The Periodic Fever, Aphthous stomatitis, Pharyngitis and cervical Adenitis syndrome in children

Clinical and immunological aspects

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1 Preface

1.1 Acknowledgements

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My main supervisor Professor Knut Øymar at the Pediatric Department of Stavanger University Hospital, triggered my interest for medical research, when I was engaged in a small project almost 10 years ago. I’m deeply grateful for the wise guidance, continuous support and inspiration that he has offered me throughout this PhD project. His infinite patience and positive attitude made me believe that any problem can be solved, and made me focus on the goal not the obstacles.

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Randaberg, March 2015

Jostein Førsvoll
1.2 Summary of thesis

**Background** The Periodic Fever, Aphthous stomatitis, Pharyngitis and cervical Adenitis (PFAPA) syndrome, first described in 1987, is defined by clinical criteria. With onset during early childhood, the affected children experience short, regular episodes of fever accompanied by at least one of the following clinical signs: aphthous stomatitis, pharyngitis and cervical adenitis. At the beginning of this thesis, very few studies on PFAPA in Scandinavian children had been published, and no population based studies existed. The cause of the syndrome was unknown, although a dysregulation of the immune system was indicated in one study. Curation of PFAPA after tonsillectomy was reported, but the role of the tonsils in the etiology of PFAPA was unclear.

**Aim** The overall aim of this thesis was to study epidemiological and clinical characteristics of PFAPA in a population based approach, and to study immunological aspects of PFAPA in blood and tonsils.

**Methods** All children in South Rogaland diagnosed with PFAPA during 2004–2010 were evaluated clinically, and parents were interviewed systematically at the time of diagnosis. A follow-up interview was conducted at least one year after the diagnosis was set. Levels of hematologic parameters, immunoglobulins (Ig) and inflammatory proteins were measured in blood from children with PFAPA during and between febrile episodes and in control children with pneumonia during the febrile phase and at least four weeks after full recovery. Palatine tonsils from children with PFAPA were evaluated histologically, and the number of different cell types in tonsillar germinal centers was identified immunohistochemically. Tonsils from children with tonsillar hypertrophy served as negative controls.

**Result** In paper I, 46 children (32 boys; p = 0.011) were diagnosed with PFAPA. The median age of onset was 11.0 months (quartiles: 5.0, 14.8). The incidence of PFAPA was estimated to 2.3 per 10 000 children up to 5 years of age. Cervical adenitis, pharyngitis and aphthous stomatitis were present during febrile episodes in 93%, 83% and 46 % of the children respectively. Twenty children experienced spontaneous resolution; median age 60.2 months (range 24–120), and 17 children experienced
prompt resolution of febrile episodes after tonsillectomy; median age 50.9 months (range 15–128). In paper II, 22 children with PFAPA and 14 children with pneumonia were included. In children with PFAPA, serum levels of interleukin (IL)-6, CXCL10 and CCL4 were significantly increased during febrile episodes. The levels of IL-6 and CXCL10 were higher in children with PFAPA during the febrile episodes than in children with pneumonia. The levels of CXCL10 were also higher in children with PFAPA between febrile episodes compared to children with pneumonia after full recovery. The total levels of eosinophils and lymphocytes, and the level of CD4+ and CD8+ cells decreased during febrile episodes of PFAPA compared to the afebrile period. Levels of IgA, IgD, IgG and IgM did not differ between children with PFAPA and controls and were within age related normal levels. In paper III, 11 children with PFAPA and 16 children with tonsillar hypertrophy were included. Tonsils from children with PFAPA showed reactive lymphoid hyperplasia dominated by well-developed germinal centers with many tingible body macrophages. The histologic findings were unspecific, and a similar morphologic appearance was also found in the tonsils from controls. The number of CD8+ cells in tonsillar germinal centers was significantly lower in children with PFAPA (median 9 cells, quartiles: 5, 15) compared to controls (median 18 cells, 12, 33) (P= 0.001). For the other cell types, no differences were found between children with PFAPA and controls.

**Conclusions** The incidence of PFAPA was 2.3 per 10 000 children up to 5 years of age. Onset of PFAPA was frequent during the first year of life. The observed pattern of cytokines in children with PFAPA may indicate activation of the innate immune system during the febrile episodes. The decrease in levels of lymphocytes in blood may reflect redistribution of these cells to secondary lymphoid tissue. The lower levels of CD8+ cells in tonsillar germinal centers found in children with PFAPA compared to controls may be a feature linked to the etiology of the disease.
1.3 List of papers

I:
Førsvoll J, Kristoffersen EK, Øymar K.
Incidence, clinical characteristics and outcome in Norwegian children with periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis syndrome; a population-based study.

II:
Førsvoll J, Kristoffersen EK, Øymar K.
Elevated levels of CXCL10 in the Periodic Fever, Aphthous stomatitis, Pharyngitis and cervical Adenitis syndrome (PFAPA) during and between febrile episodes; an indication of a persistent activation of the innate immune system.

III:
Reduced number of CD8+ cells in tonsillar germinal centers in children with the Periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis syndrome Manuscript re-submitted as invited.
### 1.4 Abbreviations

AID: Autoinflammatory disease  
APC: Antigen presenting cell  
CAPS: Cryopyrin-associated periodic syndromes  
CD: Cluster of differentiation  
CRP: C-reactive protein  
CSF: Colony stimulating factors  
DAMP: Damage-associated molecule patterns  
ELISA: Enzyme-linked immunosorbent assay  
ENT: Ear Nose Throat  
FGF-2: Fibroblast growth factor 2  
FMF: Familial Mediterranean fever  
G-CSF: Granulocyte colony stimulating factor  
GM-CSF: Granulocyte macrophage colony stimulating factor  
GP: General practitioner  
Ig: Immunoglobulin  
IL: Interleukin  
INF: Interferon  
MHC: Major histocompatibility complex  
MKD: Mevalonate kinase deficiency  
NK: Natural killer (cells)  
NKT: Natural killer T (cells)  
NLR: Nucleotide binding and oligomerization domain-like receptors  
NLRP3: Nacht domain-, Leucine-rich Repeat-, and PYD-containing protein 3  
OVLT: Organum vasculosum laminae terminalis  
PAMP: Pathogen-associated molecular pattern  
PDGF-BB: Platelet derived growth factor BB  
PFAPA: Periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis  
PGE\(_2\): Prostaglandin E\(_2\)  
TCR: T cell receptor  
T\(_{\text{H}}\): T helper
TLR: Toll-like receptor
TNF: Tumor necrosis factor
TRAPS: Tumor necrosis factor receptor-associated periodic syndrome
T_{reg}: Regulatory T cells
VEGF: Vascular endothelial growth factor
2 General introduction

2.1 Historical background

Man has been constantly exposed to microbiological agents since he first put his foot on earth, and consequently fevers and infections have always been one of the undesirable aspects of human life [1].

Linking fever and general signs of inflammation to disease was probably one of the first accomplishments of ancient medicine [2]. Fever and the local signs of infection and inflammation were described on Egyptian papyrus rolls. The Ebers papyrus, dating back to approximately 1500 BC, contains information about the antipyretic effect of the leaves and bark from the willow tree [3].

The roman writer Aulus Cornelius Celsus who lived 1st century AD is known for his description of local inflammation [4]. In his masterpiece “De Medicina”, he described four cardinal signs of local inflammation; rubor (redness), calor (heat), dolor (pain) and tumor (swelling) [4]. A fifth sign functio laesa (compromised function) was added later by Rudolph Virchow in 1858, and together these five signs of inflammation are still learnt by rote by medical students around the world [5]. Modern medicine has taught us that fever is not merely a sign of infection, but rather a sign of an ongoing acute phase response that may occur in different types of diseases.

In 1908, Janeway and Mosenthal published an unresolved diagnostic problem describing a Jewish girl who started experiencing short, recurrent attacks of fever two weeks after birth [6]. The attacks occurred at intervals of approximately one month, and as she got older the attacks were often accompanied by severe abdominal pain and headaches. It has later been suggested that this could be the first published case describing a patient with Familial Mediterranean Fever (FMF), now known as an autoinflammatory disease (AID) [7].

From the end of the 1940s, the American doctor Hobart Ansteht Reimann published several articles on periodic disease [8-10]. He gave thorough descriptions of different disease patterns and he defined periodic fever as one out of eight different forms of periodic disease [9]. The majority of Reimann’s case reports deal with
periodic disease in adulthood, but some pediatric cases are also presented. In 1962, he described a benign course of periodic fever with onset during early childhood in a female patient [11]. With new advances in medicine, many of the patients Reimann described most likely would have received distinct diagnosis today; however his pioneer work on periodic diseases is still of interest.

In 1987, Marshall et al. described twelve children with recurrent attacks of fever and a similar set of associated symptoms that included malaise, chills, aphthous stomatitis, pharyngitis, headache, cervical adenopathy, abdominal pain and nausea and vomiting (Figure 1) [12]. Some aspects of the disease resembled cyclic neutropenia, but the children had stable neutrophil counts and they were not prone to serious infections. The febrile episodes were unaffected by antibiotics, but a prompt response to prednisone with termination of the attacks were documented in three children. In 1989, this new entity was denoted with the acronym PFAPA referring to the most pronounced clinical traits: Periodic Fever, Aphthous stomatitis, Pharyngitis and cervical Adenitis [13].

**Figure 1**

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University of Louisville School of Medicine
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During the two decades following the first description of PFAPA, several large case series presented observational data supporting the assertion that this disease is a true entity [14-16]. They reported both local and general signs of inflammation during febrile episodes, and no confounding microbial agent, a prompt effect of one single dose of a systemic glucocorticoid and a resemblance to other autoinflammatory diseases [14-16]. This led to the assumption that PFAPA was caused by a dysregulated immune system, although proof thereof was lacking [17].

The first study indicating a dysregulated immune system in PFAPA was published in 2006. Stojanov et al. concluded that their observed pattern of cytokines indicated continuous inflammation and a reduced anti-inflammatory response [18].

The first report of tonsillectomy as a possible cure for PFAPA came in 1989 [19], and later this observation has been supported by several other studies [14-16, 20-23].

### 2.2 The origin of the thesis

During the workup of children with recurrent fever at Stavanger University Hospital in the years after 2000, we became aware of PFAPA as a common cause for this clinical condition. We also realized that PFAPA was not well recognized, either by pediatricians or general practitioners (GPs). Therefore, from 2004 children with recurrent fever at SUS were systematically worked up, and those diagnosed with PFAPA were prospectively registered. A case series of the 22 first children diagnosed with PFAPA at Stavanger University Hospital was published in 2007, and an incidence for the disease in our region was suggested [24]. At that time, no studies from Scandinavia describing the clinical characteristics of children with PFAPA had been published, and no other studies worldwide had evaluated the incidence of PFAPA. We also recognized that the febrile episodes of PFAPA were accompanied by clearly elevated levels of C-reactive protein (CRP), and in 2007 this observation was also published [25].

