Paediatric Autoinflammatory Diseases: Conceptual, Clinical and Mechanistic Dimensions

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1. Abstract

This thesis focuses on three paediatric autoinflammatory diseases in Sweden today; familial Mediterranean fever (FMF), periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA) and synovitis, acne, pustulosis, hyperostosis and osteitis (SAPHO). In addition to describing autoinflammation with reference to these diseases, the thesis explores the concept of autoinflammatory disease by examining four existing definitions. A modified definition is then proposed in the light of this analysis.

The aim of the first study (paper I) was to characterize FMF in western Sweden. Patients with autoinflammatory diseases were continuously registered at five hospitals and case records were analysed retrospectively. Population data on immigration were retrieved from Statistics Sweden. Thirty-seven patients with FMF were identified in the records from the years 2000 to 2008. For the majority of patients, disease onset occurred during childhood. In western Sweden, the prevalence of FMF among immigrants from the eastern Mediterranean Basin was of the same order as for their country of origin.

The second and third study (papers II and III) aimed to advance the pathophysiological understanding of PFAPA, as an initial step towards identification of molecular and cellular mechanisms important in disease pathogenesis, as well as facilitating the definition of biomarkers. Levels of blood cells, serum cytokines and functional features of neutrophils were investigated during afebrile and febrile phases in children with typical PFAPA episodes. The results show oscillations in the concentration of blood cells between the afebrile and febrile phases of PFAPA. Upon onset of fever, there were modest levels of pro-inflammatory serum cytokines, together with increased levels of the IFN-γ-induced chemokine IP10/CXCL10. Further, neutrophils were analysed for functional features such as apoptosis, production of reactive oxygen species (ROS) and priming status (paper III). The results show that neutrophils from patients with PFAPA are primed, show decreased apoptosis and generate increased amounts of intracellular ROS during febrile attacks, whereas the afebrile phase was characterized by increased apoptosis. How these molecular and cellular features may affect disease pathogenesis is discussed in the thesis.

The fourth study (paper IV) investigated whether deficiency in neutrophil intracellular production of NADPH-oxidase-derived ROS is a disease mechanism in SAPHO, as suggested in a previous case report. Cells from four patients with SAPHO showed normal production of ROS, both intracellularly and extracellularly, contradicting the previous finding and showing that the SAPHO syndrome is not necessarily associated with deficient neutrophil intracellular ROS production.

This thesis gives new insights into a group of diseases that has been largely overlooked in the context of immune function, as each disease involves newly defined mechanisms important for innate immune regulation. The thesis also attempts to advance the definition of autoinflammatory diseases, complementing the previous definitions with the possible activation of the adaptive immune system and an association with other immune dysfunctions.

2. List of publications

This thesis is based on the following papers referred to in the text by their Roman numerals:

I. Wekell P, Friman V, Balci-Peynircioglu B, Yilmaz E, Fasth A, Berg S. Familial mediterranean fever – an increasingly important childhood disease in Sweden. Acta Paediatrica (2013) 102: 193-198.

II. Brown KL*, Wekell P*, Osla V, Sundqvist M, Sävman K, Fasth A, Karlsson A, Berg S. Profile of blood cells and inflammatory mediators in periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA) syndrome. BMC Pediatrics (2010) 10:65.

III. Sundqvist M, Wekell P, Osla V, Bylund J, Christenson K, Sävman K, Foell D, Cabral DA, Fasth A, Berg S, Brown KL, Karlsson A. Increased intracellular oxygen radical production in neutrophils during febrile episodes of periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis syndrome. Arthritis and Rheumatism (2013) 65: 2971-2983.

IV. Wekell P*, Björnsdottir H*, Björkman L, Sundqvist S, Christenson K, Osla V, Berg S, Fasth A, Welin A, Bylund J, Karlsson A. Neutrophils from patients with SAPHO syndrome show no signs of aberrant NADPH-oxidase dependent production of intracellular reactive oxygen species. (Submitted manuscript)

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Appendix

Brown K, Wekell P, Karlsson A, Berg S. On the road to discovery in periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA) syndrome. Proceedings of the National Academy of Sciences USA (2011) 108:E525.

3. Table of Contents

4. Populärvetenskaplig sammanfattning

Denna avhandling fokuserar på tre autoinflammatoriska sjukdomar hos barn i Sverige idag; familjär medelhavsfeber (FMF), periodisk feber, afte, pharyngit och adenit (PFAPA) och synovit, akne, pustulos, hyperostos och osteit (SAPHO). Autoinflammatoriska sjukdomar är ett relativt nytt samlingsnamn för en grupp sjukdomar som ofta karaktäriseras av återkommande attacker av feber och inflammation kopplat till sjukdomskänsla, buksmärtor, hudutslag och inflammation i skelettet. Innan diagnosen ställs söker dessa personer sjukvård upprepade gånger, bemöts ofta med bristande kunskap och förståelse samt behandlas med antibiotika utan effekt. Under 2008 bildades en translationell autoinflammatoriskt forskargrupp i Västsverige, ett samarbete framförallt mellan avdelningen för pediatrik och avdelningen för reumatologi och inflammationsforskning vid Göteborgs universitet samt barn- och ungdomskliniken i NU-sjukvården idag med ett flertal nationella och internationella samarbetspartners. Denna avhandling är ett resultat av denna translationella insats. Förutom att utforska dessa tre sjukdomar analyserar avhandlingen begreppet autoinflammation genom att undersöka fyra befintliga definitioner och i ljuset av denna analys föreslås ny modifierad definition.

Det har nyligen visats att autoinflammatoriska sjukdomar orsakas av obalans i det medfödda immunförsvaret. Kunskapen om det medfödda immunförsvaret har i grunden förändrat synen på vårt immunförsvar och till detta har kunskapen om inflammatoriska processer och autoinflammatoriska sjukdomar givit ett stort bidrag. Än mer fascinarande är insikten att obalansen i det medfödda immunförsvaret kan ge sjukdom i det förvärvade immunförsvaret i form av immundefekter och autoimmunitet, men också att autoinflammatoriska sjukdomar kan vara förenat med sådana tillstånd.

Utan adekvat behandling löper patienter med FMF risk för att utveckla livshotande proteininlagringar i inre organ (amyloidosis). Syftet med den första studien (artikel I) var att undersöka klinisk bild, genetik och förekomst av FMF i Västsverige. Patienter med FMF registrerades kontinuerligt vid fem sjukhus och registret analyserades i efterhand. Befolkningsuppgifter hämtades från Statistiska centralbyrån. Trettiosju patienter med FMF identifierades under åren 2000 till 2008. Det stora flertalet patienter hade en sjukdomsdebut som barn och förekomsten av sjukdomen hos individer från östra Medelhavsområdet var i samma storleksordning som i deras ursprungsland.

Periodisk feber, aftös stomatit, pharyngit & adenit (PFAPA) är den vanligaste autoinflammatoriska sjukdomen hos barn i Sverige och i de flesta delar av världen. PFAPA debuterar vanligen före fem års ålder och karaktäriseras av återkommande,

ofta påtagligt regelbundna feberepisoder vanligen 4-5 dagar långa med ett intervall av 4-6 veckor. Feberepisoderna är kopplade till symtomen i akronymen. Diagnosen ställs kliniskt med stöd av kriterier. I utvalda fall opereras halsmandlarna bort vilket kan göra att alla tecken på sjukdomen försvinner utan att vi förstår varför. Det finns ingen risk för amyloidos hos barn med PFAPA och sjukdomen läker i de flest fall ut inom 3-5 år. I studie två (artikel II) och tre (artikel III) frågar vi oss vilka sjukdomsmekanismer som är involverade vid sjukdomen och försöker identifiera diagnostiska markörer för sjukdomen. I artikel II undersökte vi förändringar i koncentrationen av olika blodkroppar och cytokiner under olika faser av PFAPA. Arbetet beskriver svägningar i blodbilden och identifierar den IFN-γ inducerade cytokinen IP10/CXCL10 som en potentiell biomarkör. Resultaten tyder på att såväl det medfödda som det förvärvade immunsystemet är engagerat vid PFAPA. I arbete III studerade vi neutrofilfunktionen vid PFAPA och fann att grundläggande aspekter av neutrofilfunktionen är påverkad inklusive naturlig celldöd, aktivering och produktionen av intracellulära syreradikaler.

I den fjärde studien (artikel IV) tog vi reda på om brist på produktion av intracellulära syreradikaler är en sjukdomsmekanism vid SAPHO, vilket en tidigare studie hade föreslagit. Celler från fyra patienter med SAPHO visade normala produktionen av syreradikaler, både intracellulärt och extracellulärt, vilket strider mot tidigare publicerade resultaten och visar att SAPHO inte nödvändigtvis är förenat med bristfällig intracellulära ROS produktion hos patienternas neutrofiler.

5. Abbreviations

AGS	Aicardi-Goutières syndrome
APLAID	autoinflammation and PLAID
CAMPS	CARD14-mediated psoriasis
CANDLE	chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature
CAPS	cryopyrin associated periodic syndromes
CGD	chronic granulomatous disease
CINCA	chronic infantile neurological cutaneous articular syndrome
CL	chemiluminescence
CRP	C-reactive protein
DAMP	damage-associated molecular pattern
DIRA	deficiency of IL1 receptor antagonist
DITRA	deficiency of IL-36 receptor antagonist
ecROS	extracellular ROS
EO-IBD	early-onset inflammatory bowel disease
ESR	erythrocyte sedimentation rate
FCAS	familial cold autoinflammatory syndrome
FMF	familial Mediterranean fever
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HPF	hereditary periodic fever
HIDS	hyperimmunoglobulinemia D with periodic fever syndrome
icROS	intracellular ROS
IFNγ	interferon γ
IL	interleukin
IL1Ra	IL1 receptor antagonist
IP10	interferon γ induced protein 10
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MKD	mevalonate kinase deficiency
MPO	myeloperoxidase
mtROS	mitochondrial ROS
MWS	Muckle-Wells syndrome
NET	neutrophil extracellular trap
NF-κB	nuclear factor-κB
NLRP3	NOD-like receptor family, pyrin domain containing 3
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
NOMID	neonatal-onset multisystem inflammatory disease
NSAIDs	non-steroidal antiinflammatory drugs
PAMP	pathogen-associated molecular pattern

PAPA	pyogenic arthritis, pyoderma gangrenosum and acne
PFAPA	periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis
PLAID	PLCγ2-associated antibody deficiency and immune dysregulation
PLC	phospholipase C
phox	phagocyte oxidase
PKC	protein kinase C
PMA	phorbol 12-myristate 13- acetate
PMN	polymorphonuclear leukocytes
PRR	pattern recognition receptor
ROS	reactive oxygen species
SAA	serum amyloid A
SAPHO	synovitis, acne, pustulosis, hyperostosis and osteitis
SOD	superoxide dismutase
TLR	toll like receptor
TNFα	tumor necrosis factor α
TRAPS	TNF receptor-associated periodic syndrome

6. Prologue

Innate immunity has for long been seen simply as a basic, non-complex way for the body to defend itself against microbes, by eliciting an inflammatory reaction accompanied by activation of the body's foot soldiers, neutrophils. The more complex immune mechanisms involved in long-term immunity, immune deficiency and autoimmunity were attributed entirely to the adaptive immune system, which has been the primary focus for immunologists for decades. However, for these researchers focusing on the adaptive immune system, there was a clue to a coming paradigm shift right before their eyes: vaccines that were used to provide long-term adaptive immune protection did not function without adding adjuvants to the antigen. Thus, vaccines needed an innate as well as an adaptive signal.

Charles Janeway first foresaw the existence and function of innate immunity in a classical article of 1989, "Approaching the asymptote? Evolution and revolution in immunology" (1). In this article he proposed the idea of pattern recognition, a general principle of innate immune recognition, and his work provided a conceptual framework for the integration of innate and acquired immunity (2). Later researchers in the field, Bruce Beutler and Jules A. Hoffmann, were awarded the Nobel Prize in 2011 "for their discoveries concerning the activation of innate immunity", indicating that the innate immune system is presently a hot topic in the field of immunology (3).

As for all biological systems, dysfunction or dysregulation may lead to disease. In innate immunity, the field of autoinflammatory diseases has been central to the remarkable development of knowledge. Not only has research about these diseases led to an increased understanding of the disease mechanisms in autoinflammatory disease *per se*, but it has also been indispensable for the understanding of innate immunity as a whole, as well as shedding light on the importance of these mechanisms in other disease conditions. Today, researchers at one end of the spectrum in the autoinflammatory field discuss rare monogenic conditions, while discussions at the other end of the spectrum concern the role of these inflammatory pathways in common diseases such as diabetes, Alzheimer's disease and atherosclerosis (4, 5). This is a development that no one could have foreseen fifteen years ago, when the concept of autoinflammation was coined.

7. The children with autoinflammatory diseases in this thesis

Three children have been truly inspirational to me in writing this thesis, and they will serve as illustrations of different aspects. The descriptions are actually amalgams of many patient histories from my clinical experience and not of specific individuals. We will call the three persons Sabina, William and Johan. Sabina represents the starting point for the epidemiological study of *familial Mediterranean fever* (FMF) in western Sweden that is the basis of paper I. William corresponds to part of the patient cohort in the translational studies of *periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis* (PFAPA), that are reported in papers II and III. With the help of Johan, we were able to test a pathognomonic hypothesis in the pathogenesis of *synovitis, acne, pustulosis, hyperostosis and osteitis* (SAPHO) syndrome in paper IV. At this point I would like to express my sincere thanks to all the individuals represented by Sabina, William and Johan, and their parents, for their contribution. Eventually all the children were diagnosed with specific autoinflammatory diseases, diagnoses that few paediatricians could or should have made when the children presented at the paediatric emergency room for the first time:

> Sabina, 6 years old, presents with fever, severe abdominal pain and increased inflammatory markers. The consulted surgeon cannot exclude a diagnosis of appendicitis and decides to carry out a laparoscopy.
>
> William, 2 years old, has high fever for three days with tonsillitis and increased C-reactive protein. He is discharged from the emergency room with oral antibiotics.
>
> Johan, 15 years old, develops fever and severe pain in his right tibia. He is admitted to the paediatric ward with a preliminary diagnosis of bacterial osteomyelitis.

You will meet Sabina, William and Johan again as this thesis advances.

8. Introduction

Inflammation can be regarded as the innate immune system's response to danger, such as infections, environmental agents, or waste from dying and damaged cells. The innate immune response is a first line of defence, rapid and short-lived, that recognises exogenous and endogenous danger by a limited number of germline-encoded evolutionarily conserved receptors. These receptors include pattern recognition receptors (PRRs) such as extracellular and intracellular toll-like receptors (TLRs) as well as intracellular RIG-I-like receptors (RLR) and NOD-like receptors (NLRs), activated by pathogen-associated molecular patterns (PAMPs) or endogenous damage-associated molecular patterns (DAMPs). Important cells for the innate immune response are granulocytes (basophils, eosinophils and neutrophils), monocytes, macrophages or innate lymphoid cells (ILCs), including natural killer (NK) cells (6). In addition, the full immunological response depends on the adaptive immune system, interlinked and crosstalking with the innate immune system. The adaptive immune response is considerably slower than the innate response when initially exposed for danger by means of an antigen. The cells of the adaptive immunity, T and B cells, undergo somatic mutations and clonal expansion as they recognize an antigen by their non-self receptors, leading to fine-tuning of the receptor specificity and a very efficient effector response when exposed to the same antigen again. This indicates that the system is highly specific and enables immunological memory. Today, we know that the innate and adaptive immune systems are closely linked in a network that evolved in close proximity to microbes in the context of the evolutionarily ancient innate immune system.

The effectiveness of the innate immune system is impressive and from an evolutionary perspective one may ask why we need an adaptive immune system at all. It has been claimed that the reason is that the adaptive system is more energy-efficient and therefore has a survival advantage, given that the adaptive immune response avoids eliciting the inflammatory response that is a hallmark of the innate immune response, causing fatigue, sickness, organ damage, or even death.

8.1 What defines autoinflammatory diseases?

This thesis focuses on three clinically important paediatric autoinflammatory diseases in Sweden today: two periodic fever syndromes, the monogenic FMF, as depicted in Sabina's story, and the polygenic (multifactorial) PFAPA, as illustrated by William, and finally autoinflammatory bone diseases exemplified by SAPHO, as represented by Johan.

Classical autoinflammatory diseases (periodic fever syndromes) are characterized by recurrent episodes of fever, systemic inflammation, and symptoms such as skin rash, abdominal pain, thoracic pain, lymphadenopathy, or arthritis. More recently defined autoinflammatory diseases often have symptoms similar to the classical diseases but continuously rather than in episodes, or with milder inflammation (or both).

During the last twenty years, a remarkable development has led to more accurate diagnosis of autoinflammatory diseases, and their pathophysiology and genetic background are better understood. However, although the concept of auto-inflammation has been established and refined since it was coined, there are still reasons to discuss the defining characteristics of an autoinflammatory disease.

Here, the concept of autoinflammatory disease will be explored by means of four definitions and adjoining models that have been proposed as the field has evolved (4, 7-11). Limitations and merits of the different definitions and models will be presented, together with the major scientific achievements linked to the respective definitions. This comparison will be concluded by the proposition of a modified definition of autoinflammatory disease, a definition that will provide a framework for this thesis as it examines the three conditions FMF, PFAPA and SAPHO.

8.1.1 The first definition of autoinflammatory diseases: McDermott

When Michael McDermott and Daniel Kastner coined the concept of Autoinflammatory Disease in 1999, it marked a paradigm shift as it depicted an entirely new group of immunological diseases. These diseases were defined as "*conditions characterized by seemingly unprovoked episodes of inflammation, without high-titer of autoantibodies or antigen-specific T-cells"* (7, 12). This first definition of autoinflammatory conditions was suggested in the same article that described the genetic background of *TNF receptor-associated periodic syndrome* (TRAPS) and also linked to the identification two years earlier of mutations that cause FMF (7, 13, 14). The definition proposed by McDermott made a clear distinction between autoinflammation and autoimmunity. This demarcation, although plausible at the time, may appear simplistic today, bearing in mind the emerging understanding that innate and adaptive immunity are closely linked and that autoinflammatory conditions may have an adaptive or autoimmune component (11, 15-18). McDermott acknowledged the limitation of his definition when he and his colleague McGonagle seven years later proposed that immunological diseases ought to be conceived as a continuum with "pure monogenic autoinflammatory diseases" at one end and "pure monogenic autoimmune diseases" at the other, as illustrated in Figure 1 (8).

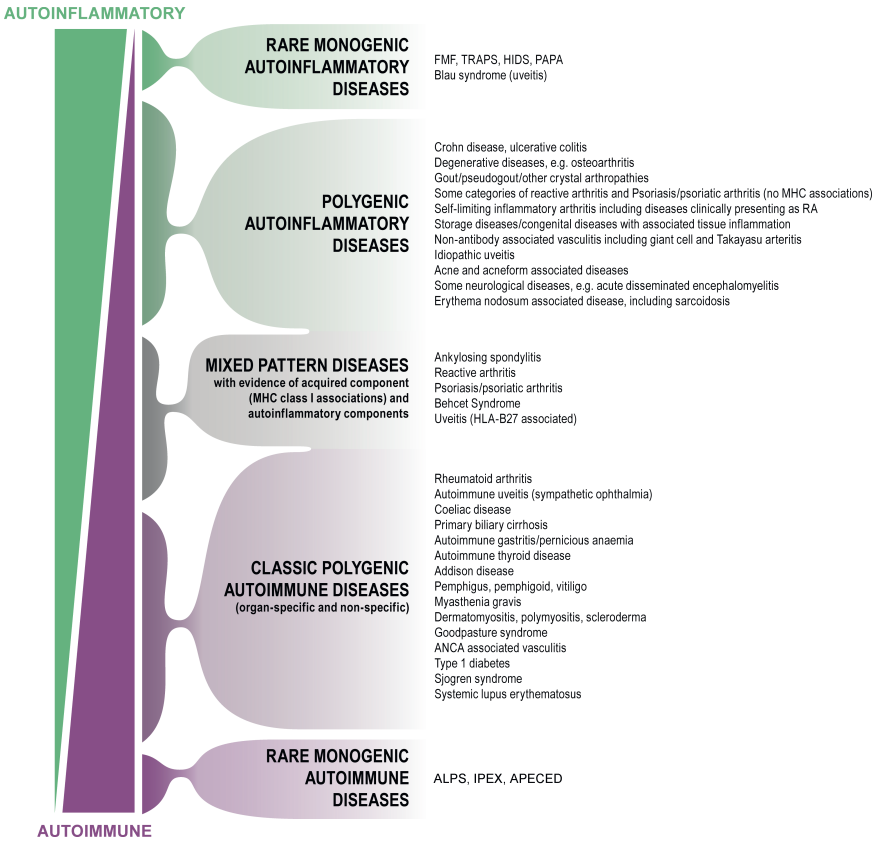

Figure 1. The immunological Disease Continuum. McGonagle & McDermott. PLoS Med. 2006;3(8):e29. With permission.

