement for diagnosis, but can assist in making the diagnosis. Based on our experience we suggest a set of clinical characteristics that may serve as guidelines in the rational ordering of genetic tests (table 8). A diagnosis of HIDS should be entertained in patients with 1. recurrent attacks of fever of at average 3-7 days persisting more than 6 months AND 2. elevated serum IgD (>100 IU/l) *or* sibling with confirmed HIDS *or* first recorded attack after childhood vaccination *or* 3. at least three of the following symptoms during attacks: cervical lymphadenopathy, abdominal pain, vomiting or diarrhoea, athralgia or arthritis of large peripheral joints, aphthous ulcers, and skin lesions. Applying these criteria to our cohort, all patients would

Recurrent fever episodes lasting 3-7 days persisting more than 6 month

AND One or more of the following

1. Sibling with genetically confirmed HIDS
2. Elevated serum IgD (>100 IU/l)
3. First attack after childhood vaccination
4. Three or more of the following symptoms during attacks:
 - Cervical lymphadenopathy
 - Abdominal pain
 - Vomiting or diarrhoea
 - Arthralgia or arthritis of large peripheral joints
 - Aphthous ulcers
 - Skin lesions

Table 8 Clinical guidelines when genetic testing for HIDS should be considered.

be identified. However, this suggested guideline has not been tested yet for its discriminative power in unselected patients presenting with recurrent fever.

In this study we did not find a relationship between the four most prevalent mutations and the severity of the phenotype. A possible bias could be that we only include patients in the database that have a HIDS phenotype, therefore excluding patients with the most severe phenotype associated with MVK mutations, i.e. mevalonic aciduria. Mevalonic aciduria is, in addition to fever attacks, characterized by psychomotor retardation, progressive cerebral ataxia, dysmorphic features, and progressive visual impairment (18). Mandy

et al could differentiate mevalonic aciduria and HIDS patients on the basis of their genotype (25): MVA patients never possess a V377I allele. However, in a patient group restricted to HIDS, Cuisset et al also failed to find a genotype-phenotype relationship (8). We observed a gradual decrease in the frequency of attacks during life, although after the age of 20 years half the patients still have attacks at least every other month.

Long-term complications in our patient cohort consisted of renal amyloidosis (n=3), joint contractures (n=4) and abdominal adhesions (n=10). The occurrence of adhesions in the absence of prior surgery indicates that the abdominal pain in HIDS attacks may be due to sterile peritonitis, analogous to that in other autoinflammatory syndromes. The incidence of amyloidosis is remarkably low compared to other periodic fever syndromes (33, 35). Recent developments have improved the treatment of HIDS. The data in this cohort indicate that it may be a reasonable strategy to try the efficacy of prednisone in a HIDS patient, starting at the first signs of attack. When this is ineffective or insufficient, treatment with a biological agent should be considered. A dose of 100 mg anakinra or 25 mg etanercept subcutaneously at the first signs of an attack has recently shown beneficial effects in a number of case reports (2,3,6,34). However, randomized trials are lacking. If either Anakinra or Etanercept is not effective, a switch to the other can be considered, since some patients have good response to anakinra but not etanercept and vice versa. In mildly affected patients, simvastatin can safely be tried to reduce the number of days of illness without side-effects (31), although the present study highlights its limited efficacy. There does not appear to be a role in the treatment of HIDS for other immunosuppressive and immunomodulatory agents, or continuous antibiotics.

In this study we show that HIDS adversely influences several aspect of quality of life. Although patients were capable of performing physical activities as measured by physical

functioning scale, they did experience limitations in daily activities due to their disease. Furthermore, HIDS has an unfavourable effect on the social functioning of patients. In a study by Buskila *et al*/on the quality of life in FMF patients similar results were found. Although Buskila *et al* used a different instrument to measure quality of life, they found a negative effect on social functioning and independence (5).

It is known that parents of chronically ill children tend to overprotect their sick child (28). Chronic diseases in children often increases their dependence on caregivers and decreases the participation in peer- and school activities (33). This may explain the impairment in social development and the development of autonomy as we found. But since this questionnaire was developed for and validated in adolescents and young adults (16-30 years), we could only include 14 patients in the analyses of these items and although there is a significant difference between patients and controls we should be cautious about drawing too firm conclusions.

No less than 42.9% of patients indicated that HIDS prolonged their school career and 26.6% of patients were unemployed. It cannot be inferred from these data whether the lack of educational and occupational achievement might result in part from neurocognitive impairment due to the metabolic defect itself. In any case, the febrile attacks do interfere with a normal school career and if this can be prevented by effective anti-inflammatory therapy, this might improve educational and social outcome.

The results of the present study of quality of life are relevant to clinical practice. Knowledge about possible gaps in the development in the course of life and about dimensions of child development that are affected by HIDS enables physicians to focus attention to these subjects, and offer counselling where necessary. For example, paediatricians could help parents to stimulate and encourage the independence of their child.

In conclusion, HIDS is a severe disease that starts early in life with lifelong recurrent attacks of fever accompanied by a variety of symptoms, including lymphadenopathy, abdominal pain, arthralgia, vomiting and diarrhoea, skin lesions, and aphthous ulcers. In this series of 103 patients we found a considerable delay in diagnosis (median 9.9 years). During life there is a gradual decrease in the number of attacks, although half the patients over 20 years of age continue to have more than 6 attacks per year. Prednisone, anakinra and etanercept can be effective in a part of the patients in reducing severity of attacks. HIDS adversely affects several aspects of the quality of life and interferes with educational achievements and employment status. Infrequent but severe complications of HIDS include amyloidosis (2.9%), joint contractures (3.9 %) and abdominal adhesions (9.7%).

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Defective apoptosis of peripheral-blood lymphocytes in hyper-IgD and periodic fever syndrome

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Chapter 3

Abstract

Hereditary periodic fever syndromes are characterized by incapacitating attacks of fever and generalized inflammation. While the mutated genes for the major syndromes in this group are known, the pathogenesis remains unclear. The aim of this study was to investigate apoptosis in patients with periodic fever as a possible pathogenic factor. We measured anisomycin-induced apoptosis with annexin-V flow cytometry and caspase-3/7 activity in peripheral-blood lymphocytes from symptom-free patients with hyper-IgD and periodic fever syndrome (HIDS; n=10), TNF-receptor-associated periodic syndrome (TRAPS; n=7), and familial Mediterranean fever (FMF; n=2). HIDS lymphocytes showed a decreased percentage of apoptosis during remission by both methods compared with controls (17.8% vs 55.4%), whereas no difference was observed in TRAPS or FMF lymphocytes. This defective apoptosis of lymphocytes may be a central pathogenic mechanism in HIDS, since dysfunction of one of the inhibitory mechanisms to curtail the immunologic response could cause an unbridled generalized inflammation after a trivial stimulus.

Introduction

Hereditary periodic fever syndromes, also known as autoinflammatory disorders, are characterized by incapacitating attacks of fever and generalized inflammation. Patients present with a long history of recurrent episodes with spiking fever, abdominal discomfort, diarrhea, vomiting, chest pain, or arthralgia. The most prevalent examples of this group of syndromes are familial Mediterranean fever (FMF), TNF receptor associated periodic syndrome (TRAPS), and hyper-IgD and periodic fever syndrome (HIDS) (1). Although the genetic defects are known, the mechanisms by which these syndromes cause inflammatory attacks are largely unclear (1,2). Factors known to precipitate an attack are infection, trauma, vaccination, and both physical and psychological stress. It is hypothesized that the normal immunologic response to relatively trivial insults is not stopped in time, but instead, leads on to an overwhelming and generalized inflammatory attack. This may be caused by a lack of inhibitory signals to down-regulate the inflammation, or an inability to respond to such signals. Apoptosis plays an important role in down-regulation of the inflammatory response, for example, by reducing the lifespan of activated lymphocytes. We hypothesized that a defect in apoptosis regulation is the cause of the exaggerated inflammatory response in periodic fever patients. This was investigated in peripheral-blood lymphocytes of patients with periodic fever syndromes.

Patients, materials, and methods

Ten patients with HIDS (2 female, age range 18-44 years), 7 patients with TRAPS (6 female, age range 20-66 years), and 2 patients with FMF (both male, ages 24 and 36 years) were included in this study. All patients showed the relevant pathogenic mutations (Table 1). Nineteen healthy unrelated persons served as controls (13 females, age range 20-51 years). The study was approved by the local ethics committee, and written informed consent was obtained from all participants. Patients and healthy volunteers signed informed consent forms in accordance with the Declaration of Helsinki. Peripheral venous blood from patients in a symptom-free interval and from controls was collected simultaneously and processed in pairs. Peripheral-blood mononuclear cells (PBMCs) were isolated using Ficoll gradient separation (Ficoll-Paque Plus, Amersham Biosciences, Amersham, United Kingdom). Cells were resuspended at a concentration of 5.0×10^6 /mL in RPMI 1640 medium (Dutch modification; Invitrogen, Paisley, United Kingdom) and incubated at 37°C. The protease inhibitor anisomycin (Sigma-Aldrich, St Louis, MO) was added in a concentration of 20 µg/mL to induce apoptosis. At different times (0, 2, 5, and 24 hours) the reaction was stopped. At 0, 5, and 24 hours cells were stained with annexin-V-FITC and PI (Apoptest-FITC; VPS Diagnostics, Hoeven, The Netherlands) and analyzed by flow cytometry (Coulter XL; Beckman Coulter, Fullerton, CA) The lymphocyte population was selected based on forward and side scatter and staining with labelled antibodies to CD14, CD3, and CD19 (3) At 2 hours cells were permeabilized and the profluorescent caspase-3/7 substrate was added (Apo-One Homogenous Caspase-3/7 Assay; Promega, Madison, WI). Fluorescence

was measured using a fluorometer (POLARstar Galaxy; BMG Labtech, Offenburg, Germany) at excitation 485 nm and emission 520 nm. Results shown are corrected for background emission. Results were analyzed using the unpaired *t* test by GraphPad Prism, version 4.00 (GraphPad Software, San Diego, CA).

Results and discussion

After 24 hours of incubation with anisomycin, lymphocytes from 10 patients with HIDS showed a significantly smaller percentage of apoptosis than healthy controls, as shown by annexin-V staining (17.8% vs 55.4%, $P < .001$; Figure 1B,E). This difference was not observed in unstimulated cells (Figure 1A). There were no differences in the percentages of apoptosis in lymphocytes from patients with TRAPS (n=7) and FMF (n=2) compared with those of healthy donors, whether stimulated with anisomycin or unstimulated (Figure 1C-D). At all times, PI binding of cells was less than 0.5%, indicating that the amount of necrotic or late apoptotic cells was negligible.

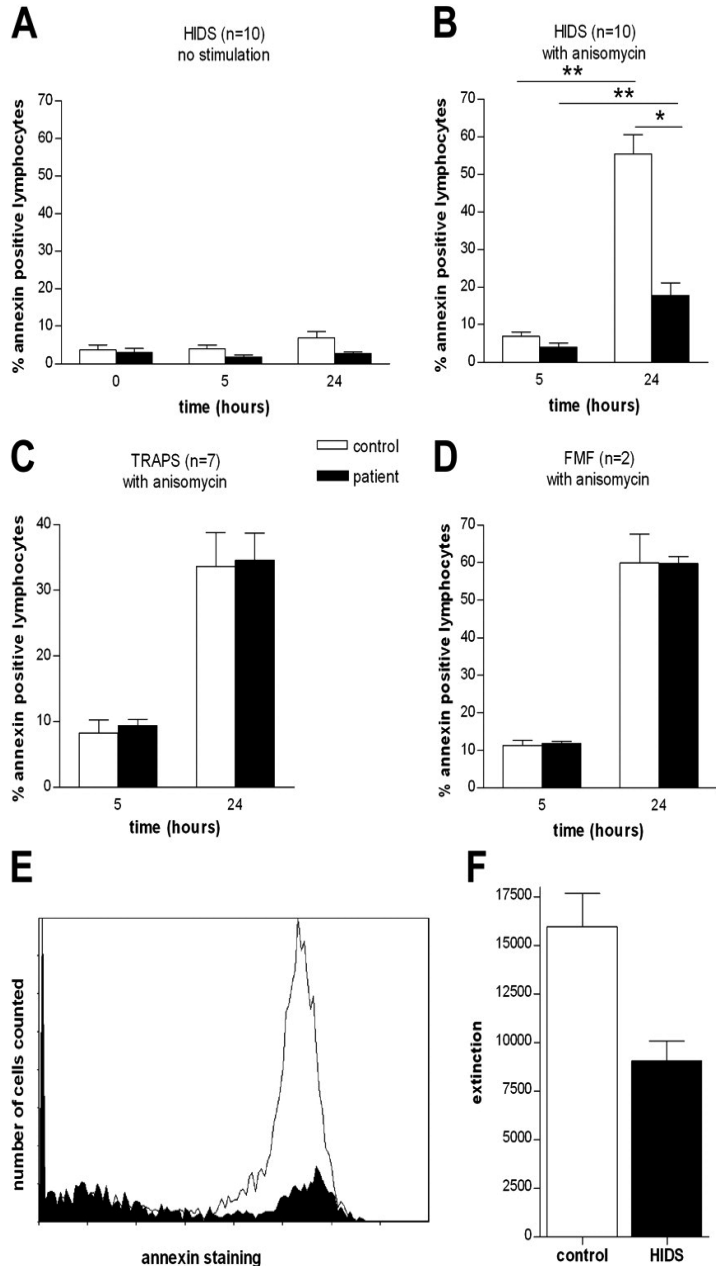
Table 1. Genotypes of study participants

Protein, gene (syndrome)	No. of patients
Mevalonate kinase, <i>MVK</i> (HIDS)	
V377I/V377I mutation	1
V377I/I268T mutation	1
V377I/H20P mutation	1
V377I/del mutation	1
P167L/I268T mutation	2
V377I/417insC mutation	4
TNF-receptor type 1, <i>TNFRSF1A</i> (TRAPS)	
C43Y mutation	1
Y38C mutation	2
C29F mutation	2
C70Y mutation	2
Pyrin, <i>MEFV</i> (FMF)	
M694V/M694V mutation	2

A caspase-3/7 activity assay confirmed the difference in apoptosis found with annexin-V staining in patients with HIDS compared with controls (9060 vs 15 971, $P = .026$; Figure 1F). Decreased apoptosis in stimulated lymphocytes in HIDS may well be central to the pathogenesis of the inflammatory attacks: dysfunction of one of the inhibitory mechanisms to curtail the immunologic response can explain the unbridled generalized inflammation seen after a trivial stimulus. The causative mutations in HIDS are located in the gene encoding mevalonate kinase (MVK), an enzyme in the isoprenoid metabolism, the end-products of which include cholesterol, protein isoprenylation (including the prenylated Ras/Rho proteins), dolichol, and ubiquinone (1). The exact pathogenesis of HIDS is unclear. However, several of the isoprenoid end-products have been linked with apoptosis (4). A link between the isoprenoid pathway and apoptosis was also suggested by Nagashima et al, (5) who showed that inhibition of this pathway by statins induced apoptosis in rheumatoid arthritis synoviocytes. This might offer an explanation for the beneficial effect of statins seen in patients with HIDS (6). Other periodic fever syndromes

Figure 1. Lymphocyte apoptosis in periodic fever patients.

Apoptosis of lymphocytes is expressed as a percentage of annexin-V-positive lymphocytes (PI negative); white bars represent controls, black bars patients. (A) Patients with HIDS versus controls without anisomycin stimulation; neither group shows apoptosis. (B) Incubation of lymphocytes with anisomycin results in a significant increase of apoptosis after 24 hours when compared with unstimulated lymphocytes in both controls ($P < .001$) and patients ($P = .005$), but the increase in HIDS lymphocytes is significantly less than in controls ($P < .001$). Lymphocytes from patients with (C) TRAPS or (D) FMF show as much apoptosis as controls. (* $P = .005$; ** $P < .001$). (E) Healthy control (white) versus a patient with HIDS (black) after 24 hours of exposure to anisomycin. Representative result from 10 experiments. (F) Apoptosis of lymphocytes of patients with HIDS expressed as caspase-3/7 activity after 2 hours of anisomycin stimulation. Patients show decreased caspase-3/7 activity compared with controls. Data represent mean values \pm standard error of the mean.



are caused by mutations in molecules that contain a death domain, death-effector domain, and/or CARD, involved in the protein-protein interactions required for apoptosis or signaling through NF κ B. These include the TNF-receptor type 1 (TNFRSF1A) in TRAPS, pyrin in FMF, cryopyrin in cryopyrin associated periodic syndrome (CAPS), and NOD2/CARD15 in Blau syndrome. A central role for an apoptosis defect in these syndromes has therefore been hypothesized and such a defect has been found in

vitro; that is, decreased apoptosis of macrophages from pyrin-mutant mice (7). However, available data on apoptosis measured in patient cells is limited. Two studies in Turkish patients with FMF show normal apoptosis of peripheral-blood leukocytes in symptom-free patients but increased apoptosis of neutrophils and monocytes during inflammatory attacks (8) or elevated serum soluble FAS concentrations (9). A third study showed decreased TNF-induced neutrophil apoptosis in patients with TRAPS (10). We included 2 symptom-free patients with FMF who showed no defect in lymphocyte apoptosis. We did not find an apoptosis defect in 7 patients with TRAPS. Preliminary studies from our laboratory suggest that the apoptotic potential of lymphocytes in TRAPS is not affected by an attack (data not shown). Conceptually it would be interesting to measure apoptosis in patients with HIDS during inflammatory attacks. However the profound lymphopenia that occurs early during attacks so far has hampered these measurements. At recovery from an attack, we measured restored apoptotic activity in 2 patients (59.8% early apoptosis after 24-hour stimulation with anisomycin) compared with controls. This might mean that apoptotic pathways are activated at that stage to end the attack. An explanation for the difference between HIDS and the other syndromes found in our study may be the cell type studied. Reasoning from the clinical phenotype, we chose to investigate apoptosis of lymphocytes in HIDS. Lymphadenopathy, lymphocytic infiltrates in skin biopsies, and many B lymphocytes containing IgD in the cytoplasm in bone marrow can be observed in HIDS, (11,12) and this may be related to the lymphocyte apoptosis defect. In the other periodic fever syndromes, different cell types are more prominent and point to cell-specific defects. FMF is characterized by infiltrates that are almost exclusively composed of neutrophils, whereas the urticarial response in certain cryopyrin-associated periodic syndromes could point to a role of mast cells. This hypothesis of cell-type specificity is supported by the study by D’Ossualdo *et al*, (10) showing abnormal neutrophil apoptosis in 8 patients with TRAPS. In this study, one patient with HIDS was measured and found to have normal neutrophil apoptosis. In conclusion, we have shown decreased apoptosis of circulating lymphocytes *ex vivo* in fever-free patients with HIDS after stimulation with anisomycin. This lymphocytic defect was not seen in TRAPS or FMF. Decreased apoptosis may be central to the pathogenesis of HIDS.

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A clinical prediction rule to exclude the hyperimmunoglobulin D syndrome in patients with recurrent fever

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Chapter 4

Abstract

Objective: The hyperimmunoglobulin D syndrome (HIDS) is an autosomal recessive autoinflammatory disease caused by mutations in the mevalonate kinase gene. Our objective was to define a clinical criterion able to exclude HIDS without genetic testing in some patients.

Methods: A recursive partitioning algorithm was applied to derive the clinical criterion in 149 patients with genetic testing in a French reference laboratory, among whom 35 had HIDS. The criterion was validated in a cohort of 93 patients with genetic testing in a Dutch reference laboratory, among whom 28 had HIDS.

Results: The most discriminatory composite clinical criterion satisfied by all patients with HIDS in the derivation cohort was [onset < 5 years old OR (attacks with joint pain AND length of attacks < 14 days)]. It had a sensitivity of 100% [95% confidence interval 88 to 100%], a specificity of 28% [95% CI: 17 to 40%] and a negative likelihood ratio of 0 [95% CI: 0 to 0,44] in the validation cohort. If genetic testing had only been performed for patients with a positive composite criterion, 18 tests (19%) would have been avoided in the validation cohort without missing a single mutation-positive patient.

Conclusion: This criterion could help prevent unnecessary genetic tests, which are resource and time consuming.

Introduction

Patients with recurrent febrile attacks without an apparent infectious cause may pose a challenge for clinicians. The differential diagnosis is broad and includes the Hyper Immunoglobulinemia D and periodic fever syndrome (HIDS). HIDS is a rare autosomal recessive disease that belongs to the group of autoinflammatory syndromes.(1) A typical patient has an onset of disease in the first year of life with febrile attacks that last 3-7 days and recur every few weeks and are accompanied by cervical lymphadenopathy, arthralgia, and gastrointestinal distress. A marked elevation of polyclonal immunoglobulin D is found in the serum.(2) However, there is a considerable variation in onset of disease and in clinical manifestations. Furthermore, elevated serum IgD is not universally present in HIDS patients and it can also be found in some patients with other periodic fever syndromes.(3)

In 1999 two groups separately discovered that HIDS is caused by a mutation in the mevalonate kinase gene (*MVK*), located on the long arm of chromosome 12 (12q24).(4;5) This allowed genetic testing as a new diagnostic tool. Although genetic testing for HIDS is nowadays possible, its availability is usually limited to specialist referral centres.

Furthermore, it is a time-consuming and expensive test. The aim of this study was to develop an algorithm based on clinical features that can exclude HIDS without the use of genetic testing.

Material and Methods

Derivation cohort

Patients were considered for inclusion in the derivation cohort if they were tested for *MVK* mutation in a French reference laboratory from January 2002 to January 2007. In atypical cases, genetic testing was performed only if the *MVK* enzyme activity was low. Mevalonate kinase enzyme activity was determined in peripheral blood lymphocytes using a radiometric assay(6) and residual activity between 1 and 30% of controls was considered as indicative of HIDS.(7) Patients with mevalonic aciduria or with a family member already tested positive were excluded.

Clinical data were collected prospectively by the physicians requesting the tests, using a questionnaire that inquired for the following characteristics of attacks: age at onset, duration, and clinical features (fever, lymphadenopathy, hepatosplenomegaly, abdominal pain, vomiting, diarrhoea, joint pain, skin lesions).