In 2007, during the early stages of this thesis, the etiology of PFAPA was unknown. Although several large studies provided observational data, only one single study indicating a possible dysregulation of the immune system was present [18].
Consequently, there were several unresolved questions regarding epidemiological, clinical and immunological aspects of PFAPA. We therefore aimed to conduct a population based study of PFAPA in the South Rogaland area to address all these main issues, based on the current knowledge at that time. The presentation of PFAPA in the introduction will therefore be based on the current knowledge when the work was initiated and the basis of the thesis was planned.

More recent knowledge will be presented and discussed in light of our results in the discussion. In order to study and discuss the PFAPA syndrome, an inflammatory disease of unknown etiology, it is necessary with a basal understanding of the immune system. Therefore this thesis includes a brief introduction to the immune system with a particular focus on inflammation and inflammatory proteins.

2.3 A brief introduction to the immune system

The main task of the immune system is to defend its host from infectious agents. Human dependency on a constantly functioning immune system is illustrated by studies of the immune deficient patient. The immune system defends us from infections and it also prevents malignant disease, participates in all aspects of tissue repair and has an important role in general physiologic maintenance [26, 27]. The immune system possesses an arsenal of cellular and humoral defense mechanisms. These mechanisms are potentially harmful, if the ability to discriminate between “self” and “non-self” is disturbed or if effector pathways are bypassed life-threatening disease may occur.

Classically, the immune system is divided into two different branches, the innate and the adaptive immune system (Figure 2) [28]. The innate immune system acts as the first and almost immediate line of response. The adaptive immune system generates a more fine-tuned and targeted response that evolves over days to weeks and also includes immunologic memory [27]. However, these two branches collaborate to form a coordinated immune response. Additionally, the body is equipped with anatomical and physiological barriers, and like a moat and a castle wall they keep most unwanted invaders out.
**Figure 2** The integrated human immune system. The human microbial defense system can be simplistically viewed as consisting of 3 levels: (1) anatomic and physiologic barriers; (2) innate immunity; and (3) adaptive immunity. In common with many classification systems, some elements are difficult to categorize. For example, Natural Killer (NK) T cells and dendritic cells could be classified as being on the cusp of innate and adaptive immunity rather than being firmly in one camp.

Figure and legend reprinted with permission from Turvey SE, Broide DH. Innate immunity. J Allergy Clin Immunol. 2010; 125: S24-32. Copyright © 2010 Published by Elsevier Inc. All rights reserved.
2.3.1 The innate immune system

The innate immune system consists of factors that are present at birth, and all components of the immune system that are encoded in their mature functional form by germline genes [27]. Natural or spontaneous antibodies of IgM type also contribute to the innate immune response [29]. The innate immune system stays relatively unchanged throughout life, and it does not adapt its response after encountering harmful agents [30]. It is the first line of defense and in many respects the most important one. Most of the infectious agents the body encounter are repelled by the innate immune system before an infection is established [31].

The innate immune system consists of humoral and cellular components. Humoral immunity (from Latin: “humor” meaning “moisture”) includes all immunological active substances in the body fluids including the blood, spinal fluid, tears, saliva and other secretions. Both the innate and the adaptive immune system contribute to humoral immunity [27, 31]. The humoral defense mechanisms of the innate immune system include the antimicrobial peptides, the complement system, cytokines and other acute phase proteins [31, 32]. Natural antibodies secreted by naïve B cells are usually also referred to as an innate humoral defense mechanism.

Cytokines are small, soluble proteins crucial for immune cell signaling. Early release of cytokines from activated innate immune cells initiate, direct and escalate the response. Cytokines have key roles for both the innate and the adaptive immune system and they will be discussed in further detail later.

2.3.1.1 The acute phase proteins

An acute phase protein may be defined as a plasma protein that has a change in concentration by at least 25 percent during an inflammatory response [33, 34]. Most acute phase proteins are positive (their concentration increases), but examples of negative acute phase proteins (as for instance albumin, transferrin and alpha-fetoprotein) also exists [33].

Many of the acute phase proteins are immunologically active proteins, and for some their role in inflammation is not fully understood [33]. The acute phase proteins
include the proteins of the complement system, CRP, fibrinogen, serum amyloid protein A, haptoglobin, procalcitonin and many more [33, 35, 36].

CRP is a member of the pentraxin family [27]. It was the first acute phase protein to be identified as early as in 1930, and it may increase 1000-fold during inflammatory processes [33, 37]. The name was assigned due to the observed reaction with pneumococcal C-polysaccharide. CRP increases rapidly during infections and after tissue injury. The measured level of CRP shows relatively good correlation to the severity of the acute phase reaction, and may to some extent distinguish between viral and bacterial infections [38, 39]. It is therefore widely used in clinical practice. CRP is a pattern recognition molecule, binding to conserved molecular patterns on pathogens or other molecule configurations that are exposed as a result of tissue damage [27, 38]. CRP has many different immunological functions including the ability to enhance phagocytosis (opsonization) and to activate complement [38].

The complement system acts as a cascade, in which more than 40 proteins are involved. When the cascade is activated it exerts important humoral immunological functions including direct attack on bacteria or infected cells and the release of potent mediators of inflammation [32, 40].

2.3.1.2 Recognition and presentation of antigens

Cells of the innate immune system include circulating monocytes, macrophages, mast cells, neutrophils, eosinophils and natural killer (NK) cells [31]. Dendritic cells and natural killer T (NKT) cells have key roles in both adaptive and innate immunity (Figure 2).

In order to neutralize harmful pathogens, the innate immune cells need to recognize them. In contrast to the adaptive immune system where receptors for antigen-recognition are produced randomly in great variety, the innate immune system relies on a relatively small number of receptors [31]. These receptors recognize conserved molecular structures that different microbes have in common. These structures are usually referred to as pathogen-associated molecular patterns (PAMPs) [27, 31]. Some receptors of the innate immune system can also sense danger by recognizing other signals. These signals are called damage-associated molecule
patterns (DAMPs) and are endogenous, non-infective factors from tissues that are exposed as a consequence of damage, inflammation or infection [31, 41].

The Toll-like receptors (TLRs) have their name from their similarity to the TOLL protein crucial for the immunity in fruit flies, and are the most extensively studied PAMP recognition system in humans [28, 31]. TLRs are located in and on a variety of cells including circulating monocytes, macrophages, dendritic cells, neutrophils and epithelial cells [27, 32]. There are at least ten different TLRs in humans, expressed as homo- or heterodimers and they recognize different distinct PAMPs [31, 32]. For example, TLR4 recognize bacterial lipopolysaccharide and TLR5 recognize bacterial flagellin [31, 32]. TLR-stimulation results in synthesis of cytokines. The cytokines then play a crucial role in activating and recruiting other components of the innate and the adaptive immune system. Another receptor system called the nucleotide binding and oligomerization domain (NOD)-like receptors (NLR) survey the intracellular compartment detecting PAMPs and DAMPs [28, 31, 32, 42].

The elimination of microbes is essential in an immune response, and the neutrophils, macrophages and monocytes are the most important phagocytes. These cells ingest microbes and contain them in intracellular vacuoles where they are degraded by toxic effector molecules including superoxide and enzymes [27]. After phagocytosis the ingested peptide fragments may be presented to other cells of the immune system. The cells performing antigen presentation is collectively termed antigen presenting cells (APCs) and they represent an important link between the innate and the adaptive immune system. The immunological synapsis will be further discussed below [27].

The dendritic cells are specialized APCs with a unique ability to generate strong activation signals for T cells. Dendritic cells located in the periphery easily migrate to secondary lymphoid organs when they are activated by pathogens. In the lymph nodes dendritic cells migrate to T cell areas, and thereby facilitating the meeting between themselves and the one of the few unique T cells with affinity for the exact same antigen [43].
2.3.2 The adaptive immune system

The T- and B cells constitute the adaptive branch of the immune system. Through extensive rearrangement of antigen receptor gene elements, these cells produce a large repertoire of unique antigen receptors. Only a few cells of the adaptive immune system will have the ability to recognize a given antigen, and the activated cells need time to proliferate before an efficient immune response is achieved. In contrast to the innate immune system, the adaptive immune system adjusts its response and it has immunologic memory; with repeated encounter of an antigen the adaptive immune system will react faster and more efficiently [27].

The T cell receptors (TCRs) and the antigen binding sites of immunoglobulin (Ig) (i.e. the B cell receptor) are produced in a similar way. Genetic elements are combined randomly by the help of recombinase enzymes enabling a few hundred gene elements to give rise to millions of different antigen receptors [27]. A strict control system is mandatory to avoid autoimmunity. The T cells mature in the thymus, and only cells carrying a functional TCR without autoimmune affinity are released to the periphery [27].

2.3.2.1 T cells

The TCR is a cell surface complex, and if the variable $\alpha$ and $\beta$ chains recognizes antigen bound to a major histocompatibility complex (MHC), associated invariant chains initiate signaling [27]. The associated invariant chains constitute the cluster of differentiation (CD) 3 complex, and all TCR bearing cells can therefore be referred to as CD3+ cells [27].

The T cells are divided further into subsets on the basis of function and expression of certain types of CD molecules. The two major subsets of T cells are either CD4+ or CD8+, and these molecules play an important role when the TCR interacts with MHC molecules forming the immunological synapsis. An immunological synapse consists of molecules on the T cell-side; TCR-CD4 or CD8, CD28 co-stimulatory molecules and lymphocyte function-associated antigen-1 adhesion molecule binding to cells on the APC-side; MHC, CD80 or CD86 co-stimulatory and intercellular adhesion molecule-1, respectively [27, 44].
Based on their role in the immune system, most of the CD4 bearing (CD4+) T cells are referred to as T helper (T<sub>H</sub>) cells, and most of the CD8 bearing (CD8+) T cells are referred to as cytotoxic T cells. T cells constitute approximately 65% of the lymphocytes in peripheral blood with a CD4:CD8 ratio of 1.3 during the ages of one to six years [45].

MHC class I molecules are present on almost all nucleated cells. A selection of peptides of intracellular origin is constantly being displayed in a groove on this molecule. The CD8 molecule acts as a co-receptor binding to the MHC class I and is crucial for the formation of the immunological synapsis. In this way cytotoxic T cells perform constant immune surveillance, and if foreign or altered peptides are recognized an immune reaction towards infected or malfunctioning cells may occur [27].