McGonagle and McDermott's continuum model moved the understanding of immunological diseases forward by integrating the concept of autoinflammation with that of autoimmunity. Secondly, it applied the concept of autoinflammation not only to monogenic diseases (diseases that are the result of mutation(s) in a single gene) but also to polygenic diseases (diseases that are influenced by more than one gene). Finally, the continuum model recognized that a single disease can have both an autoinflammatory and an autoimmune component. In this way McGonagle and McDermott challenged the clear distinction between autoinflammatory and autoimmune disease that was part of McDermott's first definition. At the time, they envisaged that non-infectious inflammatory conditions could be accommodated within the spectrum of their continuum model (8).

8.1.2 The second "definition" of autoinflammatory diseases: Dinarello

In 2007, Charles Dinarello proposed a second definition of, or rather criterion for, autoinflammatory disease. This was more than twenty years after his paramount discovery of interleukin-1 (IL-1), a signature cytokine in inflammation (9, 19). Dinarello was aware of the emerging understanding of the overlap between autoimmunity and autoinflammation when he suggested that *"the best criterion for identifying an autoinflammatory disease is that the clinical, biochemical, and hematologic manifestations are rapidly and impressively reversed upon initiation of treatment with IL-1 blockade"* (9). Today there are several different strategies for IL-1 inhibition, including anakinra (IL-1Ra), rilonacept, and canakinumab. Anakinra blocks the IL-1 receptor and thereby obstruct the effect of both IL-1α and IL-1β; rilonacept traps IL-1α and IL-1β before they bind to the IL-1 receptor and canakinumab similarly captures IL-1β (20).

Although IL-1 is necessary for the disease pathology in many autoinflammatory conditions, increased serum concentrations of IL-1 are rarely measurable and, as the definition rightly points out, the only way to demonstrate the pathogenic role of IL-1 in a specific autoinflammatory disease *in vivo*, is to block IL-1. The shortcomings of Dinarello's definition are evident today. Firstly, there are autoinflammatory diseases that have a poor response to IL-1 blockade, for example NF-κB activation disorders such as *familial cold autoinflammatory syndrome 2* (FCAS2) (21, 22), *Blau syndrome / paediatric granulomatous arthritis* (BS/PGA) (23-25) and *deficiency of IL-10Ra in early-onset enterocolitis* (EO-IBD) (26, 27). Secondly, there are diseases that respond to IL-1 blockade that are not considered autoinflammatory diseases, for example systolic heart failure after acute myocardial infarction (28), stroke (29) and type 2 diabetes mellitus (30). Hence, although the definition or criterion by Dinarello has its merits, particularly in that it highlights the role of IL-1 and IL-1 blockade in many autoinflammatory conditions, it lacks both specificity and sensitivity.

The importance of the balance between IL-1 and IL-1Ra was underlined in 2009 by the characterisation of the autosomal recessive disease *deficiency of IL-I receptor antagonist* (DIRA), with a disease mechanism that is caused by unopposed IL-1β and IL-1α, contrasted by the hypersecretion of IL-1β in *cryopyrin-associated periodic syndromes* (CAPS) (4, 5, 31, 32). This difference in pathogenesis may also explain the different clinical features in DIRA and CAPS, with pustular rash and sterile osteomyelitis in DIRA and urticarial-like rash and bony overgrowth in CAPS.

8.1.3 The third definition of autoinflammatory diseases: Kastner

In 2010, a third definition was proposed by Daniel Kastner, stating that autoinflammatory diseases are *"clinical disorders marked by abnormally increased inflammation, mediated predominantly by cells and molecules of the innate immune system, with a*

significant host predisposition" (11). This definition was closely linked to the identification and pathophysiological understanding of variants in the pattern recognition receptor NOD-like receptor family, pyrin domain containing 3 (NLRP3) that causes the autoinflammatory CAPS syndrome (33-35). The definition also recognizes that cells and molecules of innate immunity, including NLRs, are fundamental in autoinflammatory diseases. The definition is in keeping with the model that McGonagle and McDermott proposed, in which immunological diseases ought to be conceived as a continuum with "pure" monogenic autoinflammatory and autoimmune diseases as endpoints, although Kastner did not explicitly recognize this aspect in his definition (11).

8.1.3.1 THE MECHANISM BEHIND CAPS

Patients with CAPS have gain-of-function mutations in the *NLRP3* gene that lead to three, sometimes overlapping, phenotypes: *familial cold autoinflammatory syndrome* (FCAS), *Muckle–Wells syndrome* (MWS), and *chronic infantile neurological, cutaneous and arthritis* (CINCA) – also known as *neonatal-onset multisystem inflammatory disease* (NOMID), in order of increasing severity (33, 35-38).

Under healthy conditions, NLRP3 is auto-repressed through interaction between the NACHT domain and the leucin-rich repeat domain (LRR). This auto-repression is removed through cellular activation by PAMPs and DAMPs (Figure 2) (39). As a result, NLRP3 unfolds itself and the NACHT domain is exposed. This leads to oligomerization of NLRP3 that recruits apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase 1, inducing activation of caspase 1, which in turn activates IL-1 and IL-18.

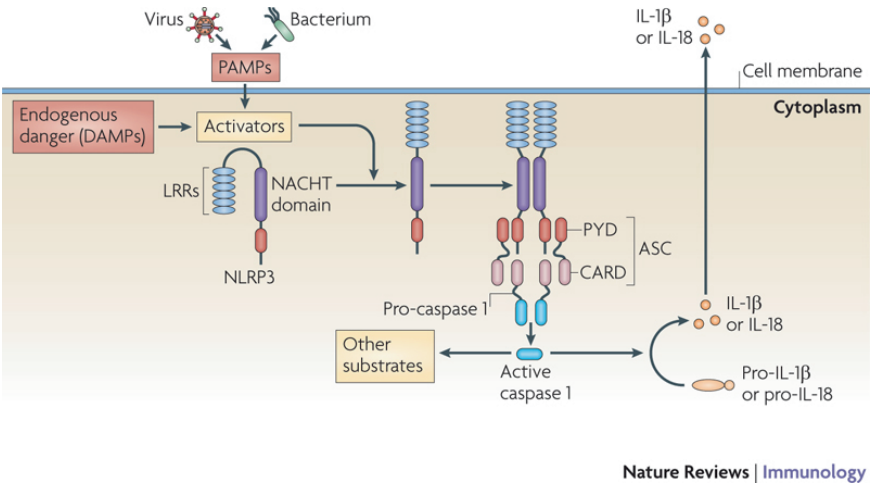

Figure 2. NLRP3 inflammasome activation. Tschopp & Schroder K. Nat Rev Immunol. 2010;10(3):210-5. With permisson.

Intriguingly, the same CAPS-associated mutation can give rise to several different disease phenotypes (FCAS, MWS or CINCA), even in members of the same family. Further, a significant proportion of patients with a CAPS phenotype have been found to be mutation-negative altogether, which has puzzled the clinical and scientific community since the gene was defined (37). Today, we know that a number of patients previously defined as mutation-negative have somatic mutations only in a fraction of the investigated cells, namely, somatic mosaicism of the *NLRP3* mutation (40-42). This was an important discovery that fundamentally changed the way we think about autoinflammatory disease aetiology.

8.1.3.2 Disease classification according to the Kastner definition

In 2009, the Kastner group proposed a classification in which six categories of autoinflammatory disease were defined, based on molecular pathophysiology: IL-1β activation disorders (inflammasomopathies, e.g. CAPS, gout and asbestosis), nuclear factor-κB (NF-κB) activation syndromes (e.g. FCAS 2), protein misfolding disorders (e.g. TRAPS, spondyloarthropathies), complement regulatory diseases (e.g. age-related macular degeneration; AMD), disturbances in cytokine signalling (e.g. cherubism), and macrophage activation (e.g. familial *haemophagocytic lymphohistiocytosis*; familial HLH) (4). Note that this classification includes asbestosis and silicosis among the autoinflammatory conditions, despite the fact that asbestos and silica clearly are external factors. Although they trigger NLRP3 activation, these conditions do not primarily depend on a predisposition of the host and therefore ought to be considered as NLRP3-mediated conditions and not autoinflammatory conditions.

8.1.3.3 Other inflammasome- and IL1-related autoinflammatory diseases

Improved mechanistic knowledge of NLRP3-initiated inflammation has increased our understanding not only of CAPS but also of several other autoinflammatory conditions, including FMF, *mevalonate kinase deficiency* (MKD), and *pyogenic arthritis, pyoderma gangrenosum and acne* (PAPA) (5, 11). However, the role of NLRP3 in these other diseases is still to be settled. As stated above, the understanding of the NLRP3 inflammasome and the balance between IL-1 and IL-1Ra was emphasized by the discovery of DIRA (section 8.1.2) (4, 5, 31, 32).

8.1.4 The fourth definition of autoinflammatory diseases: Grateau

The fourth, more clinically based, definition was proposed by Gilles Grateau in 2013, stating that *"autoinflammatory diseases are diseases with clinical signs of inflammation, associated with elevated levels of acute-phase reactants, which are attributable to dysfunction of the innate immune system, genetically determined or triggered by an endogenous factor"* (10).

Grateau rightly points out that it may be problematic to define autoinflammatory diseases based on disease-causing mutations and their functional consequences. For example, the fact that one *NLRP3* mutation can give rise to several different phenotypes in the CAPS spectrum makes it difficult to satisfactorily characterize a disease on genetic or functional bases without also describing the phenotype (4, 10).

By his definition, Grateau restricts the term 'autoinflammatory disease' to conditions with *"elevated levels of acute-phase reactants"*. Thus, Grateau excludes several inflammasome-related conditions, for example type 2 diabetes, arteriosclerosis and age-related macular degeneration. He also rejects the possibility that there could be autoinflammatory conditions that show a local inflammation without displaying increased systemic inflammatory markers, because such conditions would not be autoinflammatory according to his definition. Another example is the newly defined interferon (INF)-mediated autoinflammatory conditions that are associated with increased levels of C-reactive protein (CRP) only when the patient suffers from severe flares; consequently, Grateau would exclude patients with only moderate flares from receiving a diagnosis of autoinflammatory disease (43). Before these issues have been resolved it is inadvisable to go down the narrow path proposed by Grateau.

The definition by Grateau includes the statement that autoinflammatory diseases may be triggered by an endogenous (internal) factor. In certain monogenic autoinflammatory diseases, accumulation of endogenous is known to cause cellular stress. One example is the accumulation of misfolded TNF receptor retained in the ER, causing activation of mitochondrial ROS production and ultimately leading to TRAPS. Another mechanism is the upregulation of intracellular sensors, which can then be triggered by various stressors such as cold in CAPS and menstruation in FMF. There are also several examples of endogenous triggers for NLRP3, and to what extent the resulting diseases should be considered to be autoinflammatory can be discussed. Such triggers include drusen (tiny yellow-whitish accumulations of proteins and lipids in the eye) in AMD, amyloid-beta (which may form plaques) in Alzheimer's disease, cholesterol crystals in atherosclerosis and free fatty acids in metabolic disorders. Grateau does not answer the above question, and to some degree he even avoids the problem by the exclusion of several of these conditions as they lack increased inflammatory markers. It is far beyond the scope of this introduction to further clarify these issues.

In his article, Grateau addresses some of the limitations of the previous definitions and models by the proposition of a bidimensional model that incorporates dysfunction of the innate and adaptive immune systems (Figure 3) instead of a continuum, as proposed by McGonagle and McDermott (Figure 1). The graphic representation proposed by Grateau depicts diseases with over-activation or deficiency of the adaptive immune system in one dimension and an over-activation or deficiency of the innate immune system in the other (10). As pointed out in the publication, the model does not allow for the graphic representation of conditions that show over-activation and deficiency in the same (innate or adaptive) system. It is somewhat enigmatic that Grateau does not take the chance to embrace the bidimensional nature of immunological diseases and address the limitations of his model by proposing a broader definition of autoinflammatory diseases instead of a narrower one, and this leaves the Kastner definition as the most useful thus far.

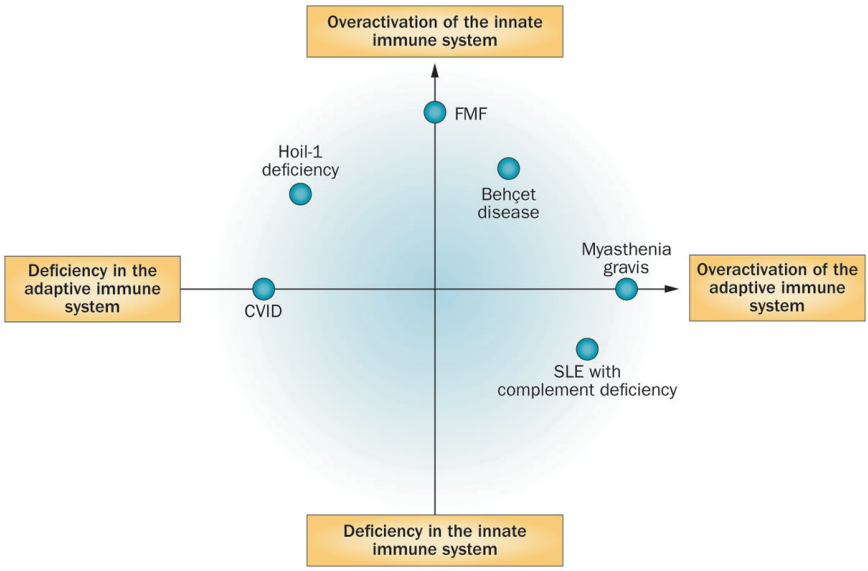

Figure 3. Incorporating dysfunction of the innate and adaptive immune systems in disease classifications. Grateau et al. Nat Rev Rheumatol. 2013;9(10):624-9. With permission.

8.2 The emerging paradigm for defining autoinflammatory conditions today

As outlined above, several definitions of autoinflammation have been launched in close relation to new discoveries and understandings in the autoinflammatory field, with prominent examples being the identification of disease-causing genes in FMF,

TRAPS, and CAPS, as well as the conceptualization of autoimmune and autoinflammatory immunological diseases as a continuum. Today, the circumstances for defining autoinflammatory diseases have changed yet again, thanks to recent insights about the complex network that constitutes our immune system as a whole. We also have a better understanding of the close link between the innate and the adaptive immune system, which has evolved in the context of the ancient innate system. Hence, dysregulation of the innate immune system can rarely be regarded in isolation, because it will generally affect other parts of the immune system, including the adaptive system. Furthermore, a paradigm shift is currently in progress: newly defined monogenic diseases are distorting established disease categories, as they have immunological phenotypes with autoinflammation in combination with immunodeficiency or autoimmunity, or both.

8.2.1 Does dysregulation of innate immunity in "pure monogenic autoinflammatory diseases" have effects on the adaptive immune system?

It can be expected that dysregulation of the innate immune system even in pure monogenic autoinflammatory conditions, for example CAPS, DIRA and FMF, with an increased production of pro-inflammatory cytokines such as IL-1β, IL-18 and IL-6, will also affect the adaptive immune system (44-46). The most prominent example is $CD4^+$ T cell differentiation, as proinflammatory cytokines are important for this process. To what extent dysregulation of innate immunity under these conditions leads to autoimmunity or to other types of immunological diseases needs to be further investigated (11).

8.2.1.1 THERE IS DIFFERENTIATION OF $CD4^+$ CELLS IN CAPS, DIRA AND FMF

Increased expression of pro-inflammatory cytokines in monogenic autoinflammatory diseases such as CAPS, DIRA and FMF is likely to affect $CD4^+$ T cell differentiation. When naive $CD4^+$ T cells differentiate into different subsets, for example Th1, Th2 and Th17, these processes are induced by subset-specific cytokines and transcription factors, mainly generated by antigen-presenting cells and the responding T cells themselves. It is well established that pro-inflammatory cytokines like IL-1β, IL-18 and IL-6 are important for directing these processes towards the different subsets. These subsets are in turn geared to combat particular types of pathogens, but may also contribute to the development of autoimmunity.

The monogenic autoinflammatory diseases CAPS, DIRA and FMF all affect $CD4^+$ T cell differentiation. CAPS and DIRA involve Th17 differentiation while FMF affects both Th17 and Th1 differentiation of CD4+ T cells (31, 47-49). In CAPS, differentiation of CD4+ Th17 cells has been reported in the urticaria-like rash, with increased IL-17 levels in serum, indicating an innate immune regulation of adaptive immune function (47). Decreased IL-17 serum levels and Th17 frequency was observed upon treatment with IL-1 blockade in both humans and mice (47, 50),

indicating a direct coupling between the adaptive and innate system. Patients that suffer from the autosomal recessive disease DIRA, with pustular rash and sterile osteomyelitis, also have increased numbers of CD4+ Th17 cells and show enhanced IL-17 expression in the inflamed skin (31). Data from FMF is conflicting, as some studies support a Th1 differentiation (48, 51, 52), whereas others support a Th17 differentiation (49, 53, 54) of CD4+ T cells.

8.2.1.2 There is no significant identifiable increase in autoantibodies in CAPS, TRAPS or FMF

Autoimmunity can be understood as an organism's immune reaction against its own cells and tissues, mediated by the adaptive immune system. This results in the development of immune reactivity due to loss of tolerance towards endogenous antigens, defined by autoreactive $CD8^+$ T cells or autoantibodies. The Th17 response seen in IL-1 driven autoinflammatory diseases as well as in infections with extracellular bacteria and fungi, is also known to mediate tissue damage in autoimmune disease. In classical monogenic autoinflammatory diseases, polyclonal hyperglobulinemia is reported (11); however, this does not seem to be associated with a loss of tolerance, as all attempts to identify autoantibodies have been unsuccessful until now (11, 55, 56). Neither autoreactive $CD8^+$ T cells has been described in classical monogenic autoinflammatory diseases.

8.2.1.3 FMF, but not CAPS or TRAPS, can be linked to other immunological diseases

The presence of other immunological diseases associated with CAPS, TRAPS and FMF can be discussed with regard to the autoinflammatory–autoimmune disease spectrum as defined by McGonagle and McDermott (8).

In CAPS and TRAPS there are no signs of increased occurrence of manifestations of classical polygenic autoimmune diseases, however, vasculitis has occasionally been described in the CAPS diseases NOMID/CINCA as well as in TRAPS. Neither in FMF has an increased occurrence of classical polygenic autoimmune diseases been observed, including rheumatoid arthritis, although *MEditerranean FEver gene* (*MEFV)* may modify rheumatoid arthritis severity (57-59). A coexistence of FMF and juvenile idiopathic arthritis is very rare but has been described (60, 61). FMF is however associated with the polygenic autoinflammatory disorder *Crohn´s disease* (62, 63), and with the mixed pattern diseases *Behçet´s syndrome* (64, 65) and *ankylosing spondylitis* (66, 67), as well as with vasculitis such as *Henoch–Schönlein purpura* (64, 68-70) and *polyarteritis nodosa* (71). One can speculate whether these associations reflect the complex role that pyrin has in the disease mechanism of FMF, including activation of other pathways than IL-1-dependent pathways, in particular the enhancement of NF-κB activation (72).

8.2.2 'New' monogenic conditions distort the immunological disease categories

As depicted above (8.2), recently defined monogenic conditions have phenotypes that combine autoinflammation with immunodeficiency and/or autoimmunity. Hence, in order to include these complex conditions among the autoinflammatory diseases, an updated definition needs to be formulated. Below, examples will be chosen from such conditions that have a predominant autoinflammatory phenotype over a immunodeficiency phenotype, rather than those dominated by immunodeficiency over autoinflammation, for example *chronic granulomatous disease* (CGD).

8.2.2.1 APLAID AND HOIL-1 COMBINE AUTOINFLAMMATION AND ADAPTIVE IMMUNODEFICIENCY

A few very rare diseases defined by mutations in the innate immune system and leading to a phenotype that *combines* autoinflammation with deficiency in the adaptive immune system have been described recently (73, 74). One such disease is *autoinflammation and PLCγ2-associated antibody deficiency and immune dysregulation* (APLAID), which has a clinical manifestation that combines autoinflammatory features, such as recurrent blistering skin rash, interstitial pneumonitis and ocular inflammation with immunodeficiency, in combination with recurrent sinopulmonary infections due to low concentrations of IgA and IgM. The condition is caused by a dominantly inherited gain-of-function mutation in the *PLCG2* gene that codes for the enzyme phospholipase Cγ2 (PLCγ2) (74, 75). This enzyme is involved in several immunological pathways, both in cells of the innate and adaptive immune system (74-76).

The second condition in this group, *heme-oxidized IRP2 ubiquitin ligase 1 deficiency* (HOIL-1 deficiency), was reported in 2012. This disease has a phenotype that combines autoinflammation with immunodeficiency (73, 77), resulting in recurrent episodes of fever and systemic inflammation, hepatosplenomegaly and lymphadenopathy, as well as severe recurrent bacterial infections due to fewer memory B cells and impaired response to pneumococcal polysaccharides. The disease is caused by a loss-of-function mutation in the *RBCK1* gene coding for HOIL-1, a component of the linear ubiquitination chain assembly complex (LUBAC). Interestingly, the immunological consequence of the mutation is different in different cells: on the one hand, HOIL-1-deficient lymphocytes and fibroblasts show compromised activation of NFκB signalling in response to IL-1β, in keeping with the described immunodeficiency. On the other hand, HOIL-1-deficient monocytes display enhanced sensitivity to IL-1β and produce large amounts of IL-6 and MIP-1α in response, which can explain the autoinflammatory manifestations (78).