Derivation method

Mutation-positive patients (two mutated *MVK* alleles) were compared to mutation-negative patients (zero or one mutated *MVK* alleles) with the Mann Whitney and the Fisher tests. All variables were entered in the recursive partitioning algorithm to find the most specific composite clinical criterion satisfied by all mutation-positive patients.

Step by step, the algorithm found the best variables (and best cut-off value for quantitative variables) to distinguish mutation-positive from mutation-negative patients. It was manually overridden when appropriate, to ensure clinical meaning and applicability of the classification rule. When two variables were close candidates at one step, several characteristics were taken into account to choose between them: robustness of the association between the variable and the mutation status (strong association also in univariable analysis), reliability of the variable (objective measure), and number of missing values (threat to the reality of the association). At this stage, the imputation method for missing values was the best-case hypothesis: patients with missing values were allocated to the group they best fit into.

Validation cohort

The validation cohort consisted of all patients that were seen or consulted about in a large tertiary care centre for evaluation of recurrent fever in whom genetic testing was ordered from January 2004 until June 2007. Patients were excluded if a family member had already tested positive. Clinical data were extracted from the international HIDS database (www.hids.net) in which data from patients with suspected and proven HIDS are collected. (8)

Validation method

In the validation population, missing data were imputed according to the worst-case hypothesis: when genetic testing was negative, missing data were assigned in order to have a positive composite criterion if possible, and vice versa. Sensitivity, specificity and negative likelihood ratio of the composite clinical criterion were assessed in the validation cohort. The potential impact of the rule was evaluated with the number of genetic tests that could have been avoided by using the composite criterion in the validation cohort.

Statistical software

Statistical analyses were performed with Stata 8.2 (StataCorp) and recursive partitioning with JMP 5.1 (SAS Institute).

Results

The French derivation cohort included 149 patients with genetic testing, among whom 35 (23%) were mutation-positive. Characteristics of patients and of their attacks are given in table 1. Comparison of mutation-positive and mutation-negative patients is displayed in table 2. The composite criterion [*onset < 5 years old OR (attacks with joint pain AND length of attacks < 14 days)*] was identified by recursive partitioning as the most specific among perfectly sensitive criteria.

The Dutch validation cohort included 93 patients with genetic testing, among whom 28 (30%) were mutation-positive (table 1). According to the worst-case analysis in this cohort (figure 1), the composite criterion had a sensitivity of 100% [95% confidence interval 88 to

	All patients				Mutation-positive patients					
	Derivation cohort (N=149)		Validation cohort (N=93)		Derivation cohort (N=35)		Validation cohort (N=28)			
	Available data	Result	Available data	Result	Available data	Result	Available data	Result		
Age of onset (years)	134	3 (1-12)	93	2 (1-11)	35	2 (0.5-3)	28	1 (1-1.5)	0.77	0.50
Length of attacks (days)	122	4 (3-7)	84	5 (4-7)	31	4.5 (3.5-7)	27	6 (5-7)	0.01	0.04
Males	149	73 (49%)	93	42 (45%)	35	15 (43%)	28	12 (43%)	0.60	1
Fever	138	135 (98%)	93	93 (100%)	34	34 (100%)	28	28 (100%)	0.28	1
Abdominal pain	134	100 (75%)	80	56 (70%)	34	28 (82%)	26	22 (85%)	0.53	1
Joint pain	136	98 (72%)	90	59 (66%)	33	29 (88%)	28	24 (86%)	0.31	1
Diarrhoea	130	51 (39%)	88	32 (36%)	35	21 (60%)	26	18 (69%)	0.78	0.59
Vomiting	51	23 (45%)	87	40 (46%)	18	8 (44%)	27	22 (81%)	1	0.02
Rash	135	70 (52%)	81	39 (48%)	34	20 (59%)	26	17 (65%)	0.67	0.79
Hepatosplenomegaly	119	27 (23%)	81	15 (19%)	33	17 (52%)	27	9 (33%)	0.60	0.20
Lymphadenopathy	116	61 (53%)	81	56 (69%)	30	22 (73%)	28	25 (89%)	0.03	0.18

Table 1. Comparison of the derivation (French) and validation (Dutch) cohorts. Results are given as median (interquartile range) or number of patients (percentage) and compared with the Mann-Whitney and Fischer test.

	Mutation-negative patients (N=114)		Mutation positive patients (N=35)		
	Available data	Result	Available data	Result	p
Age of onset (years)	99	5 (1.5-14)	35	2 (0.5-3)	0.001
Length of attacks (days)	91	4 (3-6.5)	31	4.5 (3.5-7)	0.22
Males	114	58 (51%)	35	15 (43%)	0.44
Fever	104	101 (97%)	34	34 (100%)	0.43
Abdominal pain	100	72 (72%)	34	28 (82%)	0.26
Joint pain	103	69 (67%)	33	29 (88%)	0.03
Diarrhoea	95	30 (32%)	35	21 (60%)	0.004
Vomiting	33	15 (45%)	18	8 (44%)	1
Rash	101	50 (50%)	34	20 (59%)	0.43
Hepatosplenomegaly	86	10 (12%)	33	17 (52%)	< 0.001
Lymphadenopathy	86	39 (45%)	30	22 (73%)	0.01

Table 2. Comparison of mutation negative and mutation positive patients in the derivation (French) cohort. Results are given as median (interquartile range) or number of patients (percentage) and compared with the Mann-Whitney or Fischer test.

100%], a specificity of 28% [95% CI: 17-40] and a negative likelihood ratio of 0 [95% CI: 0-0,44]. If genetic testing had only been performed for patients with a positive composite criterion, 18 tests (19%) would have been avoided without missing a single mutation-positive patient.

Discussion

In this study we present a classification rule based on clinical criteria that can prevent the use of unnecessary testing for MVK without missing a single mutation-positive patient. We used recursive partitioning to derive this classification rule. This hypothesis-free multivariable method natively explores interactions between predictor variables. It is also easy to understand by clinicians and well suited to find a classification of positive patients with maximal sensitivity, rather than a classification of positive and negative patients with maximal overall accuracy. (9;10)

Moreover, the value of the classification rule is probably underestimated in the validation cohort. Since we used worst-case imputation for missing values, the true negative predictive value is conceivably higher. The value of the classification rule is further underestimated by the selection of the cohorts. In the derivation cohort patients, atypical patients needed to have a reduced enzyme activity for genetic testing to be performed, while patients in the validation cohort were tested only after consultation by experts in the

field. A higher yield in preventing unnecessary testing can be expected in unselected patients with recurrent fever. Indeed previous research has shown that systematical genetic testing for HIDS in patients with recurrent fever has a very low yield. In a cohort of 60 patients with recurrent fever and negative testing for the most likely auto-inflammatory syndrome other than HIDS, no *MVK* mutation could be identified.(11) In another study of patients with recurrent fever, negative testing for FMF, and at least one of three features compatible with HIDS (aphtosis, post-vaccinal reactivity, cervical lymphadenopathy), only 2% of them had *MVK* mutations.(12) Both these studies highlight the need for guidance in order to rationalise the ordering of genetic studies in patients with recurrent fever. Clinical feature of mutation-positive patients were close in both cohorts and very similar to the largest published series.(13) Some features, like joint pain, diarrhoea, hepatosplenomegaly or lymphadenopathy, were more prominent during attacks in mutation-positive than in mutation-negative patients. However, no single clinical feature was perfectly sensitive and able to exclude the diagnosis of HIDS when missing. Although elevated serum IgD values were initially considered diagnostic, when genetic testing became available many patients with a compatible clinical picture did not have mutations in *MVK*.(14) Furthermore, it was recently shown that elevated serum IgD concentrations in patients with suspected HIDS has very poor predictive value.(15) *MVK* activity testing should be the diagnostic gold standard for HIDS. However, it is very time consuming and labour intensive, making it at least as costly as genetic testing. Moreover, radiometric assays are performed only in selected laboratories with adequate radioprotection measures.

This study also has some limitations. First, HIDS is a rare condition and both cohorts contain a limited number of patients. Nevertheless, this is one of the largest clinical studies performed on HIDS to date. Second, because of the retrospective nature of the study, many data were missing on several features. With complete data, we might have generated a classification rule with higher negative predictive value. Third, since this study was designed to evaluate when genetic testing for *MVK* mutations has no value, we cannot recommend on which patients genetic testing should be considered. A prospective study, preferably multi-centre, with clear inclusion criteria is needed to validate the classification rule and to further guide clinicians to decide when to use genetic testing for *MVK* mutations.

In conclusion, genetic testing for HIDS might be limited to patients with recurrent fever whose attacks began before the age of 5, or are associated with joint pain and last less than 14 days. Such a rule, once prospectively validated, could prevent at least 20% unnecessary genetic tests, which are resource and time consuming.

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Effect of etanercept and anakinra on inflammatory attacks in the hyper-IgD syndrome: introducing a vaccination provocation model

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Chapter 5

Abstract

Background: Hyper-IgD and periodic fever syndrome (HIDS) is an hereditary autoinflammatory syndrome, characterised by recurrent inflammatory attacks. Treatment of HIDS is difficult, although simvastatin is beneficial and etanercept might be effective. Studying the treatment of a rare periodic syndrome is complicated by the varying frequency and severity of symptoms and low prevalence. Our aim was to develop a system of clinical observations to evaluate effectiveness of treatment-on-demand.

Methods: Seven fever episodes in three HIDS patients were monitored, with and without administration of etanercept or anakinra. We developed a clinical score, which includes 12 symptoms. In one patient, inflammatory attacks were provoked by vaccination.

Results and conclusions: At the onset of an attack, all patients reported a clinical score between 20 and 25. The score was used to quantify severity and define the end of an attack. Reproducible monitoring of inflammatory episodes was difficult, even in this pilot study. The effect of early administration of etanercept was variable. In one patient, a fever episode could be readily provoked within 12 to 24 hours by vaccination. In this patient, the IL-1ra analogue anakinra was more successful in aborting the inflammatory attack than etanercept. We propose that this vaccination model will allow evaluation of treatment-on-demand in a controlled setting.

Introduction

The hyper-IgD and periodic fever syndrome (HIDS, MIM#260920) is an autosomal recessively inherited autoinflammatory syndrome, caused by deficient enzyme activity of mevalonate kinase, an enzyme in the isoprenoid pathway. Patients present with a long history of recurrent fever attacks, lasting three to seven days, accompanied by chills, headache, generalised lymphadenopathy, arthralgia, skin lesions, abdominal pain and diarrhoea. Laboratory analysis reveals an intense acute-phase response during fever attack; *ex-vivo* production of tumour necrosis factor (TNF)- α and interleukin (IL)-1 β by monocytes and macrophages is significantly higher at the time of attack.¹ An attack is usually preceded by a well-recognisable prodromal phase of malaise, headache, and musculoskeletal symptoms.² Sometimes, a (trivial) stimulus can be identified as a trigger of the attack, and most HIDS patients experience a severe inflammatory episode after any vaccination. In between two attacks patients are asymptomatic, although the acute-phase response may sometimes persist.

Until now, treatment of HIDS patients is largely supportive and very difficult. Various standard anti-inflammatory drugs (including colchicine, NSAIDs, steroids and thalidomide) have failed to suppress the attacks.^{2,3} We have already shown that simvastatin, an inhibitor of HMG-CoA reductase, can be beneficial in reducing the number of days of illness when taken continuously.⁴ Reports on the response to etanercept, an inhibitor of TNF α , in HIDS have been mixed: favourable in two children,⁵ uncertain in one,⁶ and no effect in another child.⁷ Studying the effectiveness of a treatment for a rare periodic syndrome is complicated by its variability of frequency and severity of symptoms and its low prevalence. We have tried to develop a system of rigorous clinical observations to evaluate the effectiveness of treatment-on-demand.

Methods

Three female Dutch HIDS patients gave written informed consent for participation in this study; the study was approved by the local medical ethical committee. The patients were not on any medication apart from that mentioned in the case observations. They were instructed to contact us at the first indication of a fever attack. When that happened, the patient was admitted to hospital for close monitoring, which included regular measurement of body temperature and blood sampling for C-reactive protein (CRP). We asked the patients to complete a daily symptom score card, rating the absence or presence of 12 different symptoms and their severity on a scale from 0 to 10. These 12 symptoms were lymphadenopathy, nausea, myalgia, arthralgia, aphthous ulcers, abdominal pain, skin lesions, headache, sore throat, tiredness, diarrhoea and nasal congestion. This was used to develop a clinical scoring system to help better delineate the duration and severity of a fever episode. By adding the scores on the individual symptoms, a daily score could thus range from 0 to 120. At the height of a fever episode, patients experienced between 7 and 12 of these 12 symptoms, while the maximal documented

score ranged from 45 to 88 points. Despite an anticipated variability in scores between patients and between separate attacks in the same patient, all patients reported a total score between 20 and 25 at the time of presentation at the start of a fever attack. Thus, a score of 20 or higher was taken to represent the presence of a fever attack, while the time point of the first score below 20 was taken as the end of the attack.

Case observations

Patient 1

A 35-year-old woman had experienced characteristic febrile attacks since the age of 3 months. At age 33, HIDS was finally diagnosed, confirmed by mevalonate kinase mutation analysis (table 1). During childhood, vaccinations used to trigger febrile attacks but the administration of hepatitis A immunoglobulin at the age of 34 years did not precipitate any symptoms. She was closely monitored during an attack which started with a sore throat, myalgias, fatigue, nausea and headache (figure 1A). Her body temperature was 36.6°C at presentation. Over the next few days symptoms increased and her body temperature quickly rose to 39.5°C, with a maximum CRP of 102 mg/l. The symptoms of the attack lasted four days although serum CRP concentration was still elevated at seven days (figure 1A). The next time she was admitted at the start of a fever attack, two doses of etanercept (25 mg) were administered subcutaneously at 12 and 36 hours after presentation (figure 1B). There was no noticeable difference in her clinical symptoms as expressed in the clinical score, body temperature or decrease in CRP concentration between this fever episode and the previous, untreated one (figure 1).

Patient 2

In this 26-year-old woman the diagnosis of HIDS was made three years ago, after she had experienced fever episodes since two months after birth. Childhood vaccinations consistently precipitated febrile attacks. The diagnosis was confirmed by mutation analysis, and an immeasurably low mevalonate kinase enzyme activity (table 1). The attack during which she was monitored appeared to be uncommonly severe with respect to the accompanying symptoms. She did not receive treatment during this attack which lasted

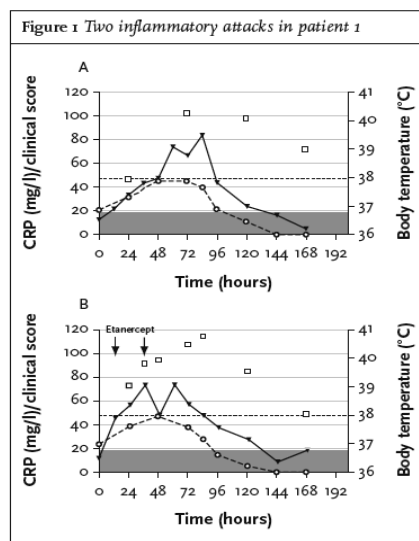


Figure 1: □ = Serum CRP concentration (mg/l) on left Y-axis; ▼ = body temperature (°C) on right Y-axis; ○ = total clinical score on left Y-axis; grey area depicts area of clinical score below 20 points. a) untreated inflammatory episode; b) inflammatory episode treated with etanercept at 12 and 36 hours after onset of symptoms (denoted by arrows).

Table 1 Patient characteristics

Case	Age (years)	Age at onset (months)	Mevalonate kinase genotype	Mevalonate kinase enzyme activity*	Serum IgD (U/ml) [†]
1	35	3	V377I/G202R	ND	2330
2	26	2	V377I/417insC	<0.1%	745
3	38	1	V377I/1268T	10.7%	616

*Expressed as % of normal; [†]reference value IgD <100 U/ml; ND = not determined.

seven days; she experienced massive cervical and iliac lymphadenopathy and developed oral and vaginal aphthous ulcers. CRP concentration rose to a maximum of 315 mg/l after 72 hours, while her body temperature was maximally 38.4°C (figure 2A).

A subsequent fever episode is depicted in figure 2B. The patient only reached our hospital some 48 hours after onset of symptoms, and immediately after admission etanercept treatment was initiated. At that time, serum CRP concentration was similar to that at 48 hours into the first attack (263 vs 252 mg/l), as was her clinical score (74 vs 71 points) (figure 2). After institution of treatment, the CRP concentration declined steadily, and within three days her symptoms had disappeared (figure 2B). Thus, etanercept seemed to shorten this attack in comparison with the previous one. The patient maintained that the second attack was milder and she was unconvinced of a beneficial effect.

Patient 3

Patient 3 is a 38-year-old woman with fever episodes since birth (table 1). She was admitted to the hospital for observation of an untreated fever episode. On admission, she complained of myalgia, arthralgia, abdominal pain, and skin lesions. Her body temperature was 36.8°C, but rose to above 39°C within two days (figure 3A). The symptomatic attack persisted for six days, with a concomitant rise and fall of CRP concentration. Because of a planned trip to China she required several vaccinations, and given her earlier childhood experiences of fever attacks after vaccinations, we decided to admit her for administration of the injections. We gave her one vaccination for hepatitis A (a primo vaccination in her case) and one (repeat) DTP toxin vaccination, separated by one month. Some 24 to 48 hours after administration of each vaccine she developed a characteristic HIDS attack with fever, abdominal pain, myalgia and fatigue. In both instances this was accompanied by an intense acute-phase response (maximum CRP 166 mg/l; figures 3B

Figure 2 Two inflammatory attacks in patient 2

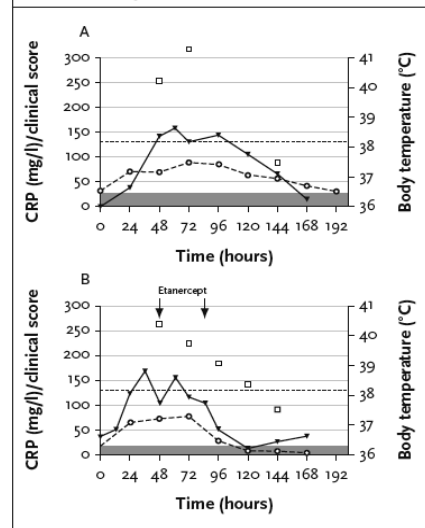


Figure 2: □ = Serum CRP concentration (mg/l) on left Y-axis; ▼ = body temperature (°C) on right Y-axis; ○ = total clinical score on left Y-axis; grey area depicts area of clinical score below 20 points One untreated inflammatory episode (a), and one inflammatory episode treated with etanercept (b, denoted by arrows).

and 3C). We used this model of vaccination-precipitated HIDS attack to test the efficacy of early institution of treatment to abort the fever episode. In the first attack, two doses of etanercept (25 mg, subcutaneously) were administered 72 and 86 hours after vaccination (48 and 72 hours after onset of the first symptoms). The symptomatic attack lasted for 5.5 days in total, with maximum CRP concentration reaching 36 hours after the first dose of etanercept (figure 3B). At the next vaccination one month later, we administered anakinra, a recombinant selective IL-1 receptor antagonist (100 mg per dose, subcutaneously), at three time points from 72 hours after vaccination, with an interval of 24 hours. Body temperature normalised and symptoms disappeared within 17 hours after the first injection, peak CRP concentration was reached at 12 hours after the first injection and CRP gradually returned to baseline values (figure 3C). Thus, anakinra aborted the attack after some three days of symptoms, in contrast to the 5.5 and 6 days of the other witnessed attacks. Antibody titres were found to be adequate after both vaccinations (data not shown), thus demonstrating that the administration of cytokine antagonists 72 hours after vaccination did not have an influence on the effectiveness of the vaccinations.

Discussion

In this pilot study monitoring fever attacks in HIDS, we closely followed seven fever episodes in three patients, with and without intervention. To quantify the accompanying symptoms of the inflammatory attack, we developed a clinical scoring system consisting of the total scores on a visual analogue scale on a range of 12 symptoms and signs. At the moment the patients felt the onset of a fever attack, they documented a total score of between 20 and 25. Since concentration of CRP and some of the symptoms, most notably tiredness, may persist for a longer period, the end of a HIDS attack may be difficult to define. We found that the proposed clinical score may help with this as well: we suggest

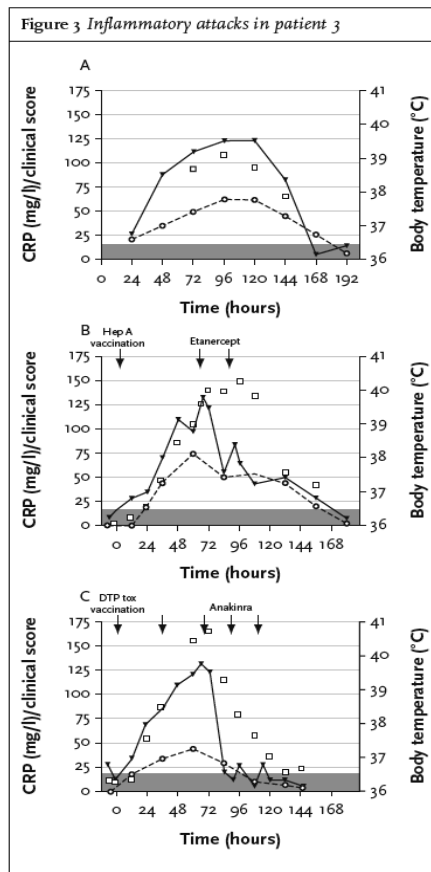


Figure 3: □ = Serum CRP concentration (mg/l) on left Y-axis; ▼ = body temperature (°C) on right Y-axis; ○ = total clinical score on left Y-axis; grey area depicts area of clinical score below 20 points. a) untreated inflammatory episode; b) inflammatory episode provoked by hepatitis A vaccination, and treated with etanercept (denoted by arrows); c) inflammatory episode provoked by DTP vaccination, and treated with anakinra (denoted by arrows).

defining the end of the attack as the moment at which the clinical score decreases to 20 or less.