MHC class II molecules can be highly expressed on all APCs, including dendritic cells, monocytes, macrophages and B-cells. The peptides present in the groove of the MHC class II molecules are mostly generated from endocytosed material. The CD4 molecule acts as a co-receptor to facilitate the formation of the immunological synapsis between the TCR and the MHC class II molecule. Recognition of an antigen presented may activate the T<sub>H</sub> cell [27, 46]. Lymph nodes provide a unique environment for contact between APCs and the lymphocytes of the adaptive immune system, in which a single dendritic cell is in contact with approximately 500 cytotoxic T cells and 5000 T<sub>H</sub> cells in the course of one hour [43].

Upon activation, a formerly naïve T<sub>H</sub> cell develops towards one of different possible functional ends. This process is termed T<sub>H</sub> cell differentiation [27, 46]. The cytokines present during the activation influence the direction of differentiation, and when a T<sub>H</sub> cell has reached its functional end-stage it can be characterized by the cytokine panel it produces and other features such as expression of certain receptors [46].

Classically, two different subsets, namely T<sub>H</sub>1 - and T<sub>H</sub>2 –cells were defined, with T<sub>H</sub>1 cells predominantly producing the cytokines INF-γ and interleukin (IL)-2 and T<sub>H</sub>2 cells predominantly producing IL-4, IL5 and IL-13 [46]. INF-γ release from T<sub>H</sub>1 cells results in classic activation of macrophages, B cell activation and differentiation
to IgG switch, further Th1 differentiation and up-regulation of MHC class II. Th1 cells are linked to induction of autoimmune diseases. Th2 cells mediate immune responses directed towards extracellular parasites including helminthes. Th2 cells influence B cells and thereby promoting IgE or IgG4 production. A Th2 cell predominance is linked to induction and persistence of atopic disease including asthma and allergies [27, 46-49].

Although the fundamental discovery that the B cells required help from cells of thymic origin in order to activate and start Ig production was made many years ago, the knowledge of this process is still expanding. Th1 and Th2 are no longer the only known variants of T helper cell differentiation. In fact, the activation of B cells may rely most on a more recently discovered subset of Th cells, namely the T follicular helper cells. These cells are of outmost importance for the formation of germinal centers in secondary lymphoid organs and subsequent Ig production. T follicular helper cells also plays a role in immune memory, and if dysregulated they may contribute to autoimmunity [46, 48].

Th17 cells, which produce IL-17, play an important role in defending the body from extracellular bacteria and fungi. As for dysregulation of Th1 and T follicular helper cells, the dysregulation of Th17 cells is also linked to autoimmunity [46].

Another subset of CD4+ T cells have an important role in down-regulating the immune response [27]. These cells are termed regulatory T cells (or T_{Reg}) and they are subdivided into two groups. One of the groups is developed as regulatory cells in the thymus and is characterized by expressing both CD4 and CD25 antigens. Their regulatory function is characterized by the secretion of the immune-modulatory cytokines transforming growth factor-β and IL-10 [50]. They are thought to downregulate self-reactive cells, and are also called natural T_{Reg}s. The second group of regulatory cells is induced T_{Reg} cells and their differentiation is governed by the presence of IL-10 upon activation [27]. T_{Reg} cells may be involved in regulation of autoimmune and atopic activity and in down-regulation of the specific immune response [27].
2.3.2.2 The B cells

The B cells constitute about 25% of the lymphocytes in peripheral blood from the ages of one to six years [45]. Ig is built up of two heavy chains and two light chains, and serves both as the B cell receptor and as secreted immune effector molecules. Ig has two identical variable domains acting as antigen binding sites known as the fragment antigen-binding (Fab) regions. The two Fab regions are connected to one constant domain conveying effector functions, the fragment crystallizable (Fc) region [27, 51]. Igs are divided into subclasses, namely IgM, IgD, IgG, IgA and IgE on the basis of the heavy chain isotypes.

The naïve B cell expresses the Ig-receptors IgM and IgD. Although IgM has a low affinity for antigen, it is more polyreactive than other isotypes and thereby allowing naïve B cells to respond quickly to different antigens [51].

Naïve B cells are present in large numbers in lymph node follicles, and their activation depends on several concurrent events, including binding of antigen to surface IgM and co-stimulatory signals from subsets of CD4+ T cells. Upon activation several different subsequent events may occur. Activated B cells may mature to plasma cells and secrete large amounts of Ig, but they may also perform other tasks including antigen presentation and clonal expansion with formation of immune memory. Isotype switching where the B-cell switches from IgM and IgD production to IgG, IgA or IgE production is a key aspect of B cell activation. Cytokine exposure during activation decides Ig type. Enhanced Ig antigen affinity is provided by somatic mutations occurring along with isotype switching. B cell clones with superior affinity get proliferative advantages and clones with poor affinity undergo apoptosis due to the lack of stimulatory signals [27, 52].

IgD is found both in a membrane-bound form on naïve B cells and in serum where it constitutes only a microscopic fraction of the total serum Ig [53]. Although IgD was discovered as early as 1965, its physiologic function has remained a puzzle for decades [53-55]. Usually, IgD production is down-regulated upon B cell activation, but apparently a small fraction of activated B cells produce IgD [56]. IgD bound to basophils is involved in human immune responses, and IgD is also present in respiratory, salivary, lacrimal and mammary secretions, indicating a role in mucosal
immunity [56]. In animal studies, IgD has been shown to function as an “activation backup system” for naïve B cells depleted of IgM [56, 57].

2.4 Cytokines

In order to perform its tasks the immune system needs means of communication. Although some of the signals crucial for an immune response depend on direct cell to cell contact; soluble signaling proteins called cytokines are involved in virtually all activities of the immune system. Cytokines are involved from ignition to resolution of an immune response [58].

Cytokines involved in the ignition and escalation of the inflammatory process are referred to as pro-inflammatory cytokines, and cytokines counteracting inflammation and inducing resolution are referred to as anti-inflammatory cytokines. This classification is somewhat oversimplified because a cytokine may have both pro- and anti-inflammatory properties. In inflammation the pro-inflammatory effects of cytokines include cell activation, proliferation and recruitment. Cytokines are mainly released from leucocytes, but may also be released from a wide variety of other cells, and are therefore not exclusive to the immune system [58, 59].

Cytokines convey their actions by binding to specific receptors on the surface of target cells. A cytokine may act on the same cell it was secreted from (autocrine), cells in the immediate surroundings (paracrine), and if sufficiently stable it may enter the bloodstream and act on distant cells (endocrine) [60].

Most cytokines share the following key features [59, 60]:

- They have multiple biological actions (pleiotropy).
- They have functional overlap with other cytokines (cytokine redundancy).
- They act in synergy with other cytokines
- They may induce other cytokines and create a cytokine cascade.
- They are potent and may induce their actions at relatively low concentrations.
- Soluble receptors and inhibitory cytokines may counter-act their effect
The nomenclature of cytokines reflects different historical approaches, and due to pleiotropy a given cytokine often received multiple names reflecting its different functions [61]. A nomenclature system was introduced to help this problem, and each new discovered member was assigned the name interleukin or IL and a new number, and thereby a neutral name [59]. This is in many ways a rational system, but it lacks structure because the numbers are simply assigned in ascending order and functional similarities are not taken into account [59]. Not all cytokines have been included in the “IL system” and some cytokines still have names reflecting their function. For example the colony stimulating factors (CSFs) and the INFs [61]. A sub-group of cytokines, the chemokines (from: chemotactic cytokines) have their own nomenclature system described below.

Cytokines showing considerable functional overlap or antagonistic actions in the same pathway are grouped in cytokine families [61]. For example, the IL-1-family includes more than 10 members that all exhibits related pro-inflammatory and anti-inflammatory functions [62].

More than 300 cytokines, chemokines and growth factors have been described, and their functions are not restricted only to the immune system [58]. Together, the cytokines constitute a vast and overwhelming network of signaling proteins. Only a few selected and relevant pro-inflammatory cytokines will be presented here.

### 2.4.1 Interleukin-1β

IL-1β is an important pro-inflammatory cytokine with numerous key functions in the inflammatory process. The major sources of IL-1β are monocytes, macrophages and dendritic cells. It is an alarm cytokine initiating and coordinating the early response to endogenous and exogenous danger sensed by the immune system [30]. IL-1β is involved in every aspect of the acute phase of inflammation, both locally at the site of injury and systemically [63]. IL-1β acts on a variety of different cells and induces production of many cytokines and acute phase proteins [30]. It is a potent pyrogen and it also induces increased pain sensitivity, vasodilatation, hypotension and sickness behavior, for example lethargy and anorexia [58, 63]. Secreted IL-1β recruits
and activates inflammatory cells and it is the most potent pro-inflammatory cytokine known [58].

The production and secretion of IL-1β is under strict control and depends on a complex multistep process including the assembly of a large macromolecular complex often referred to as the inflammasome. Following an inflammatory stimulus, a pathway including activation of the transcription factor nuclear factor kB leads to increased transcription of different proteins including pro-IL-1β, an inactive precursor of IL-1β [28]. NLRP3 (from: Nacht domain-, Leucine-rich Repeat-, and PYD-containing protein 3) is a protein encoded by the NLRP3 gene and a member of the aforementioned NLR-family. NLRP3 was formerly denoted cryopyrin, and upon activation it associates with other proteins forming the NLRP3 inflammasome. Caspase-1, a specialized converting enzyme, is contained and activated within the NLRP3 inflammasome and cleaves pro-IL-1β producing active IL-1β [28, 30, 64, 65]. Loss of control over IL-1β production or inactivation is involved in the pathogenesis of different AIDs, and due to its prominent role IL-1β is sometimes referred to as the gatekeeper of inflammation [64].

IL-1 receptor antagonist (IL-1ra) is an important regulator of the effects of IL-1β [66]. By binding to the IL-1 receptor it inhibits the actions of IL-1β and thereby modulating the extent of the inflammatory response [66]. IL-1ra is induced by IL-1β and other pro-inflammatory cytokines [67]. IL-1ra is available as a recombinant drug, and it is used in the treatment of different rheumatic and autoinflammatory diseases [62].

2.4.2 Tumor necrosis factor

Tumor necrosis factor (TNF)-α and TNF-β are two of the members of the TNF family. Originally, the TNFs were discovered as peptides with the ability to stimulate anti-tumor immunity. TNFs have direct cytotoxic effects on cancer-cells and they also induce anti-tumor immune activity [58, 61].

TNF-β is produced by lymphocytes. TNF-α is predominantly produced by monocytes and tissue macrophages, but it may be induced in many cells including neutrophils, NK cells and other lymphocytes during inflammation [58]. TNF-α is
processed in the form of a membrane-bound protein, and can be released in its active form by cleavage of the extracellular domain [58]. TNF-α has a central role in inflammation. It activates and attracts various immune-competent cells to the site of inflammation and induces the production of many other cytokines. It activates the vascular endothelium and promotes formation of adhesion molecules [68].