8.2.2.2 TYPE I INTERFERONOPATHIES COMBINE AUTOINFLAMMATION WITH AUTOIMMUNITY

The very rare monogenic type I (INF-α and INF-β) interferonopathies comprise a group of diseases with heterogeneous phenotypes that are brought together by

mutations that lead to chronic type I interferon secretion and immunological dysregulation that combine autoinflammation with autoimmunity, although the molecular mechanisms are still to be fully understood (17). It is well known that INFs have a broad immunomodulatory function that enhances antigen presentation in denditric cells, activates T- and B-cells and restrains production of pro-inflammatory cytokines. That these functions are dysregulated in type I interferonopathies could be expected. Surprisingly, however, type I interferonopathies also have an autoinflammatory phenotype, despite the fact that interferon inhibits synthesis of proinflammatory cytokines, including IL-1 (43), which indicates that the autoinflammatory component of the disease is not IL-1 driven. The autoinflammatory phenotype in these diseases is not as pronounced as in IL-1 mediated diseases; fever is not always present, increased CRP is often restricted to severe flares, and disease flares are frequently associated with lymphopenia or leukopenia and not, as for IL-1-mediated diseases, with neutrophilia and increased inflammatory markers intrinsic to flares (43).

A model type I interferonopathy is the *Aicardi–Goutières syndrome 1* (AGS1) that has an early-in-life onset with a clinical picture of autoinflammation of the brain in addition to autoimmune systemic symptoms. In many ways AGS1 mimics congenital viral infections (79).

Another IFN-mediated condition, *chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature* (CANDLE), is caused by a recessive mutation in the *PSMB8* gene, coding for one of the proteasome β subunits, leading to a loss of proteasome function. In addition to the features that are described in the acronym, patients with CANDLE have panniculitis, and hepatosplenomegaly (80). The disease is also referred to as *JMP syndrome, proteasome-associated auto-inflammatory syndrome* or *Nakajo Nishimura syndrome* (81, 82).

8.2.2.3 PLAID COMBINES AUTOINFLAMMATION WITH ADAPTIVE IMMUNODEFICIENCY AND AUTOIMMUNITY

Another very rare disease, *PLCγ2-associated antibody deficiency and immune dysregulation* (PLAID), is caused by a different dominant mutation in the *PLCG2* gene than the one causing APLAID (74). The clinical features of PLAID are even more intriguing than in APLAID, as the phenotype is associated with autoinflammation (cold urticaria) in combination with immunodeficiency (*common variable immunodeficiency*; CVID) *and autoimmunity* (thyroiditis and antinuclear antibodies).

8.2.3 Organising monogenic autoinflammatory conditions according to mode of innate immune dysregulation

Today, there is a broad spectrum of monogenic autoinflammatory conditions that can be classified according to different principles (5, 10, 83, 84). Firstly, they can be organized according to clinical features: 1) periodic fever diseases (e.g. FMF, MKD, CAPS and TRAPS), 2) diseases with pyogenic lesions (e.g. DIRA and PAPA), 3)

diseases with granulomatous lesions (e.g. Blau syndrome), 4) diseases with psoriasis (e.g. *deficiency of IL-36 receptor antagonist*; DITRA), 5) autoinflammatory bone disorders (e.g. Majeed syndrome), 6) diseases with panniculitis-induced lipodystrophy (e.g. CANDLE) and 7) others (e.g. APLAID) (83).

Recently it has been proposed that monogenic autoinflammatory diseases also can be classified according to pathogenic mechanisms (Figure 4), in other words, in terms of how the molecules of innate immunity are dysregulated: 1) intracellular sensor function defects, as in CAPS (IL-1β), FMF (IL-1β), AGS7 (IFN type 1) and *CARD14-mediated psoriasis;* CAMPS (Nf-κB); 2) accumulation of intracellular triggers that cause cell stress and activation of intracellular sensors, as in TRAPS (IL-1 and others), CANDLE (IFN type 1), AGS1 (IFN type 1) and Majeed syndrome (IL-1); 3) loss of a negative regulator of inflammation, as in DIRA (IL-1), DITRA (IL-36), and EO-IBD (IL-10); and 4) effects on signalling molecules that upregulate innate immune cell function as in APLAID (IL-1 and others) (5).

Alternatively the diseases can be classified according to the defining cytokine, for example IL-1 (CAPS, FMF, Majeed syndrome, DIRA), IL-1 and others (TRAPS), NF-κB (CAMPS), type I interferons (AGS1, AGS7, CANDLE), IL-36 (DITRA) or IL-10 (EO-IBD) (5).

In clinical practice, classification according to clinical features is necessary in order to facilitate the diagnostic process, as the autoinflammatory disease spectrum gets more and more complex. However, for increased understanding of patho-physiology and identification of specific treatments for these complex conditions, it is on the other hand necessary to organize the conditions according to several modalities including disease mechanisms, defining pathways and cells (cytokines), as well as the genetic background. In addition, immunological diseases could be organized according to how they pertain to and combine different types of immunological disease modalities, such as autoinflammation, autoimmunity and immune deficiency.

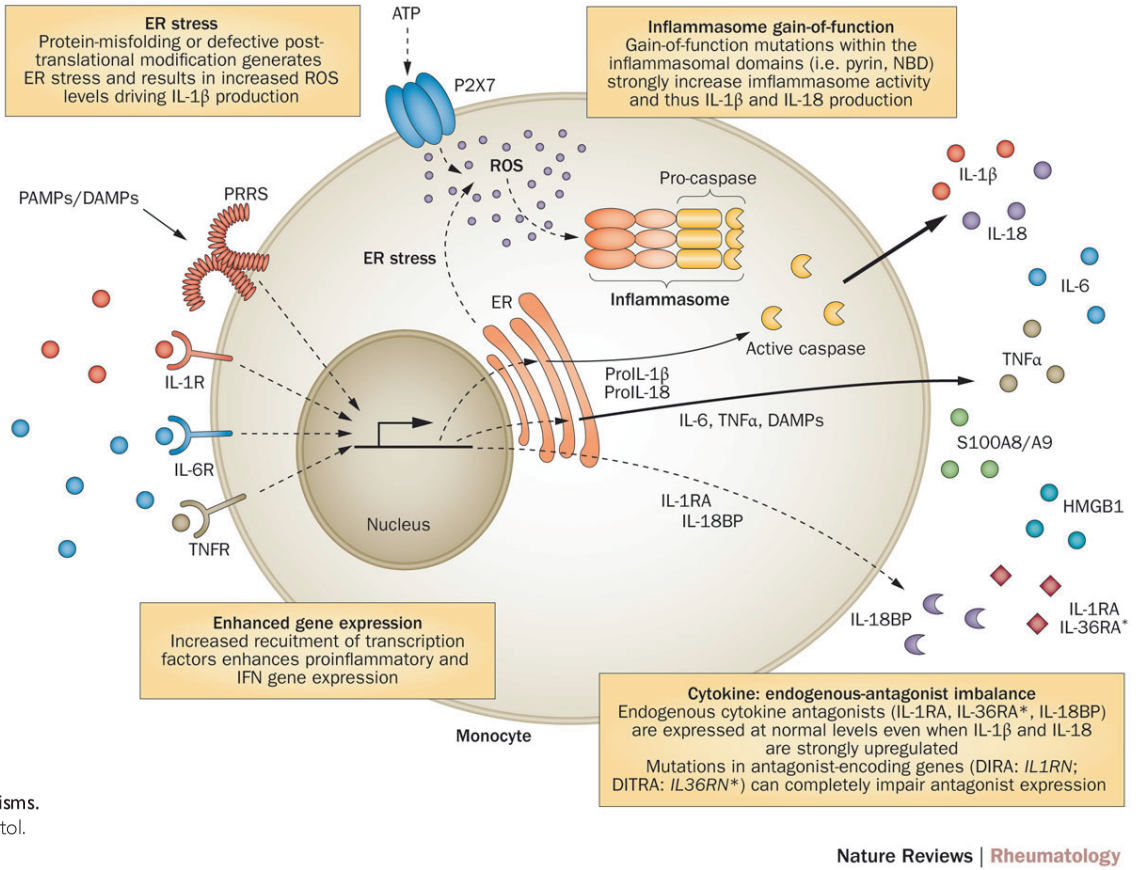

Figure 4. Key principles of autoinflammatory pathomechanisms. Holzinger et a.. Nat Rev Rheumatol. 2015. In press. With permission.

8.3 A proposed modified definition

As described above, the prerequisites for a definition of autoinflammatory conditions have changed considerably since the term was first coined almost twenty years ago. This pertains firstly to the discovery that polygenic and mixed pattern diseases have both an autoinflammatory and autoimmune component. Secondly, there is an increased understanding of the close connection between innate and adaptive immunity, and the fact that monogenic autoinflammatory conditions frequently activate adaptive immunity. Finally, recently defined monogenic autoinflammatory conditions have phenotypes that combine autoinflammation with immunodeficiency or autoimmunity.

Hence, a relevant definition of autoinflammatory diseases needs to reflect this complexity and place autoinflammation and innate dysregulation in the context of an immunological network in order for the definition to be complete, congruent and precise. A definition needs to embrace the fact that autoinflammatory conditions can be associated with dysfunctions of other parts of the immune system and allow for specific characterization of the respective immune dysfunction. The above analysis has led me to propose a modified definition of autoinflammatory diseases:

> *Autoinflammatory diseases are immunological diseases defined by increased inflammation (phenotype), driven by dysregulation of molecules and cells of the innate immune system (mechanism) with a host predisposition (genetic: monogenic, polygenic, epigenic or acquired) as necessary and sufficient criteria, with frequent activation of the adaptive immune system and a possible association with other immune dysfunctions (autoimmunity or immunodeficiency*).
>
> MODIFIED FROM KASTNER (11)

The above definition will form the structure for the continuation of this thesis when discussing the autoinflammatory conditions FMF, PFAPA and SAPHO with respect to phenotype, mechanism, host predisposition and activation of the adaptive immune system.

9. Clinical phenotypes with regard to increased inflammation

Sabina's appendix was normal during laparoscopy. She continues to have attacks of fever with severe abdominal pain or chest pain, lasting for approximately two days, with a marked increase in C-reactive protein. Sabina is diagnosed with FMF, which was suspected by the paediatrician given that Sabina's parents are from Lebanon. Sabina improves after initiation of daily treatment with colchicine.

In the year following his visit to the emergency room, William develops febrile episodes lasting for four to five days every fourth week. The attacks are associated with pharyngitis, tonsillitis and lymphadenitis. At the age of four, William is diagnosed with PFAPA. By this time, he has been treated for urinary tract infection, streptococcal infections, and pneumonia. He is referred for a tonsillectomy review by an otolaryngologist.

After Johan is admitted to the ward, he develops pain from multiple sites in addition to his right tibia, including the left clavicle, sternum and sacrum. He is treated with intravenous antibiotics upon suspicion of bacterial osteomyelitis. Due to severe acne, he is referred for consultation with a dermatologist, who proposes the diagnosis of SAPHO. When the MRI shows lesions compatible with osteomyelitis and the cultures from the biopsy are negative, the diagnosis of SAPHO is established. Antibiotics are discontinued and non-steroidal anti-inflammatory drugs are introduced.

9.1 FMF - familial Mediterranean fever

Before the efficacy of colchicine treatment for FMF was discovered in 1972, the disease caused enormous suffering, developed into amyloidosis and led to early death for many patients (85, 86). The childhood onset, the severity of the attacks and the often grave natural course is important to bear in mind when healthcare is planned and provided in Sweden for children whose parents originate from countries where FMF is a common disease (paper I) (83, 87).

9.1.1 Clinical Manifestations

The FMF phenotype is characterized by recurrent febrile attacks with duration of between 12 hours and three days. It is associated with serositis, predominantly peritonitis, pleuritis and arthritis, with a frequency of 92%, 22% and 11%, respectively, in a Swedish cohort (paper I). Severe abdominal pain is caused by peritonitis that often mimics appendicitis and, indeed, a significant proportion of patients have undergone laparotomy before they are diagnosed with FMF (85, 88-90). The pleuritic chest pain is almost always unilateral, but the pain can shift side between attacks. It is not uncommon that the pain during an attack starts in the chest and subsequently moves into the abdomen (85). Arthritis primarily engages large joints of the lower limbs (hips, knees and ankles) and may last longer than the febrile episode (91). The differential diagnosis is mainly septic arthritis, for which arthritis caused by FMF is often mistaken (91, 92).

Rarely, patients with FMF have attacks that are associated with pericarditis and orchitis (93-97). No pericarditis was reported in the cohort in paper I.

A typical feature of FMF is the erysipelas-like erythaema that consists of a tender plaque with sharply demarcated advancing borders, usually located on the dorsum of the foot or in the ankle region (98, 99). In paper I, skin rash was reported in 5% of the patients, probably erysipelas-like erythaema in most cases.

In children, the intensity of the abdominal pain can vary, from mild abortive attacks to severe peritonitis; the inflammatory reaction also slows peristalsis and constipation is not uncommon (85, 100). Below the age of two, the disease can have an onset without the specific features that would suggest that the child has FMF; this underlines the difficulties of diagnosing FMF in very young children, especially if they are heterozygous for disease-causing mutations in the *MEFV* gene (see below) (101-103).

During an attack there is a significant rise in inflammatory markers such as neutrophilia as well as increased CRP and serum amyloid A protein (SAA). Subclinical inflammation, for which increased SAA is a sensitive marker, is common between attacks in untreated patients, most likely contributing to the risk of developing amyloidosis (see below) (104).

Between attacks, children with FMF are often well, but exertional leg pain is quite common. Exertional leg pain is not prevented by colchicine prophylaxis but can be treated with non-steroidal anti-inflammatory drugs (NSAID) (105). Another manifestation of FMF that occurs independently of the attacks is prolonged febrile myalgia. This disorder presents with low-grade fever and myalgia that lasts for several weeks (106-108). The symptoms are probably caused by a vasculitis that is steroid-responsive (106). For other long-term chronic conditions associated with FMF, the reader is directed to the introduction of this thesis (section 8.2.1.3).

The most serious complication of FMF is amyloid A amyloidosis (AA amyloidosis) that most often presents with renal amyloidosis first indicated by proteinuria. Renal AA amyloidosis in FMF is associated with a significant long-term risk of renal failure. The precursor of the amyloid deposit in FMF is the inflammatory marker SAA, which rises in concentration during attacks of FMF. In the attack-free periods, SAA decreases but often remains elevated. To our knowledge, none of the patients among the Swedish cohort in paper I had amyloidosis. Although rare, patients without symptoms can present with amyloidosis before the onset of attacks or with amyloidosis as the only disease manifestation (85, 109).

9.1.2 Epidemiology

FMF is the most common monogenic autoinflammatory condition in the world (83, 87). It is particularly common in individuals with an origin in the eastern Mediterranean Basin, such as Turks, Arabs, Armenians and Jews. In these populations the prevalence is as high as 100–200 per 100 000. This high prevalence is relevant for countries that receive large number of immigrants from these regions, for example Germany (110) and Sweden (paper I). In the Swedish cohort reported in paper I, the prevalence was at the same level as in the country of origin; the prevalence among Swedish inhabitants of Turkish origin was 173 per 100 000, among those of Lebanese origin it was 124 per 100 000 and among those of Syrian origin 86 per 100 000. FMF is however not limited to individuals from the eastern Mediterranean region, and an increasing number of patients from other parts of the world, including Greece, Italy, Japan, India, China and the United Kingdom, have been diagnosed (111-115). In the cohort described in paper I, none of the patients originated from Sweden, which was expected.

9.1.3 Diagnosis

In 1997, Livneh and co-workers proposed an update of the classical Tel Hashomer diagnostic criteria for FMF, including the simplified Tel Hashomer criteria (Figure 5) (116).

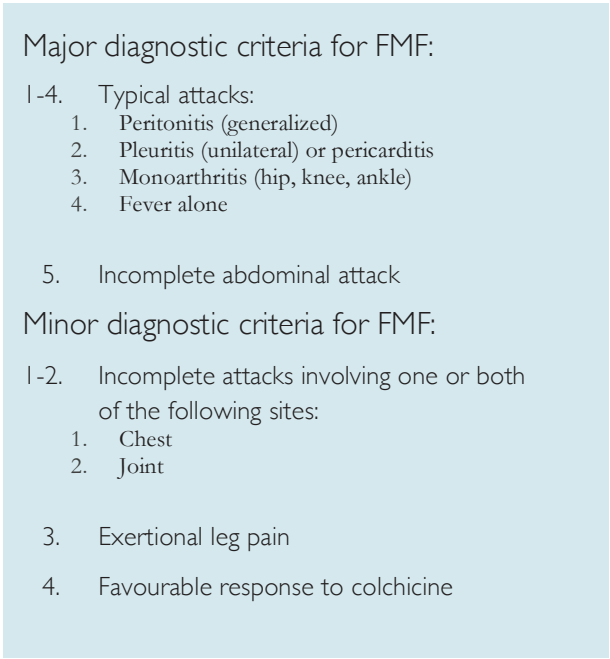

Figure 5. Simplified Tel Hashomer criteria for familial Mediterranean fever (FMF). The requirements for a diagnosis are to fulfil at least one of the major criteria or at least two of the minor criteria. Typical attacks are defined as recurrent (at least three of the same type), febrile (≥ 38°C) and short (lasting between 12 hours and three days). Livneh et al. Arthritis Rheum. 1997;40(10):1879-85.

Approximately 90% of patients with FMF have onset during childhood, as described in a multicentre study from Turkey, where the mean age of onset ± 1 SD was 9.6 ± 8.6 years and the mean age of diagnosis ± 1 SD was 16.4 ± 11.6 years (93). In our cohort from Sweden, the median age of onset was four years (range three months to 37 years) and the median age of diagnosis was 10 years (range 2–44 years) (paper I). Despite the fact that almost all patients with FMF have an onset during childhood, it took until 2009 before anyone proposed specific diagnostic criteria for children (83, 85, 117). At least two of the following characteristics are required for diagnosis: fever (lasting 6–72 h, three or more attacks), abdominal pain (lasting 6–72 h, three or more attacks), chest pain (lasting 6–72 h, three or more attacks, unilateral), arthritis (lasting 6–72 h, three or more attacks, monoarthritis), exertional leg pain and family history of FMF (117). In a Turkish population, the fulfilment of two or more of the five characteristics gave a diagnosis of FMF with a

sensitivity of 86% and a specificity of 94% (117). In a mixed French cohort, two of the characteristics did not improve the diagnostic process compared to the Tel Hashomer criteria (118). If three characteristics in the criteria for children were used instead of two, the same study found a sensitivity of 77% and specificity of 95% (118).

It is often claimed that FMF is a clinical diagnosis because the genetic analyses can only confirm the disease but not exclude it entirely (paper I) (83). In establishing a clinical diagnosis, the above diagnostic criteria can support the clinician in this decision (116, 117). A clinical diagnosis of FMF is not always possible, which is highlighted by the very young children with febrile episodes as the only manifestation of FMF (101-103). At this very young age the only way to progress towards a FMF diagnoses, apart from expectation, is by genetic testing. Furthermore, many clinicians and parents feel a need to confirm a clinical diagnosis with genetic analyses (83). In the globalized world of today, many paediatricians care for children with FMF and other periodic fever syndromes in multi-ethnic populations as described in paper I, which is an even larger challenge. In such contexts, it becomes clear that it is often a difficult task to diagnose PFAPA in children from populations with high prevalence of FMF and to diagnose FMF in children from populations with low prevalence of FMF. Under such circumstances the paediatrician needs to combine all diagnostic modalities to provide the best possible care for children with periodic fever syndrome. This requires a clinical workup that includes a meticulous history, clinical examination during episodes, evaluation of inflammatory markers in and out of episodes, a fever diary, genetic testing when indicated and in some cases a trial of colchicine for suspected FMF and corticosteroids for suspected PFAPA. In applying such a diagnostic workup, the clinician needs to be aware of the shortcomings and merits of each of these diagnostic methods.

9.1.4 Management

As discovered by Goldfinger in 1972, colchicine is an effective treatment for FMF that dramatically changes the life of affected patients by substantially decreasing the risk of amyloidosis, prolonging life expectancy and improving quality of life (86, 120, 121). The aim of colchicine treatment is to reduce the severity and number of attacks, control inflammation in the attack-free periods and prevent amyloidosis.

In addition to preventing attacks, colchicine treatment aims at normalizing the serum levels of SAA between attacks (83, 104). A threshold SAA level, below which there is no long-term risk for amyloidosis, has never been established, but prophylactic colchicine treatment efficiently prevents amyloidosis in almost all compliant patients (121, 122). In addition to colchicine treatment and compliance, the risk of developing amyloidosis is associated with the *MEFV* mutation M694V/M694V (109, 123, 124) (section 10.1), country of residence (88, 125, 126)and SAA1 alpha/alpha genotype (93, 127-129).

In cases of poor response to colchicine treatment, non-compliance needs to be considered (83, 87). Although it is rare that patients do not respond to colchicine or cannot tolerate the medication, such patients can effectively be treated with IL-1 blockade (83, 130-132).