The results of monitoring fever episodes in patients 1 and 2 demonstrate the variability of this disorder. It was our aim to start with intervention at the earliest possible moment and we therefore instructed patients to contact us as soon as possible after the start of symptoms. However, even in our small pilot study there was a difference of 36 hours between the time of first administration of etanercept in the two patients. The wide variation seriously impedes the comparability of results in a larger trial set-up. Part of the delay was caused by the fact that we admitted patients to our clinic, which might also induce a selection bias towards more severe attacks. However, treatment at home would make it more difficult to objectively observe symptoms, body temperature and CRP concentrations. A HIDS patient who needed vaccinations allowed us to observe the power of these vaccinations to provoke a fever episode. In both instances, vaccination resulted in a fever episode within 12 to 24 hours. These attacks were comparable with attacks not precipitated by vaccinations. There are some advantages to using this provocation model: it is simple, easy to use and offers an opportunity to closely monitor the onset of the attack from the very beginning, and thus to standardise the time to starting treatment. Since patients are hesitant to receive (necessary) vaccinations because of the risk of a febrile attack, a closely monitored setting will be helpful to ensure that they do receive them. With respect to the observations of effect of treatment-on-demand in these patients: these first results appear to be mixed, and this pilot study does not allow us to draw firm conclusions on that point. Anakinra seems to be more successful than etanercept; this warrants further examination.

HIDS is part of a group of hereditary autoinflammatory syndromes,⁸ whose common pathogenic background seems to involve IL-1 signalling. Recently several groups have reported treating hereditary autoinflammatory syndromes successfully with the recombinant form of IL-1ra, anakinra.⁹⁻¹³ It remains difficult to get solid evidence on treatment efficacy in orphan diseases, especially when periodic and variable in phenotype, such as HIDS. Most published reports concern clinical observations in one or two patients. Drug trials set up on established lines such as randomised controlled trials will often remain underpowered because of too few patients and too few episodes of illness.^{3,4} Also, because the frequency of fever attacks in adult HIDS patients will usually diminish to between 6 and 12 per year, patients will be more interested in an on-demand treatment which shortens an attack than in continuous treatment (and continuous life-long risk of side effects), unless this continuous treatment will abolish all further symptoms. We suggest that the vaccination provocation model in combination with the clinical score described in this pilot study will offer an opportunity for more rigorous and standardised study of on-demand treatment in HIDS.

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Hereditary periodic fever and reactive amyloidosis

J.C.H. van der Hilst, A. Simon, J.P.H. Drenth

Chapter 6

Abstract

Hereditary periodic fever syndromes (HPF) are a group of diseases characterised by recurrences of fever and inflammation separated by symptom-free intervals. Familial Mediterranean fever (FMF) is the most frequent entity within this group of disorders which further consists of hyperimmunoglobulinaemia D and periodic fever syndrome (HIDS), tumour necrosis factor receptor-associated periodic syndrome (TRAPS), and cryopyrin-associated periodic syndrome (CAPS). In recent years the causative genes have been identified. Reactive amyloidosis is a severe complication of HPFs. This is caused by deposition of fibrils that consist of the proteolytically cleaved acute phase protein serum amyloid A (SAA). Several factors have been identified that modulate the risk for developing amyloidosis, including SAA concentrations, polymorphisms in the SAA gene and ethnic origin. Furthermore, the risk of developing amyloidosis varies widely between the different HPFs. Colchicine is the cornerstone in the management of FMF, as it reduces the severity and frequency of attacks and is also effective in preventing amyloidosis. In the other HPFs, the introduction of anticytokine-based therapies is a promising new option in treating these inflammatory conditions and they potentially can prevent amyloidosis.

Introduction

Hereditary periodic fever (HPF) syndromes are a group of genetic diseases clinically characterised by recurrent febrile attacks lasting in length from a few days to a few weeks. By definition, these episodes of fever are separated by symptom-free intervals of variable duration. During attacks patients have vigorous inflammation, with leukocytosis and elevated concentrations of acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) (1). So far, at least 4 different genetic HPFs have been well defined at the clinical and genetical level. The best known HPF is familial Mediterranean fever (FMF), but three other entities have been identified as well: hyperimmunoglobulinaemia D and periodic fever syndrome (HIDS), tumour necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) and the cryopyrin-associated periodic syndrome (CAPS), which encompasses Muckle–Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS), and chronic infantile neurological cutaneous and articular syndrome (CINCA). The most devastating complication of the HPFs is reactive (type AA) amyloidosis (2). This is caused by accumulation of amyloid fibrils in the extracellular spaces of various organs and tissues, most notably the kidneys, liver and spleen, leading to organ failure (3). Not all patients with elevated SAA concentrations will develop amyloidosis. Several genetic and environmental factors modify the risk for reactive amyloidosis. In this review we will focus on amyloidosis in the group of HPF and genetic and clinical aspects of the HPFs.

AA amyloidosis

Pathogenesis of AA amyloidosis

Amyloidosis is a general denominator for a group of diseases that are characterised by extracellular deposition of fibrils of aggregated proteins (4). These fibrils consist of polymers in a β sheet configuration of a precursor protein. To date, at least 21 different proteins have been described that have the ability to form amyloid fibrils, including A β 42 in Alzheimer's disease and immunoglobulin light chains in AL amyloidosis (5). The amyloidosis type in HPF is reactive amyloidosis, also known as AA amyloidosis. The precursor protein in reactive amyloidosis is SAA. SAA is an acute phase protein that is mainly produced in the liver upon stimulation with various pro-inflammatory cytokines. It is found in plasma as an apolipoprotein of HDL cholesterol. During active inflammation serum concentrations beyond 1000 mg/l can be reached, which is 1000-fold higher than the constitutional concentration (6–8). The conserved homology of the protein among a wide range of species suggests that SAA has an important biological function (9). SAA has been implicated in leukocyte chemoattraction, (10, 11) opsonisation of Gram-negative bacteria (12), and cholesterol metabolism (13–15). However, to date, the exact function of SAA remains speculative. Although the size of the SAA protein produced by the liver is 104 amino acids, amyloid fibrils found in patients with AA amyloidosis mainly consist of an

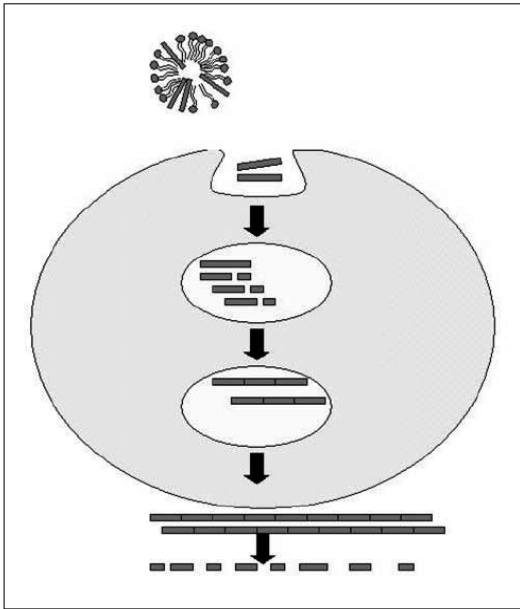


Figure 1

Schematic overview of amyloidogenesis.

1. After dissociation from HDL-cholesterol, SAA is taken up by the macrophage and transported to the lysosome.
2. In the lysosome, the c-terminal part of the SAA protein is cleaved. The intermediate AA protein can take on a β sheet configuration.
3. The AA protein polymerises to form a fibril.
4. The fibrils are deposited in the extracellular-space.
5. Binding of glycosaminoglycans, lipids and serum amyloid P stabilises the fibril. To some extent, the amyloid fibrils are degraded

accumulation of the 76 N-terminal amino acids of this protein (16–18), although proteins of different length have been reported (19, 20). The C-terminal portion of SAA is cleaved off by macrophages. After dissolution of HDL, SAA is absorbed by macrophages and transported to the lysosome (21–24) where it is cleaved by a group of enzymes called cathepsins (25–27) (Fig. 1). In normal circumstances SAA is completely degraded. In patients with amyloidosis, the process of degradation is thought to be impaired, leading to the accumulation of the 76-amino acid intermediate (28). The acidic environment of the lysosome facilitates the formation of these intermediates into a β -sheet structure and subsequent polymerisation. After deposition of these accumulated intermediates in the extracellular space, several glycosaminoglycans, serum amyloid P and lipid components bind to the fibril, and confer resistance to proteolysis (29–31).

Clinical manifestations of AA amyloidosis

Clinical symptoms of reactive amyloidosis are usually non-specific and depend on the organ involved. Several organs can be affected by AA amyloidosis, but the kidneys are most frequently involved. Reactive amyloidosis therefore usually presents as proteinuria with or without renal impairment. Renal involvement is found in >90% of patients (32, 33). If the underlying process cannot be controlled, renal failure will ensue, necessitating renal replacement therapy or kidney transplantation. Gastrointestinal involvement is seen in about 20% of patients with reactive amyloidosis, and may present as diarrhoea, malabsorption, or gastrointestinal pseudo-obstruction (32–34). This has become more common with the availability of haemodialysis and renal transplantation, which has increased the life expectancy and thus duration of amyloid accumulation in these patients. Amyloidotic goitre, hepatomegaly, splenomegaly, and polyneuropathy are less frequently

encountered features of reactive amyloidosis (32, 35–37). In contrast to other types of amyloidosis, cardiac involvement is rare in reactive amyloidosis (38).

Diagnosis of amyloidosis

A diagnosis of amyloidosis can be confirmed by detection of amyloid fibrils in biopsy material. Amyloid fibrils can be identified by staining tissues with Congo Red. Under polarised light microscopy this gives a typical apple-green birefringence (Fig. 2). Immunohistochemistry can subsequently differentiate between different types of amyloid. A direct biopsy of the affected organ (usually kidney) can be used, but because a biopsy carries the inherent risk for procedure-related complications, alternatives have been sought (39). One of those alternatives which are easier to execute and less prone to complication is a subcutaneous fat biopsy, as this has been shown to have a sensitivity of 66%–82% in patients with a high index of suspicion for amyloidosis (40–43). Another possibility is a rectum biopsy, which has a similar sensitivity (32, 44, 45). As patients with a negative rectum biopsy can have a positive fat biopsy, and vice versa, a biopsy of the other tissue in case of a negative result is useful to increase the yield of detection. Bone marrow biopsies and gingival biopsies can also be used, but have a lower sensitivity (46, 47). If all biopsies are negative and there is still a clinical suspicion, biopsy of the affected organ is indicated as the gold standard (39, 48).

Prognosis of amyloidosis

AA amyloidosis is a severe condition with high mortality. The median survival as taken from several studies varies between 24 and 53 months from time of diagnosis (32, 33, 49–51). Complications related to renal failure and renal replacement therapy are the main cause of death. It must be stressed that the progression of type AA amyloidosis and survival is strongly dependent on the ability to control the underlying inflammatory process (48,52). If the inflammation can be controlled and, thereby, SAA concentrations are reduced to values below 10 mg/l, a regression of amyloid mass as well as a reduction or reversal of nephropathy can be expected (53). In the following paragraphs we will discuss the HPFs with an emphasis on amyloidosis as a complication.

Familial Mediterranean fever

FMF is an autosomal recessive condition and it is the most prevalent of the HPF. Worldwide more than 10 000 patients are affected. FMF mainly affects ethnic groups originating from countries lining the Mediterranean basin. An exceptionally high prevalence has been found in Sephardic Jews, oriental Arabs, Turks and Armenians (54).

Genetic features and pathogenesis

FMF is caused by a mutation in the *MEFV* gene. Although some 90 mutations have been described (www.fmf.igh.cnrs.fr/infervers/), the four most prevalent (M694V, M680I, M694I, and V726A) account for over 80% of cases (55–57). *MEFV* encodes for pyrin/marenostrin, which is expressed in myeloid cells, particularly in mature granulocytes and to a lesser extent in monocytes, but not in lymphocytes, in a tissue-restricted manner. It can be up-regulated by several cytokines: IFN α , IFN γ and TNF α (58). Pyrin was the first of a novel class of proteins discovered that are involved in apoptosis and inflammation (59). It influences the process of NF- κ B activation and IL-1 secretion (60, 61).

Clinical manifestations

An inflammatory attack of FMF typically lasts 12 hours to 3 days. It begins abruptly, reaches its peak within a few hours and subsides rapidly (62–65). In between attacks patients feel completely well, although most have signs of subclinical inflammation (66). In the vast majority of patients FMF becomes clinically apparent before the age of 20. Signs of painful serositis accompanying the fever are the hallmark of the disease. Ninety-five percent of patients experience abdominal attacks. Rebound tenderness, abdominal wall rigidity, and diminished bowel sounds are often present. Synovitis is the second most common form of attack. Some 50%–75% of patients have episodes of arthritis, mainly affecting the large joints of the lower extremity (62, 65). Pleural inflammation presenting as unilateral pleural pain is experienced by 40% of patients. Pericarditis is rare and occurs in less than 1% (67).

Amyloidosis in FMF

Before the advent of colchicine, amyloidosis was relatively frequent. It occurred in up to 60%–75% of patients over the age of 40, and the incidence varied among different ethnic groups (68). Rather counter-intuitively, occurrence of amyloidosis is not directly associated with the severity or frequency of the inflammatory attacks (62, 69). Furthermore, it has been suggested that amyloidosis can be the presenting symptom in patients who have never experienced a classical FMF attack (phenotype II), although this is very rare (62, 65, 70–72). Not all FMF patients will develop amyloidosis, which suggests the presence of other contributing factors. The role of genetic background was established by comparing the incidence of amyloidosis in Jewish patients from different ethnic origins. It was shown that Jewish patients with a North African ancestry (Sephardim) have a much higher chance of developing amyloidosis than those originating from Iraq or eastern Europe (Ashkenazim) (73). The incidence of amyloidosis in different ethnic groups is presented in Table 1. Apart from ethnicity, several other genetic risk factors have been defined. The M694V mutation has been shown to be a strong risk factor of developing amyloidosis in different ethnic groups in many (74–81), but not all studies (82–85). Although the frequency of attacks does not generally correlate with any specific mutation, patients that

have the homozygote for M694V do have a more severe phenotype with a higher frequency of arthritic involvement, younger age of onset and a need for higher dosage of colchicine to control attacks (65, 74, 77, 86). Furthermore, the ethnic groups with the highest prevalence of M694V mutation (Sephardic Jews, Turks) also have the highest incidence of amyloidosis (Tables 1 and 2). Another factor that modulates the risk of developing amyloidosis is the SAA1 gene haplotype. Single nucleotide polymorphisms in the gene coding for SAA define 3 haplotypes: 1.1, 1.3 and 1.5. Patients with a 1.1/1.1 genotype have an increased risk for amyloidosis of 3–7-fold, independent of MEFV genotype (77, 79, 82, 87). Further evidence of genetic involvement in amyloidosis is the 4.5–6-fold increased risk of developing amyloidosis in affected family members of FMF patients who have already developed amyloidosis (65, 69, 88). Moreover, male patients have a 1.2–4-fold higher risk for developing amyloidosis compared to females (77, 79, 88). Environmental factors have been implicated in the risk of acquiring amyloidosis based on the initial observation that amyloidosis was found in 24% of FMF patients living in Armenia, while it was absent in Armenian FMF patients living in the USA (89). Later reports demonstrated that Armenians in the USA will develop amyloidosis, although the prevalence is lower (81).

Table 1 Prevalence of amyloidosis in patients with FMF in different ethnic groups

Ethnic origin	<i>n</i>	Amyloidosis, %	Publication year	Remarks	Ref.
Sephardic Jews	470	26.5	1967	15 patients non-Sephardic origin	[68]
Sephardic Jews	95	12	1970		[162]
Sephardic Jews	516	34.3	1982	Including Bagdadi Jews	[73]
Sephardic Jews	121	8.2	2000		[74]
Non-Sephardic Jews	44	2.3	1982		[73]
Non-Sephardic Jews	51	3.9	2000		[74]
Turks	25	61.9	1969		[163]
Turks	605	29.7	1997		[88]
Turks	253	19.8	2004		[69]
Turks	2436	12.9	2005		[65]
Turks	401	5.5	2005		[164]
Armenians	100	0	1974		[89]
Armenians	150	33.3	2000	10.3% in Armenians living in USA	[74]
Armenians	90	17.9	2000		[79]
Arabs	175	1.7	1986		[63]
Arabs	476	0.4	1999	Only children included	[165]

Table 2 Distribution of M694V homozygosity in different ethnic groups

Ethnic origin	M694V homozygote, %	References
Sephardic Jews	50–73	[74, 166, 167]
Turks	28–42	[65, 74, 85, 167]
Armenians	21–29	[74, 81]
Arabs	4–14	[77, 80, 167]
Ashkenazi Jews	0	[77, 166]

Therapy

The introduction of colchicine as the mainstay therapy for FMF in the late 1970s dramatically changed the course of the disease for many patients. With a dose of 1 mg/day, most patients become asymptomatic or have a markedly decreased number of attacks. Furthermore, amyloidosis is almost completely prevented in patients adherent to colchicine treatment, even in patients that continue to have attacks (90). Amyloidosis and subsequent renal failure can still develop in the long term, even in compliant patients (91, 92). In patients who have proteinuria or nephritic syndrome because of renal amyloidosis on presentation, a dose of 1.5–2 mg colchicine/day is advisable, as this can stabilise or even reverse nephropathy (93, 94). In patients who already have renal failure, colchicine has no beneficial effect on renal function (88, 91, 94, 95). In patients with end-stage renal disease, both haemodialysis or peritoneal dialysis can be instituted (96, 97). In eligible patients, renal transplantation is a therapeutic option with good prognosis (98, 99). Colchicine prevents the recurrence of amyloidosis in most, but not all, patients who have received a renal transplant (65, 100–102). Because colchicine is so effective in preventing amyloidosis, all FMF patients should be advised to take colchicine, including those with mild disease. As regression of amyloid is only seen in patients that achieve low SAA levels, it is advisable to measure SAA at regular intervals and adjust the dose of colchicine if indicated (103).

TNF receptor-associated periodic syndrome

This dominantly inherited disorder was first described in a large family of Irish/Scottish ancestry and hence named familial Hibernian fever (104). Later it emerged that this condition occurred in families throughout the world including Japanese, Dutch, Puerto Rican, Arabs, Ashkenazi and Sephardic Jews (105–107). After identification of the incriminated gene the name was changed to TRAPS (108).

Genetic features and pathogenesis

The involved gene encodes for one of the two known receptors for the pro-inflammatory cytokine TNF (TNFRSF1A). To date at least 64 mutations have been identified (www.fmf.igh.cnrs.fr/infervers/). Incomplete penetrance has been described (107, 109). The biological actions of the TNFRSF1A include activation of adhesion molecule expression, induction of cytokine production, pyrexia, and cachexia (110). After activation the receptor is cleaved and shed into the circulation where it acts as an inhibitor of TNF α . Because all known mutations are in the extracellular domain of the receptor, it has been hypothesised that TRAPS mutations interfere with the shedding of the TNF receptor (108). This would lead to deficiency of anti-inflammatory soluble TNF receptors. Reduced levels of soluble TNF-receptor have been found in patients, as well as a defect in shedding *in vitro*. However, a shedding defect is not ubiquitous to all mutations (105).

Clinical manifestations

The age of onset varies widely, but most patients become symptomatic within the first decade of life. Attacks persist for a minimum of 3 days, but usually last longer, up to several weeks (111, 112). Localised myalgia and painful erythema are the hallmark of the disease. Myalgia has been found in virtually all patients (112). Patients describe it as a deep cramping muscle pain, often severely disabling. Cutaneous manifestations are present in 87% of patients during attacks (113). Most patients exhibit localised erythematous macules and patches that tend to migrate to the distal parts of the extremities. Abdominal pain, often accompanied by vomiting, constipation and bowel obstruction, is common and occurs in the majority of patients. Conjunctivitis and periorbital oedema, with or without red discoloration, are other distinctive features of TRAPS (113, 114).

Amyloidosis in TRAPS

An estimated 14%–25% of TRAPS patients develop reactive amyloidosis (105, 108, 112). Duration and severity of inflammatory attacks correlate with the risk of reactive amyloidosis, but other genetic or environmental factors may also modulate the risk. An association of mutations involving cystein residues has been proposed (115), but this concept was challenged by others (116). In some families all adult patients are affected while in other families no cases of amyloidosis are found (117). Affected family members of TRAPS patients with amyloidosis are at increased risk and it is advisable to screen urine samples at regular intervals for proteinuria.

Therapy

In contrast to FMF, the attacks in TRAPS do not respond to colchicine treatment, and this treatment cannot prevent amyloidosis (112). Mild cases can be treated symptomatically with NSAIDs. For more severe cases, corticosteroid therapy can be given to attenuate the severity and length of attacks, but prolonged courses of high doses are often required. Other immunosuppressive therapies have been tried with only fairly limited success, including methotrexate, thalidomide, azathioprine, and cyclosporine (118, 119). Recent advances in the biological therapy of rheumatoid arthritis have led to exploration of a novel treatment for TRAPS. Etanercept, a construct of soluble TNF receptor, reduced the length and number of attacks (118, 120). Furthermore, etanercept reversed nephrotic syndrome in a patient with established amyloidosis (121). We have reported favourable results with anakinra, a synthetic form of the soluble receptor antagonist of the pro-inflammatory cytokine IL-1, in a TRAPS patient resistant to etanercept (122).

Cryopyrin-associated periodic syndrome

CAPS comprises 3 phenotypically distinct autosomal dominant syndromes: MWS, familial cold auto-inflammatory syndrome (FCAS), also known as familial cold urticaria, and

CINCA, also known as neonatal-onset multisystem inflammatory disease (NOMID). Flares of fever, typical urticarial skin rash and arthralgias characterise all three syndromes. Although each syndrome has its distinct clinical manifestations, they are caused by mutations in the same gene.

Genetic aspect and pathogenesis

FCAS, MWS and CINCA are caused by mutations in the *CIAS1* gene that encodes for cryopyrin (115, 123, 124). As with pyrin, the protein involved in FMF, cryopyrin is involved in the regulation of IL-1 production and NF- κ B activation (125, 126). Remarkably, it has been reported that some of the same *CIAS1* mutations can lead to either of the three clinical syndromes that make up CAPS (115, 127).