Bacterial lipopolysaccharide and different pro-inflammatory cytokines are important inducers of TNF-production, and many of the physiological changes in septic shock can be attributed to TNF-α [32, 35]. TNF-α is an important mediator of inflammation in juvenile rheumatoid arthritis, and treatment with TNF-inhibitors have a beneficial effect on clinical outcome [68].

2.4.3 Interferon-γ

Although the names of the interferons (INFs) reflect their ability to interfere with viral replication, INF-γ shows only modest antiviral ability [58]. The main sources of INF-γ are NK cells, NKT cells and activated T H1 cells, but may also be induced in other cells such as dendritic cells, macrophages and B cells [69-71].

INF-γ shows great pleiotropy, and it may influence the expression of hundreds of genes important for both innate and adaptive immune response [70]. Stimulating increased MHC I and MHC II expression, activation of a wide variety of immune cells from macrophages to T cells, promoting the production of many different chemokines and adhesion molecules is only a selection of immunomodulatory actions that can be attributed to INF-γ [69, 70]. INF-γ has paramount importance for cell mediated immunity, including the defense against intracellular microbes and tumor surveillance. As stated above, INF-γ skews T H differentiation towards T H1, and in turn T H1 cells produce significant amounts of INF-γ that classically activates macrophages and induces IgG isotype switch in B-cells. [72, 73].

2.4.4 Interleukin-6

Innate immune cells like macrophages, monocytes and dendritic cells are the most important source of IL-6. Additionally, T and B cells and a variety of non-leucocytes such as endothelial cells, fibroblasts and hepatocytes may produce IL-6. IL-
6 secretion is induced by stimuli that represent cell damage or stress and cytokines like IL-1, IL-2, TNF and interferon can induce its synthesis [58, 74].

IL-6 is an important pro-inflammatory cytokine. In many aspects there is an overlap in function between IL-6 and IL-1β, and the two cytokines often cooperate in a synergistic manner. IL-6 is considered the most important inducer of hepatocytic production of acute phase proteins including CRP [38].

IL-6 also mediates activation and differentiation of T cells and stimulates differentiation of B cells into mature plasma cells [58]. Due to its important role in the regulation of inflammation IL-6 is also a target for therapy, and an IL-6 receptor blocking antibody, Tocilizumab, is a treatment option in rheumatoid arthritis [74].

2.4.5 Chemokines

The chemokines are a group of small (8-12 kilo Dalton) chemotactic cytokines with related function and structure. The chemokines have the ability to induce directed cell migration (chemotaxis) in different cell types such as neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts and keratinocytes. Chemotaxis and activation of lymphocytes is the cardinal feature of the chemokines, but they also have homeostatic or housekeeping functions providing normal tissue maintenance [58, 75].

The chemokines all have a similar structure. The chemokines are divided into four subfamilies denoted XCL, CCL, CXCL and CX3CL on the basis of the spacing between conserved cysteine residues near the amino terminus of the peptide [75]. Many of the chemokines were discovered prior to the development of the standard nomenclature system, and therefore they have been given new names. For example, IL-8 is now denoted CXCL8, interferon-gamma-inducible-protein-10 or IP-10 is now denoted CXCL10 and macrophage inflammatory protein-1β is now denoted CCL4 [75].

The relationship between chemokines and chemokine receptors is complex. Some chemokines activate more than one type of chemokine receptors, and one type of chemokine receptors may be activated by more than one type of chemokines (Figure 3) [75].
Figure 3. Chemokines and chemokine receptor families

Most chemokines can interact with multiple receptors, and a single receptor can interact with multiple chemokines. This is the case for most CC (red) and CXC (green) chemokines. Decoy receptors (black) can also bind multiple chemokines. On the other hand, a minority of receptors (blue) have only one ligand.

Figure and legend reprinted with permission from Lazennec G, Richmond A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. Trends Mol Med. 2010; 16: 133-44. Copyright © 2010 Published by Elsevier Inc. All rights reserved.
2.5 Inflammation

Inflammation is derived from the Latin word *inflammō* and it translates ignite. In medical terms, inflammation describes the complex response the immune system implements in reaction to harm, and the result is a coordinated delivery of blood components to the site of infection or injury. The process of inflammation was reviewed by Medzhitov in 2008 and 2010 and the presentation in chapter 2.5 is mainly based on those publications [5, 76]. Many of the important recognition strategies and signaling pathways involved in inflammation have been discussed above, and therefore the concept of inflammation will only be briefly presented here.

2.5.1 Initiation of inflammation

A wide variety of exogenous and endogenous inducers of inflammation may initiate the process. The exogenous inducers of inflammation can be divided into infectious and non-infectious agents. The infectious agents can be either PAMPs or virulence factors. An example of a virulence factor is the pore-forming exotoxins that Gram positive bacteria may produce, and its presence is detected by the NLRP3 inflammasome. The non-infectious agents include allergens, toxic substances and foreign bodies. Endogenous inducers include trigger signals that are produced by stressed or damaged tissue [76].

2.5.2 The inflammatory pathway

Inflammatory mediators including cytokines are released when the inducers are recognized by the sensors of the immune system. The mediators subsequently act on target cells locally and in the periphery, and orchestrate the physiologic changes that characterize the inflammatory process. Different pro-inflammatory cytokines such as IL-1, TNF and IL-6 have the ability to act systemically, both directly and through the induction of other acute phase proteins. The physiologic effects that occur as a result of circulating pro-inflammatory cytokines are numerous and include sickness behavior and fever [5].

An example of an inflammatory pathway is initiated when a gram negative bacterium breaches a physical barrier. Lipopolysaccharide from the bacterial wall is
recognized by TLR4 located on resident macrophages. The activated macrophages release pro-inflammatory cytokines acting locally on cells in the surroundings, including the endothelial cells of the adjacent vessels, and peripherally on distant organs including the liver that in turn produces acute phase proteins (Figure 4) [76].

Simply, the main goal of inflammation is the deposition of leucocytes and other blood components at the site of injury. The blood vessels close to the site of injury are affected by inflammatory mediators inducing activation of the endothelium and hemodynamic changes [5]. Altered properties of the endothelium and changes in the blood flow leads to leakage of plasma into the surrounding tissue [5]. The activated endothelial cells produce chemo-attractants and express adhesion molecules on their luminal surface. Transendothelial migration of leukocytes is a complex multistep-process. Once located in the extracellular space, the leucocytes move further towards the site of injury aided by chemotaxis [27, 77].

Inflammation is a fundamentally protective response. The goal is disposal of the harmful agent and repair of damaged tissue. Resolution of inflammation is an active and controlled process that usually occurs when the inducer is eliminated [5]. Nevertheless, inflammation is the key ingredient in many diseases, and if the process is inappropriately triggered or uncontrolled it may be harmful.
Figure 4. The Inflammatory Pathway

The inflammatory pathway consists of inducers, sensors, mediators, and target tissues. Inducers initiate the inflammatory response and are detected by sensors. Sensors, such as Toll-like receptors (TLRs), are expressed on specialized sentinel cells, such as tissue-resident macrophages, dendritic cells, and mast cells. They induce the production of mediators, including cytokines, chemokines, bioactive amines, eicosanoids, and products of proteolytic cascades, such as bradykinin. These inflammatory mediators act on various target tissues to elicit changes in their functional states that optimize adaptation to the noxious condition (e.g., infection or tissue injury) associated with the particular inducers that elicited the inflammatory response. The specific components shown represent only a small sample of the myriad different sensors, mediators, and target tissues involved in the inflammatory response.

Figure and legend reprinted with permission from Medzhitov R. Inflammation 2010: new adventures of an old flame. Cell. 2010; 140: 771-6. Copyright © 2010 Published by Elsevier Inc. All rights reserved.
2.6 Fever in children

The normal body temperature has a circadian variation and it is at its lowest in the early morning and reaching a maximum 0.5 to 1°C higher in the late afternoon or early evening [78].

The body temperature is regulated by different thermo-regulatory circuits, with the preoptic region of the anterior hypothalamus as the most important control center [78, 79]. Thermo-sensitive neurons sense the body temperature continuously and different temperature regulatory mechanisms for heat loss or heat gain are available [78]. Blood can be redirected to or from subcutaneous areas. Sweating or shivering can be activated. Behavioral changes, as for instance alterations of clothing or seeking a cooler or warmer environment, are induced by the perception of undesired temperature [78].

The term fever or pyrexia describes a state of elevated body temperature due to an elevation of the thermal balance point [78]. In contrast, heatstroke or hyperthermia describes a situation where the body temperature is elevated although the thermal balance point is unaltered [78].

A rectal temperature gives the best estimate of core temperature. Oral and axillary measures of temperatures are less accurate, but more convenient and better tolerated, especially in older children [78, 80]. Insufficient agreement between rectal and infrared ear thermometry has been demonstrated in different studies; and in one systematic review, infrared ear thermometry failed to diagnose fever in 30-40 percent of febrile children [81, 82].

The cut off temperature for fever is not uniformly defined in different studies [83, 84], but for practical purposes fever can be defined as a body temperature above 38 °C when measured rectally or above 37.5 °C when measured axillary regardless of the time of day [85].

Bioactive substances that can elevate the thermal balance point causing fever, are termed pyrogens [78]. Endogenous pyrogens are produced by the host itself, and it has been argued that pyrogenic cytokines would be a more appropriate name for these substances, since this term reveals their true nature [86]. The most important
endogenous pyrogens are the cytokines IL-1β, IL-6, TNF-α and INF-γ [78]. Exogenous pyrogens are not produced by the host. Primarily, they are parts of or complete microbiological agents or microbiological products including toxins [78]. Both endogenous and exogenous pyrogens cause fever by inducing enzymes (including cyclooxygenase-2) for biosynthesis of Prostaglandin E$_2$ (PGE$_2$) from arachidonic acid [67, 79]. PGE$_2$ is a small molecule that is not hindered by the blood-brain barrier [78]. PGE$_2$ acts on the thermal control center in hypothalamus elevating the thermal balance point [78].

The most significant anatomical area for pyrogens is the organum vasculosum laminae terminalis (OVLT), also called the circumventricular organs [86]. The OVLT is an area consisting of fenestrated endothelium located on both sides of the preoptic region of the anterior hypothalamus [86]. Receptors for pyrogenic cytokines and TLR’s for exogenous pyrogens are present on the vascular side of this endothelium, and receptor activation triggers a pathway leading to PGE$_2$ release [86]. Although the pathway involving the OVLT is thought to be dominant in the induction of fever, cytokines may cross the blood-brain barrier and act on the hypothalamus in a more direct fashion [78, 86]. Animal studies on infections have demonstrated that production of PGE$_2$ from peripheral organs like liver or lungs may be responsible for the early phase of fever [78]. PGE$_2$ synthesis can be diminished by antipyretics like ibuprofens and aspirin by the inhibition of the cyclooxygenase-2 enzyme [67].