9.1.5 Prognosis

Today, when colchicine treatment is available for patients with FMF in most parts of the world, life expectancy is comparable to the general population and the risk of amyloidosis is confined to non-compliant patients (133, 134). Neither FMF nor colchicine treatment is associated with an increased risk of malignancy or cardiovascular disease (134, 135).

9.2 PFAPA– periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis

Children with PFAPA are identified by the stereotypical attacks of fever associated with signs and symptoms in the throat in the absence of upper respiratory tract infections. In contrast to FMF, PFAPA is not considered a lifelong disease or associated with amyloidosis or other long-term complications. Marshall and co-workers established the acronym PFAPA in 1989, two years after first having described the syndrome (136, 137).

9.2.1 Clinical Manifestations

Patients who suffer from PFAPA have recurrent attacks of fever and symptoms associated with the signs forming the disease acronym: aphthous stomatitis, pharyngitis and adenitis in the absence of upper respiratory tract infection. Between the episodes, children with PFAPA are healthy, with normal growth and development as delineated in the classical diagnostic criteria (Figure 6) (138).

The duration of the fever attacks is usually three to seven days (most commonly four to five days), with an interval of two to eight weeks (most commonly three to six weeks) between the attacks (137-141). At some stage of the disease, the episodes characteristically occur with a regular interval, "sometimes to a point that the parents can predict the time for an episode and inform their employer" (142). The regularity may disappear over time and the child then experiences longer intervals, and shorter or milder episodes (or both) (83). According to our clinical experience, children with PFAPA have fewer viral infections than other children as long as their PFAPA is active, but the viral infections seem to re-emerge as the child grows out of the episodes (unpublished observation).

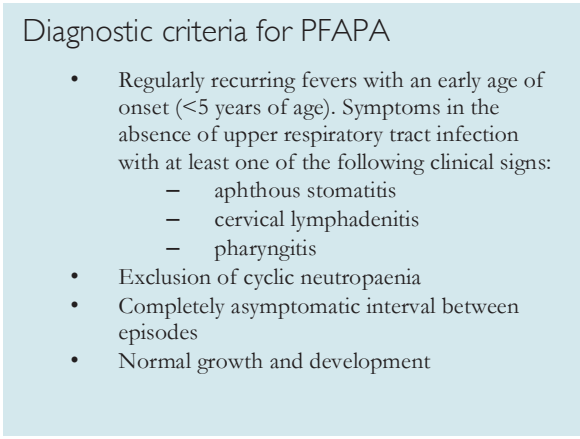

Figure 6. Classical diagnostic criteria for PFAPA as defined by Thomas and co-workers. Thomas et al. The Journal of Pediatrics. 1999;135(1):15-21.

In addition to the signs and symptoms included in the classical criteria, children with PFAPA often have mild stomach ache, leg pain, as well as nausea and vomiting during episodes (138, 140). The spectrum of additional symptoms was even more striking in a recent study by Hofer and co-workers, that rightly stressed the importance of establishing new disease criteria with better specificity (143). Even though children with PFAPA by definition are free of symptoms between episodes, aphthous stomatitis is not confined to the episodes and a few children with very frequent attacks do not recover completely between attacks (144).

Inflammatory variables are significantly increased during attacks, but both CRP and SAA normalize between episodes (paper II and III), the latter in contrast to children with FMF.

9.2.2 Diagnosis

The diagnosis of PFAPA is still largely based on recognition of the clinical features delineated in the classical PFAPA criteria (Figure 6) (138). These criteria are not totally exclusive to other conditions, including hereditary (monogenic) periodic fevers (HPFs) and need to be refined to improve specificity in particular but also sensitivity (145). Until a "gold standard" has been defined, for example in terms of genetic predisposition or disease mechanism, it is hard to see how the validity of different sets of clinical criteria can be accurately evaluated. Gattorno and co-workers have proposed a diagnostic score with the aim of predicting the likelihood that a child with PFAPA-like symptoms could instead have a hereditary periodic fever syndrome (146). It is important to remember that the accuracy of such prediction will always depend on the prevalence of relevant conditions in the

population, for example the prevalence of PFAPA and FMF in Sweden. In addition, it is not clear how somatic mosaicism of monogenic autoinflammatory disorders will influence the validity of the diagnostic score developed by Gattorno.

The shortcomings of the diagnostic criteria with regard to specificity influences not only the clinical situation but also affects PFAPA research, as researchers may include PFAPA patients that differ in clinical specification from one cohort to another. In paper II and III of this thesis, we tried to mitigate some of the inadequacies of the classical criteria by attempting to define a cohort of children with "typical PFAPA"; our definition excluded children with the additional symptoms that may suggest HPFs: skin rash, arthritis, severe abdominal pain, diarrhoea, thoracic pain and splenomegaly, fever episodes longer than seven days, a history of hearing loss or symptoms secondary to cold.

Included in the classical criteria is the exclusion of the very rare disease cyclic neutropenia. In cyclic neutropenia, the blood neutrophils typically oscillate with a 21-day interval. On occasion, cyclic neutropenia cannot be excluded on clinical grounds as the nadir of neutrophil count may occur before the onset fever. In such cases, analysing the neutrophil elastase gene (*ELANE*) or repeated neutrophil counts three times a week for six weeks can confirm the diagnosis, and thus differentiate it from PFAPA (147). Recurrent infections also need to be considered as a differential diagnosis, including repeated streptococcal infections or urinary tract infections as well as viral infections that involve the throat and cause increased inflammatory markers, for example adenovirus. Over time, however, several characteristics of PFAPA will exclude recurrent infections as the aetiology of the child's symptoms, in particular, the often clockwork periodicity, presence of aphthous stomatitis, lack of response to antibiotics, distinct response to corticosteroids and the absence of spread of the infection among other family members, as well as negative culture when appropriate.

PFAPA also needs to be distinguished from monogenic periodic fever syndromes such as FMF, MKD, and TRAPS, and the diagnosis of PFAPA should be challenged in children who fulfil the criteria but show additional signs and symptoms suggestive of HPFs as discussed above (119, 146, 148). For this purpose, the above-mentioned diagnostic score could be useful (146). The diagnostic workup for PFAPA can be particularly challenging in children with an origin in a population with a high prevalence of FMF, as discussed above. Another HPF to bear in mind is MKD, also called HIDS (*hyperimmunoglobulinaemia D with periodic fever syndrome*). During the last 20 years, no MKD diagnosis has been established in western Sweden in children of Swedish origin (unpublished data) and MKD may not exist at all in Sweden. In this context, it is important to note that hyper-IgD is not a specific indicator for HIDS, as increased IgD concentrations are also seen in other inflammatory conditions, including autoinflammatory diseases such as FMF, TRAPS and PFAPA (139, 149, 150). Instead, increased excretion of mevalonate acid *during* an inflammatory episode is a valid screening test for MKD, provided that the laboratory can detect very low concentrations of the substance in urine. If indicated, MKD is diagnosed by the identification of a mutation in the *MVK* gene.

9.2.3 Epidemiology

In early studies it was estimated that no more than one case of PFAPA would be diagnosed during an entire paediatric career. Today, PFAPA is diagnosed at a higher frequency. The syndrome is also recognized in older children and even in adults (83, 151, 152). Although PFAPA has been described in many parts of the world, the only report of incidence is from Norway, where it was estimated to an annual incidence of 2.3 per 10 000 children up to five years of age (141). In western Sweden, approximately 300 children were given a definite or probable diagnosis of PFAPA during a 10-year period (unpublished data). This corresponds to an annual incidence of at least 3 per 10 000 children below the age of five years, which is similar to the estimation from Norway. A predominance of boys has been described in several PFAPA cohorts, most pronounced, at 70%, in the Forsvall cohort (141, 143, 153).

9.2.4 Management

NSAIDs are more efficient than paracetamol for reducing fever during PFAPA episodes (154). Corticosteroids abort an attack with such efficiency, usually giving fever resolution within hours, that you can question the PFAPA diagnosis if the effect fails to materialize (138, 140, 144, 153, 155). For unknown reasons, a significant proportion of children experience a shortening of the intervals after treatment with corticosteroids (140, 151, 153).

In western Sweden, steroid treatment is primarily used to postpone a febrile episode that occurs at an unsuitable time for the child, whereas in some parts of the world the chosen approach is to treat each episode with corticosteroids (156). Tonsillectomy has turned out to be the most attractive treatment alternative, with a resolution of the episodes in 80-90% of cases in the initial case series (157). This was confirmed in a limited randomized control trial (158) and in meta-analyses (159). Since the treatment with tonsillectomy is based on clinical experience and so far no pathophysiological ground has been disclosed, it is important to carefully evaluate, in discussion with the parents, the risk–benefit balance for each child, bearing in mind the age of the child as well as the length, intensity and frequency of the episodes, and the likely time to resolution without treatment.

Cimetidine, a histamine type 2 receptor antagonist, has previously been reported to induce remission (138), but a recent report found it to be ineffective in the majority of patients (156). Colchicine (the first choice of treatment for FMF) has been evaluated in a few patients with PFAPA, resulting in an increased interval between the episodes, but this needs to be further investigated (160). We tend to use colchicine in children with "atypical" PFAPA (that is, children who have additional symptoms but are not suffering from HPF) or in children who did not improve following tonsillectomy. In our experience, both often have a similar atypical phenotype. One small case series indicates that PFAPA flares are responsive to IL-1 blockade, an approach that has to be further evaluated (161).

9.2.5 Prognosis

Children with PFAPA are usually healthy between the attacks, and the symptoms disappear within three to five years after disease onset or in adolescence (137-139, 154, 155). It is important to note that remissions are well described in PFAPA and we have seen late relapsing of episodes after several years in our clinic (140) (unpublished observation). Despite the good prognosis, our clinical experience and preliminary data from parental interviews indicate that the disease considerably influences the quality of life of the child and the situation of the family as a whole as long as the episodes persist (manuscript in preparation).

9.3 SAPHO syndrome - an autoinflammatory bone disorder

Autoinflammatory bone disorders are characterized by dysregulation of innate immunity that causes inflammation in typically sterile bone (162-164). Today, there are several genetically defined autoinflammatory bone disorders, e.g., *deficiency of interleukin-1 receptor antagonist* (DIRA, affected gene *IL1RN*), *Majeed syndrome* (affected gene *LPIN2*) and *cherubism* (affected gene *SH3BP2*) (4, 31, 164-168).

Other autoinflammatory bone disorders are most probably polygenetic and thus defined by clinical phenotype or clinical criteria, for example, *chronic non-bacterial osteomyelitis* (CNO), *non-bacterial osteitis* (NBO), *chronic recurrent multifocal osteomyelitis* (CRMO), and *SAPHO* (4, 162, 163, 168). These conditions lack genetic and functional definitions and are not mutually exclusive; for this and other reasons they have obvious conceptual shortcomings (164, 168, 169).

9.3.1 Diagnosis

Chamot proposed the notion of SAPHO syndrome in 1987 (170). The term is more often used in adults than in children, where CRMO is more frequently used (164, 171). In 1994, Kahn suggested three diagnostic criteria for SAPHO syndrome: (i) chronic recurrent multifocal osteomyelitis with or without skin manifestation, (ii) acute or chronic sterile arthritis associated with either pustular psoriasis or palmoplantar pustulosis, or severe acne and (iii) sterile osteitis in the presence of one of the skin manifestations (172). According to Kahn, one of these three criteria is sufficient to diagnose SAPHO. In Paper IV we used the criteria proposed by Khan in a narrow sense, by only including patients with both skin symptoms (acne or psoriasis) and bone lesions, the two distinct clinical feature of SAPHO, corresponding to criterion (iii). The notion of SAPHO as a disease of both skin and bone is in keeping with a recent review (164).

9.3.2 Clinical Manifestations

The disease course in SAPHO varies considerably, from being a monophasic disease to showing relapsing–remitting features or taking a chronic, non-relapsing course (171, 173). Inflammatory markers, including CRP and ESR, are not always but often increased, and then to very variable degrees (164, 173, 174), but increased inflammatory markers at the onset of disease has been associated with a prolonged disease course (173). Interestingly, we have indications that the acute phase reactant SAA, often thought to parallel CRP fluctuations and therefore not evaluated very frequently, may in fact be a more sensitive marker of inflammation than CRP in SAPHO (paper IV), as for FMF between attacks.

The bone lesions in SAPHO represent a clinical spectrum from self-limiting single or oligolesions to chronic lesion(s) or recurrent multifocal osteomyelitis, as in the description of patient A-D in paper IV (171, 173). Typical locations are the clavicle, anterior chest wall, vertebrae, and pelvis, exemplified in paper IV (171, 172, 175). The histopathology of the lesions mimics infectious osteomyelitis but is typically sterile (175, 176), that is, bacteria are isolated in a considerable proportion of patients, but are referred to as contaminations (or possible triggers; see below).

Dermatologic manifestations in SAPHO are characterized by neutrophil dermatosis, namely, severe acne, palmoplantar pustulosis and psoriasis (164, 170). Of patients with neutrophil dermatosis, up to 60% have palmoplantar pustulosis and around 25% have acne conglobata or acne fulminans, the latter with a male predominance, exemplified in paper IV (177). Onset of the skin lesions can occur simultaneously with the osteomyelitis, or before and after initiation of bone engagement.

9.3.3 Epidemiology

The incidence and prevalence of SAPHO is not known. The syndrome is more often described in adults than in children and a female predominance has been suggested in recent studies (164, 171, 177).

9.3.4 Management

The first-line of treatment for SAPHO is NSAIDs. SAPHO is also treated with corticosteroids, methotrexate (MTX), TNF-α antagonists, bisphosphonates and IL-1 blockade (164, 178, 179). In our small cohort (paper IV), this range of treatments is demonstrated; patient A was treated with NSAIDs only, patient B with NSAID, prednisolone and MTX, patient C with NSAID and MTX, and patient D with NSAID, MTX and infliximab. Initially, a significant proportion of patients with SAPHO receive antibiotics before bacterial osteomyelitis has been ruled out. This is exemplified by the NBO cohort investigated by Jansson et al., in which 74% of all patients received antibiotic, compared to 44% in a cohort from western Sweden (180) (unpublished data). This is also illustrated by patient A and B in paper IV.

9.3.5 Prognosis

Despite the lack of clear case definition and due to the variable courses that SAPHO can take, most researchers seem comfortable with the view that SAPHO in general has a favourable prognosis and treatability, and with disabling complications being very rare (172, 174, 177).

10. Host predisposition as a necessary and sufficient cause (genetics)

Sabina's genetic analysis reveals only one mutation instead of the two expected in an autosomal recessive disease. Sabina and her parents become agitated and question the diagnosis of FMF, a lifelong heritable disease that is unheard of among their relatives.

William's maternal grandmother recalls that William's mother had similar febrile episodes during her preschool years. The parents therefore wonder whether PFAPA is in fact a heritable condition, in contrast to what several physicians have told them.

Johan's uncle and aunt have psoriasis and the parents ask whether that could have contributed to Johan developing SAPHO.

10.1 Autosomal recessive mutations in *MEFV* cause FMF

FMF is regarded as an autosomal recessive inherited disease that is caused by mutations in the *MEFV* gene (OMIM*608107), identified in 1997 on chromosome 16p13 by positional cloning (13, 14). The gene consists of 10 exons, with the greater part of the disease-associated mutations residing on exon 10, which encodes the B30.2 domain of pyrin. (for functional implications, see chapter 11)

The two research groups that identified the *MEFV* gene in 1997 described four FMF associated mutations: M680I, M694V, V726A and M694I (13, 14). These are still the most common disease-associated mutations in populations with a significant FMF prevalence (181). Today, approximately 300 *MEFV* variants have been identified, of which 80–90 are associated with an FMF phenotype, leaving 200 variants with unknown significance or to be regarded as polymorphisms occurring in more than 1% of the general population, the latter including E148Q and P369S, both screened for in paper I (182). A single variant of these polymorphisms does not usually present with an FMF phenotype and they are most plausibly regarded as variants that indicate an increased susceptibility to inflammation (181, 182).

When patients with clinical FMF are screened for the most common mutations, approximately two thirds are homozygous or compound heterozygous, that is, they

have two disease-causing mutations, as expected for an autosomal recessive disease (183). This is in keeping with paper I, where two FMF-associated mutations were found in 75% of the patients screened for 10 mutations (F479L, M680I (G/C), M680I (G/A), I692del, M694V, M694I, K695R, V726A, A744S, R761H) and two polymorphisms (E148Q, P369S). Hence, a significant proportion (≈ 25%) of patients with clinical FMF either carry a heterozygous mutation or lack mutations entirely, a fact that puzzles the scientific community (181). The simplest explanation is that the screening procedure has missed the second mutation. However, this has *not* been verified by several studies that used advanced techniques, including sequencing of the entire *MEFV* gene and its promoter region (184, 185). Another possible explanation is that a second somatic mosaic mutation contributes to the disease expression among "heterozygotes". In mutation-negative patients it has been suggested that a genetic defect upstream or downstream in the pyrin pathway could explain the FMF phenotype (186). Two studies indirectly indicated that other genes may be involved (187, 188).

Other reports question the claim that FMF is always an autosomal recessive disease. Several studies in fact suggest an autosomal *dominant* trait (189-192), for example, in families of British origin, patients with FMF either had one mutation alone (*ΔM694*) or a compound variant with two mutations on one allele (*E148Q/M694I*)(192).

The interpretation of genetic analyses in FMF and hereditary recurrent fevers is complex and guidelines for these purposes have been developed (193). Today, there is a need to develop recommendations that combine clinical and genetic diagnosis (182). An approach that needs to be further evaluated is the one proposed by Shinar et al. and adapted by Ozen, which recommends investigating whether compound heterozygous mutations that are known to occur on the same allele are located on different alleles in the patient (83, 193). This would prove that the finding is in keeping with compound heterozygosity and not a consequence of a complex allele (83, 193). The same recommendation holds in the case of one pathogenic and one uncertain mutation (83, 193).

It has been suggested that the only possible explanation for the high frequency of *MEFV* mutations in certain populations is that heterozygous carriers have a survival advantage, possibly in the form of an increased resistance to an undetermined infection. In addition, country of residence influences the severity of FMF, that is, patients from the Eastern Mediterranean region have a milder disease phenotype once they migrate to Europe, suggesting that environmental factors may be at play in disease expression in general and in heterozygous disease expression in particular (194). A recent study by Xu et al. suggested that pyrin is activated by pathogen-mediated modifications of host proteins, which may be a key to this undetermined infection and to the fact that country of residence is important for the disease expression (195).

10.2 Indications that PFAPA is a polygenic disease or a mixed pattern disease

PFAPA was regarded as a non-hereditary condition for a long time (139). However, many parents related their experience that they had a similar history of symptoms themselves or among other relatives. Recent data show that a heritage-positive family history is reported by 10% in the Eurofever Registry and by 27% in a large cohort of PFAPA patients (115, 143). In a study by Cochard et al., 45% of patients had a family history that was positive for recurrent fever and in 12% of these families a physician confirmed a history of PFAPA (196). Today, due to the familial clustering, PFAPA is most adequately described as a polygenic disease with a predominantly non-Mendelian inheritance. Nevertheless, as long as no disease-associated genes have been established (see below), proper studies of familial frequency have to be postponed until children with a reliable PFAPA diagnosis have children and grandchildren of their own.

Mutations in classical monogenic periodic fever syndromes (in the *MEFV, TNFRSF1A, CARD15/NOD2* and *NLRP3* genes) have been analysed in cohorts of PFAPA patients, without finding a higher frequency of these mutations than in the general population (197, 198). The R92Q mutation in the *TNFRSF1A* gene is regarded as a polymorphism and, when disease is present, the phenotype is associated with a high rate of spontaneous resolution of the recurrent fever episodes, similar to what is seen in PFAPA rather than the usual progression in TRAPS (199). In a study from 2013, 20% of children with PFAPA were shown to have a polymorphism in the *NLRP3* gene. When all identified variants (R488, V198M and Q703K) were taken into account, the frequency was significantly higher than expected, suggesting that the NLRP3 inflammasome might in fact be involved in PFAPA (200). In contrast, a recent follow-up study did not find the V198M mutation in *NLRP3* to be associated with PFAPA (198). The same study screened genes involved in other known autoinflammatory syndromes and genes that encode components of inflammasomes, but was unable to identify any single variant that on its own could explain the pathogenesis of PFAPA (198).

In conclusion, with regard to the familial clustering with a predominantly non-Mendelian inheritance, PFAPA is best regarded as a polygenic disease, although an environmental factor is difficult to completely rule out, taking into account the often favourable outcome of tonsillectomy (83, 198, 201) (appendix).

10.3 Genetic background to SAPHO

So far, no gene has been associated with SAPHO, and familial occurrence of the syndrome is rare (172, 173). In a cohort of 38 patients, in which 89% had skin lesions, there was no association of SAPHO with either *PSTPIP2* (a gene that causes inflammatory bone disease in a murine model), *NOD2/CARD15* (a gene

that occurs in Crohn's disease) or *LPIN2* (a gene that causes Majeed syndrome, a very rare autosomal recessive disorder with early-onset recurrent non-infectious osteomyelitis) (202). Another study examined the immunogenetic resemblances between psoriatic arthritis and SAPHO, but did not find any associations between SAPHO and HLA-Cw6, HLA-B27, or HLA-DR antigens (203). A gene that so far has not been investigated in SAPHO is the disease-causing gene *ILRN* in DIRA, which is characterized by systemic inflammation, pustular skin rashes, and multifocal osteomyelitis, in common with SAPHO.