Clinical manifestations FCAS is characterised by attacks of fever, urticarial skin rash, arthralgia and conjunctivitis after exposure to cold. An episode starts 2–3 h after exposure and generally subsides within 24 h (128, 129). Attacks are accompanied by an intense acute phase response, as evidenced by high leukocyte counts in peripheral blood. MWS has a similar phenotype as FCAS except that attacks are not provoked by cold exposure. Furthermore, sensorineural hearing loss, a frequent complication of MWS, is not seen in FCAS (130–132).

CINCA is a more severe disease with a neonatal onset. Disabling arthritis, especially of knees, and chronic meningitis leading to headaches, cerebral atrophy, and seizures as well as lymphadenopathy and splenomegaly are features of this condition (133). The distinction between the 3 subtypes is not always clear. Different phenotypes within a single family have been described (134), and patients thought to be affected by one syndrome can have symptoms of another syndrome (131, 132, 135).

Amyloidosis in CAPS

Although MWS was originally described as a triad of deafness, urticaria and amyloidosis, not all patients with MWS develop amyloidosis (136). Later it was estimated that approximately one-third of patients suffer from amyloidosis, and there is familial clustering (130). This supports the notion that other regulatory genes are involved in the pathogenesis of amyloidosis. Several cases of reactive amyloidosis have been reported in FCAS and CINCA (129, 133). Although studies are too small to make an accurate estimate, the incidence seems to be lower in FCAS and CINCA than in MWS.

Therapy

In CINCA and MWS, corticosteroid therapy can be useful in selected patients (133, 137). One patient with MWS who used corticosteroids, azathioprine, and later cyclophosphamide therapy for the prevention of graft rejection after renal transplantation for reactive amyloidosis had complete resolution of articular and cutaneous symptoms (138). Until recently, no effective therapy for FCAS was known. Colchicine is ineffective in CAPS as it cannot prevent amyloidosis nor has it been beneficial in established amyloidosis

(139). Hopeful results have been obtained with anakinra. It can prevent attacks in patients with FCAS in response to a cold stimulus (140). A recent report has also shown promising results in MWS (131). Anakinra gave dramatic clinical response within hours after start of treatment. Moreover, in 2 patients with nephropathy due to amyloidosis, anakinra substantially decreased proteinuria (141). Similar positive results have been obtained in CINCA syndrome (142). Although these data are preliminary, and long-term consequences are unknown, this novel therapy looks very promising.

Hyper IgD syndrome

HIDS was identified as a separate disease entity in 1984 (143). It is inherited as an autosomal recessive trait (1). So far the International HIDS Registry (www.hids.net) contains 96 patients with known MVK mutations. Although most patients are of Dutch or other Western European origin, patients have been reported from all continents.

Genetic aspects and pathogenesis

HIDS is caused by a mutation in the gene MVK, which leads to a deficiency of the enzyme mevalonate kinase (144, 145). Mevalonate kinase is an enzyme in the isoprenoid biosynthesis pathway. In this pathway cholesterol is produced in addition to a number of nonsterol isoprenoids, which include ubiquinone, heme A, farnesyl and geranyl (146). How reduced activity of mevalonate kinase leads to an autoinflammatory condition is not known. It has been speculated that either a lack of isoprenoids or an excess of the substrate of mevalonate-kinase, mevalonic acid, may contribute to the inflammatory phenotype. What has been shown is that that pro-inflammatory cytokine production by mononuclear cells of patients with HIDS is greatly enhanced (147–149).

Clinical manifestations

The first clinical episode usually begins before the end of the first year of life. Attacks start with chills, followed by a sharp rise in body temperature accompanied by cervical lymphadenopathy and abdominal pain. Hepatomegaly, splenomegaly, arthralgias, skin rash, diarrhoea and vomiting are common symptoms (137, 150). After 4–6 days symptoms gradually fade. An attack can be provoked by minor trauma, vaccination, or stress. The attacks usually recur every 4–6 weeks, but there is considerable inter- and intra individual variation. A marked elevation of polyclonal immunoglobulin D is found in the serum. However, this is not specific, as it can also be found in some patients with other periodic fever syndromes.

Amyloidosis in HIDS

Until recently no cases of amyloidosis were seen in HIDS, setting it apart from other periodic fever syndromes (151). Recently, Obici *et al*/reported the first patient with AA amyloidosis as a complication of HIDS (152). A second case was reported separately to the HIDS registry. Strikingly, both patients had clinical symptoms of HIDS for more than 20

years before the advent of amyloidosis. Still, the incidence is remarkably low compared to other periodic fever syndromes. SAA concentrations are high during attacks, and many patients have elevated levels when asymptomatic. Furthermore, SAA polymorphisms are normally distributed among patients with HIDS (153). This suggests that mevalonate kinase deficiency might protect against amyloidosis.

Therapy

Corticosteroids are ineffective in preventing or treating attacks. Thalidomide, as a TNF-inhibiting drug, was also unsuccessful in preventing attacks and had considerable side-effects (154). A recent trial with simvastatin showed some therapeutic effect in reducing the number of days of illness (155). Simvastatin inhibits HMG-CoA reductase, the enzyme proximal to mevalonate kinase in the isoprenoid pathway (156). Preliminary investigations demonstrated a mixed result of etanercept (157–159) and anakinra (160).

Future perspectives

Recent advances in genetics have greatly enhanced our knowledge of the pathogenesis of periodic fever syndromes (161). On the other hand, the identification of affected proteins in CAPS and FMF has offered us further insight in the mechanisms of inflammation. It has become clear that TNF α and interleukin play important roles in the pathogenesis of all the periodic fever syndromes. Biologicals specifically aimed against these proinflammatory cytokines are promising agents in the treatment of TRAPS, HIDS, and CAPS. Furthermore, it is to be hoped that amyloidosis may be prevented by these therapies. However, despite new therapeutic options, amyloidosis will remain a complication of HPF. A recent Turkish study showed a prevalence of 12% among FMF patients, despite the availability of a good prophylactic agent (65). In general HPFs have extended our knowledge of inflammatory mechanisms; they may also shed further light on the mechanisms of amyloid formation.

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Serum amyloid A serum concentrations and genotype do not explain low incidence of amyloidosis in Hyper-IgD syndrome

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Chapter 7

Abstract

Background. Hyper-IgD and periodic fever syndrome (HIDS) is an autosomal recessively inherited disorder characterized by recurrent episodes of fever and inflammation. Unlike other chronic inflammatory conditions, amyloidosis is very rare in HIDS. For deposition of amyloid of the AA type, high concentrations of SAA are a prerequisite, together with certain SAA1 gene polymorphisms. The SAA1.1 genotype predisposes for amyloidosis, while SAA1.5 genotype exerts a protective effect.

Aim of the study. To determine if SAA concentrations and SAA1 gene polymorphisms could explain the virtual absence of amyloidosis in HIDS patients.

Methods. We measured SAA and CRP concentrations in serum of 20 HIDS patients during an attack and during the asymptomatic phase. Genotype of SAA1 gene was determined in 60 HIDS patients.

Results. SAA serum concentrations during attacks were very high (median 205 mg/l; range 75–520 mg/l, normal <4.2 mg/l). During attack-free periods 45% of patients still had elevated SAA concentrations. The distribution of the genotype of SAA1 gene in HIDS was similar to healthy controls (SAA1.1 0.41 vs. 0.50 $p=0.32$).

Conclusion. Patients with HIDS have high SAA during attacks and show sub-clinical inflammation when asymptomatic. The low incidence of amyloidosis cannot be explained by a predominance of non amyloidogenic SAA related genotypes.

Introduction

Reactive (AA) amyloidosis refers to the systemic deposition of insoluble fibrillar amyloid proteins in the extra-cellular space in a number of different organs, most notably the kidney. The first manifestation of the AA type amyloidosis is proteinuria, which progresses to nephrotic syndrome and finally renal failure (1). The AA protein that forms the amyloid fibrils in type AA amyloidosis is a degradation product of serum amyloid A (SAA), an acute phase protein produced in response to inflammation. It is generally accepted that high SAA serum concentrations during a long period of time are a prerequisite for the development of AA amyloidosis (2). AA type amyloidosis has been described in chronic inflammatory disorders such as juvenile chronic arthritis, inflammatory bowel disease, and in hereditary periodic fever syndromes.

Hereditary periodic fever syndromes or autoinflammatory syndromes are a group of genetic disorders characterized by recurrent episodes of fever with vigorous acute phase response separated by symptom-free intervals (3). The best known representative of this group is familial Mediterranean fever (FMF), but over the last two decades five other disorders have been described: the Hyper-Immunoglobulinemia D and periodic fever syndrome (HIDS), TNF-receptor-associated periodic syndrome (TRAPS), Muckle–Wells syndrome (MWS), familial cold auto-inflammatory syndrome (FCAS), and lastly Chronic Infantile Neurological Cutaneous and Articular syndrome (CINCA). Type AA amyloidosis is a frequent complication of most hereditary periodic fever syndromes. For example, it was found in up to 60% of FMF patients before the advent of colchicine treatment (4), and it has been reported in 25% of TRAPS patients (5,6), and in up to 35% of patients with MWS (7). Although the prevalence of amyloidosis among the hereditary periodic fever syndromes is high, current data indicate that it is a very rare event in HIDS. HIDS is an autosomal recessively inherited disorder characterized by recurrent attacks of fever, bilateral cervical lymphadenopathy, and by abdominal pain and diarrhoea. In its classical form, it is caused by mevalonate kinase (MVK) gene mutations that lead to a deficiency of mevalonate kinase, a central enzyme to the isoprenoid metabolism. HIDS earned its name because patients have markedly elevated serum concentrations of polyclonal IgD (8,9). Patients with HIDS seem to have the pre-requisites for development of amyloidosis. It is a chronic inflammatory condition; symptoms of HIDS start typically in the first year of life, patients suffer from frequent attacks and during attacks they exhibit a vigorous acute-phase response. Despite a thorough 20-year follow-up, there are only 2 documented cases among 92 patients with genetically proven HIDS held at The International Hyper-IgD syndrome Registry (www.HIDS.net), of which one has recently been published (10). In view of these facts, the low incidence of amyloidosis in HIDS patients is remarkable. A prolonged high plasma level of SAA in chronic inflammation is considered necessary for deposition of AA proteins in tissues. However, a high concentration of SAA alone is not sufficient for the

development of reactive type AA amyloidosis. SAA1 gene polymorphisms have been identified as additional risk factors. The presence of 2 single nucleotide polymorphisms within exon 3 of the SAA1 gene defines 3 haplotypes (1.1, 1.3, 1.5) (11). In Caucasians, the 1.1 allele exhibits a pro-amyloid phenotype, while 1.5 allele seems to protect (12,13). The present study was performed to investigate whether SAA concentrations and genotype prevalence could explain the low incidence of amyloidosis in HIDS.

Patients and methods

Cohort study

Twenty HIDS patients known in our clinic were enrolled in this first part of our study (Table I). All patients carried MVK gene mutations. Sampling of plasma and serum was performed at two time points during a 6-month period, during fever attack and during remission. A fever attack was defined as (1) raised body temperature (>38°C), (2) at least one of the following symptoms and signs: lymphadenopathy, abdominal pain, arthritis, and/or skin rash and (3) no clinical indications for the presence of infection. Remission was defined as the absence of symptoms for at least 1 month. No medication was allowed during the study period. Close follow-up of the patients did exclude bacterial and/or viral infections during the course of the investigation. We sampled for CRP, SAA, serum creatinine, and performed urinalysis. SAA and CRP were measured with enzyme linked immunosorbent assays (14) (normal SAA 54.2 mg/l; normal CRP 53.7 mg/l; detection limit for both assays is 0.001 mg/l).

Table 1. Characteristics of 20 HIDS patients in the cohort study.

Sex (male:female)	11:9
Disease duration	29.5 (13-50)
Mutations	
V377I/unknown	5
V377I/H20P	4
V377I/I268T	4
V377I/W62X	2
P167L/I268T	2
V377I/G309S	1
P167L/G202R	1
V377I/V377I	1

SAA genotype study

We collected DNA samples from 60 HIDS patients from the Nijmegen HIDS registry. All patients had MVK gene mutations. DNA was extracted by standard methods and we included 50 healthy Dutch blood donors as controls. SAA1 genotypes were determined by polymerase chain reaction followed by restriction fragment length polymorphism analysis

as described elsewhere (15). We established the presence of 2 single-nucleotide polymorphisms within exon 3 of the SAA1 gene that define 3 haplotypes corresponding to SAA1.1 (Val52, Ala57), SAA1.3 (Ala52, Ala57), and SAA1.5 (Ala52, Val57). The ethical committee of our institution approved the study protocol, and all patients gave informed consent.

Statistical analysis

The statistical significance of differences between groups was calculated either by Chi-square test for categorical data or Fisher's exact test where appropriate.

The distribution of each allele frequency among the control population was tested whether it fitted the Hardy–Weinberg equilibrium.

Results

Cohort study

The distribution of the 20 patients in the cohort with respect to MVK genotypes, gender, age, and duration of disease is presented in Table I. Renal function was normal in all patients (mean serum creatinine 64 mmol/l, range 53–86) and none had proteinuria, the first sign of nephropathic amyloidosis. During attacks, all HIDS patients exhibited high concentrations of SAA (median 205 mg/l; range 75–520 mg/l) and CRP (210 mg/l; 67–385 mg/l) (Figure 1). During the asymptomatic stage, 9 of the 20 patients (45%) still had SAA concentrations above normal. SAA concentrations during attacks and symptom-free intervals did not correlate with age, gender, or type of MVK mutation. Also CRP was elevated in the majority of patients (10 mg/l; 0.1–240 mg/l).

Genotype study

Figure 2 displays the SAA1 genotype of 60 HIDS patients and 50 healthy controls. The distribution of alleles fitted the Hardy–Weinberg equilibrium. There was no difference in distribution of amyloidogenic (1.1) and protective (1.5) genotypes between patients and controls ($P = 0.28$). Neither the concentrations of SAA nor the SAA/CRP ratio was significantly correlated with one of the SAA1 genotypes.

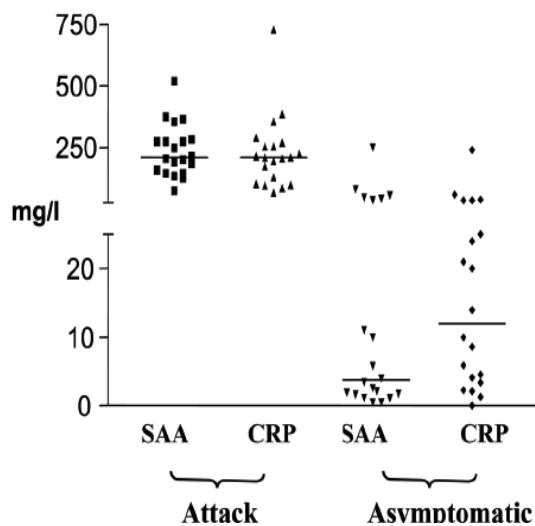


Figure 1: SAA and CRP concentrations during an attack and when asymptomatic in 20 HIDS patients. Thick bar represents the median. SAA normal <4.2 mg/l CRP normal <3.7 mg/l.

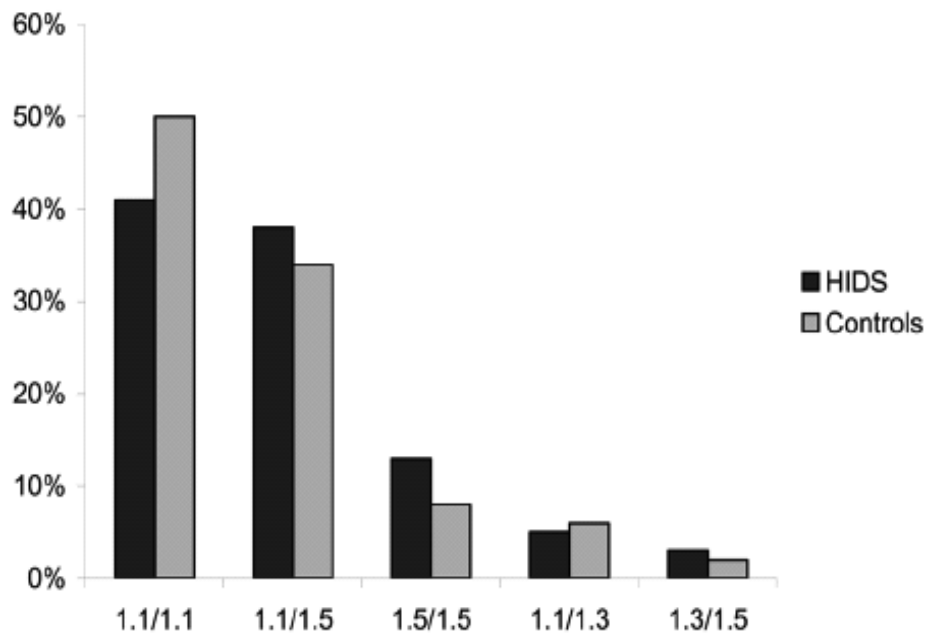


Figure 2: Genotype of SAA1 gene in 60 patients and 50 controls.

Discussion

Despite strongly elevated SAA concentrations during attacks and persistently raised SAA concentrations in between attacks, HIDS patients do not show a high incidence of amyloidosis. From the available data in the literature we could calculate that the incidence of developing amyloidosis is approximately 1.8/100 patient years in untreated FMF (16). This is 20 times higher than the incidence of amyloidosis in HIDS (0.09/100 patient years). Although median SAA concentrations in FMF are in some studies reported to be higher than those we found in HIDS, this does not seem to be sufficient explanation (17). Even in systemic juvenile chronic arthritis, a condition that tends to regress during life, the incidence of amyloidosis is 5–7 times higher after 15–29 years of follow up (0.50–0.65 cases/100 patient years) (18,19). Our study demonstrates that the low incidence of amyloidosis is not explained by the distribution of SAA1 genotype: The amyloidogenic SAA 1.1 genotype being present in 41% of patients. The risk of developing amyloidosis with the SAA1.1 genotype in an autoinflammatory condition such as FMF is strongly elevated (12,13). The odds ratio of acquiring amyloidosis in FMF patients with SAA1.1 genotype was 2.99 (12). In other inflammatory disorders associated with type AA amyloidosis such as juvenile chronic arthritis, the SAA1.1 genotype was detected in 80.5% of cases complicated by amyloidosis compared to 12.5% in cases free from amyloidosis (20). Recently Terai et al. found similar results in Finnish patients with rheumatoid arthritis (21).

The question is how to explain the protection against amyloidosis in HIDS. In classical HIDS, the defect concerns mevalonate kinase (MVK), the enzyme phosphorylating mevalonate. Mevalonate is the precursor of isoprenoid groups that are incorporated into an array of end-products, such as ubiquinone, Rho, Ras, haem A, and dolichol (22). One possibility to explain the decreased incidence of amyloidosis is that the excess of mevalonate might influence amyloidogenesis in a still obscure way. A more attractive hypothesis concerns a possible shortage of metabolites downstream, interfering with isoprenylation of proteins that protect against amyloidosis (23). A variety of regulating proteins, like Ras, are dependent on isoprenylation to be activated. Decreased isoprenylation of these proteins could interfere with amyloidogenesis on different levels. For example, the expression of lysosomal proteases has been shown to be regulated by Ras (24). Since lysosomal SAA degradation is seen as a central process in amyloidosis, decreased isoprenylation could influence this process. Furthermore, a role for matrix metalloproteinases (MMP) could be envisaged. MMPs have been shown to degrade SAA and amyloid fibrils (25) and MMP expression is regulated by isoprenylated proteins (26). Another possibility is that the decreased isoprenoid metabolism alters HDL cholesterol to which SAA is bound as an apolipoprotein. This could interfere with SAA uptake in macrophages, leading to decreased processing of SAA to AA proteins. If the latter hypotheses are true it might be expected that treatment with HMG-CoA reductase inhibitors would interfere with amyloidogenesis. More investigation in this area is needed

to find out whether these drugs are effective in prevention and perhaps treatment of secondary amyloidosis.

In conclusion, although HIDS patients have all the prerequisites for developing amyloidosis, including high SAA concentrations and normal distribution of SAA1 genotype, amyloidosis is only a rare complication. Thus, the causative defect in HIDS, mevalonate kinase deficiency, seems to interfere with amyloidogenesis.

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Lovastatin inhibits formation of AA amyloid

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Chapter 8

Abstract

AA amyloidosis is a severe complication of many chronic inflammatory disorders, including the hereditary periodic fever syndromes. However, in one of these periodic fever syndromes, the Hyper Ig-D and periodic fever syndrome, amyloidosis is rare despite vigorous, recurring inflammation. This hereditary syndrome is caused by mutations in the gene coding for mevalonate kinase, an enzyme of the isoprenoid pathway. In this study we used a cell culture system with human monocytes to show that inhibition of the isoprenoid pathway inhibits amyloidogenesis. Inhibition of the isoprenoid pathway by lovastatin resulted in a dose dependent reduction of amyloid formed (53% at 10 μ M ($p=0.01$)) compared to mononuclear cells that are exposed only to Serum Amyloid A. The inhibitory effects of lovastatin is reversible by addition of farnesol, but not geranylgeraniol. Farnesyl transferase inhibition also inhibited amyloidogenesis. These results implicate that the isoprenoid metabolism could be a potential target for prevention and treatment of AA amyloidosis.

Key words: Amyloidosis, SAA, Hyper IgD syndrome, Monocyte, Isoprenoid

Introduction

Type AA, or reactive, amyloidosis is a serious, potentially life-threatening complication of chronic inflammatory conditions. The kidneys are most often affected, but other organs, such as the intestines or heart can also be involved(1).