Fever is a sign of an acute phase reaction, and although infections are the most frequent cause of fever it may be present in many non-infectious illnesses. Almost every child experiences a febrile illness during childhood, and it is one of the most common reasons for children to see a doctor [83]. Fever may be a beneficial physiological response shortening the duration of infectious diseases [87]. When treating with antipyretics the goal is to improve the child’s general condition, and it should therefore be reserved for children with apparent signs of discomfort [84].
2.7 Periodic fever

The term *recurrent fever* is often used in pediatric medical literature and simply describes a situation of reoccurring febrile episodes.

Periodic fever may be more narrowly defined and describes an illness where recurrent fever is the cardinal symptom and each episode has a predictable and similar clinical course lasting days to weeks [17]. Each episode should be separated by a symptom-free period where the child is completely well. The symptom-free interval between the febrile episodes may range from weeks to months [17]. The febrile episodes may have an almost fixed interval, but they may also be more sporadic in nature [88, 89]. The febrile episodes occur during all seasons. The child should not be contagious during febrile episodes, and the febrile episodes should not appear as the result of interactions with sick, febrile contacts [17].

2.8 The PFAPA syndrome

During the two decades following the discovery of PFAPA by Marshall et al., more than 20 papers presenting patients with the disease were published [13-16, 18-23, 90-101], and it was recognized as a clinically defined periodic fever syndrome of unknown etiology.

This overview of the knowledge on PFAPA during the emergence of this thesis is based on three large studies including a total of 176 patients [14-16]. Short reports, letters and studies regarding limited aspects of PFAPA has been left out from the general presentation, but they are included when appropriate.

2.8.1 Diagnostic criteria

In a letter to the editor of the Pediatric Infectious Disease Journal printed in September 1989, Marshall et al. presented the acronym PFAPA and a set of diagnostic criteria for the syndrome [13]. In January 1999, Thomas and Edwards presented a revised set of diagnostic criteria for PFAPA [102], modified later the same year and published together with the presentation of 94 children with PFAPA (Table 1) [15]. Although these criteria for PFAPA have been widely used in the literature, there has been no formal international consensus. Several publications on PFAPA deviate from
these criteria, especially the age criterion. In recent publications a more stringent approach to setting the PFAPA diagnosis has been proposed, which will be featured in the discussion.

Table 1
Diagnostic criteria for PFAPA according to Thomas et al. [15].

<table>
<thead>
<tr>
<th>Criteria</th>
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<tbody>
<tr>
<td>1) Regularly recurring fevers with an early age of onset (&lt;5 years of age)</td>
</tr>
<tr>
<td>2) Constitutional symptoms in the absence of upper respiratory infection with at least one of the following clinical signs:</td>
</tr>
<tr>
<td>a. Aphthous stomatitis</td>
</tr>
<tr>
<td>b. Cervical lymphadenitis</td>
</tr>
<tr>
<td>c. Pharyngitis</td>
</tr>
<tr>
<td>3) Exclusion of cyclic neutropenia</td>
</tr>
<tr>
<td>4) Completely asymptomatic interval between episodes</td>
</tr>
<tr>
<td>5) Normal growth and development</td>
</tr>
</tbody>
</table>

PFAPA: Periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis

2.8.2 Clinical presentation and prognosis

   In the three large early publications, the observed median age of onset ranged from two to four years, and male predominance was consistently reported [14-16]. The occurrence of the cardinal symptoms vary somewhat from study to study, but pharyngitis and cervical adenitis were generally more frequent than aphthous stomatitis.

   During the febrile episodes the fever rises abruptly, reaching high temperatures of 39 to 41°C. In addition to the cardinal symptoms a number of associated symptoms have also been reported, with chills, headache, abdominal pain and nausea as the most frequent ones. The febrile episodes lasts 4 to 5 days and occur approximately every fourth week. The febrile episodes of PFAPA often reoccur at almost fixed intervals and most children experience no seasonality. Over time the symptom-free period may
become longer and the febrile episodes shorter. PFAPA may relapse after the disease has been absent for months or years.

PFAPA usually resolves during childhood, and the mean duration of the syndrome was 4.5 and 8.0 years in two studies reporting cessation in 34 and 9 children respectively [14, 15].

2.8.3 Laboratory tests

During the febrile episodes, white blood cell count, neutrophil count and erythrocyte sedimentation rate are moderately elevated [14, 15, 18, 19]. In 2007, we found that elevated levels of CRP during febrile episodes were a consistent finding in children with PFAPA, with a mean maximum level of 185 mg/L [25].

The laboratory tests must be regarded as unspecific. However, in 2007, Yoshihara et al. suggested that measuring procalcitonin might distinguish febrile episodes of PFAPA from bacterial infections; as they found undetectable levels in all samples from PFAPA patients and elevated levels in all samples from febrile controls [101].

In 1999, Padeh et al. found elevated levels of serum IgD in 12 of 18 children with PFAPA, but in contrast Thomas et al. found normal IgD levels in 15 of 15 children with PFAPA and elevated IgE levels in 8 of 16 children with PFAPA [14, 15]. In the latter study, negative antinuclear antibody was found in 29 of 30 children with PFAPA, and negative rheumatoid factor and antistreptolysin O was found in all 12 and 14 tested children with PFAPA respectively.

2.8.4 PFAPA as a pathological entity

At the beginning of 2008, more than 300 children with PFAPA were described in the English literature. Although there was a growing body of observational data on children fulfilling clinical criteria for this type of periodic fever, the present data was inadequate to understand the pathogenesis of the disease and maybe also inadequate to be sure that PFAPA was a distinct pathological entity.

The diagnostic criteria presented by Marshall et al. in 1989, modified by Thomas et al. in 1999, states that signs of upper airway infection must be absent during the febrile attacks. Additionally, the almost clockwork periodicity in many of
the children, an abrupt effect of systemic glucocorticoids, the lack of seasonality, no
significant relation to daycare attendance and the non-contagious nature of the
episodes speaks strongly against a viral or bacterial etiology in PFAPA [14-16, 103].

However, the apparent effect of tonsillectomy in children with PFAPA has led
some to consider that microbiologic agents may play a part in the pathogenesis [103].
Padeh et al. diagnosed PFAPA in children only if they experienced no effect of
antibiotics, either as treatment during a febrile episode or as prophylaxis; and if they
had negative throat cultures during the attacks [14]. Thomas et al. and Tasher et al. had
a somewhat less systematic approach, but their findings are comparable with no effect
of antibiotics in 92% and 98% of children respectively [15, 16]. Thomas et al. reported
negative bacterial cultures from blood in 105 of 105, from urine in 110 of 111 and
from throats in 255 of 284 of the samples collected during febrile attacks of PFAPA
[15].

Cazeneuve et al. performed screening of the gene MEFV involved in Familial
Mediterranean fever (FMF) in six children with PFAPA. The analysis revealed no
FMF mutations and the authors concluded that this argued against FMF involvement
in PFAPA [94]. These results coincides with Padeh et al.’s study from in 1999
reporting no homozygote or double heterozygote FMF mutations in Israeli children
diagnosed with PFAPA [14]. In 2006, Maschio et al. reported that they found no
inflammatory bowel disease protein 1 gene polymorphisms, related to Crohn’s disease,
in 40 children with abdominal pain and diarrhea as associated symptoms to their
PFAPA [97].

The first description of cytokine measurements in children diagnosed with
PFAPA was a reference to unpublished data in the article by Thomas et al. from 1999
[15]. These data has to our knowledge remained unpublished, but it was stated that
INF-γ, tumor necrosis factor and IL-6 were elevated during febrile attacks of PFAPA.
In 2006, Stojanov et al. reported cytokine measurements from six children diagnosed
with PFAPA. They concluded that their results indicated a dysregulation of the innate
and adaptive immune system in PFAPA with both continuous pro-inflammatory
cytokine activation and a reduced anti-inflammatory response [18]. This was the only
paper published prior to 2008 reporting measurement of cytokines in PFAPA. It substantiated that the disease may be caused by a dysregulation of the immune system.

In parallel to our study, more extensive projects have studied the pathogenesis of PFAPA, with several major publications during the recent years. These will be featured in the discussion.

2.8.5 Medical and surgical treatment

An abrupt effect from a single dose of a systemic glucocorticoid resulting in termination of the febrile episode was observed in several early studies on PFAPA [13-16]. Thomas et al. recommended prednisone or prednisolone at doses of 1 mg/kg once daily for two days, and 0.5 mg/kg on day three and four if the attack was still ongoing [15]. In 2006, Tasher et al. reported termination of the febrile episode in 51 out of 54 children after a single dose of prednisone with a mean dosage of 0.59 mg/kg [16]. The use of glucocorticoids is generally well tolerated, but an increased frequency of febrile attacks after administration has been reported [14-16].

Cimetidine is a H2-receptor antagonist and although it is primarily used to reduce the production of gastric acid, it also has immune-modulatory properties [104]. Cimetidine was reported effective as prophylactic therapy in six patients with PFAPA in 1992 [105]. In 1999 it was reported that Cimetidine induced remission in 8 of 28 children [15].

As early as in 1989, cessation of febrile episodes resembling PFAPA after tonsillectomy and adenoidectomy was described in four boys [19]. Complete remission after tonsillectomy with or without adenoidectomy has later been reported in several studies, although the procedure is not successful for all children [14-16, 20-22, 96].

In 2007, the first randomized controlled trial investigating the effect of tonsillectomy for PFAPA was published [23]. They concluded that tonsillectomy appeared to be an effective treatment for PFAPA. The authors have been criticized for not using the proper diagnostic criteria [106, 107], and the large percentage of children presented as PFAPA-cases having fever as their only clinical symptom is unsettling.
2.9 Differential diagnoses to PFAPA

A variety of different diseases may cause recurrent fever. The diagnostic criteria for PFAPA have unknown specificity, and relevant differential diagnoses must be considered when a child with periodic fever is evaluated. If a PFAPA diagnosis is given incorrectly, this may delay diagnosis and treatment of a potentially harmful disease. Conditions that may resemble PFAPA are presented below, with a focus on clinical similarities and differences.

2.9.1 Infections

Children have more frequent infections than adults, most commonly in the youngest age groups [108]. In clinical practice, recurrent acute infections will be the most likely preliminary diagnosis in all children until the diagnosis of PFAPA is made.