There are indications of association between the SAPHO-like disease *non-bacterial osteomyelitis* (NBO) and psoriasis; in a study of 89 patients, 20% had first-degree and second-degree relatives with psoriasis (180). Several genes responsible for monogenic psoriatic disease were recently defined. These include an autosomal recessive loss-of-function mutation in *IL36RN* that causes deficiency of the IL-36 receptor antagonist (DITRA) (204, 205), with a phenotype characterized by generalized psoriasis as well as fever, fatigue and increased inflammatory markers. Another gene associated with psoriatic disease is the autosomal dominant gain-of-function mutation in *CARD14* that causes CARD14-mediated psoriasis (CAMPS), with symptoms such as plaque or pustular psoriasis as well as familial pityriasis rubra pilaris (206, 207). Finally, a cohort of patients with pustular psoriasis with a heterozygous mutation in the *AP1S3* gene, but no mutations in *IL36RN* and *CARD14* has been described (208). Variants in all of these genes have so far not been investigated in SAPHO, but genetic characterization could provide important information about a possible genetic predisposition for the disease. With regard to the overrepresentation of first-degree and second-degree relatives with psoriasis in NBO in general and SAPHO in particular, variants in the genes *IL36RN, CARD14* and *AP1S3*, in addition to *IL1RN* are pending investigation.

11. Dysregulation of the innate immune system (mechanism)

Sabina's altered pyrin function causes intense FMF attacks with peritonitis, characterized by a massive influx of neutrophils into the abdominal cavity.

In the case of William, the dysregulation of innate immune function gives rise to regular febrile attacks that respond exceptionally well to corticosteroids. During William's attacks of PFAPA, his neutrophils show an inflammatory phenotype, consisting of increased intracellular oxygen production, delayed apoptosis and priming.

In Johan, diagnosed with SAPHO, the neutrophils show normal production of intracellular oxygen radicals (icROS). In the inflammatory state his neutrophils are pre-primed with increased icROS production compared to the non-inflammatory state.

The identification of disease-causing mutations in a number of monogenic autoinflammatory conditions has lead to a remarkable molecular understanding of the innate immune system and the regulation and dysregulation thereof (4, 5). Less is known about the roles of specific cell types in the different conditions, although it has been claimed that the cell type most affected by a particular mutation may determine the clinical phenotype (5). In this chapter, the molecular and neutrophil dysregulation will be discussed for the three conditions that form the basis of this thesis, FMF, PFAPA and SAPHO.

11.1 Molecules

Although the disease-causing mutations in FMF were discovered almost 20 years ago, there are still considerable knowledge gaps regarding the molecular disease mechanisms. Over time, it has also been shown that it is difficult to delineate the mechanisms of innate dysregulation in polygenic or multifactorial diseases, as exemplified by PFAPA and SAPHO, but there are some clues as to which molecular mechanisms are involved.

11.1.1 Dysregulation of innate immunity mediated by altered pyrin in FMF

When disease-causing mutations in the gene for pyrin (*MEFV)* were identified as causative for FMF almost 20 years ago, it was expected that this would, as a spinoff, give fundamental insights into the physiological and pathophysiological function of pyrin (13, 14). It has been more difficult than expected to clarify the role of wild-type and mutated *MEFV*, and the function of the protein is still largely unknown (5). Recent studies suggest that pyrin may be a specific immune sensor for bacterial inactivation (glycosylations) of Rho GTPases, leading to caspase-1 activation and subsequent IL-1 and IL-18 activation (195). This may be beneficial not only for the clarification of pyrin function in immune regulation *per se*, but also for the mechanism behind dysregulation of pyrin function in FMF (see below) (5, 195). These new data may lead to the development of new treatment options to complement the standard treatment with colchicine.

11.1.1.1 Loss-of-function mutation in pyrin leads to increased NLRP3 inflammasome activation

Analysis of *MEFV* expression on mRNA level in leukocytes from bone marrow and peripheral blood shows that pyrin is expressed in neutrophils, eosinophils and monocytes, but not in lymphocytes (209). In granulocytes, pyrin levels are higher in FMF patients than in both healthy controls and patients with other causes of active inflammation (184). There are no significant differences in pyrin levels between patients who have one compared to two mutations (184).

The traditional understanding is that wild-type pyrin interacts with and inhibits the NLRP3 inflammasome, and that *MEFV* mutations give rise to a loss of function in pyrin, that is, FMF-associated mutations in *MEFV* cause decreased inhibition of the NLRP3 inflammasome compared to wild-type pyrin, leading to increased production of IL-1β, and thus enhanced proinflammatory symptoms associated with the disease (210). A recent study in human monocytes support the idea that increased IL-1β secretion is NLRP3-dependent in FMF, although the results of this study are also consistent with the idea that disease-causing mutation in *MEFV* is a gain-of-function mutation (see below) (211).

11.1.1.2 Gain-of-function mutation in pyrin leads to direct activation of caspase-1 and increased IL-1 production

The hypothesis that pyrin interacts with NLRP3 and inhibits the NLRP3 inflammasome was challenged by studies in mice that show that some *MEFV* mutations in fact give rise to a gain-of-function pyrin, resulting in increased IL-1β secretion (212). Although these findings are elegant and intriguing, corresponding mechanisms have yet to be investigated in humans. In addition to the potential differences between humans and mice, it has been suggested that the function of pyrin may vary between different human cell types (5).

It is likely that pyrin has other roles in addition to the regulation of IL-1β, including the possibility that caspase-1 cleaves pyrin itself and that the N-terminal fragment translocates to the nucleus and enhances nuclear factor (NF)-κB activation (72). By this hypothesis, pyrin produced as a result of *MEFV* mutations would be cleaved more efficiently by caspase-1 in leukocytes, leading to increased enhancement of NF-κB activation and thus increased inflammation (72).

11.1.1.3 OTHER MECHANISMS REGULATING PYRIN FUNCTION

A recent study demonstrated that pyrin is activated by pathogen-mediated modifications of small G proteins, Rho GTPases, induced by bacterial toxins from, for example, *Clostridium difficile*, *Vibrio parahemolyticus* and *C. botulinum* (195). As stated above, this might give clues to a possible survival advantage of FMF heterozygous individuals, but may also be of relevance to the fact that country of residence influences disease severity (4). For example, individuals with FMF who have moved from their home countries in the Mediterranean Basin to Germany, have milder disease compared to patients in their country of origin (194).

11.1.1.4 MECHANISMS BEHIND COLCHICINE TREATMENT OF FMF

The underlying mechanism by which colchicine treatment is successful is not completely understood. Colchicine reduces the levels of inflammatory cytokines in serum, including IL-6, IL-8 and TNF-α (213). As gene transcription is unaltered by colchicine in FMF, it is suggested that the drug inhibits translocation, processing or secretion of cytokines (214).

It is known that colchicine accumulates in neutrophils (215). One early study suggested that colchicine alters the number and distribution of selectins on both endothelial cells and neutrophils, and thereby interferes with neutrophil extravasation and inflammation (216). It is known that colchicine binds to free tubulin dimers, which, when incorporated into budding microtubules, disrupt further microtubule polymerization. The ending of microtubule polymerization inhibits vesicle transport, cytokine secretion, phagocytosis, and migration (217-219), all mechanisms of great importance for the maintenance of a neutrophil-driven inflammatory response. Accordingly, colchicine-treated neutrophils have been shown to be less elastic compared to untreated neutrophils, which may restrain migration through small pores, a function that is essential for extravasation to serosal cavities (220).

The above findings could all be related to effects on the microtubules and, together with the fact that colchicine accumulates in neutrophils, this gives a reasonable explanation until other hypotheses possibly evolve to explain the efficacy of colchicine as a treatment for FMF.

11.1.2 Molecules involved in the dysregulation of innate immunity in PFAPA

The PFAPA phenotype has all the features of a classical periodic fever syndrome, with stereotypical unprovoked febrile episodes associated with localized inflammation and an often clockwork periodicity (145). Even though recent studies have shed new light on the pathophysiology of PFAPA, the disease mechanisms are largely unknown. The disease intrigues the paediatric and autoinflammatory research community with regard to its regular episodes, exceptionally good response to corticosteroids and, as observed by the attentive clinician, the reduced number of upper respiratory infections. Activation of an innate immune response during PFAPA episodes is supported by production of proinflammatory cytokines (161, 200, 221-224), an increase of neutrophilic leukocytes (neutrophilia) (138, 139, 153, 223, 224), as well as an increase in acute phase reactants (221, 223).

During PFAPA episodes, neutrophilia and monocytosis, as well as lymphopenia and eosinopenia, are found (paper II and III) (161, 200, 223, 224). Neutrophilia linked to lymphopenia is in keeping with emergency granulopoesis, in which expansion of bone marrow myelopoesis is paralleled by a decrease in bone marrow lymphopoesis (225), which leads to an increased neutrophil–lymphocyte ratio; this is currently being investigated as an inflammatory marker in a number of conditions, including FMF (226, 227). In FMF, the neutrophil–lymphocyte ratio might serve as an alternative marker to monitor the response to colchicine treatment (226). In PFAPA, such a ratio may also potentially be used as a complement to CRP and SAA to detect inflammation during episodes and exclude inflammation between episodes.

Acute-phase inflammatory variables (CRP, ESR and SAA) are significantly increased during attacks of PFAPA, and both CRP and SAA typically normalize between episodes, as exemplified in paper II and III. CRP is a well-established, nonspecific systemic inflammatory marker that is synthesized by hepatocytes secondary to IL-6 stimulation. SAA is also produced in the liver upon stimulation by proinflammatory cytokines and binds high-density lipoprotein (228). SAA is a useful inflammatory indicator in the management of children with autoinflammatory conditions, firstly, to measure low-grade inflammation between attacks, secondly, to monitor effectiveness of anti-inflammatory treatment and, thirdly, to estimate the risk of developing amyloidosis (104, 229). In PFAPA, a raised SAA during attacks does not add much information to the increase in CRP. Between attacks of PFAPA, SAA is valuable for verifying that there is no inflammation in the attack-free interval, as is expected in PFAPA syndrome.

Procalcitonin is a pro-peptide of the hormone calcitonin that increases in bacterial infections. It is produced in the thyroid gland and is used to identify severe bacterial infections such as sepsis (230, 231). Except for studies of procalcitonin in PFAPA and some minor studies in FMF, this peptide has not been investigated in autoinflammatory diseases (232-234). In PFAPA, two studies have indicated that procalcitonin is not elevated during febrile episodes (235, 236). In the cohort presented in paper II, procalcitonin serum levels in both febrile and afebrile

patients, as well as in healthy controls, were below the upper reference level of 0.2 μg/L, in other words, serum procalcitonin did not show the same marked increase during attacks as CRP and SAA. Nonetheless, there was a significant increase in procalcitonin during the febrile phase compared both to the afebrile phase and to healthy controls. These results parallel the findings in FMF, in which a slight but significant increase in procalcitonin levels was observed during inflammatory attacks as compared both to attack-free periods and to healthy controls (232).

The S100 proteins A8/9 and A12 are cytosolic calcium-binding proteins that, when released by granulocytes and monocytes at the site of inflammation, function as DAMPs (237). As such, they lead to activation of TLR4 on monocytes, which stimulate secretion of proinflammatory cytokines such as IL-18 and IL-6 (237). High levels of S100 proteins are characteristic of active systemic juvenile idiopathic arthritis and FMF (238, 239). One study indicated that S100A8/A9 and A12 are significantly elevated in febrile PFAPA, afebrile PFAPA and febrile controls compared to afebrile controls (200). The increase of S100A8/A9 and S100A12 in febrile PFAPA is confirmed in Paper III, although the finding that these inflammatory markers are increased in afebrile patients could not be verified. The normal levels during the afebrile phase indicate that S100A8/A9 and S100A12 are not specific markers of PFAPA, as previously suggested by others.

Galectin-3 is an endogenous β-galactoside-binding lectin produced by macrophages upon stimulation with DAMPs and PAMPs (240). Increased plasma concentration of galectin-3 is a marker of inflammation in infections and non–infectious inflammatory conditions (241). In paper III, plasma concentrations of galectin-3 were shown *not* be increased in febrile attacks of PFAPA, that is, they were at the same level as in afebrile intervals and afebrile controls. This is in contrast to the increased serum levels of galectin-3 during attack-free periods in FMF (242), but also to the increase in *systemic lupus erythematosus* (SLE) (243), *Behçet's disease* (244), *rheumatoid arthritis* (245) and *juvenile idiopathic arthritis* (246), as well as in *Crohn's disease* and *ulcerative colitis* (247). Furthermore, a recent study of plasma galectin-3 showed increased concentrations in sepsis but not in viral infections, nor in attacks of non-determined autoinflammatory syndromes, as compared to healthy controls (241). If increased levels of galectin-3 in certain diseases and low levels in PFAPA are verified, this can serve as a tool for distinguishing these conditions from each other, thus helping to solve this clinical problem, particularly with regard to young children with an origin in regions with high prevalence of FMF. Hence, galectin-3 should be further evaluated both in FMF and in PFAPA, as well as in other defined autoinflammatory conditions.

The fever-mediating cytokine IL-1β has been examined in a number of studies of PFAPA. Stojanov and co-workers reported the unexpected observation that serum IL-1β is increased during PFAPA episodes (221), which is rarely the case in IL-1-driven diseases. This conflicts with several studies showing no increase in serum concentrations of IL-1β during episodes (paper II) (161, 200). Another study of whole blood gene expression on the mRNA level showed increased expression of the IL-1-related genes *IL1B*, *IL-1RN*, *CASP1* and *IL-18RAP* during PFAPA episodes (161). Studies of *ex vivo* LPS-stimulated PBMCs and purified monocytes

from febrile patients with PFAPA show that the cells had an increased IL-1β secretion compared to cells from afebrile patients (200). Finally, one small case series suggested that PFAPA flares are responsive to IL1-blockade; the latter is often the only way to prove that a disease is IL-1-driven (161). In conclusion, the role of IL-1 during PFAPA episodes is still to be settled. A well-designed study of IL-1 blockade would be an important step forward, regardless of result.

A number of other proinflammatory cytokines in addition to IL-1 have been evaluated in PFAPA with the expectation that inflammatory mediators are increased during the febrile episode. The proinflammatory cytokine IL-6 is indeed increased in serum during disease flares (paper II) (141, 161, 200, 221). One study also showed increased IL-18 levels during febrile episodes (161). In addition, IFN-γ (221) and the INF-γ-related cytokine IP-10/CXCL10 were increased in PFAPA attacks compared to the afebrile phase (paper II) (161, 200, 224), as were MIG/CXCL9 (161) and MIP-1β/CCL4 (161, 224). In the Stojanov study from 2006, IL-12 was elevated in a very early febrile phase, as compared to afebrile controls (221). However, a second study by Stojanov could not verify this finding, possibly due to that the samples were taken later during the episodes (161). In summary, this cytokine pattern supports an increased INF-γ secretion somewhere during the PFAPA cycle with an adjoining Th1 differentiation of CD4+ T cells (further discussed in Chapter 13, Association with additional immune dysfunctions).

The resolution of fever in PFAPA is followed by a period during which the patient is basically healthy. However, some studies found an increased level of cytokines, and the question of an increased inflammatory activation between febrile episodes has yet to be settled (161, 221, 224). Increased IL-6 between periods has been reported in two studies (161, 221), but this finding was not verified by others, including us (paper II) (200). Furthermore, IP-10 was not significantly increased during the afebrile phase of PFAPA compared to healthy controls in almost all studies (paper II) (161, 200). Conversely, the study by Førsvoll et al. showed an increase in IP-10 during the afebrile phase of PFAPA compared to children who recovered from pneumonia; however, children with PFAPA were sampled after at least 10 days without fever, whereas children recovering from pneumonia were sampled after four weeks (224). One study showed a significant increase in MIG/CXCL9 and MIP-1β during the afebrile phase as compared to healthy controls (161), but this was not found in Paper II. To improve the understanding of the cytokine dysregulation during the different phases of PFAPA, future studies need to include homogeneous, well-defined cohorts and control groups that are sampled at defined time points, if possible in a serial manner.

Dysregulation of innate immunity in PFAPA is suggested by the combination of phenotype, increase of proinflammatory cytokines, prompt response to corticosteroids, lack of response to antibiotics and the possible response to IL-1 blockade. Nonetheless, the disease mechanism is still to be established. The role of the tonsils is highlighted by the often positive response to tonsillectomy, and the possible presence of a trigger in the tonsils needs further evaluation (201). The differentiation of CD4+ T cells towards a Th1 response that is indicated above will be further discussed in section 13.1.

11.1.3 Molecules involved in the dysregulation of innate immunity in SAPHO

Recently, the identification of disease-causing mutations in monogenic autoinflammatory bone disorders has advanced the pathogenic understanding of these conditions, for example in *Majeed syndrome (LPIN2),* DIRA (*IL1RN*), and *cherubism* (*SH3BP2*). In DIRA, there is an absence of IL-1Ra, which leads to unopposed IL-1α and IL-1ß signalling and clinical features such as skin rash and multifocal osteomyelitis responsive to IL-1 blockade (31). In Majeed syndrome, the disease mechanism is not understood, but a few patients have been treated with IL-1 blockade with a favourable outcome (248). The pathophysiology of cherubism is also unknown, but mice with a mutation in *SH3BP2* develop TNF-α-dependent bone lesions, and one speculation is that the oral bacterial flora is responsible for the predilection of hyperinflammatory bone lesions in the maxilla (5). The disease mechanisms in non-monogenic cases of autoinflammatory bone disorders, including CNO, NBO, CRMO and SAPHO, are largely unknown. In SAPHO in particular, a better understanding of the interplay between the immune response and microbial triggers could form the basis of an interesting disease model, with the potential to advance the current understanding of the role of microbes in autoinflammatory conditions.

There is a general agreement that SAPHO is not an infectious disease (169). This is supported by the fact that antibiotics are ineffective or have only a transient effect in SAPHO (249). Still, a number of studies report positive cultures from bone lesions in patients with SAPHO, as illustrated by one of the patients in paper IV (171, 249-251). In particular, the significance of *Propionibacterium acne* is intriguing, isolated in 42% of cases in a recent review of 90 cases, and in 67% in another recent study of 21 patients with SAPHO (249, 252). In an older study, *P. acne* was isolated from six surgically obtained consecutive bone specimens in the same patient with SAPHO (251). Hence, many studies indicate that *P. acne* functions as a disease trigger in SAPHO. The pathways that *P acne* activates may provide a clue to the disease mechanism of SAPHO. In mice, *P acne* was shown to activate TLR9, leading to a phenotype with splenomegaly, intrahepatic granuloma formation, hypersensitivity to TLR ligands, and enhanced resistance to infection (169, 253, 254). Other studies suggest that *P. acne* induces caspase-1 activation in neutrophils, generating both IL-1β and IL-18 production (255, 256). In summary, the role of microbes, including *P. acne,* as triggers of innate immune activation in SAPHO is an interesting disease hypothesis that needs to be further investigated.

In the search for an innate disease mechanism triggered by microbes in SAPHO, the study by Ferguson et al. from 2008 is important, as it suggests that aberrant production of NADPH-oxidase-derived reactive oxygen species (ROS) by neutrophils could define the disease (257). With the study by Ferguson as a point of departure, we decided to investigate whether aberrant production of NADPH-oxidase-derived ROS was a general feature of SAPHO (section 11.2.1.1).

11.2 Cells

11.2.1 Innate immune cells in FMF, PFAPA and SAPHO, focusing on neutrophils

The innate immune system comprises several cell types of myelopoetic origin, with neutrophils being the most numerous. Neutrophils are crucial for our innate immune response and defence against microbes. When the integrity of the host is threatened, neutrophils have a remarkable arsenal of tools to recognise, locate, attack and destroy the intruder (258-260). The killing mechanisms of neutrophils include phagocytosis, frustrated phagocytosis (degranulation) and formation of neutrophil extracellular traps (NETs) (260, 261). The importance of neutrophils in host defence is illustrated by inherited defects in neutrophil function that cause immunodeficiencies, including neutropenias linked to premature apoptosis (for example, *severe congenital neutropenia* 1, SCN1 (*ELANE*) and SCN3 (*HAX1*)), defects in chemotaxis (such as *leukocyte adhesion deficiency* (LAD) *type 1–3* (*ITGB2*, *SLC35C1*, *FERMT3*)), defects in granule formation (*neutrophil-specific granule deficiency* (SGD)) and defects in production of oxygen radicals (for instance, CGD) (262, 263). On the other hand, if not adequately controlled, neutrophils can also become destructive to host cells and tissues, as seen in chronic inflammation, as well as in autoinflammatory and autoimmune disease (264-267).

Despite the significance of neutrophils in the innate immune response and, by deduction, in autoinflammatory diseases, neutrophils are rarely studied in these disorders, and their pathogenic role and molecular phenotype remain largely unknown. One possible reason for this inadvertency is that studying these terminally differentiated (and thus nonculturable) cells is difficult, due to their inherent properties and the technical struggle that this entails. Consequently, few laboratories in the autoinflammatory field have the experience in neutrophil research to adequately address these issues (chapter 14.).