Amyloidosis is caused by the deposition of insoluble fibrils in the extracellular matrix of organs and tissues (2,3). Amyloid fibrils in AA amyloidosis are derived from C-terminally cleaved fragment of the acute phase protein serum amyloid A (SAA). SAA is an acute-phase protein produced in the liver in response to pro-inflammatory cytokines (4). It is transported in the plasma as a component of high density lipoprotein (HDL)(5). Although the SAA protein produced by liver is 104 residues in length, amyloid fibrils consist mainly of fragments of SAA containing the 76 N-terminal residues (6,7). The process of C-terminal cleavage and assembly into amyloid fibrils is referred to as amyloidogenesis. It is generally accepted that macrophages are central to this process (8-11). After internalization by the macrophage, SAA traffics from early to late endosomes and then to lysosomes for where proteases of the cathepsin family either fully catabolize or partially degrade SAA (8-10,12-15).

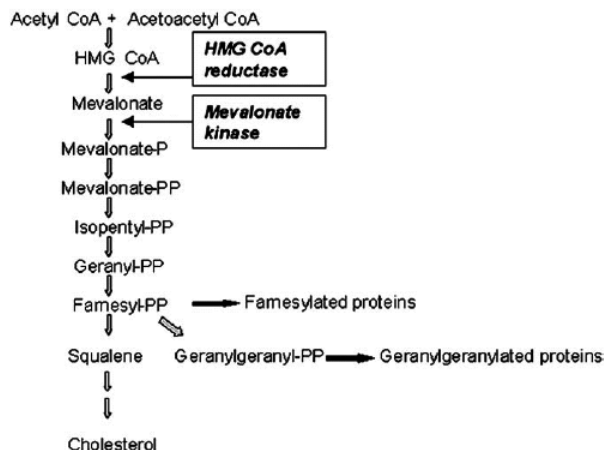
Periodic fever syndromes, nowadays also designated as hereditary auto-inflammatory syndromes, are a group of disorders characterized by recurrences of fever accompanied by vigorous acute-phase response (16). They include familial Mediterranean fever (FMF), Cryopyrin associated periodic syndrome (CAPS), TNF receptor associated periodic syndrome (TRAPS), and hyper immunoglobulin D and periodic fever syndrome (HIDS). The latter disorder is an autosomal recessive disorder characterized by recurring inflammatory attacks with a combination of one or more of the following signs: fever, skin rash, abdominal pain, arthritis, and lymphadenopathy (17). HIDS is caused by a defective mevalonate kinase, a major enzyme in the isoprenoid pathway. This pathway eventually leads to the production of cholesterol and non-sterol isoprenoids (Figure 1).

Because of prolonged SAA elevation, all patients with periodic fever syndrome are at risk for developing amyloidosis. This is illustrated by the fact that up to 60% of FMF patients developed amyloidosis in the era prior to the introduction of an effective treatment, i.e. colchicine (18). Colchicine inhibits inflammation and normalizes SAA concentrations by a yet unknown mechanism only in FMF and it is ineffective in other auto-inflammatory diseases (19). In CAPS and TRAPS, amyloidosis develops in a third to a quarter of patients (19). Remarkably, however, amyloidosis is rare in HIDS. Despite the fact that HIDS has been recognized as a specific entity since 1984, the first cases of amyloidosis in HIDS were only recently described in the literature (20,21). Using data from an international database of more than 120 well characterised HIDS patients with a follow-up of up to 23 years (www.hids.net), we have determined the incidence of amyloidosis in HIDS to be less than 3%.

Since HIDS patients have all the prerequisites for developing amyloidosis (22), we hypothesized that it is the underlying genetic defect that confers protection (23). If the

mevalonate kinase defect interferes with amyloidogenesis through a deficiency of isoprenoid products, then inhibiting the preceding enzyme, HMG CoA reductase, should also inhibit amyloid formation. In order to test this hypothesis we used a human cell culture system that allows the quantitative and qualitative study of amyloidogenesis in vitro.

Figure 1. Overview of the isoprenoid biosynthesis. 3-Hydroxy-3-methylglutaryl (HMG) CoA is metabolized into mevalonate by HMG CoA reductase. Mevalonate is phosphorylated by mevalonate kinase



Material and methods:

SAA and amyloid-enhancing factor (AEF)

Mouse recombinant SAA 1.1 was produced in *Escherichia coli* and purified as described previously (24). Purified SAA was dissolved in 4 M urea at a concentration of 7.5 µg/µl. AEF was prepared as described previously (25).

Amyloid induction

Peripheral blood mononuclear cells from normal human donors were isolated on ficoll-hypaque gradients and cultured in 96-well plates. Wells were rinsed after 4 hours to select for adherent mononuclear cells and maintained in 100 µl RPMI supplemented with 15% fetal calf serum, 1 mM pyruvate, and 1% gentamicin at 37° C in an atmosphere with 5% CO₂. Amyloid production was initiated by the addition of 2 µl SAA and 2µl AEF leading to a final concentration of 150 µg/ml and 12 µg/ml. Delipidated rSAA rapidly associates with HDL in the FSC(26). Where applicable, medium was supplemented with lovastatin (Sigma-Aldrich, St. Louis, MO) at indicated concentrations with or without 5 µM farnesol or geranylgeraniol (Sigma-Aldrich). Amyloid induction was also performed with supplementation of 200nM Farnesyl protein transferase (FPT) inhibitor II (Calbiochem, Nottingham, UK). Medium and supplements were replaced every other day. All conditions were tested in 3 independent experiments. Results are expressed as percentage of amyloid production versus cell without supplementation (100%).

Amyloid quantification

After the indicated time period, cells and cell-associated amyloid were collected and spun down on glass slides, with a cytospin centrifuge for 10 minutes at 500 rpm. Cells were fixed

in 10% neutral buffered formalin and stained for 45 minutes in Congo red prepared in 80% alkaline ethanol. After quick dips in water, cells were counterstained with Gill's hematoxylin solution (Sigma-Aldrich). They were dipped in acidified 70% ethanol, several times in water, and once in ammonium solution. After dehydration in alcohol, slides were cleared in xylene and coverslips were applied with Permount.

The presence of amyloid fibrils was confirmed by visualisation of apple-green birefringence of Congo-red material on polarized light microscopy.

The amount of amyloid was determined with a digital image analysis protocol that was previously described (27), with minor modification. Briefly, the slides were analysed using a CCD RGB camera (Sony 950 P) mounted on top of a light microscope (Axioskop 2 plus) and attached to a KS400 image analysis system (both from Karl Zeiss, Weesp, the Netherlands). A 20x objective with a numerical aperture of 0.5 was used for image acquisition, resulting in pixels with a dimension of $0.39 \mu\text{m}^2$. In each slide, 20 randomly selected fields were digitized for analysis. In each digitized RGB image, the red component was used to define the area covered with Congo red bound amyloid. The amount of background as determined by the area covered with red material in cell cultures treated with AEF, but without SAA, was less than 0.3%. In addition the number of nuclei in the selected fields was automatically assessed.

To study if lovastatin influenced the uptake of SAA, mononuclear cells were incubated with $150 \mu\text{g}/\text{ml}$ SAA with or without $5 \mu\text{M}$ of lovastatin. Aliquots of supernatant at 0, 24, and 48 h were subjected to Tris-Tricine SDS polyacrylamide gel electrophoresis. Proteins were visualized by staining with Coomassie brilliant blue. Uptake of SAA was determined by comparing the amount of SAA remaining after 0 (100%), 24, and 48 h of incubation using a densitometer (Umax, Uden The Netherlands) and Totallab TL100 software (nonlinear dynamics, Newcastle, UK). The experiment was performed in triplicate and results are expressed as mean percentage of SAA taken up \pm S.E.M.

Statistical analysis

Data were compared with unpaired Student's *t* test. A *p*-value <0.05 was considered to be significant.

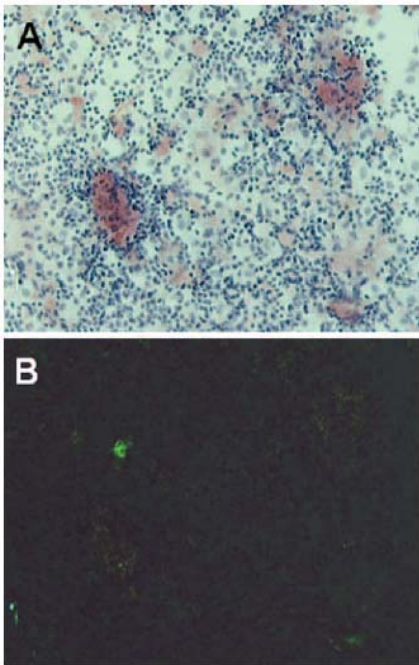


Figure 2. Extracellular Congo red deposits after 5 days of incubation with 150 $\mu\text{g/ml}$ SAA and AEF (A). The Congo red deposits show typical apple-green birefringence under polarized light (B).

Results

We were able to induce in vitro amyloidogenesis in a reproducible and consistent manner. We found that amyloidosis induction was initiated by the addition of SAA (150 $\mu\text{g/ml}$) and AEF to human monocytes. With this concentration, which is well within the range found during inflammatory attacks of AIDS patients, the first extracellular amyloid deposits appear as early as three days after the start of induction. The deposits stain with Congo-red and show typical apple-green birefringence under polarized light microscopy, indicative of amyloid fibrils (Figure 2). When induction of amyloidogenesis is prolonged, there is a gradual increase in the amount of amyloid as shown in Figure 3. For example, Congo-red material is present in approximately 10% of the culture after 9-day incubation with SAA.

Next, we investigated the effect of the HMG-CoA reductase inhibitor lovastatin on amyloid

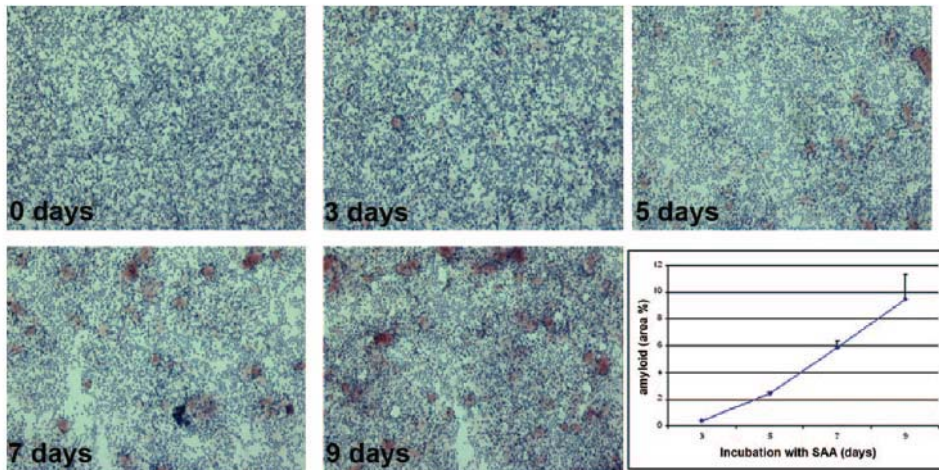


Figure 3. Deposition of amyloid during time. Mononuclear cells were incubated with 150 $\mu\text{g/ml}$ SAA and AEF. There is a linear increase in the amount of amyloid depositions during 9 days of incubation.

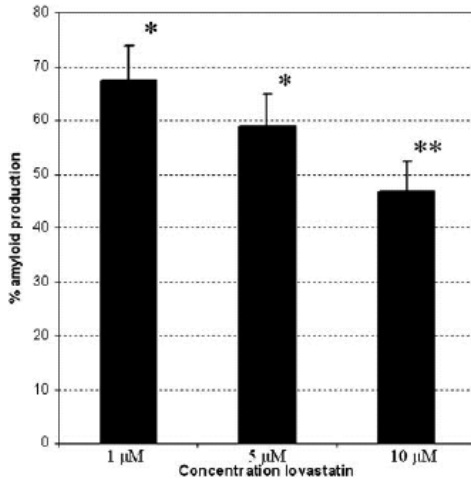


Figure 4. Effect of lovastatin on amyloidogenesis. Mononuclear cells were incubated with 150 $\mu\text{g/ml}$ SAA and AEF for 7 days. Addition of lovastatin gives a dose-dependent reduction of amyloid deposition. Error bars represent SEM. *, $P < 0.05$; **, $P = 0.01$.

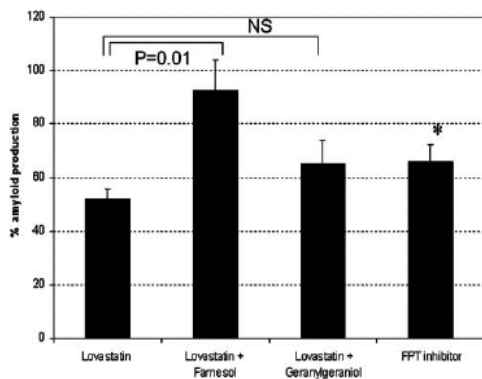


Figure 5. Effect of farnesol and geranylgeraniol on inhibitory effect of lovastatin. Addition of farnesol (5 μM) but not geranylgeraniol (5 μM) reverses the inhibitory effect of lovastatin. FPT inhibitor (200 μM) inhibits amyloidogenesis. *, $P < 0.05$, compared with control. Error bars represent SEM. NS, Not significant.

production. Cells were cultured for 7 days in the presence of SAA (150 $\mu\text{g/ml}$) and AEF plus lovastatin at different concentrations. On visual inspection cells had a uniform morphology and assessment by trypan blue exclusion showed 80 - 85% of the cells maintained viability under all conditions tested. The number of cells was similar in all conditions tested (data not shown). We found that blocking the isoprenoid pathway by lovastatin resulted in a dose dependent reduction in the amount of amyloid produced, up to 53% in the highest concentration tested (Figure 4).

To substantiate that the effect of lovastatin was mediated through inhibition of the isoprenoid pathway, we tested whether addition of farnesol or geranylgeraniol would reverse the inhibitory effects of lovastatin. Farnesol and geranylgeraniol are hydrophobic molecules that enter cells freely and are intracellularly converted to farnesyl pyrophosphate and geranylgeranyl pyrophosphate in two monophosphorylation reactions (28) (Figure 1). Addition of farnesol at a concentration of 5 μM completely reversed the inhibitory effects of 5 μM lovastatin. In contrast, geranylgeraniol did not significantly reverse the inhibitory effect of lovastatin on amyloid formation (Figure 5). Farnesyl is transferred to proteins by the enzyme farnesyl protein

transferase (FPT). Inhibition of FPT resulted in a 36% decrease in amyloid production ($p=0.03$). To test if lovastatin influenced amyloidogenesis by inhibiting uptake or increased degradation in the medium of SAA by mononuclear cells, we incubated cells with 150 $\mu\text{g/ml}$ SAA with or without 5 μM lovastatin. There was no significant difference between

cells exposed to lovastatin and controls in the disappearance of SAA from the medium at 24 hours ($34.4 \pm 3.4\%$ vs $36.8 \pm 2.3\%$ $p=0.29$) or 48 hours ($56.9 \pm 4.2\%$ vs $50.5 \pm 3.0\%$ $p=0.14$). There were no degradation fragments visible on SDS-PAGE (data not shown).

Discussion

Employing an *in vitro* system with human monocyte-derived macrophages, we show that lovastatin is able to inhibit amyloidogenesis in a dose-dependent manner. This inhibition can be reversed by the addition of farnesol but not geranylgeraniol, which suggests that the inhibitory effect of lovastatin on amyloidogenesis is dependent on farnesyl-derived isoprenoids. Clinical amyloidosis is a complex step-wise process which depends primarily on the extent and duration of SAA elevation. Steps in the amyloidogenic mechanism include SAA internalization by macrophages, C-terminal cleavage of SAA to AA, intracellular initiation of fibril formation, deposition of fibrils in the extracellular space, and association of SAA/AA fibrils with glycosaminoglycans. Previous studies have provided evidence that monocyte-derived macrophages carry out the aforementioned steps (14,26,29). On this basis, we have chosen to use the cell culture model for testing our hypothesis regarding the potential involvement of isoprenoid compounds in amyloid development.

Farnesyl precedes geranylgeranyl in the isoprenoid pathway (Figure 1). Farnesol, but not geranylgeraniol, reversed the anti-amyloidogenic effect of lovastatin. Furthermore, inhibition of farnesyl protein transferase reduced amyloid formation. This suggests that one of the farnesylated proteins has a role in the amyloidogenesis in mononuclear cells. Farnesyl is a non-sterol isoprenoid that binds to a specific amino acid sequence, a process that is catalyzed by farnesyl protein transferase (30). These proteins, including Ras, Rho, and HDJ2, require this prenylation for their proper membrane localization and activity (31,32). Isoprenylated proteins are involved in many cellular functions including cell proliferation, chaperone function, and apoptosis.

The exact role of farnesylated proteins in amyloidogenesis remains unknown, but we can speculate on a possible mechanism through the regulation of intra-lysosomal enzyme expression. A critical step in amyloidogenesis is C-terminal cleavage of SAA which may occur in the lysosomes of macrophages. In patients who develop amyloidosis, impaired degradation of SAA leads to the accumulation of 76 amino acid fragments that can adopt a β -sheet structure typical of all the amyloid fibrils. The enzymes that are capable of degrading SAA belong to the cathepsin family of proteases. Cathepsin L, B, and D have been implicated in the pathogenesis of amyloidosis through degradation of SAA (33-37). *In vitro* studies demonstrated that cathepsin B can generate potentially deleterious intermediate degradation products found in amyloid fibrils (33,35). In contrast, cathepsin D protects against amyloidosis by degrading SAA in the N-terminal portion, thereby preventing the formation of amyloidogenic intermediates (36,38). Since the expression of cathepsins is regulated by the farnesylated protein Ras (39-43), decreasing farnesylation

could alter the expression of these proteases and result in reduced activity in lysosomes, creating a micro-environment favourable to AA protein formation.

The results from our experiments on AA amyloidosis agree with data from studies of another form of amyloidosis, i.e. Alzheimer's disease. The cerebral plaques of Alzheimer's disease are composed of amyloid fibrils formed from A β protein. There is evidence that hypercholesterolemia is an early risk factor for the development of the amyloidosis of Alzheimer's disease. Indeed, a significant association was shown between total plasma cholesterol level and presence of cerebral amyloid deposition (44). The role of the cholesterol biosynthesis pathway in the pathology of Alzheimer's disease is further supported by several epidemiological studies that suggest that lowering serum cholesterol by means of HMG CoA reductase inhibitors may retard onset of Alzheimer's disease (45-49). Recently, Gellermann and colleagues showed that lovastatin can reduce the formation of amyloid-like A β plaques by human macrophages. They found that at a lovastatin concentration of 4 μ M, A β amyloid plaques were reduced by 35% (50). Collectively, these data suggest a connection between the isoprenoid pathway and amyloid fibril production. The results presented here have two important implications. First, they point to the possibility that statins are an effective therapeutic option for the treatment and/or prevention of AA amyloidosis. Second, these results offer a possible explanation for the initial observation that HIDS patients are less vulnerable to amyloidosis than the other periodic fever syndromes (22).

In conclusion, blocking the isoprenoid pathway reduces the capacity of monocytes to produce amyloid. This could be a potential target for prevention in patients at risk for AA amyloidosis, and possibly also for treatment of patients with amyloidosis.

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AL amyloidosis enhances development of amyloid A amyloidosis

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Chapter 9

Sir, In the April issue of this journal, Rekhman *et al.* describe an interesting case of a patient with concomitant AL (also referred to as primary or myeloma-associated amyloidosis) and amyloid A (AA) amyloidosis.¹ The authors state that 'the pathogenesis of dual amyloidosis is not clear nor is the relationship of dual deposits with rapid progression'. However, our recent advances in the knowledge of the pathogenesis of amyloidosis may explain both enigmas.

The amyloidoses constitute a group of disorders that are characterized by deposition of protein fibrils in organs and tissues leading to organ dysfunction.² The fibrils are aggregates of a precursor protein that has a typical β -pleated-sheet conformation. So far at least 23 different precursor proteins have been identified that can aggregate into fibrils, including $A\beta$ peptide in Alzheimer plaques and $\beta 2$ microglobulin in haemodialysis-associated amyloidosis.³ Amyloidosis develops when the precursor protein is over-expressed (e.g. increased expression of acute phase protein serum amyloid A protein (SAA) during inflammation in AA amyloidosis) or when a mutation in a constitutively expressed protein leads to a greater tendency to aggregate (e.g. in familial ATTR amyloidosis). In type AA amyloidosis SAA has to be proteolytically cleaved into AA amyloid fragments before these can be incorporated into fibrils.

The kinetics of amyloidosis is characterized by a lag phase during which all the prerequisites are present but no fibrils are formed. Once a critical nucleus of amyloid is formed, the conditions change to favour aggregation with very fast kinetics.⁴ The lag phase can last from weeks to years. For example, in a mouse model for AA amyloidosis, amyloid fibrils are formed in the spleen in response to injection of an inflammatory stimulus after a lag phase of 3–4 weeks. It has been known for a long time that this lag period can be shortened dramatically to 3 days by the simultaneous injection of extracts of spleen from amyloidotic mice.⁵ The activity of this amyloid-enhancing factor (AEF) has been shown to depend on small molecules with a β sheet structure. It acts as a template for amyloid fibrillogenesis to begin, similar to a snowflake that starts growing from a speck of dirt.⁶ Not only AEF generated from AA fibrils can thus shorten the lag period, but other types of amyloid fibrils have also been shown to act as AEF in AA amyloidosis, including AL amyloid fibrils.^{7,8}

Therefore, we suggest the following cascade of events in this patient with dual amyloid deposits. First, plasma cell dyscrasia induced amyloid fibrils of the AL type. These fibrils of the AL type act as an AEF for AA amyloidogenesis, making the patient far more susceptible to any other type of amyloidosis. The systemic inflammation secondary to mucocutaneous bullous amyloidosis will have resulted in elevated serum concentration of SAA in this patient. Because of the presence of AL type amyloid fibrils acting as AEF, this rapidly resulted in AA amyloid deposition.

To test this hypothesis we used a well-defined cell culture model of AA amyloidosis in which isolated human monocytes are incubated with recombinant SAA (150 mg L^{-1}) for 7 days, with or without mouse spleen-derived AEF.^{9,10} Monocytes were isolated from a patient suffering from AL amyloidosis and four healthy volunteers. Amyloid fibrils were

detected by staining the cells with Congo red. As shown in Figure 1, after 7 days of incubation cells of healthy volunteers make amyloid fibrils only when they are simultaneously exposed to AEF. However, monocytes from the patient with AL amyloidosis showed extensive AA amyloid deposition even without co-incubation with AEF. This suggests that the previous exposure to AL amyloid fibrils acts as an AEF to enhance AA amyloid formation. In conclusion, AL amyloid fibrils may act as AEF for AA amyloidogenesis.