Healthy children who attend daycare or have siblings may have up to 10 self-limiting viral diseases during one year [17, 108]. During these febrile episodes there are usually clear signs of a respiratory tract infection on examination, and the clinical picture will vary between episodes. According to the diagnostic criteria (Table 1), PFAPA should not be diagnosed if the recurrent febrile episodes are accompanied with signs of a respiratory tract infection including wheezing, cough, rhinorrhea or otitis. Recurrent infections usually show clear seasonal variation, and the child will tend to have good periods when kept away from daycare.

2.9.1.1 Pharyngitis

Pharyngitis or pharyngotonsillitis is an infection affecting the palatine tonsils with concurrent involvement of the lingual tonsils, the adenoid and the pharyngeal wall [109]. The term “infectious”, “viral” or “bacterial” pharyngitis is used here to distinguish pharyngitis of viral or bacterial origin from the clinical sign of pharyngitis present in PFAPA. All the cardinal signs of PFAPA may be present in viral or bacterial pharyngitis. Recurrent infectious pharyngitis is therefore an important differential diagnose to PFAPA [15, 109].

Infectious pharyngitis is usually characterized by fever, sore throat, redness of tonsils and pharynx, tonsillar exudate and cervical adenitis [110]. Presence of
additional symptoms from the respiratory tract including coughing or rhinorrhea is indicative of a viral rather than a bacterial origin [110]. Most cases of infectious pharyngitis are caused by viruses, especially during the first years of life. Beta-hemolytic group A streptococci are the most common bacterial agent, and they are responsible for approximately 15-30% of all cases of infectious pharyngitis. Bacterial pharyngitis due to beta hemolytic group A streptococci is uncommon in children younger than three years. Infectious pharyngitis shows a clear seasonal variation with the highest incidence during the winter [109, 110].

A modern rapid antigen diagnostic test for beta hemolytic group A streptococci provides a result within a few minutes. Unlike a rapid antigen diagnostic test, a throat swab culture test will detect different types of bacteria; and it is often referred to as the gold standard with high sensitivity and specificity when used at signs of illness [109]. Noteworthy, isolated positive throat swab culture tests are not always easy to interpret, as some children are healthy carriers of pathogenic bacteria [111].

In children with signs of recurrent infectious pharyngitis, a proper clinical evaluation and diagnostic testing should be made. This includes a rapid antigen diagnostic test for beta hemolytic group A streptococci. If this test is negative, a throat swab bacterial culture test should be performed. If there is periodic fever with no sign of a concurrent respiratory tract infection and negative tests, the child should be evaluated further considering a PFAPA-diagnosis.

2.9.1.2 Chronic infections with recurrent fever:

Recurrent fever that may resemble PFAPA have been described both in children with chronic Epstein-Barr infection and chronic Mycobacterium chelonae infection [112, 113]. However, these case studies probably represent seldom and peculiar patterns of disease.

A strain of the Borrelia spirochetes, Borrelia recurrentis is known to cause outbreaks of louse-borne relapsing fever in developing countries [114]. The disease show a clear geographical distribution, and the clinical picture is severe and does not constitute a relevant differential diagnosis to PFAPA [115]. Other strains of Borrelia spirochetes, including B. Hermsii, B. turicatae and B. hispanica may cause tick-borne
relapsing fever, a disease with similar signs and symptoms as louse-borne relapsing fever, but with a lower mortality rate [116, 117].

2.9.2 Cyclic Neutropenia

Cyclic neutropenia is a rare, autosomal dominant, hematological disorder characterized by regular oscillations of the neutrophil count [118]. Sporadic cases occur due to new mutations [118]. In affected patients, the peak level of neutrophils is in the normal or subnormal range and during the nadir of the cycle the level of neutrophils may approach zero [118]. The average duration of a full cycle is 21 days [118, 119]. The disease often presents during early childhood. During nadir, the children can develop pharyngitis, mouth ulcers and lymphadenopathy, and they are at risk of life threatening infections due to the severe neutropenia [119].

In cyclic neutropenia, a mutation in the \textit{ELANE} gene causes a misfolding of the protein neutrophil elastase. This has a dramatic impact on the differentiation of progenitor cells in the bone marrow, but the mechanism is not fully understood [118].

In its mildest form cyclic neutropenia may mimic PFAPA and therefore it should be excluded before setting the PFAPA-diagnosis [12, 15, 119]. Cyclic neutropenia is diagnosed by demonstrating oscillations in the neutrophil count together with the typical clinical picture. A diagnosis should be confirmed by a mutation analysis [89]. Cyclic neutropenia may be excluded by measuring levels of neutrophils two times weekly over a period of six to eight weeks. Measuring neutrophils in the normal range two times weekly between two subsequent febrile episodes is considered sufficient to rule out cyclic neutropenia in patients evaluated for PFAPA [89].

2.9.3 Childhood malignancies

Fever, (non-tender) adenopathy and constitutional symptoms may be the presenting signs of childhood leukemia and lymphoma [120, 121], and one isolated report of acute lymphatic leukemia presenting with periodic fever exists [122]. Although the initial manifestation of a childhood malignancy may resemble some features of PFAPA, additional and chronic symptoms indicative of this severe differential diagnosis are usually present [17]. These symptoms may include fatigue, pallor, bleeding, bruising, weight loss, bone or joint pain, a lump, petechiae, anemia,
thrombocytopenia, leukocytosis, hepato- and splenomegaly [120, 121, 123]. Malignant disease should always be kept in mind when evaluating a child with unexplained fever. According to the diagnostic criteria, a PFAPA diagnosis should not be made unless the child is completely asymptomatic between febrile episodes.

2.9.4 Autoimmune diseases

Autoimmune disorders will at presentation usually not represent a differential diagnosis for PFAPA as a true pattern of periodic fever with completely asymptomatic intervals in-between is unlikely [103]. A thorough medical history and clinical examination with a focus on rashes and joint symptoms including swelling, pain and stiffness is important in children with unexplained fever [124, 125].

Behçet’s syndrome is an inflammatory disorder of unknown etiology, characterized by vasculitis that usually affects multiple organ systems. Recurrent oral ulceration is the most common clinical feature, but in contrast to PFAPA the ulcerations are very painful and may result in scarring. In addition to the oral ulcerations, other manifestations including genital ulcers, eye and skin lesions, musculoskeletal and gastrointestinal may be present [126]. Initially, the recurrent oral ulcerations and exacerbations may resemble PFAPA, but as the disease progresses the burden of symptoms in Behçet’s syndrome will distinguish it from PFAPA.

The Behçet’s syndrome is listed as an AID in some publications [127], but it is included here among the rheumatic disorders because auto-reactive T cells and autoantibodies has been implicated in the pathogenesis of the disease [126].

2.9.5 Autoinflammatory diseases

The term autoinflammatory disease (AID) was introduced by McDermott et al. in 1999 when the genetic background for Tumor necrosis factor receptor-associated periodic syndrome (TRAPS) was discovered [128]. Recognizing that a genetic defect may cause uncontrolled inflammation gave new insights “old” diseases with unknown etiology, and laid grounds for the discovery of others.

Initially, AIDs were defined as disorders with seemingly unprovoked inflammation without high-titer autoantibodies or antigen-specific T lymphocytes [128, 129]. However, due to advances in understanding of these diseases and the
innate immune system, a new definition for AIDs was proposed in 2010: “Clinical disorders marked by abnormally increased inflammation, mediated predominantly by the cells and molecules of the innate immune system, with a significant host predisposition” [130]. This definition takes into account that triggers may provoke attacks and that there is a continuum rather than a strict boundary between the autoinflammatory and the autoimmune diseases. For some of the AIDs the pathogenesis is largely understood, and the loss of control over IL-1β production or inactivation is involved in the pathogenesis for several of the diseases in this category [30, 127].

The first AIDs to be recognized were the hereditary periodic fever syndromes, but the category has expanded and now includes a wide spectrum of diseases [127, 131]. Some of these have a monogenic etiology, but others have a more complex or unknown mode of inheritance [127]. As featured in the discussion, based on resent results PFAPA is now considered an AID, although the etiology is not fully understood [127, 130].

The systemic AIDs with chronic activity, extensive morbidity and often characteristic disease patterns do not represent actual differential diagnosis to PFAPA. These diseases include among others deficiency of IL-1 receptor antagonist or DIRA, pyogenic sterile arthritis, pyoderma gangrenosum and acne syndrome, Majeed syndrome, Blau syndrome and Schnitzler syndrome. They will not be discussed further.

A genetically defined subgroup of AIDs, FMF, Mevalonate kinase deficiency (MKD) and TRAPS have periodic fever as their common cardinal feature, and are often referred to as the classical monogenic periodic fever syndromes. Although they differ somewhat in clinical characteristics and may show distinct geographical distribution, they all represent important differential diagnosis to PFAPA and are discussed in further detail below.

Cryopyrin-associated periodic syndrome (CAPS) is a collective term for three diseases that arise from mutations in the same gene [132]. Although bursts of inflammation and fever may accompany all three diseases, they do usually not
represent a differential diagnosis to PFAPA due to their distinct clinical presentation and severity. The CAPS-continuum will be presented in short below.

2.9.5.1 Familial Mediterranean fever

Familial Mediterranean fever (FMF) is the most common of the monogenic periodic fever syndromes [133]. The gene responsible for FMF is located on the short arm of chromosome 16 and named MeFV (from: MEditerranean FeVer). It was simultaneously discovered by two independent research groups in 1997 [134, 135]. The disease is usually inherited in an autosomal recessive fashion; although patients with a FMF phenotype who have a favorable response to colchicine have been described with no or only one detectable mutation [127, 136]. In ethnic groups where FMF is endemic the diagnosis remains clinical, and the Tel-Hashomer criteria presented by Livneh et al. are often used to set the diagnosis [137]. In populations where FMF is rare genetic testing is advisory, but interpretation of the results may sometimes be difficult [138].

The disease occurs worldwide, but as indicated by its name, the disease is most frequent in populations living around the Mediterranean basin. Cases of FMF has been reported from USA, European countries and Asia, but it is rare in other ethnic groups than Armenians, Turks, Arabs and non-Ashkenazi Jews [139]. Due to immigration the prevalence of FMF increases in the Nordic countries [140].

Disease onset occurs usually in early childhood, and during the first decade in 80% of cases [133]. The FMF episodes are generally irregular and short, lasting one to three days on average. The attacks are often spontaneous, but emotional stress, vigorous physical activity, viral disease and some drugs are reported as triggers [138]. In addition to fever, abdominal, chest and joint pain due to serositis are the most predominant symptoms of FMF, and during early childhood recurrent fever may be the only manifestation of the disease [132, 136]. An erysipelas-like rash on the shins or dorsum of the foot is also common [138].