In FMF, as illustrated in the case of Sabina, the central role of neutrophils in disease pathogenesis is indicated by the neutrophilia that can be seen as part of a rapid acute phase response that leads to peritonitis with a massive influx of neutrophils into the abdominal cavity (85, 91, 268, 269). In PFAPA, as illustrated by William's case history, the inflammatory phenotype of circulating neutrophils consists of increased intracellular ROS production, delayed apoptosis and priming, as delineated in paper III. In SAPHO, as Johan's case history demonstrates, the notion that the disease mechanism is linked to a decrease in intracellular ROS production is challenged in paper IV.

Below, the role and functions of neutrophils in FMF, PFAPA and SAPHO will be discussed. Some central notions in cellular biology of neutrophils and the methods applied in papers III and IV are discussed in Chapter 14.

The point of departure here is the mature, circulating neutrophils, studied at a stage when they are terminally differentiated and have lost the capacity to divide (260, 270). In this situation, the possibility for phenotypic variation of the neutrophil population as a whole is limited. Instead, phenotypic modifications occur in the individual neutrophil throughout its short life span (271).

11.2.1.1 Production of oxygen radicals by neutrophils in FMF, PFAPA and SAPHO

Production of reactive oxygen species (ROS) is essential for neutrophil bacterial killing and intracellular signalling (258). Activated membrane NADPH-oxidase transfers electrons from NADPH in the cytoplasm across the membrane to molecular oxygen. Oxygen is reduced to superoxide anion, successively forming other ROS. When the NADPH-oxidase is assembled in the plasma membrane, ROS are released extracellularly (extracellular ROS; ecROS) and when it is assembled in the phagosomal membrane or in granular membranes, the radicals are designated intracellular ROS (icROS) (see figure 9 in section 13.8).

The pathophysiological consequences of defect ROS production are best illustrated by the disorder chronic granulomatous disases (CGD) (272). In this rare but serious disease, decreased ROS production is associated with immunodeficiency, leading to aberrant defence against bacteria. Also, patients with CGD often suffer from colitis or other sterile inflammatory disorders (266, 272, 273) indicating that normal NADPH-oxidase-derived ROS production seems to not only contribute to bacterial killing but also to be involved in the control of aseptic inflammation (273-276). In one study, a novel variant of CGD showed that a specific deficiency in the intracellular production of ROS (icROS) is enough to give both the immunodeficiency and the proinflammatory phenotype, indicating that icROS are of importance for immune regulation (277).

In 2008, Ferguson et al. proposed that SAPHO could be a second disease in which decreased icROS production is a mechanism behind (over)activated innate immune mechanisms. They presented a case study of a patient with SAPHO-like features, and their hypothesis was received with interest in the neutrophil field (257, 278). Although convincingly shown in that paper, we could not verify the idea that SAPHO is linked to decreased icROS production (paper IV); in our study, both ecROS and icROS production were normal in four well-defined SAPHO patients, two of whom were studied both in the inflammatory and non-inflammatory state.

Paper III demonstrated that icROS production is significantly increased in neutrophils from patients with PFAPA during febrile episodes, as compared to afebrile phases and healthy controls. However, the production of ecROS was not altered in paired febrile and afebrile samples, or when compared to their respective controls. In contrast, a previous study found that unstimulated neutrophils from patients with FMF produced higher levels of ecROS during febrile attacks than neutrophils from healthy controls (279). One early study on PMNs from patients with FMF demonstrated increased ROS production after phagocytosis (280). ROS

in that experimental setup was most probably of intracellular origin, thereby concurring with our findings for PFAPA syndrome (280).

In conclusion, with regard to NADPH-oxidase-derived icROS production as a regulator of inflammation, the decreased icROS production in CGD gives rise to increased sterile inflammation. This stands in contrast to the association of increased icROS production in PFAPA episodes and in the inflammatory phase of SAPHO. How an enhanced inflammatory response can be associated with both increased and decreased icROS production remains an intriguing question. One could speculate that the explanation is the unbalanced icROS production that is the common denominator of these divergent findings and that this unbalance results in increased inflammatory signalling. An alternative explanation could be that decreased icROS production is a necessary and sufficient cause of hyper-inflammation in CGD, whereas an increase icROS production in PFAPA and SAPHO is a consequence of a dysregulated innate immune response.

In TRAPS, mitochondria from both neutrophils and monocytes produce increased amounts of ROS, which are associated with increased pro-inflammatory cytokine secretion, including increased activation and hyperresponsiveness to LPS (281). This is in keeping with our data on neutrophils from afebrile PFAPA patients, where we showed an increased mitochondrial ROS (mtROS) production compared to healthy controls. This was not the case in the febrile PFAPA phase, in which mtROS was lower than in febrile controls and comparable to the afebrile phase in the same individual (paper III). The importance of these findings has to be established through more detailed investigations.

11.2.1.2 Priming of neutrophils in FMF, PFAPA and SAPHO

Neutrophil activation occurs in several steps, from neutrophils cruising the circulation in a dormant state in a healthy host, to activated cells at a site of full-blown infection or aseptic inflammation (258, 259). There are several activation steps between these two extremes, reflected by differences in cellular phenotype (section 13.6). Having met the first stimulus, neutrophils are primed, that is, set in a state of high alert that permits further activation as they encounter additional stimuli. In the normal inflammatory response, priming appears to take place during the extravasation process, keeping the cells with increased reactivity out of the blood stream, thereby protecting the host from dissemination of these cells in circulation. During an acute attack of PFAPA, however, neutrophils are primed in the circulation, indicated by increased CD11b expression but preserved L-selectin/CD62L, as we show in paper III. This is coherent with data from FMF attacks, in which CD11b expression is increased without any change in L-selectin/CD62 expression, unlike healthy controls (282). Likewise in SAPHO, neutrophils seem to exist in a primed state with increased expression of CD11b and unchanged L-selectin/CD62L, based on the two patients we studied during an inflammatory phase (paper IV).

It has been proposed that to achieve a fully primed phenotype, L-selectin also has to be shed, which is not the case in circulating neutrophils either in PFAPA, FMF

or SAPHO (283). A priming state exhibiting increased CD11b expression together with preserved L-selectin expression can be labelled pre-priming. This is based on the fact that CD11b expression can be increased by the simple procedure of isolation of the cells; when CD11b is studied in whole blood without neutrophils going through the isolation procedure, CD11b expression may not be increased to the same extent. Accordingly, the term pre-primed has been proposed to describe cells that appear as resting in whole blood but that upregulate their CD11b more easily than control neutrophils, and thus appear to be primed after isolation (paper III and IV). In this thesis, the terms primed and pre-primed are not used in a coherent manner, as the term primed is used in paper III and the term pre-primed in paper IV for labelling the same neutrophil phenotype, namely, increased CD11b expression with preserved L-selectin expression after cell isolation. Regardless of whether the term primed or pre-primed is used for the phenotype of these cells, it is important to stress that in the end they behave differently compared to control cells, as shown in both paper III and paper IV.

In FMF, neutrophils are important for the disease mechanism, as illustrated by the massive influx of neutrophils into serosal cavities, and by the effective treatment of the disease with colchicine, which inhibits chemotaxis of these cells (220). Neutrophil priming is an important step for neutrophil migration, as the process upregulates the integrin CD11b and other adhesion-mediating receptors, and a primed neutrophil phenotype in circulation might thus enhance the transmigration of these cells into serosal cavities, promoting the FMF phenotype. How priming of neutrophils is to be understood in PFAPA and SAPHO needs to be further investigated.

11.2.1.3 Neutrophil apoptosis in FMF, PFAPA and SAPHO

Apoptotic neutrophils are non-functional cells with preserved membrane integrity that signal that they are ready to be cleared from the blood circulation or extra-vascular tissue by exposure of phosphatidylserine. By going into apoptosis, the neutrophils avoid activation of the inflammatory response and instead contribute to resolution of inflammation (260). Conversely, neutrophils that die from necrosis or NETosis (violent cell death) lose membrane integrity, release toxic products and trigger proinflammatory responses. Apoptotic neutrophils that are not promptly cleared may undergo secondary necrosis and thus trigger an inflammatory response.

In paper III, we found that neutrophils show low rates of spontaneous apoptosis during PFAPA attacks, an effect that is likely to provide sufficient numbers of live and activated neutrophils to maintain the ongoing inflammatory response. In the attack-free intervals, neutrophil apoptosis rate is instead increased, even above the rate in healthy controls, which is liable to enhance clearance of excessive activated neutrophils, decelerate neutrophil production and promote an anti-inflammatory response; this is seen as normalization of the inflammatory phenotype of the disease.

In contrast to decreased apoptosis rate in attacks of PFAPA, the rate of neutrophil apoptosis has been shown to be significantly increased in FMF attacks compared to

controls, as well as compared to attack-free intervals, as shown in a few paired samples (284). In general, all peripheral blood leukocytes showed large variation in apoptosis in attack-free intervals. This might be anticipated, bearing in mind the subclinical inflammation seen in the attack-free period in patients with FMF (104).

11.2.1.4 Neutrophil extracellular traps (NETs)

Lately it has been shown that neutrophils can kill microbes by the release of neutrophil extracellular traps (NETs). These are DNA-based extracellular nets containing chromatin and antimicrobial peptides that can trap and kill microbes. Upon release of NETs, neutrophils undergo NETosis, which, in contrast to apoptosis, triggers inflammation.

There is very little data on NET formation in autoinflammatory disease. A recent study suggests that neutrophils play a pivotal role in triggering attacks in FMF by increased production of IL-1β, subsequently released during NETosis, as NETs coated with IL-1β were found extracellularly (285). The study also suggests that NETosis is then downregulated during the attack-free period, thereby facilitating resolution of the attack.

11.2.1.5 Neutrophil-modulating cytokines in PFAPA

A number of soluble molecules that affect neutrophil function have been studied in autoinflammatory diseases, without necessarily focusing on their effects on neutrophils. In PFAPA, the profile of cytokines and chemokines is complex and not completely understood, as previously discussed (Section 11.1.2). During the febrile phase, an increase in proinflammatory and IFN-γ-related cytokines are suggested, among others, IL-1, IL-18, IL-6, IFN-γ and granulocyte colony-stimulating factor (G-CSF), as well as IP-10, MIP-1β and MIG. The neutrophil phenotypes that these mediators give rise to have not been explored. This section is an attempt to discuss the importance of some of these cytokines for neutrophil function, focusing on PFAPA and with reference to papers II and III, as these two papers investigate cytokines, inflammatory markers, and neutrophil function in PFAPA.

The cytokine IL-1 is central to activation of innate immunity and a necessary and sufficient cause in many autoinflammatory diseases. Nonetheless, IL-1 is rarely measurable in serum and the only way to demonstrate the pathophysiological importance of IL-1 is by blocking its action (9). In PFAPA, one small case series suggested that febrile episodes are responsive to IL-1-blockade, although the results are not clear-cut and need to be verified (161). IL-1 prolongs neutrophil survival (286, 287), but it is questioned to what extent IL-1 has a direct effect on neutrophil activation (286). That is not to say that neutrophils do not respond to IL-1 stimulation, but the response is weaker than in other cells (286, 288). This can be explained by studies that indicate that the expression of IL-1 receptors on neutrophils is dominated by the non-functional decoy receptor IL-1RII and not by the functional receptor IL-1RI (286, 288, 289). Hence, it is reasonable to hypothesize that IL-1 may have primarily indirect effects on neutrophils, for

example by the stimulation of endothelial cells to produce neutrophil-attracting chemokines, and by upregulation of adhesion molecules (286, 290). In summary, there are several reasons for caution in interpreting neutrophil function in PFAPA as a direct effect of increased serum levels of IL-1.

A number of studies have shown an increased concentration of IL-6 in serum during PFAPA flares. It is well established that IL-6 contributes to neutrophilia and synthesis of inflammatory indicators like CRP and SAA. A recent study by Wright indicates that IL-6 does not have a direct effect on neutrophil function, including apoptosis rate, respiratory burst and expression of adhesion molecules (291). This is contrary to several earlier studies (292-294). Hence, according to Wright's study it is unlikely that increased serum levels of IL-6 during PFAPA episodes are not a sufficient cause of the altered neutrophil functions, as described in paper III of this thesis.

All studies of serum cytokines in PFAPA indicate an increase in IFN-γ-related chemokines, namely, IP-10 (161, 200, 223, 224), MIG (161)) and MIP-1β (161, 224). An increased secretion of IFN-γ is supported by a study of whole-blood gene expression (161). Taken as a whole, the above findings indicate that there is an increased IFN-γ secretion somewhere during the PFAPA cycle, most probably in a very early phase of the febrile episode (or even before initiation of fever). The direct effects of IFN-γ on neutrophil function include enhanced respiratory burst and delayed apoptosis (295). This is in keeping with paper III, in which neutrophils showed an altered phenotype including decreased apoptosis and increased icROS production during PFAPA flares.

One study of children with PFAPA investigated the presence of IL-18 in serum, showing increased concentrations during the febrile phase as compared to the afebrile phase (161). In neutrophils, IL-18 triggers various responses, including both chemokine and cytokine release, enhanced activation of the respiratory burst, as well as inhibition of neutrophil apoptosis (296-298), in line with the IFN-γ effects. In fact, IL-18 also promotes IFN-γ synthesis in combination with IL-12 (299). Altogether, it seems reasonable to conclude that IL-18 may play a part in the altered neutrophil functions in PFAPA demonstrated in paper III.

11.3 Conclusion – the role of neutrophils in FMF, PFAPA and SAPHO

The autoinflammatory field has gone through a remarkable development, with improved molecular understanding of monogenic autoinflammatory diseases as a consequence. Less attention has been given to the role and function of different cell types; in this section the role and function of neutrophils in the three autoinflammatory diseases FMF, PFAPA and SAPHO has been discussed. In conclusion, the role of reactive oxygen species as modulators of inflammation in autoinflammatory diseases is a highly relevant research area, both when derived

from the neutrophil NADPH-oxidase, and when produced as a by-product from mitochondrial respiration (281, 300). The role of ROS needs to be further investigated in PFAPA and FMF, but also in autoinflammatory diseases in general. Only when cell functional data from diseases with different phenotypes are compared in consistent way conclusions can be drawn as to where altered mechanisms should be sought.

12. Activation of the adaptive immune system and association with other immune dysfunctions

During Sabina's attacks of FMF, a Th17 and Th1 differentiation of $CD4^+$ T cells indicates that adaptive immunity is activated.

In William's PFAPA attacks, the Th1 differentiation of $CD4^+$ T cells demonstrates that the adaptive immune system is involved.

In Johan's SAPHO, an association with a Th17 differentiation of $CD4^+$ T cells has been suggested.

In Chapter 8, an activation of the adaptive immune system in monogenic autoinflammatory diseases was suggested, as evidenced by differentiation of $CD4^+$ T cells into different subsets in CAPS, DIRA and FMF (31, 47-49). Autoimmunity, on the other hand, was not recognized in terms of autoantibodies in these IL-1-driven conditions (11). McGonagle and McDermott applied the concept of autoinflammation to polygenic and mixed pattern diseases, and recognised that these conditions can have both an autoinflammatory and an autoimmune component (8). Today, several monogenic conditions have been described that combine phenotypes of autoinflammation with other immune dysfunctions, including autoimmunity and immunodeficiency, for example in APLAID, HOIL-1, PLAID as well as in interferonopathies such as AGS and CANDLE (43, 73, 74). Neither autoantibodies nor autoreactive $CD8^+$ T cells have been described in FMF, PFAPA or SAPHO. This chapter will focus on activation of the adaptive immunity in terms of $CD4^+$ T cell differentiation in the two polygenic conditions PFAPA and SAPHO, and the monogenic disease FMF.

12.1 $CD4^+$ T cell differentiation

Naive $CD4^+$ T cells can differentiate into several subsets, for example, Th1, Th2, Th17 and regulatory T cells (Figure 7). The different $CD4^+$ T cell subsets mediate specific adaptive immune responses that combat different types of pathogens and

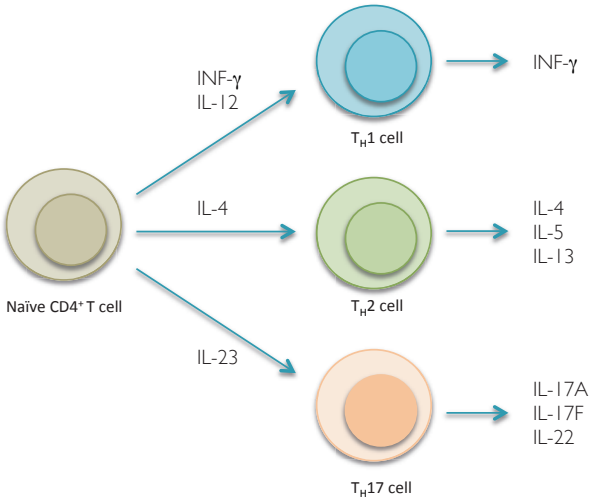

Figure 7. T cell differentiation. Sub-set specific cytokines (left) and signature cytokines (right) are shown. Adapted from Dong, Nat Rev Immunol (2006) 6:329.

regulate the immune response, but can also contribute to the development of autoimmunity or allergy. $CD4^+$ T cell differentiation is induced by subset-specific cytokines and transcription factors, mainly generated by antigen-presenting cells and the responding T cells themselves. Upon activation, each subset produces cytokines that define the respective subset (signature cytokines). In general, the signature cytokines promote the development of that same subset and suppress development of other subsets.

12.1.1 Th1 subset differentiation

The subset-specific transcription factors for a Th1 response are T-bet, STAT1 and STAT4, and the subset-specific cytokines are IL-12 and IFN-γ. A Th1 response is adapted to fight intracellular microorganisms such as *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Leishmania* species, executed by activation of cytotoxic $CD8^+$ T cells, NK cells and activated macrophages. The signature cytokine of a Th1 response is IFN-γ.

12.1.2 Th17 subset differentiation

The specific transcription factors for a Th17 response, directed to combat extracellular bacteria and fungi, are RORγT and STAT3, and the subset-specific cytokines are IL-6, IL-1, IL-23 and TGFβ. The signature cytokines of a Th17 response are IL-17A, IL-17F and IL-22. A Th17 response initiates neutrophil

inflammation and is thought to mediate the inflammatory pathology in many auto-immune diseases as well as in IL-1 driven autoinflammatory conditions (31, 47, 301).

12.1.3 The pleiotropic role of IL-18 in CD4 differentiation

CD4 T cell differentiation can be discussed from the perspective of IL-18, which can pleiotropically promote a Th1, Th17 or Th2 response, depending on the cytokine environment. In the presence of IL-12, IL-18 induces IFN-γ and promotes a Th1 differentiation. On the other hand it should be noted that IL-1β, IL-18 or IL-6 in the presence of IL-23 promotes a Th17 with a subsequent block of Th1 response.

12.2 Normal $CD4^+$ T cell differentiation in response to innate immune stimulation in early life

The response of the adaptive immune system to innate stimulation undergoes changes during childhood. *In vitro* stimulation of TLRs in monocytes from babies born at term up to two months of age activates a high production of IL-10, IL-6 and IL-23. Consequently, there is an increased anti-inflammatory response (IL-10) as well as a Th17 $CD4^+$ T cell differentiation (IL-6 and IL-23) (302-304). At this age there is also a low production of type-1 interferon, INF-γ and IL-12, the latter two leading to an impaired Th1 response (302-306). The period up to two months coincides with an increased susceptibility to *Listeria monocytogenes*, group B streptococci, and severe *Herpes simplex virus* infection, for which a Th1 response and type-I interferon are essential (304, 305, 307). Th1 response to *in vitro* stimulation of TLRs later increases and reaches adult levels between the age of two and five years (304, 305, 307). The period of low Th1 response coincides with an increased susceptibility to intracellular pathogens such as *Mycobacterium tuberculosis* and *Salmonella* species (304, 305, 307).

12.3 Differentiation of $CD4^+$ T cells in FMF, PFAPA and SAPHO

12.3.1 Th1 subset differentiation in PFAPA

In paper II, a Th1 differentiation was indicated in PFAPA, suggested by an increase in the serum concentration of the IFN-γ related chemokine IP-10/CXCL10 during

febrile episodes, a finding that has later been verified in several studies (161, 200). The Th1 response is also supported by increased IFN-γ and the INF-γ related cytokines MIG/CXCL9 and MIP-1β (161, 200) in febrile PFAPA episodes as compared to afebrile phases. Further, IL-12, a subset-specific cytokine promoting Th1 response was shown to be elevated in the early febrile phase as compared to afebrile controls (221).

The Th1 differentiation in PFAPA may be understood from the perspective of IL-18, which, in the presence of IL-12, induces IFN-γ and promotes a Th1 response (221). In a study by Stojanov and co-workers, the effect of IL-1 blockade was evaluated by measurement of several cytokines before and after treatment, resulting in a decrease in IP-10 and MIG- 1 but no effect on IL-18, which was continuously elevated (161). Again, these results underline the importance of reinvestigating the clinical and immunological response to IL-1 blockade in PFAPA.

Despite this clear Th1 response, it does not appear to protect the patients from allergy (140, 155).