This could well explain the dual expression of AA and AL amyloidosis in the same patient and the rapid progressive course.

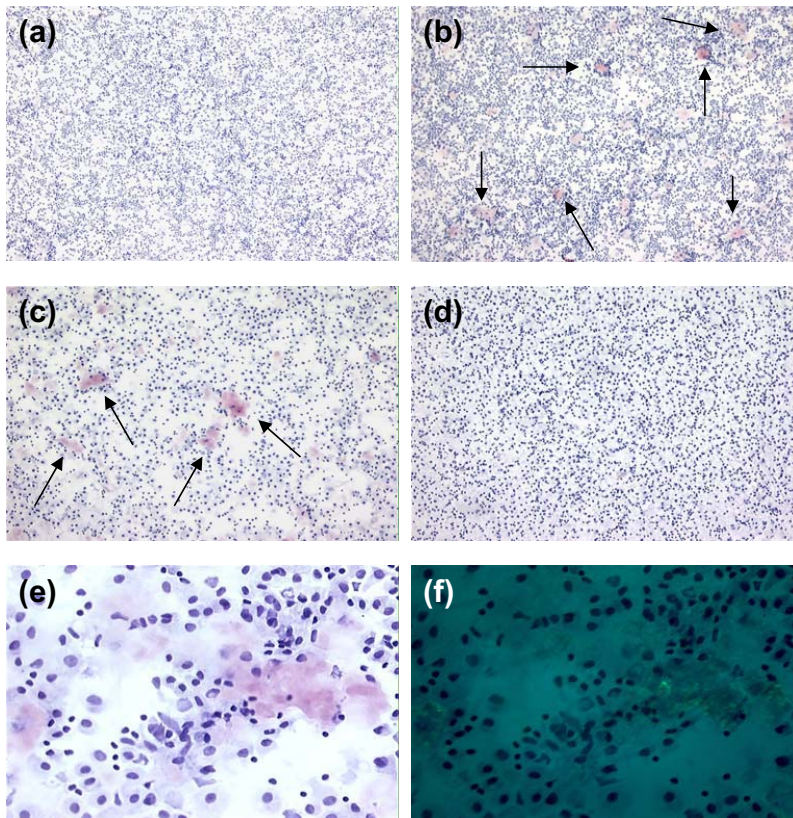


Fig 1. Congo red and haematoxylin staining of cultured monocytes. (a) and (b) Monocytes derived from healthy volunteer cultured for 7 days with 150 mg L^{-1} SAA (a) and AEF (b), showing amyloid (arrows) only when cells are simultaneously exposed to AEF. (c) and (d) Monocytes derived from a patient suffering from AL amyloidosis incubated only with SAA (c) showing extensive amyloid deposits (arrows). No amyloid is seen when cells are cultured in the absence of SAA (d). (e) Detailed image of an amyloid deposit from the AL patient showing typical apple-green birefringence under polarized light (f). Original magnification (a–d) $\times 10$, (e and f) $\times 40$. SAA, serum amyloid A protein; AEF, amyloid enhancing factor.

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Increased susceptibility of serum amyloid A 1.1 to degradation by MMP-1: potential explanation for higher risk of type AA amyloidosis

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Chapter 10

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Abstract

Objective Genetic polymorphisms in Serum Amyloid A (SAA) have been shown to substantially influence the risk of developing type AA amyloidosis. Recently, a role for matrix metalloproteinase-1 (MMP-1) has been suggested in the pathogenesis of AA amyloidosis. Therefore, we investigated if the SAA1 isotypes are differentially degraded by MMP-1.

Methods Degradation of different SAA isotypes by MMP-1 was assessed by immunoblotting. MALDI-TOF mass spectrometry was used to identify degradation fragments.

Results: We found that SAA1.5 is more resistant to degradation by MMP-1 than SAA1.1. This difference is caused by the capacity of MMP-1 to cleave at the site of the polymorphism at position 57.

Conclusion These results may explain the higher risk of amyloidosis in patients with a SAA1.1/1.1 genotype versus SAA1.5/1.5 or SAA1.1/1.5 genotype. In addition the impaired degradation of SAA1.5 by MMP-1 could also explain the higher serum SAA concentrations in persons with a SAA1.5 genotype.

Introduction

Type AA amyloidosis is a feared complication of chronic inflammatory conditions. It is caused by the deposition of insoluble fibrillar amyloid proteins in the extracellular spaces in a variety of organs and tissues (1;2). The AA protein that forms the amyloid fibril in type AA amyloidosis is mainly derived from degradation products of serum amyloid A type 1 (SAA1). SAA1 is a 12 kDa protein of 104 amino acids that is produced mainly in the liver after stimulation by various pro-inflammatory cytokines, and is considered to be part of the acute phase response (3). The amyloid fibrils found in patients with AA amyloidosis largely consist of the N-terminal 76 amino acids of SAA, although N-terminal fragments of different lengths have been reported (4-8).

Although a prolonged elevation of SAA1 is a prerequisite for the development of amyloidosis, only a small portion of patients with chronic inflammation will ever develop type AA-amyloidosis (9). Polymorphisms in the gene coding for SAA1 have been identified as a factor that influences the risk of developing amyloidosis. Two single nucleotide polymorphisms at exon 3 constitute 3 different isotypes: SAA 1.1 (Val⁵²-Ala⁵⁷), SAA 1.3 (Ala⁵²-Ala⁵⁷), and 1.5 (Ala⁵²-Val⁵⁷). Caucasian patients with a 1.1/1.1 genotype have a higher risk of developing amyloidosis compared to patients with a 1.5 genotype, but the reason for this is unknown. Recently, a role of matrix metalloproteinases (MMPs) was suggested in the pathogenesis of amyloidosis. MMPs are enzymes that modulate the extracellular matrix and are present in AA amyloid deposits (10); SAA1 induces production of MMPs by mononuclear phagocytes and synovial fibroblasts (11;12). Furthermore, MMP-1, -2, and -3 have been shown to degrade both SAA and AA fibrils in vitro (13). Interestingly, these MMPs degrade SAA1 preferentially in the region spanning amino acids 52-58. In this study, we investigated to what extent the degradation of SAA1 by MMP1 is dependent on the SAA1 isotype.

Material and Methods

Preparation of SAA1.1 and SAA1.5

A recombinant human SAA 1.1 expression system using pET21a plasmid and BL21 *E.coli* has already been established (14). SAA 1.5 cDNA was prepared from SAA1.1 by the polymerase chain reaction-mutagenesis method (15). SAA was purified by molecular sieve chromatography followed by chromatofocussing or hydrophobic interaction chromatography (14). Purity and identity were examined after purification, by amino-terminal sequencing.

Degradation of SAA by recombinant MMP-1.

Degradation experiments were performed with minor modification as described by Stix *et al.* (13). Briefly, 5 μ l of concentrated SAA (5 μ g/ μ l, dissolved in 4M of urea) was mixed with 25 μ l of reaction buffer (20 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L CaCl₂, and 1 mmol/L ZnCl₂, pH 7.6) and 5 μ l of purified human MMP-1 (Sigma-Aldrich, St Louis, Mo) at a final concentration of 0.35 μ M. The reaction was performed at 37°C and stopped after 2,

24, and 48 hours by addition of 5 μ l of 25 mM EDTA. Incubation without MMP-1 or in the presence of 3.57 mM EDTA served as negative control. The degradation experiments were performed as three independent experiments.

SDS PAGE and Western Blotting

After degradation the samples were subjected to discontinuous Tris-Tricine SDS polyacrylamide gel electrophoresis (16). Proteins were visualized by staining with Coomassie Brilliant blue. For Western blotting, proteins were transferred from unstained gels onto a nitrocellulose membrane. (pore-size: 0.22 μ m) and incubated with anti-SAA antibodies (clone 86.1 and clone 86.5, dilution 1:500) for 4 h at room temperature. Monoclonal antibodies against SAA1 were a kind gift of Johan Bijzet, University Medical Center Groningen, Groningen, the Netherlands. The membrane was rinsed and incubated with rat anti-mouse IgG antibodies conjugated with horseradish peroxidase. Immunostaining was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB). The stained gels were scanned with a densitometer (Umax, Uden the Netherlands). Images from the densitometer were analyzed using Totallab software (Nonlinear Dynamics, Newcastle, UK).

Mass spectrometry

Degradation fragments of SAA exposed to MMP-1 were characterized by matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS, Biflex III, Bruker, FRG) using the linear mode of operation. SAA was degraded as described above and 1 μ l aliquots were removed at 2, 24, and 48 h. Aliquots were mixed with 5 μ l of trifluoroacetic acid (TFA, 0.1% v/v) and 5 μ l of matrix solution (10 mg of sinapic acid in 1 ml of a 50:50 mixture of acetonitrile and 0.1% TFA, (v/v)). A volume of 1 μ l from this mixture was spotted on the target plate. Spectra were acquired in the linear mode as described (17). The BioTools 2.0 software (Bruker Daltonics, Germany) was used for the annotation of the SAA fragments; the accepted mass tolerance was 100 ppm.

Statistical analysis

Data were analyzed by the unpaired Student's *t*-test using SPSS 14.0 for windows.

Results

The first experiment was performed in order to assess the difference in the rate of degradation of SAA1.1 and SAA1.5 by MMP-1. Purified human recombinant SAA1.1 and SAA1.5 were incubated with $0.35\mu\text{M}$ of MMP-1 for 24 h and 48 h and subsequently subjected to SDS-PAGE electrophoresis. Quantitative analysis showed that both isotypes were susceptible to degradation, but SAA1.1 was degraded to a greater extent (figure 1). At 48h MMP-1 degraded SAA1.1 to 48.9% of the total amount added, while full-length SAA1.5 was only reduced to 70.8% ($p<0.05$). In addition, there was a different pattern of degradation. SAA1.1 coincubation with MMP-1, results in appearance of two degradation products of ~ 5 kDa and ~ 6.5 kDa. These fragments do not appear after treatment of SAA1.5 (figure 1b). Immunoblot analysis showed that the two bands reacted with anti-SAA antibody. In addition, immunoblot analysis, which has a higher sensitivity than SDS-PAGE, shows three faint bands in the degradation of SAA1.5 at approximately 9 kDa, 8 kDa, and 5 kDa (figure 1c).

Next, we used MALDI-TOF mass spectrometry to further investigate the degradation profile of SAA1.1 and SAA1.5 (figure 2). We were able to identify the two degradation products of ~ 6.5 kDa as SAA1.1 fragment 1-57 and ~ 5 kDa as 58-104 indicating MMP 1 cleaves SAA1.1 between residue Ala⁵⁷-Ile⁵⁸. These fragments most likely correspond to the 6,5 kDa and 5 kDa bands identified by Coomassie staining and immunoblotting.

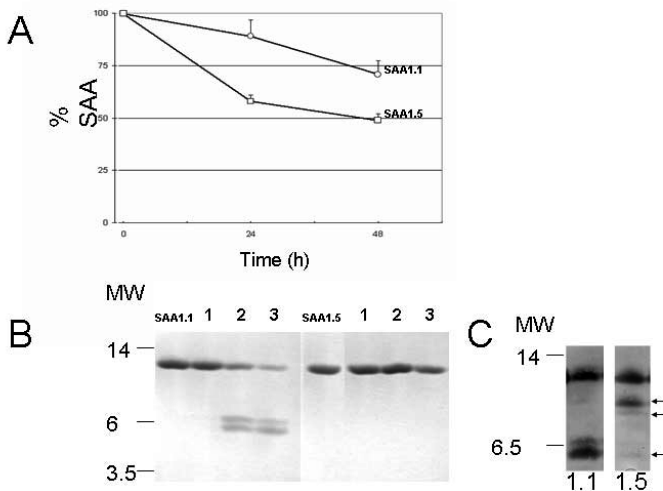


Figure 1. A. Degradation of SAA1.1 and SAA1.5 by MMP-1. Degradation was assessed by SDS-PAGE and densitometry as indicated in material and methods. Each point represents the mean value \pm SEM of three independent experiments. **B.** SDS-PAGE of SAA1.1 and SAA1.5 degradation by MMP-1. after 2 h (lane 1), 24 h (lane 2), and 48h (lane 3) incubation with $0.35\mu\text{M}$ MMP-1 reveals appearance of two bands in SAA1.1. Incubation without MMP-1 served as control (lanes SAA 1.1 and SAA1.5). Western blot analysis of the degradation after 24h (**C**) reveals three additional bands during degradation of SAA1.5 (arrows).

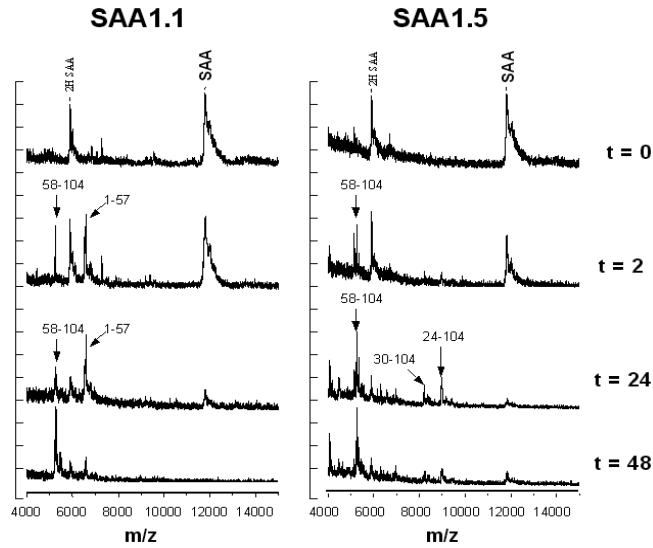


Figure 2 MALDI-TOF MS analysis of the degradation products from SAA1.1 and SAA1.5. The degradation with 0.35 μ M of MMP-1 was stopped with EDTA at 0 h, 2 h, 24 h, and 48 h. Different profile were observed for SAA1.1 and SAA1.5. At t=0 single (11,815 Da) and double (5,911 Da) charged peaks representing the SAA protein are indicated.

In the degradation of SAA1.5, we could identify 3 fragments: 58-104, 30-104, and 24-104. These fragments most likely correspond with the three faint bands of 5 kDa (58-104) , 8 kDa (30-104), and 9 kDa (24-104) identified by immunoblot analysis (figure 1c).

Discussion

This study shows that SAA1.5 is largely resistant to degradation by MMP-1, contrary to SAA1.1. This difference is determined by the capacity of MMP-1 to cleave at residues 57 and 58 of the protein, which are either Val⁵⁷-Ile⁵⁸ (SAA1.5) or Ala⁵⁷-Ile⁵⁸ (SAA1.1). This results in the emergence of different degradation products (Figures 1,2).

The difference in capacity of MMP-1 to degrade the two isoforms of SAA1 could explain the differential risk of developing amyloidosis. Based on sequencing of AA amyloid fibril proteins, SAA1.1 was proposed as a risk factor for AA amyloidosis (18). Numerous studies confirmed that patients with an SAA1.1/1.1 genotype have a strongly increased risk of developing amyloidosis not only in patients with rheumatoid arthritis, but also in patients with familial Mediterranean fever, juvenile chronic arthritis, and Behçet's disease (19-26). Caucasian patients with a 1.1/1.1 genotype have a three-to seven fold increased risk of amyloidosis compared to other genotypes (20;22;26;27). The SAA1.3 isotype, found to have a 4.5-fold increased risk of amyloidosis in the Japanese population (10;29), has an alanine at position 57 similar to SAA1.1, the increased risk of amyloidosis in patients with SAA1.3 could be caused by the same mechanism as in SAA1.1.

Our data suggest a putative role of MMP-1 in the pathogenesis of amyloidosis. This is further supported by the observation that MMPs are found in close proximity of amyloid fibers, specifically intracellularly in cells surrounding amyloid deposits (10). Furthermore, MMP-1 has been shown to degrade SAA *in vitro* (13).

We failed to detect the 30-104 and 24-104 degradation fragments of SAA 1.1 by MALDI-TOF (Figure 2). We hypothesise that this is caused by further degradation of these fragments as SAA1.1 is readily degraded at ala⁵⁷-ile⁵⁸, while the val⁵⁷-ile⁵⁸ of SAA1.5 is largely resistant to degradation. Furthermore, MALDI-TOF mass spectrometry is only a semi-quantitative analysis measuring relative abundance of various fragments in a sample. The much higher concentrations of 1-57 and 58-104 in the degradation of SAA1.1 could further limit the detection of small amounts of the 30-104 and 24-104 fragments. Figure 3 shows a schematic representation of SAA1.1 and SAA1.5 degradation.

Since amyloid fibrils are composed of N-terminal part of SAA, it is tempting to speculate that the 1-57 fragments that are produced in large quantities by MMP1 from SAA1.1, are directly incorporated into amyloid fibrils. Fragments of different length have been found in amyloid fibrils. Next to the AA protein that is composed of the first 76 amino acids of SAA, fragments of 5 kDa to 12 kDa have been found (4-8). However, to our knowledge, fragments that end at position 57 as part of AA amyloid deposits have not been described in the literature.

An alternative explanation for the increased risk of SAA1.1 genotype could be that the 1-57 fragments act as a source of Amyloid Enhancing Factor (AEF). Amyloidosis is considered a two-step process (1). First, a critical mass of monomers that have a β -sheet conformation has to be formed that can act as a nucleus (lag phase). Once the nucleus is formed there is rapid extension of fibrils. In mice, the lag phase, which takes 2-3 weeks, can be dramatically

shortened to 24-48 hours by administration of AEF (28-31). AEF are small molecules with β sheet propensity that can act as a nucleus for the generation and growth of AA amyloid fibrils similar to the action and propagation of prions (32-35). Liu *et al* (36) showed that synthetically produced N-terminal fragments of SAA have strong AEF activity, which was also shown by others (37). Fragment 1-57 can be expected to conform to a β sheet configuration (38), and thus act as AEF. Therefore, fragments resulting from MMP-1 mediated degradation of SAA1.1 could be more amyloidogenic than fragments from SAA1.5.

Interestingly, Migita *et al* found circulating fragments derived from SAA at much higher concentrations in rheumatoid arthritis patients with amyloidosis compared to rheumatoid arthritis patients without amyloidosis (39). While both groups had similar serum SAA concentrations, in the amyloidosis patients there was an considerable amount of SAA fragments that have a molecular weight of ~6 kDa, similar to the 1-57 fragment we found. These fragments may be produced by SAA degradation by MMPs.

In addition, our results may explain the earlier observations that rheumatoid arthritis patients with an SAA 1.5 genotype have higher serum SAA concentrations relative to CRP than patients with SAA 1.1 genotype (40). Also, in healthy subject basal serum SAA concentrations are significantly higher in persons that carry a SAA 1.5 allele, and is the highest in persons homozygote for SAA 1.5 (41). Furthermore, when injected in mice, SAA1.1 and SAA1.3 are more rapidly cleared from the circulation than SAA1.5 (15). These observations combined with our data might fit with a role for MMP-1 in the clearance of SAA from the circulation.

In conclusion, SAA1.1 is more susceptible to *in vitro* degradation by MMP-1 than SAA1.5, resulting in higher production of the 1-57 fragment from SAA1.1. This may explain the higher risk of AA amyloidosis in patients with a SAA1.1 genotype, and the higher serum SAA concentration in persons with an SAA1.5 genotype.

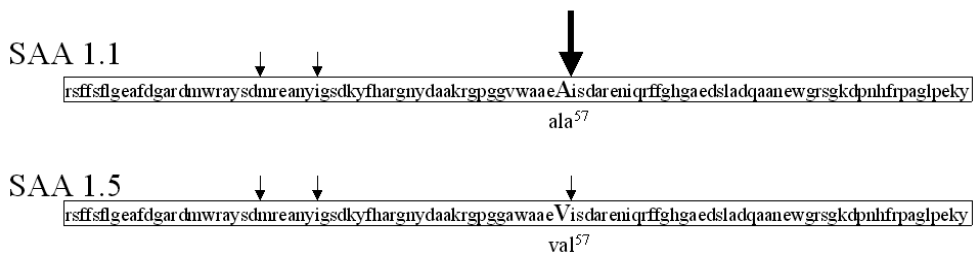


Figure 3 Schematic representation of SAA1.1 and SAA1.5 degradation by MMP-1. SAA1.1 is readily degraded at Val⁵⁷-Ile⁵⁸, while SAA1.5 is largely resistant to degradation at this position. There is also minor degradation at Asp²³-Met²⁴ and Tyr²⁹-Ile³⁰.

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Cathepsin D activity protects against development of AA amyloidosis

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Chapter 11

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Abstract

Background The extracellular, fibrillar deposits of reactive (secondary) amyloidosis are composed of amyloid A (AA) protein, a proteolytically-derived fragment of the acute phase protein serum amyloid A (SAA). While complete degradation of SAA precludes amyloid formation, limited cleavage which generates AA protein is considered part of the pathogenic mechanism.

Materials and methods In this study we investigated SAA degradation by lysosomal enzymes cathepsins B, D, and K and assessed the impact of cathepsin activity on AA amyloid formation in a cell culture model.

Results Lysates of human mononuclear cells were capable of degrading SAA. Degradation was significantly reduced by inhibition of cathepsin D with pepstatin A. Inhibition of cathepsin B or cathepsin K, however, had no effect. The SAA fragment pattern generated by mononuclear cell lysates was similar to that produced by incubating SAA with purified human cathepsin D. Consistent with *in vitro* findings, amyloid formation in human monocyte cultures was increased by 43% when cathepsin D was inhibited, but was unaffected by inhibition of cathepsin B or cathepsin K.

Conclusion These data provide evidence that cathepsin D but not cathepsin B or cathepsin K is physiologically important in SAA degradation and hence in preventing SAA from accumulating and serving as precursor of AA amyloid fibrils.