Secondary (AA) amyloidosis is a common complication in untreated FMF. The occurrence of amyloidosis differs among ethnic groups, and this may partly depend on differences in disease mutations [136, 141].
2.9.5.2 Mevalonate kinase deficiency

MKD was first denoted Hyper Immunoglobulin D Syndrome when described by Jos van der Meer et al. in 1984 [142]. Recent studies have shown that levels of IgD are not always elevated, and MKD reflecting the underlying genetic disorder is the appropriate name [143]. MKD is inherited in an autosomal-recessive pattern, and a missense mutation in the gene $\text{MKV}$ encoding the enzyme mevalonate kinase causes impaired enzyme activity [138]. Described cases are predominately, but not exclusively, of Dutch or French ancestry [133, 138].

A severe metabolic disease, mevalonic aciduria, is related to MKD. The two diseases share the same genetic defect, but they have different severity due to differences in residual enzymatic activity [138, 144].

Disease onset usually occurs during the first two years of life, and it is seldom after the first decade [132, 133, 138]. The first attack of MKD often succeeds childhood vaccination. The children experience short attacks of fever lasting four to six days. The clinical features include generalized lymphadenopathy, an erythematous rash on the palms and soles, headache, arthralgia and arthritis, splenomegaly, aphthous stomatitis and severe abdominal pain with vomiting and diarrhea [131, 132, 138, 145].

MKD shows a significant clinical overlap with PFAPA, but skin lesions, severe abdominal pain and arthralgia or arthritis in large joints may alert the physician. The detection of mevalonaturia during febrile episodes is indicative of MKD, but a mutation analysis of the $\text{MKV}$ gene should be performed in order to confirm the diagnosis [132, 145].

2.9.5.3 Tumor necrosis factor receptor-associated periodic syndrome

TRAPS was initially called Hibernian fever. Hibernia is Latin for the island of Ireland, and TRAPS was first described in an Irish family by Williamson et al. in 1982 [146]. The gene $\text{TNFRSF1A}$ involved in TRAPS is located on the short arm of chromosome 12 and encodes the TNF receptor type 1 [147]. TRAPS is an autosomal dominant disease with onset usually during early childhood [119, 132].

The clinical picture of TRAPS is heterogeneous with great intra- and inter variation of both frequency and severity of attacks [147]. The febrile attacks of
TRAPS often last 7 days or more. Intense myalgia, abdominal pain and pleuritic chest pain are the most frequent manifestations, but other symptoms including vomiting, constipation, conjunctivitis, arthritis or arthralgia and erythematous rash may also occur [119, 132, 147]. In addition to genetic variants with high penetrance and a severe corresponding phenotype, low-penetrance mutations in the *TNFRSF1A* gene with a milder corresponding phenotype also exist [148].

Renal AA amyloidosis is a known complication of TRAPS and may affect up to one fourth of the patients [119]. Non-steroidal anti-inflammatory drugs and oral corticosteroids may reduce symptoms during attacks [147].

**2.9.5.4 The cryopyrin-associated periodic syndromes**

Familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID) are three overlapping phenotypes that together encompass the CAPS-continuum. The diseases are caused by autosomal dominant mutations of the *NLRP3*-gene. All three phenotypes may to varying degree exhibit inflammatory exacerbations with an urticarial-like rash, arthralgia, headache, and conjunctivitis [131, 132]. FCAS is the mildest phenotype, where cold exposure triggers short lasting attacks of fever accompanied by an urticarial-like rash and joint pain. In MWS, the patients experience longer attacks and also progressive sensorineural hearing loss and a risk of amyloidosis. NOMID is also known as chronic infantile neurological, cutaneous and articular (CINCA) syndrome. NOMID is a severe disease with central nervous system involvement seen as seizures, aseptic meningitis and developmental delay [131, 132]. Due to their distinct and often severe clinical manifestations the diseases of the CAPS-continuum do usually not represent a differential diagnosis to PFAPA.
2.10 Summary of the introduction

PFAPA is mainly a disease of early childhood, hallmarked by short, regular episodes of fever accompanied by aphthous stomatitis, pharyngitis or cervical adenitis or any combination of the three. The disease is not defined genetically, and the diagnosis is set according to clinical criteria. Despite a benign prognosis, the disease causes grave parental concern and has a great impact on family-life.

During the two first decades after PFAPA was described in 1987, several single- or multi center studies focusing on clinical aspects of the disease were published. Although substantial observational data on PFAPA gradually emerged, little was known about the syndrome in Scandinavia, and except from one preliminary report from our study group [24], no population based studies existed, and consequently the incidence was not known.

When our study was planned the etiology of PFAPA was unknown. One single study indicated a possible dysregulation of the immune system [18], and studies reported a lack of microbial agents associated with PFAPA, and prompt resolution of a febrile episode after a single dose of a systemic glucocorticoid [12, 14-16], together underlining the inflammatory but non-infectious origin of PFAPA.

The first report of tonsillectomy as a possible cure for the PFAPA was published shortly after the syndrome was described [19]. When our study started, one randomized controlled trial on the effect of tonsillectomy in PFAPA had been published, indicating a beneficial effect of surgery [23]. The possible effect of tonsillectomy on PFAPA is puzzling, and the role of the tonsils in the etiology of PFAPA was unclear.

Stavanger University Hospital is the only center for pediatric specialist care in South Rogaland. The area is suitable for population based studies and long term follow-up. Through a prospective inclusion of all children with PFAPA in this area, we have studied epidemiological and clinical aspects of the disease. Furthermore, we have explored immunological aspects of the disease by measuring blood cytokines and studying histological and immunohistochemical characteristics of tonsils from children with PFAPA.
3 Aims of the thesis

The overall aim of the thesis was to study epidemiological and clinical characteristics of PFAPA in a population based approach and to explore immunological aspects of PFAPA in blood and tonsils.

The specific aims were:

Paper I) Epidemiological and clinical characteristics of PFAPA

- To estimate the incidence of PFAPA in South Rogaland
- To determine the age of onset of PFAPA in South Rogaland
- To study the clinical characteristics of PFAPA during febrile episodes
- To study the outcome of PFAPA after tonsillectomy
- To study the total duration of PFAPA until resolution

Paper II) Immunological aspects of PFAPA assessed by blood tests

- To study if the levels of leukocytes, subsets of lymphocytes, cytokines, chemokines and two soluble receptors differed:
  - Between febrile episodes and afebrile periods in children with PFAPA
  - Between febrile episodes in children with PFAPA, and during pneumonia in controls
  - Between afebrile periods in children with PFAPA, and after full recovery in controls
- To study if levels of immunoglobulins in children with PFAPA and controls differed, and to compare the measured levels with age related normal values

Paper III) Immunological aspects of PFAPA studied in tonsils

- To study the histological appearance of tonsils in children with PFAPA, and to compare it to tonsils from a control group of children with tonsillar hypertrophy.
- To study if the number of different types of leukocytes in tonsillar germinal centers differed between children with PFAPA and controls.
4 Subjects and methods

4.1 Catchment area

The South Rogaland region is a coastal region with mixed rural and urban areas located in the south western part of Norway. In 2004, the population included 20,500 children up to the age of five, increasing to nearly 25,000 at the end of 2013 [149]. The Paediatric department at Stavanger University hospital receives all hospital admissions and the vast majority of outpatient referrals for children in need of paediatric specialist care in the region.

4.2 Subjects and diagnosis of PFAPA

Since January 1st 2004, all children referred to or being followed at Stavanger University Hospital due to recurrent fever with disease onset before the age of five were registered prospectively in a database. Some children with disease onset prior to January 1st 2004 were included retrospectively, but the PFAPA diagnosis was confirmed prospectively. All children referred to in paper II and III were included prospectively. The children were evaluated clinically by one of two pediatricians (JF or KØ) according to recommended guidelines [17, 150].

From the beginning of the study the Ear Nose Throat (ENT) Department had a high awareness of children with recurrent fever. Children referred to the ENT Department with symptoms that raised suspicion of PFAPA were referred to the Pediatric Department for further evaluation.

PFAPA was diagnosed according to the criteria by Thomas et al. (Table 1) [15]. Additionally, the diagnosis was considered as a diagnosis of exclusion. Consequently, the PFAPA diagnosis was not set if another disease explaining the clinical picture was present, although the child fulfilled the clinical criteria for PFAPA. Cyclic neutropenia was excluded by serial measurement of neutrophils two times weekly between two subsequent febrile attacks. Genetic tests for monogenic periodic fever syndromes or other specific tests were performed when clinically indicated, but not routinely. Resolution of PFAPA was defined as a period of at least six months without any
febrile episodes. Relapse was defined as the return of febrile episodes after resolution. For children not diagnosed with PFAPA, the final diagnosis was recorded, but they were not included in the further study.

For paper II, children admitted to Stavanger University Hospital during the inclusion period with clinical symptoms of pneumonia, a radiological verified consolidation and an initial CRP value of $\geq 150$ mg/L were diagnosed as having bacterial pneumonia and included as controls. The children should be otherwise healthy with no chronic condition making them prone to pneumonia.

For paper III, the first two children who underwent tonsillectomy due to tonsillar hypertrophy after each child with PFAPA were included as controls. Children who had experienced febrile episodes with a regular pattern or recurrent pharyngitis without documentation of bacterial origin were excluded.

### 4.3 Testing procedures

**Paper I:**

The first evaluation of children with recurrent fever included a medical history and a thorough clinical examination. If a periodic fever syndrome was not excluded at the first visit, the child was seen for a second evaluation during a febrile episode with clinical examination and tests including blood samples, throat and urine cultures.

Parents of all children investigated for PFAPA answered a questionnaire (appendix I) during the first evaluation, and they were interviewed a second time, by phone, at least one year after the diagnosis was set (appendix II).
**Paper II:**

Blood samples were taken at two occasions.

- For the children with PFAPA:
  1. As soon as possible after the onset of fever during a typical PFAPA-related episode, and always within the first 24 hours.
  2. Between two febrile episodes, after at least 10 days without fever.

- For the children with pneumonia:
  1. As soon as possible after the diagnosis was set after admission for pneumonia.
  2. At least four weeks after full recovery from pneumonia.

Non-steroid anti-inflammatory drugs (except for paracetamol/acetaminophen) were not given before the blood sample was taken, except for one child with pneumonia who had received one dose of ibuprofen 34 hours prior to admission. Systemic glucocorticoids were not administered during the study period.

All children were clinically examined during the febrile episode (by JF or KØ), and a throat culture test was taken. If the test showed pathological bacterial growth, the child was excluded from the study.

**Paper III**

Children with PFAPA did not receive systemic glucocorticoids during the last four weeks prior to surgery. Both for children with PFAPA and tonsillar hypertrophy, a tonsillectomy with or without adenoidectomy was performed using standard cold dissection. Only palatine tonsils were preserved for further studies. Parents of all children with PFAPA were contacted by phone at least 12 months after tonsillectomy in order to evaluate the outcome after surgery.
4.4 Laboratory analyses including evaluation of tonsils

4.4.1 Microbiology

Throat swabs and urine cultures were analyzed according to in-house guidelines at the Department of Microbiology, Stavanger University Hospital.