12.3.2 Th17 differentiation of CD4 T cells in SAPHO

In a small study of SAPHO, an increase in Th17 cells in the peripheral blood was suggested (308). Other studies also suggest that *P. acne* promotes Th17 (309) as well as Th17/Th1 responses in patients with acne (310). In psoriasis, Th17 cells play a pivotal role at disease onset (311). Monoclonal antibodies directed to IL12/IL23 and IL17 are licensed for the treatment of psoriasis (311). These recent reports represent significant developments that may lead to other therapeutic approaches than targeting the TNFα pathway in chronic inflammation.

12.3.3 Th17 and Th1 differentiation of CD4 T cells in FMF

In FMF, the response is more complex, with indications of both a Th1 and Th17 response but without significant development of autoantibodies (section 8.2.1) (51-54). Allergy has been shown to be less frequent in children with FMF, possibly as a consequence of the Th17 and Th1 T cell differentiation that may subsequently block the Th2 response that characterizes the development of atopy.

12.3.4 Conclusion

PFAPA has an onset in children typically below the age of five, an age when the Th1 response to innate stimulation increases from paediatric to adult levels. It is also a period when children have an increased susceptibility to intracellular pathogens, including *M. tuberculosis*. Hence, it can be speculated that PFAPA is an extreme phenotype of children who have an increased Th1 response to innate stimulation, providing them with a survival advantage against intracellular

pathogens like tuberculosis during their most vulnerable age. It is also possible that the Th1 differentiation in PFAPA is a key to a potential trigger harboured in the tonsils. These two suggestions do not have to be mutually exclusive. The idea of an exaggerated, age-depended increase in Th1 response to innate stimulation in PFAPA is particularly attractive as both vanish with increased maturation and age.

Along the same lines, SAPHO is indicated by a Th17 response, as has been shown for patients with acne, a common symptom both in SAPHO (paper IV) and in IL-1-driven conditions such as CAPS. Hence, one can speculate whether or not *P. acne* is a significant trigger in SAPHO and whether IL-1 is the driving cytokine. SAPHO has been treated with IL-1 blockade and given a short-term improvement, and it would be interesting to investigate the Th17 response before and after such treatment (312).

In FMF, the adaptive immune response launched by the innate immune activation is complex, as indicated by both a Th1 and Th17 response that seems to protect patients with FMF from developing Th2-dependent effects such as atopic sensitization, allergic rhinitis, and asthma (313). This is in keeping with that the promotion of one or two T cell subsets suppresses the differentiation of other subsets.

13. Neutrophils and how to study them

Translational studies of neutrophil function in PFAPA and SAPHO introduced me, a clinician, to a research tradition representing neutrophil biology and research methods that have emerged during decades of basic neutrophil research in the laboratory. The aim of this section is to describe some central notions of neutrophil biology including methods used in our neutrophil studies of cells from patients with PFAPA or SAPHO, as shown in papers II–IV.

It is worth pointing out that some intrinsic cellular properties of neutrophils make them difficult to study. A major disadvantage is that they are terminally differentiated and short-lived after they have entered the circulation. Hence, as neutrophils do not divide they cannot be cultured and genes cannot be manipulated to enhance or silence protein expression, methods that are often used in explorative cell biology studies. Sometimes neutrophil-like cell lines (e.g. HL-60) are used for these purposes, but they do not reflect the diverse properties of neutrophils, being devoid of several of the granule subsets characterizing the mature cell (259). In addition to these cellular constraints, mouse models are not always appropriate in neutrophil studies. For example, the proportion of neutrophils in the total leukocyte count differs significantly (30% in mice, 50-60% in humans), suggesting differences between mice and humans in the role of these cells in immune defence as a whole, and there are also differences in surface receptors and signalling pathways, supporting the claim that they may fill different functions (314).

Neutrophils are short-lived, indispensable carriers of host defence that are produced in large numbers, thus exhibiting an impressive turnover. These cells initially spend a considerable part of their lifetime in the bone marrow, then make a swift visit to the blood stream and finally die, regardless of whether they have performed as defenders of the body or not. Although neutrophils are the main effector cells of the innate immune response, these cells have also emerged as holders of a broader and more complex role in immune defence. For example, neutrophils participate in directing the innate immune response, shaping adaptive immunity and contributing to resolution of inflammation (259, 264, 267).

13.1 Neutrophil maturation in the bone marrow

Each day, approximately 2 x 10^{11} neutrophils (almost 2.5 million per second) are produced in the human bone marrow during homeostasis (260). This process is mainly controlled by growth factors such as granulocyte colony stimulating factor

(G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF). G-CSF is the main regulator of neutrophil maturation by affecting the commitment of progenitor cells to myelopoiesis, the proliferation of granulocytic precursors and the transit time through granulocytic compartments (314). Neutrophil production is largely regulated by rate of apoptosis. When apoptotic neutrophils are phagocytosed by macrophages, the macrophage production of IL-23 is reduced, leading to decreased IL-17A production by neutrophil regulatory T cells, with decreased G-CSF production as a consequence (261). The time for maturation of neutrophils in the bone marrow is 12–14 days.

The starting point of neutrophil differentiation is the pluripotent haematopoetic stem cell that under the influence of growth factors and cytokines passes through several stages of maturation, namely, the myeloblast, promyelocyte, myelocyte, metamyelocyte and band cell stages, finally forming the mature polymorphonuclear (segmented) cells (261, 315). Throughout this process, small intracellular vesicles, granules, are continuously formed and their first appearance marks the cell's transition from myeloblast to promyelocyte. Granules are traditionally divided into azurophil (primary) granules formed during the myeloblast and promyelocyte stage, specific (secondary) granules formed during the myelocyte stage and, finally, gelatinase (tertiary) granules formed during the metamyelocyte and band cell stages (316).

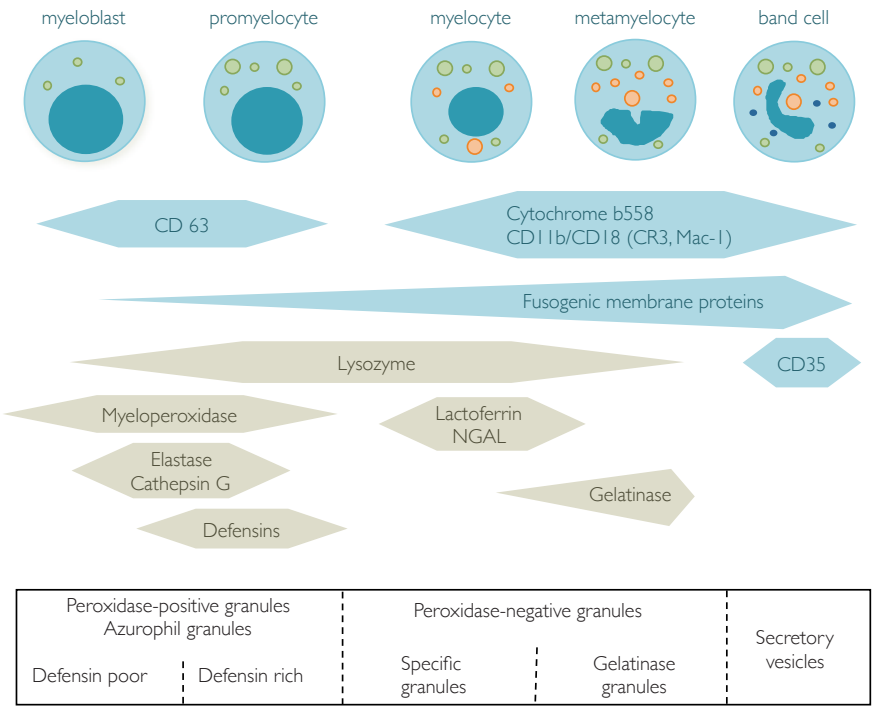

Figure 8. Granulopoiesis according to the theory of targeting-by-timing. Hager, Cowland & Borregaard. J Intern Med. 2010;268(1):25-34.

Granules are formed through a mechanism where vesicles bud off from the Golgi complex. When budded off, they are packed with the proteins that are synthesized at that particular time of cell maturation, a process termed sorting-by-timing (315, 317). Although overlap exists between the contents of the different granules, azurophil granule markers mainly include CD63, myeloperoxidase (MPO), lysozyme and elastase; specific granules are identified by their content of lactoferrin and neutrophil gelatinase-associated lipocalin (NGAL), while gelatinase granules are indentified by gelatinase (261, 316). Cytochrome b (b558), CD11b/CD18 and fusogenic membrane proteins are mainly contained in specific and gelatinase granules. The band cell stage marks the completion of granule formation. At this point, the fourth neutrophil-specific organelle, the secretory vesicle, is formed through endocytosis of the plasma membrane (258, 267, 318). As a consequence, secretory vesicles have receptors and proteins from the plasma membrane on the inner surface of the vesicular membrane and contain plasma proteins invaginated during the endocytic process, for example CD35 (261, 318). Proteins synthesized after exit of the neutrophil from the bone marrow are not packed in granules, although it is not known how and where these proteins are stored (319, 320).

13.2 Release of neutrophils from the bone marrow to the circulation

Under healthy conditions, only mature neutrophils are released from the bone marrow. Retention of immature cells is tightly controlled by signals from the receptor CXCR4 (261, 314). Throughout neutrophil maturation, the number of CXCR4 receptors decreases and at the same time the chemokine receptor CXCR2, which signals 'release', increases in number (258,314). The release of neutrophils from the bone marrow is enhanced by G-CSF (314).

As stated above, mature neutrophils that finally reach the circulation have lost the capacity to divide; they are thus terminally differentiated. Thereby the possibility of achieving phenotypic variation in the neutrophil population as a whole is limited. Instead, phenotypic modifications occur in the individual neutrophil throughout its short life span.

13.3 Neutrophils in the circulating and marginating pool

The half-life of neutrophils in circulation has been estimated to approximately eight hours at homeostasis. Recently this view was challenged in a study that estimated the average survival in circulation to approximately five days (321). However, the study has received methodological criticism and this finding thus remains to be further investigated (267, 271).

In a classical study by Mauer et al., autologous neutrophils labelled with radioactivity were injected into healthy volunteers to study turnover rate (314, 322). Surprisingly, the result showed that 50% of the cells disappeared from the free-floating pool of neutrophils in circulation. The fraction that disappeared was considered to be recoverable back to circulation and was defined as the marginating pool, consisting of neutrophils that are loosely attached to the endothelium in locations with slow blood flow, mainly in the liver, spleen and bone marrow. There is a continuous exchange of cells between the circulating and marginating pools, and a number of factors affect this exchange as well as the size of the pools (258). The marginating pool serves as a reservoir of mature neutrophils but it is also suggested that marginating neutrophils survey the respective organ for microbes and tissue damage.

13.4 Neutrophilia

Neutrophilia is defined as an increased concentration of neutrophils in a blood sample, that is, in the circulating pool. There are a number of factors that induce neutrophilia. For example, adrenaline or exercise leads to a shift of neutrophils from the marginating to the circulating pool while prednisone treatment leads to an increase in the size of the circulating pool by inhibition of adherence to the vessel wall of the marginating pool (258, 314).

During infection and many inflammatory conditions, a sometime dramatic increase in the circulating pool of neutrophils is seen. In order to evaluate this aspect in patients with PFAPA or SAPHO, complete blood count and differential including absolute neutrophil count was determined by flow cytometry (Advia Cell Counter) to identify leukocyte subpopulations by size and peroxidase staining (papers II–IV). In PFAPA, the numbers of neutrophils were increased during the febrile phase, as described in paper II and III. In line with these findings, the absolute neutrophil counts in SAPHO were increased when the patients were in an inflammatory phase as compared to a non-inflammatory phase. This indicates that there is an increased production and release of neutrophils from the bone marrow as part of the proinflammatory response.

The precise mechanisms behind such neutrophilia are not known but, in general, neutrophilia that pertains to inflammation or infection is caused by an increase of the total neutrophilic pool by an increased bone marrow production and release of cells, which increases both the circulating and marginating pools (314). Another mechanism that contributes to neutrophilia is decreased apoptosis, as indicated in paper III, where neutrophilia is correlated to delayed apoptosis during PFAPA episodes.

13.5 Recruitment of neutrophils from circulation to tissue

Neutrophil activation has mainly been studied in a context of neutrophil recruitment in response to a local inflammatory process (260). A narrative of neutrophil activation includes the claim that tissue macrophages, upon activation by PAMPS and DAMPS, produce cytokines and chemokines. Pro-inflammatory cytokines stimulate endothelial cells to produce and expose adhesion molecules (e.g. P-selectins, E-selectins, integrins and ICAMs) (260, 261, 267). P-selectin and E-selectin on the endothelium capture free-flowing neutrophils by binding to constitutively expressed selectin ligand on neutrophils (i.e. L-selectin/CD62 and P-selectin glycoprotein ligand 1). This low-affinity binding allows neutrophils to slow down, pushed along the endothelial membrane by the blood flow, resulting in what is known as rolling (Figure 9).

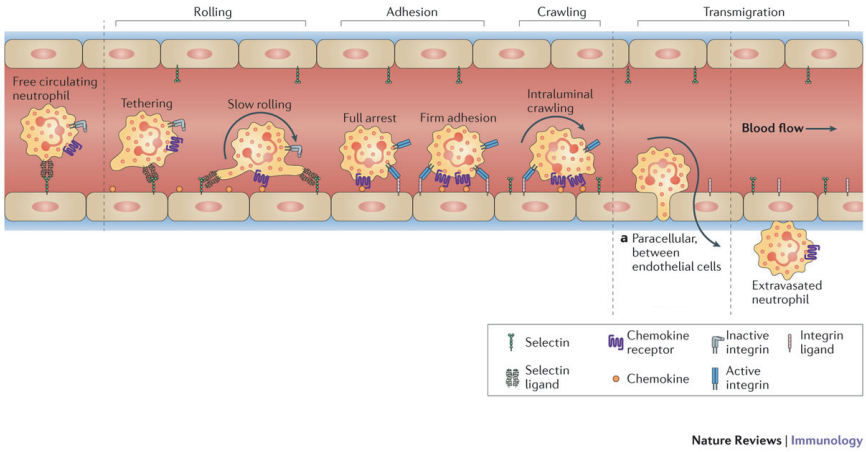

Figure 9. Neutrophil recruitment from blood to tissue.
Kolaczkowska E, Kubes P. Nat Rev Immunol. 2013;13(3):159-75.

In interaction with the endothelium, the neutrophils encounter tissue-derived cytokines and chemokines that stimulate the upregulation of integrins on the neutrophil surface by fusion of secretory vesicles and gelatinase granules with the plasma membrane. Integrin–integrin interaction between neutrophils (CD11b/CD18; Mac-1) and endothelium (ICAM) then supports arrest of the neutrophil on the endothelium, with subsequent crawling along the endothelial surface and migration through a paracellular (between endothelial cells) or transcellular (via individual cells) route, directed by a chemotactic gradient (260,, 267). Once in the tissue, neutrophils migrate through the interstitial space towards the inflammatory focus, where they become fully activated by tissue cytokines and, in the normal situation kill the intruder by phagocytosis and finally die through apoptosis and macrophage-clearance.

To what extent this narrative of neutrophil activation in the context of local neutrophil recruitment pertains to neutrophil activation in systemic inflammation needs to be further investigated.

13.6 Neutrophil phenotypes and levels of activation

There are a number of neutrophil activation steps between the two extremes of flowing freely in the blood stream and killing microbes in inflamed tissue, and these steps are reflected by differences in cellular phenotype. In circulation, the neutrophils are inert, allowing for very few interactions to take place with the endothelium and other blood cells. Having met a first stimulus, neutrophils are set in a state of alert that permits further activation as they encounter additional stimuli, that is, they are primed (260, 323). Priming is chiefly mediated by granule mobilization, increasing and altering the receptor exposure on the cell surface, but also by changes in intracellular signalling. The prevailing understanding is that priming occurs when the neutrophil traverses the vascular endothelium and migrates through the tissue to become fully activated as it reaches the infectious or aseptic inflammatory focus. The process can be initiated by several pathways, including interaction with pro-inflammatory cytokines (e.g. TNFα and IL-1β), contact with activated endothelium, exposure to PAMPs (LPS, formyl peptides), or binding of chemoattractants (IL-8 in low concentration) (259, 260). However, to what extent neutrophils can be primed (and activated) within the circulation remains an open question.

In response to stimulation, granules are mobilized in the opposite order to which they were formed, starting with the fusion of the secretory vesicles with the plasma membrane (258). Degranulation starts with fusion of secretory vesicles, the most readily mobilized organelle, which leads to increased exposure of adhesive and chemotactic receptors that are important for neutrophil recruitment to the tissue, for example integrins and selectins. The gelatinase (and specific) granules deliver yet more adhesion receptors when mobilized, as well as increased levels of cytochrome b, probably contributing to increased ROS production (see below). In addition, gelatinase granules release matrix metalloproteinases, which break down the extracellular matrix to facilitate the cell's migration towards the inflammatory focus. Finally, when azurophil and specific granules fuse with the plasma membrane, antimicrobial proteins such as lactoferrin, lysozyme and defensins, are released into the formed phagosome (or out to the extracellular space).

13.6.1 Methods to investigate neutrophil phenotype based on receptor exposure

In Papers III and IV, upregulation of CD35 (CR1) and CD11b (CR3) and shedding of CD62L (L-Selectin) were used as markers of neutrophil priming and activation, analysed by immunostaining and flow cytometry (324-326). Using this method, physical properties and antibody-mediated flourescence is detected by light emission as cells pass in a single line in front of a laser beam, giving measures of size, granularity and receptor exposure. Upon mobilization of secretory vesicles, CD35 and CD11b are upregulated, while degranulation of gelatinase (and specific) granules gives rise to a more substantial CD11b increase on the surface (327). The activation of plasma membrane-localized proteases results in cleavage of L-selectin, detected as a decreased binding of the corresponding antibody and thus fluorescence (328).

To investigate the priming status of neutrophils from patients with PFAPA and SAPHO, blood was initially left untreated or incubated with the priming agent TNFα (positive control). Leukocytes were then stained with phycoerythrin-conjugated anti-CD-35, anti-CD11b, or anti-CD62L antibodies. During attacks of PFAPA, neutrophils showed increased CD11b expression but preserved L-selectin/CD62L exposure, as depicted in paper III. Also, two patients with SAPHO, studied during an inflammatory phase in paper IV, showed an increased CD11b expression and unchanged L-selectin/CD62L expression in a similar manner as in PFAPA. This indicates that neutrophils are pre-primed or primed in a comparable manner in PFAPA and SAPHO, and that this neutrophil phenotype does not define either of the conditions. This finding also supports the theory that priming of neutrophils occurs in circulation and that primed neutrophils are not limited to extravascular tissue.

13.7 Phagocytosis

Phagocytosis is the body's main method of killing microbes and removing cell debris. Neutrophils (and other innate immune cells) engulf pathogens by an active receptor-mediated process (329). They identify opsonized microbes by complement receptors and Fc receptors, but also recognize PAMPS by pattern-recognition receptors (259, 330). The microbe is then engulfed into a phagosome formed by the neutrophil plasma membrane, and this organelle subsequently fuses with specific and azurophilic granules, transforming the phagosome into a phagolysosome. Antimicrobial proteins, in combination with toxic oxygen radicals, then kill the microbe in the phagolysosome (see below).

When a neutrophil attempts to ingest something that is too big to internalise, the phagosome cannot be completely closed. The neutrophils granules will still fuse with the plasma membrane, which lead to the release of granule-derived toxic

substances into the interstitial space, with damage to endogenous tissue as a consequence. This is often referred to *as frustrated phagocytosis* (331).