Introduction

Reactive (AA) amyloidosis is a protein deposition disease generally associated with chronic inflammatory conditions including rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, and hereditary periodic fever syndromes. The extracellular, insoluble fibrils which accumulate in this type of amyloidosis are composed of amyloid A protein, a fragment proteolytically derived from the acute phase protein serum amyloid A (SAA). While human SAA comprises 104 amino acids, the protein isolated from AA amyloid fibrils generally contains the N-terminal 76 residues, although fragments exhibiting varying degrees of C-terminal proteolysis have been described (1;2). SAA is largely metabolized in cells of monocytic origin. After uptake, SAA traffics from early to late endosomes and then to the lysosomes where it is degraded (3-9). Under normal conditions, all of the SAA is completely degraded. In patients who develop amyloidosis, however, some SAA escapes catabolism, refolds into β -sheet structure presumably in the acidic environment of the lysosome and initiates polymerization into amyloid fibrils. The exact site and sequence of events involved in amyloidogenesis, including limited cleavage of SAA to AA protein, have yet to be elucidated.

Many lysosomal enzymes, namely cathepsins, have been implicated in the degradation of SAA. Cathepsin B has been shown to cleave SAA *in vitro* with generation of an N-terminal fragment terminating at residue 76 analogous to the most common AA peptide, suggesting a role in amyloid pathogenesis (1;10). In contrast, cathepsin D could be protective by cleavage of SAA at the C-terminus (11;12). Cathepsin K completely degrades SAA *in vitro*, and expression of this protease has been detected adjacent to amyloid deposits (9;13). Although purified cathepsin B, K, and D have been shown to degrade SAA *in vitro*, the significance of their activities in human amyloidosis is hitherto unknown. The aim of this study was to assess the impact of cathepsin B, cathepsin D, and cathepsin K in *ex vivo* models of human amyloidosis.

Materials and Methods

Preparation of cell lysates

Peripheral blood mononuclear cells from healthy volunteers (PBMC) were isolated from whole blood by Ficoll-Hypac (Pharmacia, Uppsala, Sweden) gradient. PBMCs were resuspended in RPMI supplemented with 15% fetal calf serum, in a 6 well plate (5×10^6 PBMCs in 2 ml medium per well) and incubated at 37°C. After 2 hours wells were gently rinsed twice with warm PBS to select for adherent mononuclear cells. These cells were lysed in 1ml of 10 mM sodium phosphate, 1mM EDTA, 0.2% Triton X-100 buffer and centrifuged at 14000 rpm for 30 minutes. The supernatants were stored at -20°C until use.

Preparation of Serum Amyloid A and Amyloid Enhancing Factor (AEF)

Recombinant SAA 1.1 and AEF were produced as described previously (3). Purified SAA was dissolved in 4M urea at a concentration of 7,5 $\mu\text{g}/\mu\text{l}$.

Assay for SAA degradation

All reaction mixtures contained 25 μ l of cell lysate, 25 μ l of 50 mM sodium acetate, pH 4.0, and 5 μ l of purified SAA from the concentrated stock. Each reaction also contained an inhibitor or PBS (2 μ l): cathepsin B inhibitor II (10 μ M) (Calbiochem, Breda, Netherlands), cathepsin K inhibitor III (10 μ M) (Calbiochem), or pepstatin A (5 μ M) (Sigma-Aldrich, St. Louis, MO). After 24 and 48 hours of incubation at 37°C, aliquots were taken and stored at -20°C until further analysis.

Tris-Tricine SDS-PAGE

Samples were analyzed by Tris-Tricine SDS-PAGE according to Schagger and von Jagow (14). Proteins were visualized by staining with Coomassie blue, and degradation was assessed by densitometry. The blots were scanned with a densitometer (Umax, Uden, The Netherlands) and images were analyzed using Totallab software (nonlinear dynamics, Newcastle, UK).

Ex vivo amyloid induction

Amyloidogenesis was initiated using a cell model as previously described (15;16). Briefly, PBMCs from healthy human donors were isolated on Ficoll-Hypaque gradients and cultured in 96-well plates. Wells were rinsed after 4 hours to select for adherent mononuclear cells. Cells were maintained in 100 μ l RPMI supplemented with 15 % fetal calf serum, 1mM pyruvate, and 1% gentamicin at 37 °C in an atmosphere with 5% CO₂. Amyloid production as described previously was initiated by the addition of 2 μ l SAA and 2 μ l AEF leading to a final concentration of 150 μ g/ml and 12 μ g/ml. The medium was supplemented with cathepsin B inhibitor II (10 μ M), pepstatin A (5 μ M), or cathepsin K inhibitor III (10 μ M). Medium and supplements were replaced every other day. After 7 days, cells and amyloid material were collected and spun down on glass slides using a cytospin centrifuge for 10 minutes at 500 rpm. Cells were fixed in 10% formaldehyde and stained for 45 minutes in Congo red prepared in 80% alkaline ethanol. After a quick dip in water, cells were counterstained with Gill's hematoxylin solution (Sigma-Aldrich). Slides were dipped in acidified 70% ethanol, several times in water, and once in ammonium solution. After dehydration in alcohol, slides were cleared in xylene and coverslips were applied with Permount.

Amyloid quantification

The presence of amyloid fibrils was confirmed by visualisation of apple-green birefringence of Congo-red material on polarized light microscopy. The amount of amyloid was determined with a digital image analysis protocol that measures that surface covered with Congo-red material as previously described (16).

Statistical analysis.

Statistical analyses were performed with SPSS 14.02 for Windows. Data were compared

with unpaired Student's *t* test. Results are presented as means \pm SEM of three independent experiments. A *p*-value <0.05 was considered to be significant.

Results

Cathepsin D mediates degradation of SAA by lysates of human mononuclear cells.

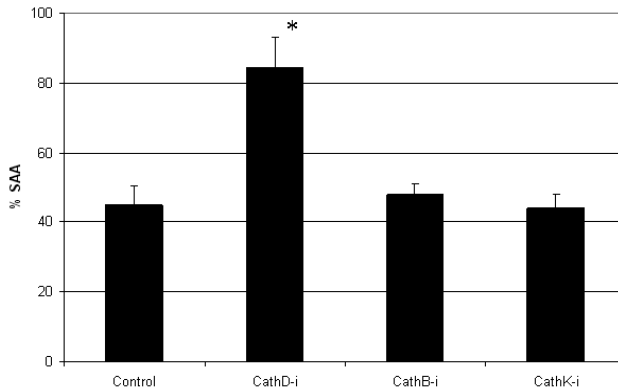


Figure 1 Degradation of SAA by lysate of monocytic cell under acidic conditions. Degradation is significantly reduced by inhibition of Cathepsin D with 5 μ M pepstatin. Error bars represent SEM. * $p < 0.05$.

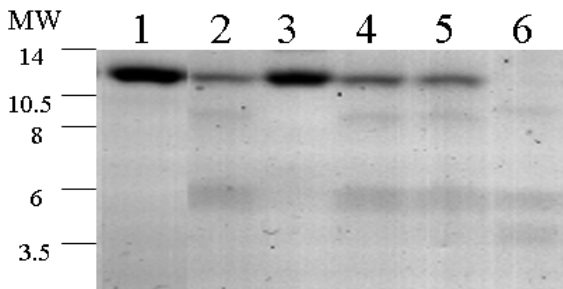


Figure 2 SDS-PAGE analysis of SAA degradation by monocytic cell lysates under acidic conditions. After 24 hours SAA is partially degraded with the appearance of 2 fragments of 6 and 9 kD (lane 2). Inhibition of cathepsin B (lane 4) or cathepsin K (lane 5) did not influence degradation. Inhibition of cathepsin D (lane 3) significantly decreased degradation. SAA without cell lysate was used as control (lane 1). Degradation was nearly complete after incubation with purified human cathepsin D (1 U/ml); fragments of ~9, 6, and 3.5 kD were generated (lane 6).

cathepsin D (1U/ml) for 24 hours. This resulted in nearly complete degradation of SAA (Fig. 2, lane 6). Faint, but distinct fragments of 9 kD and 6 kD were observed in addition to a fragment of approximately 3.5 kD (Fig. 2, lane 6).

In the first experiment we investigated the contribution of Cathepsin B, D, and -K in the degradation of SAA by human monocytes. SAA was exposed to lysates of human mononuclear cells in an acidic environment (pH 4.0) for 24h, which results in the degradation of $55 \pm 5.2\%$ of SAA (Figure 1). Significantly less degradation was seen in samples which also contained the cathepsin D inhibitor pepstatin A ($15.5 \pm 8.5\%$ vs. $55\% \pm 5.2\%$, $p = 0.02$) (Fig. 1; Fig. 2, lane 3). Co-incubation with selective inhibitors of cathepsin B or cathepsin K had no effect (Fig. 1; Fig. 2, lanes 4 and 5).

SDS-PAGE analysis of SAA degradation by cell lysates revealed two fragments of approximately 9 kD and 6 kD (Fig.2, lane 2). Lysates which had been supplemented with pepstatin A to inhibit cathepsin D lacked these two fragments. To prove that the fragments were produced by cathepsin D present in the lysate, SAA was incubated with purified human

Inhibition of cathepsin D increases amyloid production in a human cell culture model.

Next, we evaluated the effect of specific cathepsin inhibitors on amyloid formation in a human cell culture system. Inhibitors were added to cell culture medium at concentrations known to completely block enzyme activity but without cytotoxic effects. After 7 days of exposure, cell viability was unaffected by inhibitors as assessed by visual examination and trypan blue exclusion (> 85% viable cells, data not shown). Inhibition of cathepsin B or cathepsin K did not influence the amount of amyloid produced by the mononuclear cells (Fig. 3). In contrast, cell cultures maintained in 5 μ M pepstatin A to inhibit of cathepsin D contained $43 \pm 8.6\%$ more amyloid than control cultures ($p = 0.01$).

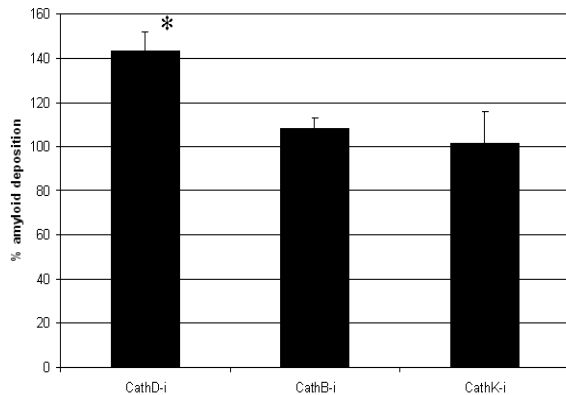


Figure 3 Effect of inhibition of cathepsins B, D, and K on amyloidogenesis. Mononuclear cells were incubated with SAA (150 μ g/ml) and AEF, with or without cathepsin inhibitors for 7 days. Cells were then fixed and stained with Congo red for quantification of amyloid. Amount of amyloid produced in absence of inhibitors was defined as 100%. Inhibition of cathepsin D gave $43\% \pm 8.6$ increase in amyloid production. Error bars represent SEM. * $p < 0.05$.

Discussion

This study demonstrates a key role for cathepsin D in SAA catabolism, and, furthermore, illustrates the importance of this enzyme in prevention of AA amyloidosis. Data showing a correlation between cathepsin D inhibition, decreased SAA degradation (Figs. 1 and 2), and increased AA amyloid formation (Fig. 3) are consistent with cathepsin D playing a critical role in decommissioning SAA as a fibril subunit precursor. This important activity was attributed specifically to cathepsin D as opposed to cathepsin B or cathepsin K, as inhibition of the latter two proteases had no effect on SAA degradation or amyloid formation by mononuclear cell lysates and cell cultures, respectively.

An important role for Cathepsin D was previously suggested by both *in vitro* and *in vivo* (mouse) studies. First, Yamada and colleagues identified sites in the amino-terminal region of SAA susceptible to cathepsin D cleavage (11). Second, the same group showed a reduced serum clearance of SAA in mice treated with the cathepsin D inhibitor pepstatin A (12). In addition, mice treated with pepstatin A while undergoing casein-induced amyloid induction exhibited amyloid deposition earlier than mice which had not received the

inhibitor (12). Consistent with this findings, mice of the CD1 strain were found to be partially resistant to amyloid induction relative to other mouse strains and also to express relatively high levels of cathepsin D (17).

A likely explanation for these observations is that Cathepsin D is important in the normal, complete degradation of SAA. There is strong evidence that the amyloidogenic potential of SAA lies within the first 10-15 amino acids of the molecule (18-21). Cleavage of SAA by Cathepsin D in the N-terminal region therefore would prevent the formation of N-terminally intact SAA peptides found in amyloid fibrils. Under conditions of reduced Cathepsin D activity, other enzymes may have opportunity to degrade SAA at the C-terminus. It is also possible that C-terminally truncated fragments have propensity to adopt a β -sheet conformation and when a critical amount of C-terminally cleaved peptides have acquired β -sheet structure, fibrillation can occur.

One candidate enzymes that has been proposed to be involved in the c-terminal cleavage of SAA is Cathepsin B. Cathepsin B is abundant in monocytic cells (22) and *in vitro* studies have shown that Cathepsin B can degrade SAA at residue 76-77 forming intermediate fragments that are found in amyloid deposits (1;10). Furthermore, Cathepsin B was shown to be spatially associated with amyloid deposits in tissue sections of affected human organs (17).

However, our results do not support a role for cathepsin B in pathogenesis of human amyloidosis. Based on inhibition studies, we attribute very little of the SAA degradation carried out by human monocytes to cathepsin B (Figs. 1 and 2). In addition, inhibition of Cathepsin B in a human cell culture system did not influence the degree of amyloid formation (Fig. 3). Our findings are consistent with those of Röcken and colleagues who found no difference in the turnover of SAA in cathepsin B knock-out mice compared to wild-type mice. Moreover, they noted no difference in the amount of amyloid deposition between the group of mice (9).

The cathepsin-K knockout mouse shows a greatly increased amount of AA amyloid deposited after silver nitrate injection compared to its wild-type littermate (9). However, cleavage of SAA by cathepsin K does not give rise to amyloid fragments (13). Furthermore, inhibition of cathepsin K did not affect the amount of amyloid deposition in our cell culture model. It is likely that the influence of cathepsin K is at the level of proteolysis of already deposited amyloid fibrils. Cathepsin K has been shown to be expressed in multinucleated cells adjacent to amyloid deposits and to be able to degrade AA amyloid deposits (13). This would not be detected in our *ex vivo* culture model.

In conclusion, Cathepsin D is a major enzyme in the normal, complete degradation of SAA and thereby plays a crucial role in preventing AA fibril formation and deposition.

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Summary and future perspectives

This thesis consists of two parts that are closely related. HIDS is one of the auto-inflammatory diseases, a group of disorders characterized by recurrent febrile attacks of non-infectious origin. The subjects of the first part of this thesis are the pathogenetic mechanisms, clinical manifestations, genetic testing, and therapeutic options of HIDS. The main complication in the auto-inflammatory diseases is reactive, or type AA, amyloidosis. However, in contrast to the other auto-inflammatory diseases AA amyloidosis is rare in HIDS. This observation served as the basis of further studies to gain more insight in the pathogenesis of AA amyloidosis, as presented in the second part.

First part

In the auto-inflammatory syndromes, the febrile attacks are accompanied by an array of inflammatory symptoms. The clinical characteristics of each of these syndromes are described in detail in **chapter one**. Recent developments have unravelled parts of the pathogenetic mechanisms that lead to inflammation. Despite the discovery of incriminating genes, clinical assessment remains the cornerstone of diagnosis. With detailed medical and family history and observation of an attack, most autoinflammatory syndromes can be distinguished.

In **chapter 2** we investigated the clinical symptoms, follow-up, and quality of life of 103 patients with genetically proven HIDS. It shows that the most frequent symptoms that accompanied attacks of fever are lymphadenopathy (87.3%), abdominal pain (85.3%), arthralgia (83.3%), vomiting (71.6%), diarrhoea (72.3%), skin lesions (68.8%), and aphthous ulcers (52%). Amyloidosis is a severe, but infrequent complication (2.9%). There is a decrease in frequency of attacks during life. However, after the age of 20, half the patients continue to have more than six attacks per year. HIDS impairs several aspects of the quality of life. Social functioning, physical role functioning, general health perception, and vitality are scored significantly lower than in controls. Since HIDS usually starts during early childhood, we also investigated the impact on social development and development of autonomy. Both are impaired in HIDS patients. In addition, HIDS negatively influenced educational achievements and employment status.

Although the causative mutation in HIDS has been described several years ago, the mechanisms that lead to inflammation remain unknown. In **chapter 3** we show that lymphocytes from HIDS patients show a defective apoptosis. We assume that this defect in apoptosis leads to an unbridled inflammatory response after a minor stimulus, as an explanation for the inflammatory phenotype of HIDS.

The discovery that HIDS is caused by mutations in the mevalonate kinase gene (*MVK*) allowed genetic testing as a new diagnostic tool. However, genetic testing is not universally available and it is a time-consuming and expensive procedure. In **chapter 4** we describe an algorithm based on clinical features that can exclude HIDS without the use of genetic testing. This decision rule was generated in a French cohort of 149 patients that were tested for HIDS and validated in a Dutch cohort of 93 patients. With the use of the

decision rule, at least 20% of tests could have been prevented without missing a single patient.

Two new therapeutic options were tested in the study described in **chapter 5**. First we developed a clinical score based on 12 symptoms to score the severity of an attacks. Then we showed that in a single patient anakinra was more effective than etanercept in preventing an attack provoked by immunization.

Second part

A severe complication of all chronic inflammatory conditions, including the auto-inflammatory syndromes, is type AA amyloidosis. In the second part of this thesis, various aspects of amyloidogenesis are investigated, which are schematically represented in the figure.

AA amyloidosis is caused by the deposition of N-terminal fragments of SAA. SAA is an acute phase protein that is produced in the liver in response to pro-inflammatory cytokines¹. It is rapidly taken up by mononuclear phagocytes. Under normal conditions, SAA is transported through the endosomal system to the lysosomes where it is completely degraded, presumably by cathepsin family of protease²⁻³. In patients with AA amyloidosis there is an impaired degradation of SAA giving rise to N-terminal fragments⁹. Fibrils mainly consist of the N-terminal 76 amino acids of SAA, but fragments of different length have been described¹⁰⁻¹⁴. In the acidic environment of the lysosomes, these fragments can adopt to a β -sheet configuration and aggregate into fibrils. After deposition of these accumulated intermediates in the extracellular space, several glycosaminoglycans, serum amyloid P, and lipid components bind to the fibril, and confer resistance to proteolysis¹⁵⁻¹⁷. A prolonged elevation of SAA is a prerequisite for the development of amyloidosis. However, only a portion of patients with continued elevated SAA eventually develop amyloidosis. A risk factor that has been identified to influence the risk of amyloidosis are polymorphisms in the SAA gene. Two single nucleotide polymorphisms at exon 3 give rise to 3 different isotypes: SAA 1.1 (Val⁵²-Ala⁵⁷), SAA 1.3 (Ala⁵²-Ala⁵⁷), and 1.5 (Ala⁵²-Val⁵⁷). Patients with a 1.1/1.1 genotype have a significantly higher risk of developing amyloidosis than patients with a 1.5/1.5 genotype.

In **chapter 6** the aspects of AA amyloidosis in autoinflammatory syndromes are reviewed. Type AA amyloidosis is a frequent complication of most hereditary periodic fever syndromes. It was found in up to 60% of FMF patients before the advent of colchicine treatment¹⁸, and it has been reported in 25% of TRAPS patients, and in up to 35% of patients with MWS¹⁹. However, in HIDS only 2.9% of patients developed amyloidosis, as described in chapter 2.

In **chapter 7** possible explanations for the low incidence of amyloidosis in HIDS patients were studied. HIDS patients did have a strongly elevated SAA during attacks. Furthermore, 45% also had elevated SAA concentrations during attack-free periods. Also, there was a normal distribution of SAA genotypes in HIDS patients (no increase in prevalence of the

so-called protective SAA genotype). So all the prerequisites for developing amyloidosis are present in HIDS, and this did not answer the question of why the incidence of AA amyloidosis in HIDS is so low.

HIDS is caused by a mutation in the gene coding for mevalonate kinase, a central enzyme to the isoprenoid metabolism²⁰. Therefore we hypothesized that the isoprenoid metabolism could influence amyloidogenesis²¹.

To further investigate the role of the isoprenoid metabolism in amyloidogenesis, a cell culture system with human monocytes was used in **chapter 8**. Inhibition of the isoprenoid pathway by lovastatin resulted in a dose dependent reduction of amyloid formed. The inhibitory effect of lovastatin is reversible by addition of farnesol, but not geranylgeraniol. Farnesyl transferase inhibition also inhibited amyloidogenesis, implicating a role for farnesylated proteins in the pathogenesis of AA amyloidosis.

In the next chapter (**chapter 9**) a possible explanation for observations of dual amyloid deposition in a single patient (both AL and AA type amyloidosis) is examined. Isolated monocytes from a patient with AL amyloidosis form amyloid fibrils when exposed to SAA, without the addition of amyloid enhancing factor(AEF). In monocytes from healthy controls amyloidosis could only be induced by the addition of SAA and AEF. This suggests that previous exposure of monocytes to AL amyloid fibrils acts as an AEF to enhance AA amyloidogenesis.

Serum Amyloid A (SAA) polymorphisms modulate the risk of developing type AA amyloidosis. Also, a role for matrix metalloproteinase-1 (MMP-1) in the pathogenesis of AA amyloidosis has been suggested²². In **chapter 10** the degradation of different SAA1 isotypes by MMP-1 is examined. SAA1.5 is more resistant to degradation by MMP-1 than SAA1.1. This difference is caused by the capacity of MMP-1 to cleave at the site of the polymorphism. These results could also explain the higher serum SAA concentrations in persons with an SAA1.5 genotype. Furthermore, MMP-1 derived fragments may act as AEF. This may explain the higher risk of amyloidosis in patients with a SAA1.1/1.1 genotype versus SAA1.5/1.5.

Cathepsin B, cathepsin D, and cathepsin K have been implicated in the pathogenesis of amyloidosis. In **chapter 11** the biological significance of three enzymes is studied. Only cathepsin D contributed significantly to the degradation of SAA by human monocytes. Furthermore, in the human cell culture system of amyloidosis, inhibiting cathepsin D resulted in an increased deposition amyloid fibrils. Inhibition of cathepsin B, and -K did not influence amyloidogenesis. This provides evidence that cathepsin D plays a central role in the pathogenesis of AA amyloidosis.

Future perspectives

To investigate the features of a disease that is as infrequent as HIDS requires an international collaboration of treating physician. This insight resulted in the establishment of the international HIDS study group and the international HIDS registry in 1994 (see website www.hids.net). Using the international HIDS registry we were able to acquire data of a substantial number of patients. It is our intention to continue with registry and with the international collaboration in order to gain further insight in the clinical characteristics and pathogenetic mechanisms of HIDS. The broad range of clinical symptoms, the frequency of attacks, and the clear impairment of the quality of life stresses the importance of effective therapies. Although we found good results with biological agents, not they are not a panacea for all patients This necessitates the continued search for better treatments. Although HIDS patients are able develop the complication of AA amyloidosis, the prevalence is remarkably low. This may provide clues to the mechanisms of amyloidogenesis. How the isoprenoid pathway influences amyloid formation merits further investigation. A next step could be testing a HMG-CoA reductase inhibitor in the mouse model of AA amyloidosis.

Although the studies presented in this thesis shed further light on the mechanisms of amyloid formation, the exact mechanisms of the 'black box of amyloidogenesis' remain to be elucidated. Figure 1 shows a schematic overview of the mechanisms involved in amyloidogenesis. A key question in this process is identifying the enzymes that cleave SAA into the fragments that are found in the deposits. A better understanding of the process of amyloid formation could direct the search for therapy for this grave complication of inflammatory diseases.

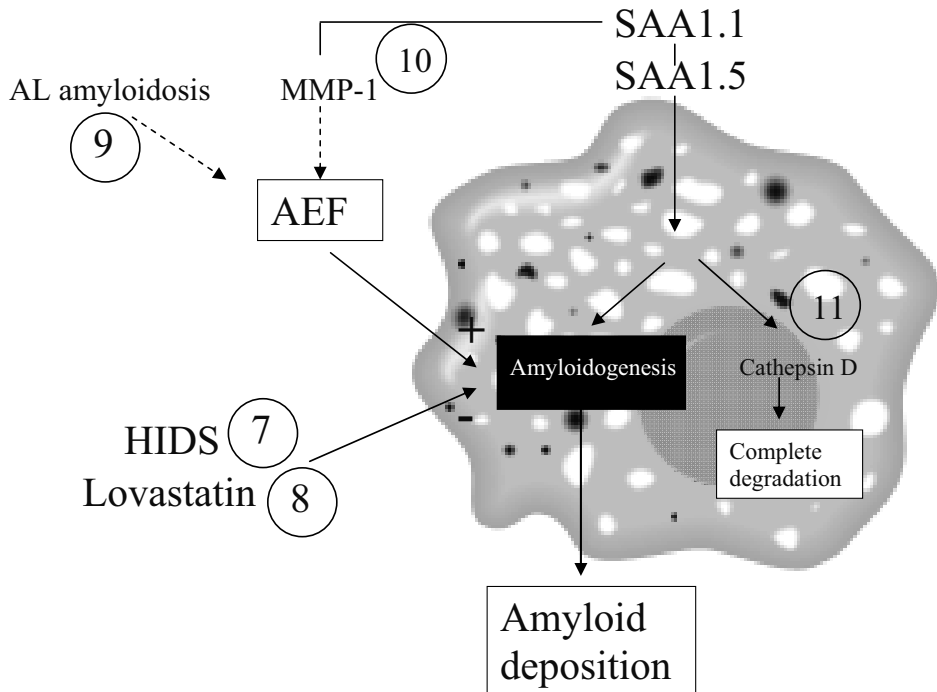


Figure 1 A schematic overview of amyloidogenesis. In the physiological situation, SAA is completely degraded in the macrophage by Cathepsin D. In AA amyloidosis there is incomplete degradation with the formation of intermediate fragments that can polymerize into a fibril. Lovastatin and HIDS prevent this process by yet unknown mechanism. Amyloid enhancing factor serves as a nucleus for the initiation of amyloidogenesis. It dramatically reduces the lag-time before amyloid deposits appear. Other types of amyloid can serve as AEF. Possibly SAA1.1 cleaved by MMP-1 can also act as AEF, proving an explanation for the increased risk of AA amyloidosis in patients carrying this isotype. The numbers refer to the chapters of this thesis

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Nederlandse samenvatting

Dit proefschrift is opgebouwd uit twee delen die nauw met elkaar samenhangen. Het hyper IgD syndroom (HIDS) behoort tot de groep van de auto-inflammatoire syndromen. Dit zijn genetische aandoeningen die worden gekenmerkt door levenslange aanvallen van koorts zonder dat er sprake is van een infectieuze oorzaak. De onderwerpen die in het eerste deel van het proefschrift worden behandeld, zijn de pathogenetische mechanismen, klinische presentatie, genetische testen en therapeutische opties bij HIDS. De belangrijkste complicatie van auto-inflammatoire ziekten is type AA amyloidose. Hoewel AA amyloidose frequent voorkomt bij de andere auto-inflammatoire ziekten is het een zeldzaamheid bij HIDS. Dit gegeven is de basis voor verdere studie naar de ontstaanswijze van type AA amyloidose, zoals beschreven in het tweede deel.

Eerste deel

Bij de auto-inflammatoire aandoeningen gaan de koortsaanvallen gepaard met diverse ontstekingsverschijnselen. De klinische presentatie van de verschillende auto-inflammatoire syndromen worden beschreven in **hoofdstuk 1**. Recente ontwikkelingen hebben nieuwe inzichten opgeleverd in de mechanismen die leiden tot ontsteking. Hoewel we inmiddels weten welke genen aangedaan zijn, blijft bij het stellen van de diagnose het klinische onderzoek toch de hoeksteen. Met een gedetailleerde anamnese, inclusief familieanamnese en observatie van een koortsaanval, kunnen de meeste auto-inflammatoire syndromen goed van elkaar worden onderscheiden.

In **hoofdstuk 2** onderzoeken we de klinische symptomen, follow-up en de kwaliteit van leven van 103 patiënten met genetisch bewezen HIDS. De meest voorkomende symptomen die samengaan met de koortsaanvallen zijn: lymfadenopathie (87,3%), buikpijn (85,3%), artralgie (83,3%), braken (71,6%), diarree (72,3%), huiduitslag (68,8%) en aften (52%). Amyloidose is een ernstige, maar zeldzame complicatie (2,9%). De frequentie van koortsaanvallen neemt af gedurende het leven. Echter, na de leeftijd van 20 jaar heeft de helft van de patiënten nog altijd meer dan 6 aanvallen per jaar. HIDS heeft een negatieve invloed op verschillende aspecten van de kwaliteit van leven. HIDS-patiënten scoren significant slechter op de schalen voor sociaal functioneren, rolbeperking door fysiek probleem, algemene gezondheidsbeleving en energie. Aangezien HIDS op jonge leeftijd begint, hebben we ook de impact op de sociale ontwikkeling en op de ontwikkeling van autonomie bestudeerd. Beide zijn gestoord bij HIDS. Bovendien heeft HIDS een negatieve invloed op zowel de schoolprestaties als op de kans op werk.

Hoewel het gen dat HIDS veroorzaakt al enkele jaren geleden is gevonden, blijft het mechanisme hoe dit leidt tot ontsteking nog onbekend. In **hoofdstuk 3** laten we zien dat witte bloedcellen van HIDS patiënten een apoptosedefect hebben. Een mogelijke verklaring voor het inflammatoire fenotype van HIDS zou kunnen zijn dat dit defect in apoptose leidt tot een ongebreidelde ontstekingsreactie in respons op een kleine stimulus.

Door ontdekking dat HIDS wordt veroorzaakt door mutaties in het mevalonaat kinase gen (*MVK*) zijn er nu genetische testen beschikbaar. Echter, genetische testen zijn niet algemeen verkrijgbaar en het is een tijdrovende en dure procedure. In **hoofdstuk 4** beschrijven we een algoritme gebaseerd op klinische symptomen waarmee HIDS kan worden uitgesloten zonder het gebruik van een genetische test. Deze beslissingsregel is gegenereerd in een Frans cohort van 149 HIDS patiënten en werd gevalideerd in een Nederlands cohort van 93 patiënten. Met het gebruik van deze beslissingsregel zouden ten minste 18% van de testen kunnen worden voorkomen, zonder één enkele patiënt te missen.

Twee nieuwe therapeutische opties zijn getest in de studie die wordt beschreven in **hoofdstuk 5**. Allereerst hebben een klinische score ontwikkeld op basis van 12 symptomen, om de ernst van een aanval te kunnen vaststellen. Vervolgens hebben we in één patiënt aangetoond dat etanercept effectiever was dan anakinra in de preventie van een aanval uitgelokt door een vaccinatie.

Tweede deel

Een ernstige complicatie van alle chronische ontstekingsziekten, waaronder HIDS, is type AA amyloidose. In het tweede deel van deze dissertatie worden verschillende aspecten van de amyloidvorming (amyloidogenese) bestudeerd. Dit is schematisch weergegeven in de figuur.

AA amyloidose wordt veroorzaakt door depositie van het N-terminale deel van SAA. SAA is een acute-fase eiwit dat in de lever wordt geproduceerd in reactie op een inflammatoire stimulus¹. Het wordt gemakkelijk opgenomen door mononucleaire fagocyten. Onder normale omstandigheden wordt SAA via het endosomale systeem naar de lysosomen getransporteerd alwaar het volledig wordt afgebroken door de cathepsine-proteases²⁻⁸. Bij patiënten met AA amyloidose is er een verstoorde afbraak van SAA wat zorgt dat er N-terminale fragmenten ophopen. Fibrillen bestaan voor het grootste deel uit de N-terminale 76 aminozuren van SAA, hoewel er ook fragmenten van andere lengte zijn beschreven¹⁰⁻¹⁴. In het zure milieu van de lysosomen kunnen deze fragmenten zich vouwen tot een β -sheet configuratie en vervolgens aggregeren tot fibrillen. Nadat deze fibrillen in de extracellulaire ruimte zijn gedeponeerd, binden er verschillende glycosaminoglycanen, serum amyloid P en lipiden aan de fibrillen waardoor deze resistent worden voor proteolyse¹⁵⁻¹⁷.

Een langdurige verhoging van SAA is een noodzakelijke voorwaarde voor het ontwikkelen van amyloidose. Echter, slechts een gedeelte van de patiënten die een langdurig verhoogd SAA hebben, ontwikkelen uiteindelijk amyloidose. Een onderkende risicofactor voor het krijgen van amyloidose is polymorfismen in het SAA-gen. Twee single-nucleotide polymorfismen in exon 3 geven 3 verschillende isotypes: SAA 1.1 (Val⁵²-Ala⁵⁷), SAA 1.3 (Ala⁵²-Ala⁵⁷) en 1.5 (Ala⁵²-Val⁵⁷). Patiënten met een 1.1/1.1 genotype hebben een hoger risico op het ontwikkelen van amyloidose dan patiënten met een 1.5/1.5 genotype.

Hoofdstuk 6 geeft een overzicht van de aspecten van AA amyloidose in de auto-inflammatoire syndromen. Type AA amyloidose is een frequente complicatie van de meeste periodieke koortssyndromen. Voordat colchicine werd ontdekt als werkzame therapie voor FMF, ontwikkelde tot 60% van de FMF patiënten amyloidose¹⁸. Bij TRAPS en Muckle-Wells syndroom patiënten komt het in 25% respectievelijk 35% voor¹⁹. Echter, bij in HIDS-patiënten was er een prevalentie van slechts 2.9%, zoals beschreven in hoofdstuk 2.

In **hoofdstuk 7** worden mogelijke verklaringen voor de lage incidentie van amyloidose bij HIDS onderzocht. Patiënten met HIDS hebben een sterk verhoogd SAA tijdens aanvallen. Bovendien had 45% van der patiënten ook een verhoogd SAA tijdens aanvalsvrije perioden. Daarbij was er een normale verdeling van het SAA genotype in HIDS-patiënten (en geen oververtegenwoordiging van het zogenaamde beschermende genotype). Dus alle voorwaarden voor het ontwikkelen van amyloidose zijn aanwezig bij HIDS patiënten en dit verklaart de lage incidentie van amyloidose niet.

HIDS wordt veroorzaakt door mutaties in het gen dat codeert voor mevalonaat kinase, een centraal enzym het isoprenoid metabolisme²⁰. Dit leidde tot de hypothese dat het isoprenoid metabolisme de amyloidogenese kan beïnvloeden.

Om dit verder te onderzoeken hebben we een celkweek methode met humane monocytyn gebruikt in **hoofdstuk 8**. Inhibitie van het isoprenoid metabolisme met lovastatine resulteerde in een dosisafhankelijke reductie van de hoeveelheid amyloid die werd gevormd. Het remmende effect van lovastatin is reversibel door de toevoeging van farnesol, maar niet door geranylgeraniol. Farnesyl transferase remming remde ook de amyloidogenese, wat een rol suggereert voor gefarnesylerde eiwitten in de pathogenese van AA amyloidose.

In het volgende hoofdstuk (**hoofdstuk 9**) dragen we een mogelijke verklaring aan voor de observatie van twee vormen van amyloidose in één patiënt (AL en AA amyloidose).

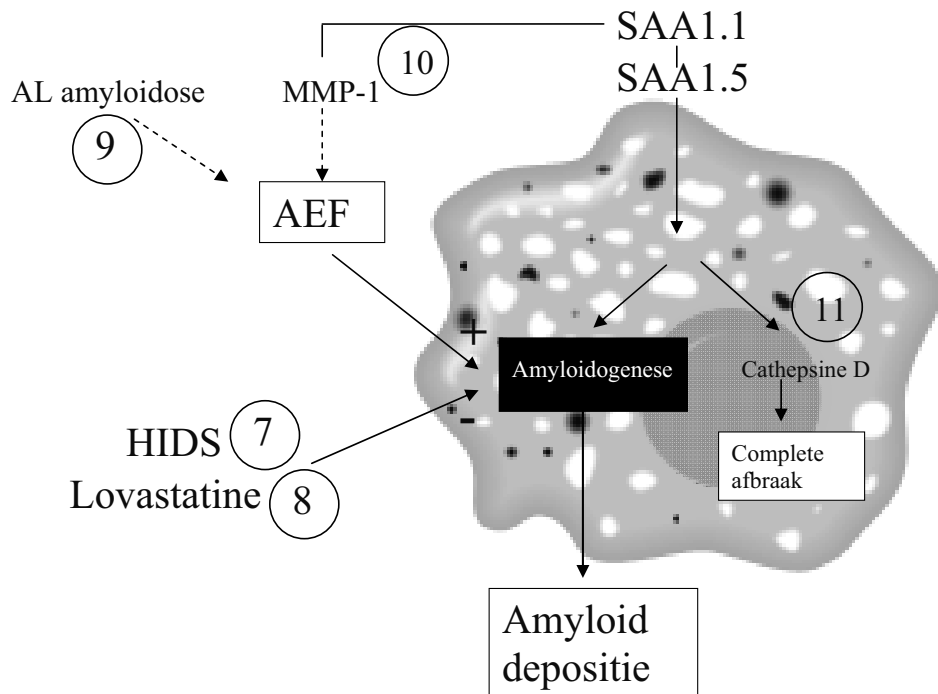
Geïsoleerde monocytyn van een patiënt met AL amyloidose vormen amyloid fibrilleren na expositie aan SAA, zonder de toevoeging van amyloid enhancing factor (AEF). In monocytyn van gezonde vrijwilligers kon amyloidose alleen worden geïnduceerd met de toevoeging van SAA én AEF. Dit suggereert dat een eerdere expositie van monocytyn aan AL amyloidose werkt als een AEF om amyloidogenese te stimuleren.

Serum amyloid A polymorfismen beïnvloeden het risico om AA amyloidose te ontwikkelen. Tevens speelt matrix metalloproteinase-1 (MMP-1) een rol in de pathogenese van amyloidose²². In **hoofdstuk 10** wordt de afbraak van de verschillende SAA isotypes door MMP-1 onderzocht. SAA 1.5 is resistent tegen afbraak door MMP-1 in vergelijking met SAA 1.1. Dit verschil wordt veroorzaakt door de capaciteit van MMP-1 om SAA te splitsen op de positie waar de polymorfismen zijn gelegen. Deze resultaten zouden een verklaring kunnen zijn voor de hogere serum SAA concentraties bij personen met een SAA 1.5 genotype. Bovendien zouden fragmenten afkomstig van de degradatie door MMP-1 kunnen dienen als AEF. Dit zou een verklaring kunnen zijn voor het grotere risico op amyloidose dat patiënten hebben met een SAA1.1/1.1.

Cathepsine B, cathepsine D en cathepsine K worden een rol toegedicht in de pathogenese van amyloidose. In **hoofdstuk 11** wordt het belang van deze enzymen bestudeerd. Alleen cathepsine D blijkt een significante rol te spelen in afbraak van SAA door humane monocyten. Bovendien geeft inhibitie van cathepsine D in het celkweek model van amyloidose een toename in de depositie van amyloid fibrillen. Inhibitie van cathepsine B en cathepsine K had geen invloed op de amyloidogenese. Dit duidt op een centrale rol voor cathepsine D in de pathogenese van AA amyloidose.

Perspectief voor de toekomst

Internationale samenwerking van behandelend specialisten is onontbeerlijk om een ziekte te kunnen onderzoeken die zo zeldzaam is als HIDS. Deze visie heeft in 1994 geleid tot de oprichting van de internationale HIDS studiegroep en het internationale HIDS register (zie website www.hids.net). Met behulp van het internationale HIDS register hebben we data van een groot aantal HIDS patiënten kunnen verzamelen. We zijn voornemens om het register en de internationale samenwerking voor te zetten om nog meer inzicht te verkrijgen in de klinische presentatie en pathogenetische mechanismen van HIDS. Het veelvoud aan klinische symptomen, de frequentie van aanvallen en de duidelijke negatieve invloed op de kwaliteit van leven benadrukken het belang van het vinden van een effectieve therapie. Hoewel we goede resultaten hebben geboekt met de biologics, zoals beschreven in hoofdstuk 5, zijn dit zeker geen panacee voor alle patiënten. Dit onderstreept het belang van het vinden van effectievere therapieën. Hoewel HIDS patiënten ook in staat zijn om amyloidose te ontwikkelen, blijkt de prevalentie van deze complicatie opmerkelijk laag. Deze observatie kan mechanismen van amyloid-vorming bloot leggen. Hoe het isoprenoid metabolisme amyloid-vorming beïnvloed dient verder te worden onderzocht. Een volgende stap zou kunnen zijn om een HMG-CoA reductase remmer in het muismodel van AA amyloidose. Hoewel de studies uit dit proefschrift meer inzicht geven in het mechanisme van amyloid-vorming blijft er een 'black-box' over die nog moet worden opgelost. Figuur 1 laat een schematisch overzicht zien van de mechanisme die betrokken zijn bij amyloid-vorming. Een sleutelvraag is welke enzymen verantwoordelijk zijn voor het klieven van SAA in fragmenten die worden gevonden in de amyloiddeposities. Een beter begrip van het proces van amyloid-vorming kan de zoektocht vergemakkelijken naar een effectieve therapie voor deze ernstige complicatie.



Figuur 1. Een schematisch overzicht van amyloid-vorming. In de fysiologische situatie wordt SAA in de macrofaag door cathepsine D volledig afgebroken. Bij AA amyloidose is er sprake van incomplete afbraak met de vorming van intermediaire fragmenten die kunnen polymeriseren tot een fibril. Lovastatine en HIDS voorkómen dit proces door een tot nu toe onbekend mechanisme. Amyloid enhancing factor vormt een kern van waaruit amyloid-vorming kan beginnen. Het geeft een aanzienlijke verkorting van de tijd voordat amyloidneerslagen ontstaan. Andere typen amyloid kunnen dienen als AEF. Mogelijk dat MMP-1 gekliefd SAA1.1 als AEF kan werken. Dit zou een verklaring kunnen zijn voor het toegenomen risico op amyloidose bij patiënten die dit isotype van SAA hebben.

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Spider webs and amyloid

The cover shows an image of the web of an *Araneus diadematus* spider. Spider webs are made of a dragline fiber that has remarkable properties. It has a tensile strength that is superior to that of high-grade steel. It is much thinner than the human hair with just a few microns in diameter and is able to keep its strength below -40°C. In addition, it is extremely lightweight: a strand of spider silk long enough to circle the earth would weigh less than 450 gram.¹ The spiders spin their silk in a highly sophisticated organ called a spinneret. It is usually on the underside of a spider's abdomen, to the rear. Most spiders have six spinnerets; some have four or two. They move independently and in concert to build webs. Recent research has shown that spider silk have strong structural similarities to amyloid fibres.² Although different species of spider, and different types of silk, have different protein sequences, a general trend in spider silk structure is a sequence of specific amino acids (usually alternating glycine and alanine, or alanine alone). Crystollographic investigation showed that the proteins in spider silk have structural conformation that is called a β -sheet.³ The assembly of spider silk threads starts with an mixture of unstructured proteins in an aqueous solution. Moving through the spinneret, the proteins adopt to a β -sheet configuration that can self-aggregate, eventually forming long fibres⁴. The conformational change has been shown to be energy dependant². All these features are thought to be characteristic for amyloid fibres. In addition, silk that is produces by the *Bombyx mori* (silkworm) which is very similar to spider silk, can be used as an amyloid enhancing factor⁵. It is therefore not surprising that Congo Red, which is used to detect amyloidosis in tissue preparation, has long been used in the textile industry as a dye for colouring silk.

Therefore the spider silk-production process, particularly the mechanisms of storing and manipulating silk proteins, might prove a valuable model system for exploring fibrillogenesis of amyloid protein.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 11 augustus 1976 te Goirle. Na het behalen van het VWO diploma in 1995 aan het Pauluslyceum te Tilburg, startte hij zijn studie geneeskunde aan de Katholiek Universiteit Nijmegen (thans Radboud Universiteit Nijmegen).

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