13.8 Production of oxygen radicals in neutrophils

A characteristic of neutrophil function is the ability to produce large amounts of oxygen radicals, formed by the phagocyte NADPH-oxidase, a multi-component enzyme assembled around cytochrome b with its subunits $p22^{phox}$ and $gp91^{phox}$. Cytochrome b is located in the plasma membrane as well as in the membranes of

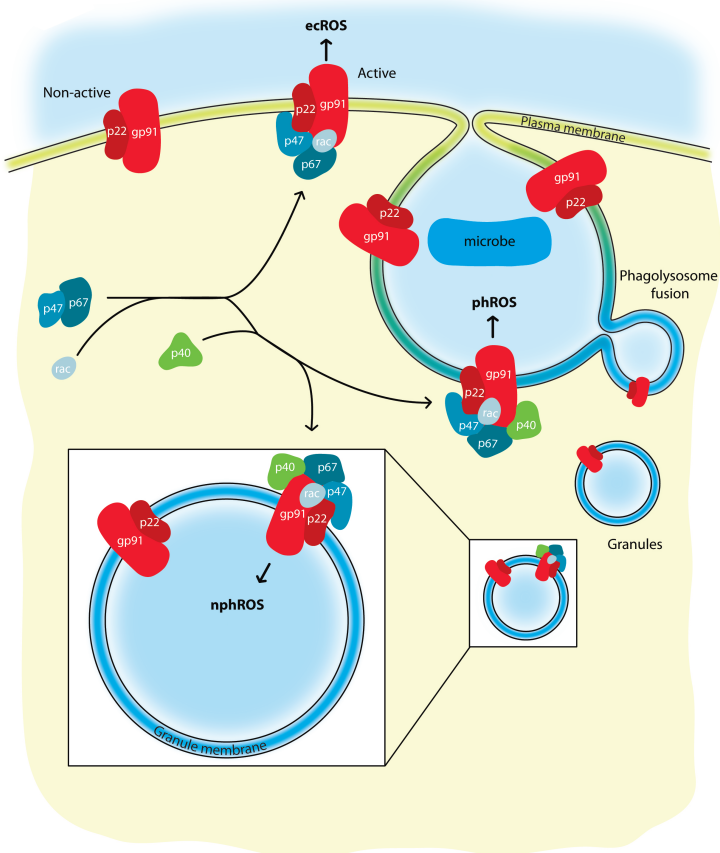

Figure 10. The neutrophil NADPH-oxidase. The NADPH-oxidase assembles upon cell activation, either in the plasma membrane, resulting in extracellular or phagosomal ROS (ecROS, phROS), or in intracellular granule membranes, resulting in nonphagosomal ROS (nphROS). Bylund et al. Free Radic Biol Med. 2010;49(12):1834-45.

the specific and gelatinase granules. When the NADPH-oxidase complex is activated, the cytosolic components $p47^{phox}$, $p67^{phox}$ and Rac translocate to cytochrome b to form a functional enzyme. When NADPH-oxidase activation takes place in the granule membranes, $p40^{phox}$ is also included in the complex (277) (Figure 10). The assembled enzyme transfers electrons from NADPH in the cytosol across the membrane to oxygen on the other side. This results in production of superoxide anion (O_2^-) that spontaneously dismutates to hydrogen peroxide (H_2O_2), and sequentially more reactive metabolites are formed, for example hydroxyl radical (OH^-). The H_2O_2 is further processed by an MPO-dependent reaction to form the highly toxic hypochlorous acid (HOCl) and peroxynitrate ($ONOO^-$), which is formed by a reaction with nitric oxide. Both HOCl and $ONOO^-$ are capable of efficiently killing microbes (273). Neutrophils themselves are protected by the expression of antioxidants such as superoxide dismutase (SOD) and catalase. The exact mechanisms whereby the killing occurs in the phagosome are still issues of debate, but ROS are highly reactive species that can be detrimental to microbes in a number of ways (273).

When the NADPH-oxidase is assembled and activated in the plasma membrane, ROS are released from the cells, producing extracellular ROS (ecROS). Correspondingly, when the oxidase is assembled in an intracellular membrane, intracellular ROS are formed (icROS). The traditional view has been that icROS are produced at the phagosome and phagolysosome membranes (phagosomal ROS, phROS). Today, however, it is increasingly accepted that icROS is also produced in the absence of phagolysosome formation, probably by NADPH-oxidase activation in granule membrane, designated as nonphagosomal ROS (nphROS). NphROS production occurs parallel to phROS production but also secondary to stimulation with certain agonists, such as the soluble protein kinase C (PKC) activator, phorbol myristate acetate (PMA), or the endogenous lectin galectin-3 (327, 332). These agonists induce the production of nphROS and ecROS but not phROS production (see methods below).

13.8.1 Methods to investigation NADPH-oxidase-derived ROS production

One of the most versatile techniques to measure neutrophil production of oxygen radicals is luminol/isoluminol-amplified chemiluminescence (CL) (333). This method is based on the fact that dyes like luminol and isoluminol emit light after being excited by ROS in the presence of a peroxidase. The plasma membrane of neutrophils is permeable to luminol, as opposed to isoluminol, which remains extracellular.

Isoluminol-amplified CL can be used to measure ROS that are released extracellularly. Isoluminol-amplified CL is peroxidase-dependent and therefore horseradish peroxidase has to be added when extracellular ROS production is measured. The method has a high sensitivity and permits kinetic measurements of ROS (O_2^-) production to be performed with high resolution. When isoluminol-

amplified CL was used in paper III to compare ecROS from PFAPA neutrophils between febrile and afebrile episodes, no differences were found. When the same method was used in paper IV, for neutrophils from patients with SAPHO, the results were in line with those of PFAPA. This reinforces the claim that the function of the NADPH-oxidase in the plasma membrane is normal and that stimulus-induced ecROS is not a proxy for the disease mechanism in either PFAPA or SAPHO.

Luminol-amplified chemiluminescence detects both extracellular and intracellular ROS production, but by addition of the extracellular scavengers SOD and catalase, the method is specific for intracellular ROS. As this reaction too is peroxidase-dependent, intracellular MPO is needed for icROS to be detected by CL. In paper III and IV quantification of MPO protein was done by ELISA in PMN homogenates, without any significant differences between the PMN sample groups. ROS produced by the NADPH-oxidase in a phagosomal membrane can be detected by CL after the phagosome has fused with the MPO-containing azurophilic granules (phagolysosome). The method however also measures ROS production in cytochrome b-containing granules (gelatinase and specific granules), and the fact that MPO has to be present for the CL reaction to take place suggests a fusion between these granules and azurophil granules to take place upon cell activation. The method has a high sensitivity and permits close detection of the kinetics of ROS (O_2^-) production. In paper III, the method was used to measure icROS in neutrophils from patients with PFAPA, with a significantly increased icROS during febrile episodes as a result. In SAPHO, icROS production was also increased in the two patients who were investigated during the inflammatory phase described in paper IV. This indicates that deficient icROS production is not a general feature of SAPHO (which has been suggested; (257) and that both PFAPA and SAPHO have an inflammatory phenotype characterized by increased icROS production. The mechanism behind the increased icROS production may be on the level of NADPH-oxidase regulation or due to alteration of signal transduction pathways, possibly linked to the (unknown) mechanisms leading to these autoinflammatory conditions.

Another method that allows for detection of both extracellular and intracellular production of neutrophil ROS is the oxidation of *p*-hydroxyphenyl acetic acid (PHPA), based on the fact that NADPH-oxidase-derived O_2^- spontaneously dismutates to H_2O_2. When reacting with H_2O_2 in the presence of an added peroxidase, PHPA becomes fluorescent and can be detected by a fluorometer. The cell membrane is impermeable to PHPA, in other words, PHPA measures H_2O_2 extracellularly only. Nonetheless, the total H_2O_2 (extracellular and intracellular) production can be measured by addition of azide; this inhibits the processing of intracellular H_2O_2 (by MPO for example) and allows for intracellular H_2O_2 to pass the cell membrane and be measured extracellularly. Intracellular H_2O_2 can thus be calculated as the total H_2O_2 production minus the intracellular H_2O_2 production. This method was used in paper IV to corroborate the findings gained by the CL technique, showing levels of total and extracellular ROS production in keeping with the results obtained by CL.

There are several different agonists that can be used to induce the production of extracellular and intracellular ROS. In paper III, the formyl peptide fMLF was used to stimulate ecROS production through activation of the chemotactic receptor formyl peptide receptor, while phorbol myristate acetate (PMA) was used to stimulate icROS production. By the activation of protein kinase C, PMA in fact activates both ecROS and icROS production, as seen in paper IV, thus, whether icROS or ecROS is measured will depend on the type of detection method used.

13.8.2 Mitochondrial ROS and how to measure them

Mitochondria are often referred to as the power factory of the cell, as they produce ATP through cellular respiration. Hence, the number of mitochondria varies according to the energy needs of different cells; for example, muscle cells have a high energy need and neutrophils have a low need, reflected by a high and low number of mitochondria, respectively. During cellular respiration in the mitochondria, oxygen is converted to water, a process that also generates ROS (mtROS) as a by-product. The mitochondria are protected from toxic ROS by mitochondrial SOD, which transforms O_2^- into the more stable H_2O_2 (334).

In paper III of this thesis, neutrophil production of mtROS was studied by staining the cells with MitoSOX Red. MitoSOX Red is a positively charged substance that selectively binds to the mitochondrial membrane. When MitoSOX Red interacts with ROS, it is oxidized into a highly fluorescent compound that can be detectable by flow cytometry (335). The number of mitochondria varies between cell types and is not even constant in neutrophils; this will affect the amount of mtROS produced. In paper III, a significant although small elevation of mtROS levels was seen in neutrophils from children with PFAPA in the afebrile phase compared to afebrile controls, although on a similar level compared to the febrile sample. This suggests that mtROS production needs to be further investigated in PFAPA, not only in neutrophils, but also in cells with a higher number of mitochondria, including monocytes. This is particularly interesting considering that the autoinflammatory syndrome TRAPS is driven by an increase in mtROS production secondary to an accumulation of a misfolded TNFR1 protein in the cytoplasma.

13.9 Neutrophil extracellular traps (NETs)

Neutrophils can kill microbes not only by phagocytosis, but also by the release of neutrophil extracellular traps (NETs). NETs are formed by neutrophils through the breakdown of their nuclear membranes and contents followed by the release of a fibrous, gluey network of chromatin and antimicrobial peptides that trap and kill the microbes (336). The scientific community initially received the early *in vitro* studies of NETs with some scepticism, as the relevance of NETs *in vivo* was hard to assess. Today, the formation and release of NETs is considered an established method for neutrophils to kill microbes together with phagocytosis and frustrated

phagocytosis. Upon release of NETs, neutrophils undergo a violent, proinflammatory cell death (NETosis), in contrast to the controlled process of apoptosis (see below).

The formation of NETs depends both on the production of ROS by the NADPH-oxidase and processing of ROS by MPO; thus, neutrophils from patients with MPO-deficiency or CGD (deficient ROS-producing capacity) do not form NETs (337, 338).

13.10 Neutrophil death and longevity

Under physiological conditions, apoptosis is the main death mechanism of neutrophils (339). Aging neutrophils undergo apoptosis after 8–48 hours in circulation and are then cleared by macrophages in the liver, spleen and bone marrow, directed towards the latter by increased CXCR4 expression (267).

Apoptotic neutrophils are non-functional cells with preserved membrane integrity preventing the release of toxic substances to the extracellular milieu. These cells signal that they are ready to be cleared mainly by the exposure of phosphatidylserine, a membrane lipid that flips from the inside to the outside membrane leaflet as part of apoptosis initiation. Apoptotic neutrophils are characterized by sequential morphological changes, such as DNA fragmentation, chromatin condensation, reduction of nuclear size, shrinkage of cells and, finally, breakdown into apoptotic bodies. Neutrophil apoptosis and clearance is a tightly regulated process that avoids activation of an inflammatory response and contributes to resolution of inflammation, the latter by the production of TGF-β and IL-10 by engulfing macrophages (260). Uptake of apoptotic neutrophils also decreases bone marrow production of neutrophils, as macrophages downregulate IL-23 production upon phagocytosis, which leads to decreased IL-17 secretion by T cells and reduced G-CSF production by fibroblasts and endothelial cells. In contrast, neutrophils that die from necrosis or NETosis lose membrane integrity, release toxic products and trigger inflammatory responses. However, apoptotic neutrophils that are not cleared promptly may undergo secondary necrosis with an inflammatory response as a consequence.

The rate of neutrophil apoptosis can be decreased or accelerated in association with an innate immune response, suggesting that the life span of neutrophils may be a mechanism to control the length and termination of the immune response (340). In an infected host, the rate of neutrophil apoptosis depends on the immune response and health of the host, but also on the microbe's immune evasion strategy (341). In general, increased neutrophil apoptosis and clearance removes activated neutrophils as well as neutrophils that have engulfed microbes. In other words, the immune response is downregulated through increased apoptosis, and production and release of neutrophils from the bone marrow are reduced (267). In contrast, decreased apoptosis ensures the presence of activated neutrophils at the site of inflammation, prolongs the inflammatory response, and increases neutrophil production in and

release from the bone marrow (270, 284). Apoptosis is delayed by cytokines such as IL-1, TNF-α, G-CSF and GM-CSF, as well as by PAMPS such as fMLF and LPS (339). IL-10 inhibits delayed apoptosis induced by proinflammatory cytokines, hence, IL-10 does not induce apoptosis by itself, but can regulate the process when initiated by proinflammatory cytokines. Neutrophil apoptosis is accelerated by a stimulus such as Fas ligand (FasL) binding to a neutrophil cell surface death receptor, CD95 (Fas) (339).

13.10.1 Methods of investigating neutrophil apoptosis

For the analysis of leukocyte cell death, a number of different characteristics can be studied, for example morphological changes, mitochondrial permeability, membrane potential, DNA cleavage, caspase activation and reorganization of the plasma membrane with exposure of phosphatidylserine (342). This summary will focus on characteristics that were studied and methods that were used in Papers III and IV.

Apoptotic neutrophils express phosphatidylserine on the plasma membrane. Annexin V (AnnV) binds specifically to phosphatidylserine and can be measured by FACS analysis when coupled to a fluorescent derivative, for example annexin V-FLOUS, as used in paper III and IV. Furthermore, late apoptotic and necrotic cells lose their membrane integrity and will then be stained by 7-aminoactinomycin D (7-AAD) that binds to exposed DNA and also fluoresces, which is used for FACS detection. Hence, viable neutrophils stain $AnnV^-$ and $7\text{-}AAD^-$, early apoptotic neutrophils stain $AnnV^+$ and $7\text{-}AAD^-$ and, finally, late apoptotic and necrotic cells stain $AnnV^+$ and $7\text{-}AAD^+$ (342).

Neutrophil apoptosis can be manipulated *in vitro*, for example in order to investigate whether apoptosis regulation is affected by disease-associated mechanisms in patient cells. The extrinsic apoptotic pathway can be induced by ligation of death receptors, for example TRAIL (TNF-related apoptosis-inducing ligand) and FAS (CD95). The latter can be activated by anti-CD95 monoclonal antibodies, which enhance clustering and cleavage of procaspase-8 that in turn activates caspase-3, leading to increased apoptosis. Conversely, neutrophil apoptosis can be delayed by antiapoptotic stimuli, for example bacterial lipopolysaccharide (LPS) that activates TLR4 (343). In paper III we found that when PMNs from PFAPA patients were exposed to anti-CD95 mAb they responded with a higher rate of apoptosis than the spontaneous rates. In response to the antiapoptotic stimulus LPS, PMNs from febrile patients with PFAPA syndrome were not further suppressed, whereas PMNs from all controls and afebrile patients with PFAPA syndrome responded to LPS with decreased apoptosis. This indicates that the proapoptotic signalling pathway is intact in PFAPA, but also that apoptosis is considerably suppressed during febrile episodes, given that it is not further suppressed by LPS stimulation. This also suggests that sufficient neutrophils are available during the inflammatory process.

14. Concluding remarks and future perspective

An increasing research interest has been directed toward autoinflammatory diseases during the last 10 years, with an escalating number of publications in the area. For FMF, PFAPA and SAPHO this has led to an improved understanding of these conditions, but there are still substantial knowledge gaps that intrigue families, clinicians and the research community. In 2008, we formed a translational autoinflammatory research group in Western Sweden, with several national and international collaborators. This thesis is a result of this translational effort. In addition to describing autoinflammation with reference to the three diseases in focus, the thesis explores the concept *per se* by examining previously suggested definitions of autoinflammatory diseases. It attempts to further advance the definition based on recent research on the immune system as a whole, as well as on evolving autoinflammatory conditions with complex phenotypes; the thesis proposes a definition of autoinflammatory diseases that complements previous definitions by including the possible activation of the adaptive immune system and association with other immune dysfunctions.

With regard to PFAPA, a broad spectrum of research challenges stand before us, including the understanding of the clockwork regularity of typical attacks, the exceptionally good response to corticosteroids, the therapeutic effect of tonsillectomy and the decreased frequency of viral infections. Our research group are currently addressing the latter in a prospective case control study comparing children who have PFAPA with healthy children from the same day care centre.

The studies of PFAPA we have reported thus far demonstrate dysregulation of molecules and cells of the innate immune system, as described in papers II and III. The study of neutrophils shows that these cells are involved in the disease process, evidenced by altered functional properties, such as ROS production, apoptosis and priming, accompanying the disease fluctuations. The importance of ROS in autoinflammation pathogenesis has recently been highlighted (300), and we have added important data to this field by showing the increase of intracellular ROS production associated with febrile episodes in PFAPA (as well as in SAPHO). The pathway (or pathways) that initiate(s) the inflammatory response in PFAPA has yet to be identified. In addition, we found an increase in mtROS production during the afebrile phase in PFAPA; given that mtROS appears to be a central disease mechanism in TRAPS, we believe that this is an important finding to explore further. Another finding from our studies is that serum levels of galectin-3 in both the febrile and afebrile phase were normal in PFAPA but not in FMF. This suggests that galectin-3 could be a valuable diagnostic marker for differentiating between these two, sometimes phenotypically very similar, diseases.

Today, PFAPA is most adequately described as a polygenic disease with familial clustering and a predominantly non-Mendelian inheritance. In an ongoing collaborative study, we are presently examining the results from the genetic analysis of a large number of genes involved in autoinflammatory and inflammatory conditions in 24 patients with PFAPA. We are using state-of-the-art technology to decipher the data and handle the substantial amount of data that the study has so far generated.

PFAPA is associated with activation of the adaptive immune system, demonstrated by a Th1 differentiation of CD4+ T cells leading to increased production of INF-γ-related cytokines during flares. The disease has an onset in children typically below the age of five, an age when the Th1 response to innate stimulation increases from paediatric to adult levels. It is also a period when children have an increased susceptibility to intracellular pathogens, such as *M. tuberculosis*. Hence, it can be speculated that PFAPA is an extreme immunological phenotype that exaggerates the normal Th1 development towards adult levels, providing the affected children with a survival advantage against intracellular pathogens such as mycobacteria at an otherwise vulnerable age. It is also possible that the Th1 differentiation in PFAPA is a key to a potential trigger harboured in the tonsils. These two hypotheses do not have to be mutually exclusive. One way to advance this question further is by studying the Th1-dependent mechanisms responsible for a regular vaccine response in children with PFAPA compared to healthy children. An educated guess would be that the vaccination response is at the higher end of the scale in children with PFAPA.

Despite the good long-term prognosis for children suffering from PFAPA, our clinical experience indicates that the disease considerably influences the child's quality of life and the family situation as a whole during active disease periods, including the frustration of numerous initial visits to the emergency room due to incorrect diagnosis and treatment. During 2015 we have initiated a study including parental interviews based on an interview guide developed on the basis of data from a Facebook group in the US and our clinical experience. Our preliminary data indicate that PFAPA, despite the good prognosis, substantially influences the family situation as a whole and the quality of life of the child (manuscript in preparation). The data will hopefully result in an in-depth understanding of these families' circumstances over time and, as a consequence, an improved understanding and recommendations of how healthcare providers can meet the child's and family's needs for medical care and support. Interestingly, data from the Facebook group underlined the fact that the PFAPA flares can be detected by slight symptoms as much as one or two days before fever initiation, as shown by comments from parents and siblings noticing grumpiness in the affected child. This inspires us to look for the initiating biological mechanisms at an earlier time point than we or any other researcher has done so far, as the initiating factors leading to an episode are so far unknown.

Paper I of this thesis, focusing on FMF, emphasizes the importance of proper identification, diagnosis and treatment for children with FMF to avoid unnecessary attacks and development of amyloidosis as a long-term consequence. The

prevalence of FMF among immigrants in western Sweden is in the same range as in their country of origin, indicating that there are well over 300 patients in Sweden today, most probably increasing as immigration from the east Mediterranean Basin increases due to the instable political situation there. We believe that close monitoring of this development and improved expertise are vital for providing children with FMF the best possible healthcare in the future.

Disappointingly, the identification of mutations in the *MEFV* gene as a cause of FMF has not led to a clear understanding of the inheritance of the disease or the physiological and pathophysiological function of pyrin. A recent study suggests that pyrin may be a specific immune sensor for bacterial inactivation (glycosylation) of Rho GTPases, leading to caspase-1 activation and subsequent IL-1 and IL-18 production. This study may be an important contribution to the understanding of pyrin function in immune regulation *per se*, but also of the mechanism behind dysregulation of pyrin function in FMF and the definition of the pyrin pathway(s). Future identification of pathways and triggers of pyrin may enable better mechanistic studies in patients with FMF, an approach in which studies of neutrophils are highly relevant, both in patients with and without colchicine treatment. Furthermore, pyrin as a sensor of bacterial modification of Rho GTPase is an interesting model for explaining the survival advantage of mutation carriers and the variation of disease expression in different contexts.

Another enigmatic fact with regard to FMF is the existence of patients who are heterozygous for the MEFV mutation, or have no identifiable mutation. However, the FMF cases in Sweden are probably too few to address these questions.

In SAPHO, the clinical features that combine skin and bone lesions are interesting from both the perspective of innate immune dysregulation leading to for example DIRA, but also from the perspective that innate immune response has evolved in close connection with microbes, highlighting the possibility of PAMPS as triggers of disease in a predisposed host. That this predisposition relates to decreased icROS production was proposed by Ferguson in 2008. In paper IV we conclude that altered ROS production is not a general feature of SAPHO syndrome and therefore other mechanisms have to be at play, possibly with a bacterial trigger, which makes *P. acne* an interesting candidate. The mechanism underlying the increased icROS activity seen in both SAPHO and PFAPA during an inflammatory active phase needs to be further investigated, and the fact that children with PFAPA in particular are available in Western Sweden allows us to pursue such an approach in our continuing research.

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