4.4.2 Blood samples (Paper I and II)

Venipuncture was performed and blood was collected in vacutainers. In addition to tubes taken for routine analyses, one tube of citrate-anticoagulated blood was centrifuged by the use of a Kubota 5930 centrifuge at 3300 rpm for eight minutes at 4°C immediately after sampling. The sample was separated and plasma was frozen at -80°C until analysis. This sample was used for analyses of cytokines, sCD25 and sCD163. The samples were thawed and divided when cytokine analysis were performed. The unused part was refrozen and stored until sCD25 and sCD163 were analyzed.

The following analyses were performed as a part of the routine at Department of Clinical Chemistry, Stavanger University Hospital:

- CRP was analyzed by immunoturbidimetric assay (CRPLX, Roche Diagnostics, Mannheim, Germany).
- White blood cell count with subgroups of neutrophils, monocytes, lymphocytes and eosinophils and thrombocytes were analyzed on a Sysmex XE-5000 (Kobe, Japan).

The following analyses were performed as a part of the routine at the Department of Immunology and Transfusion Medicine, Haukeland University Hospital:

- Lymphocyte subpopulation quantifications were performed using the BD Multitest 6-color TBNK kit with BD Trucount Tubes for relative and absolute concentration determination (BD Biosciences, San Jose, CA, USA). The samples were prepared according to the manufacturer’s instructions and analyzed on a BD Canto II flow cytometer (BD Biosciences) using BD Canto 2.1 analysis software.
• Serum IgG, IgA, and IgM were measured using a Siemens BN ProSpec Nephelometer. Serum IgD was measured by radial immunodiffusion, (IgD RID kit – NL, Binding Site, Birmingham, UK).

The following analyses were not a part of the routine, and set up exclusively for this project at the Department of Immunology and Transfusion Medicine at Haukeland University Hospital:

• Cytokines in serum were analyzed using a 27-plex cytokine panel (BioRad, CA, USA) and a Luminex-based reader (Luminex Corporation, Texas, USA). IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, CCL2, CCL3, CCL4, CCL5, CCL11, CXCL8 (IL-8), CXCL10, Fibroblast growth factor 2 (FGF-2), Granulocyte colony stimulating factor (G-CSF), Granulocyte macrophage colony stimulating factor (GM-CSF), INF-γ, Platelet derived growth factor BB (PDGF-BB), TNF-α and Vascular endothelial growth factor (VEGF) were analyzed. Samples and standards were prepared and analyzed according to the manufacturer’s instructions. Manufacturer derived detection limits ranged from 0.2-14.6 pg/ml.

• sCD25 was analyzed using a Quantikine Elisa Human Soluble IL-2 receptor alpha Immunoassay (R&D Systems, Minneapolis, USA). Samples and standards were prepared and analyzed according to the manufacturer’s instructions. The minimal detectable dose is typically less than 10 pg/ml according to the manufacturer.

• sCD163 was analyzed using a Quantikine Elisa Human CD163 Immunoassay (R&D Systems). Samples and standards were prepared and analyzed according to the manufacturer’s instructions. Sensitivity as stated by the manufacturer: Forty-six assays were evaluated and the minimum detectable dose of CD163 ranged from 0.058-0.613 ng/ml.

The cytokine panel was analyzed in duplex and sCD25 and sCD163 samples were analyzed singly.
4.4.3 Tonsils (Paper III)

Preservation and staining of tonsils for all further analysis was performed at the Department of Pathology, Stavanger University Hospital. All slides were cut as serial sections with equal slide thickness of 3 μm. Slides stained with hematoxylin and eosin from children with PFAPA and controls were evaluated collectively by a senior pathologist.

Immunohistochemical staining for CD3 (clone F7.2.38, 1:75, Dako, Glostrup, Denmark), CD4 (clone 4B12, 1:100, Novocastra, Newcastle upon Tyne, UK), CD8 (clone C8/144B, 1:50, Dako, Glostrup, Denmark), CD15, (clone MMA, 1:20, Thermo Scientific, Waltham, MA USA), CD20 (clone L26, 1:1000, Dako, Glostrup, Denmark), CD45 (clone 2B11+PD7/26, 1:500, Dako, Glostrup, Denmark), CD57, (clone NK-1, 1:25, Novocastra, Nussloch, Germany), CD163 (clone MRQ-26, 1:100, CellMarque, Rocklin, CA, USA) were used on separate slides from tonsils of children with PFAPA and controls. These slides were coded and independently evaluated by two of the authors, and the status of the child (PFAPA or control) was not known during the evaluation. Five germinal centers filling one field of vision at 40 X magnification (diameter=55μm) were selected randomly, and the absolute number of positive cells were counted.

If there was more than 20% discrepancy between the two authors for a given slide, a re-evaluation was performed; first the slide was reviewed together in order to find the reason for the discrepancy, and subsequently it was re-evaluated by both authors separately.

4.5 Ethical issues

All parts of the study were approved by the Regional Committee for Medical Research Ethics of the Western Norway, and signed statements of informed consent were obtained from parents of all participating children including the children in the control groups. The study approval was extended on one occasion to include tonsil specimens.
Children with PFAPA and children with pneumonia included in the second part of the study (Paper II) underwent two extra blood samples outside the routine. Venipuncture is a slightly painful, but safe procedure, and in order to minimalize the discomfort, local anesthetics were offered to all children.

In Norway, tonsils from children who undergo tonsillectomy are usually disposed after surgery. Preserving and studying the tonsils had no impact on the surgical procedure and was not considered as a burden for the participants.

4.6 Statistics

*Paper I*

A binomial probability distribution formula with the probability of “boy” set at 0.5 was used in order to calculate the 2 tailed p-value for the observed gender distribution. A Fisher’s exact test was used to evaluate the share of boys referred to tonsillectomy.

When calculating the incidence of PFAPA during the first five years of life, children with onset of symptoms during the years 2003 – 2009 were included. As the population increased in numbers an incidence for each of the years was first calculated, and then the average yearly incidence for children up to five years of age was estimated.

*Paper II*

When analyzing levels of cytokines, single values extrapolated below the standard range were accepted as they were. If single values were outside the detection limits the value was set as the lowest or highest value detected by the kit respectively. The level of a given cytokine for a group (febrile or afebrile, PFAPA or controls) was referred to as not detectable if the median level was below the level of detection stated by the manufacturer.

Correction for multiple testing was not performed. Differences in categorical data between groups were analyzed by the chi-square test. Differences in continuous
data between groups were analyzed by the non-parametric Mann-Whitney U test for unrelated samples, and the Wilcoxon Signed Ranks Test for related samples.

**Paper III**

Differences in categorical data between groups were analysed by the chi-square test. Differences in continuous data were analysed by either a multiple linear regression analysis or the non-parametric Mann-Whitney U test (age at surgery).

A multiple linear regression analysis was performed for the number of different cell types with group (1=PFAPA, 2= control) and observer (1=author 1, 2=author 2) as predictors estimated with the use of the method of generalized estimating equations in SPSS with sample (1,2…,10) as within subject variable, assuming an exchangeable correlating structure to adjust for correlated measures within each subject. A similar post hoc analysis was performed to study differences in numbers of CD8+ cells between children with PFAPA with or without aphthous stomatitis.

**For all papers:**

All tests were two-tailed, and a P-level < 0.05 was considered significant. Statistical analyses were performed using the latest version of the IBM-SPSS statistical package (version 18, 20 and 22) (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp).
5 Summary of results

This thesis is divided into three parts corresponding to paper I, II and III, with overlapping study populations and inclusion periods. A total of 53 children diagnosed with PFAPA were included (Figure 5).

**Paper I**  Study period: January 1\(^{st}\), 2004 to December 31\(^{st}\), 2010

- 5 children diagnosed prior to the study period
- 109 children evaluated for recurrent febrile episodes during the study period
- 46 children with PFAPA included in the study
- 68 children received other diagnosis
- 41 children

**Paper II**  Study period: January 1\(^{st}\), 2008 to August 31\(^{st}\), 2011

- 19 children included in paper I
- 3 children living outside the study area for paper I
- 22 children with PFAPA included in the study
- 14 children with pneumonia (Control group)

**Paper III**  Study period: January 1\(^{st}\), 2010 to December 31\(^{st}\), 2013

- 2 children included in paper I
- 5 children included in paper I & II
- 4 children only included in paper III
- 11 children with PFAPA included in the study
- 16 children with tonsillar hypertrophy (Control group)

**Figure 5**

An overview of the children included in in paper I, II and III. In total 53 children were diagnosed with PFAPA.
5.1 Clinical characteristics, epidemiology and outcome (Paper I):

Five children had been diagnosed with PFAPA at the Department before the study period, and they were being followed up at the outpatient clinic. During the study period, 109 children were referred and evaluated because of recurrent fever, of these 41 children were diagnosed with PFAPA, giving a total of 46 children (32 boys; P=0.011) included in the further study. Of the remaining children evaluated for recurrent fever, one girl was diagnosed with Crohn’s disease, one boy was diagnosed with Behçet’s syndrome and the other 66 children were diagnosed with recurrent viral infections.

The median age of onset of fever in children with PFAPA was 11 months (quartiles: 5.0, 14.8). The median time between the beginning of two consecutive febrile episodes was 3.5 weeks (3, 4), and the median duration of the febrile episodes was 4 days (3, 5). Twenty-five of the 46 children diagnosed with PFAPA had received 5 or more treatments with antibiotics prior to referral.

Cervical adenitis, pharyngitis and aphthous stomatitis was present during febrile episodes in 93%, 83% and 46% of the children respectively. The most commonly reported additional symptoms were malaise (87%), nausea (39%), stomach pain (39%) and headache (30%). The median value of the maximum CRP level during the febrile episodes was 172 mg/L (quartiles: 70, 235) and IgD levels were within the normal range in 38 of 39 tested children. For eight children, recurrent fever was reported in a first degree relative. The yearly incidence of PFAPA for children up to five years of age was calculated to 2.3 per 10.000.

Thirty-seven children were followed until resolution. In these children the febrile episodes of PFAPA ceased either spontaneously or after tonsillectomy. The median total duration of PFAPA for all children was 40.5 months (quartiles: 28.2, 55.4) (see errata), and the overall median age at the time of resolution was 52.1 months (quartiles: 40.3, 71.4).

All 17 children who underwent tonsillectomy experienced a prompt resolution of febrile episodes after surgery at a median age of 50.9 months (range: 15-128). In
one of these children only a partial tonsillectomy, a tonsillotomy, was performed due to young age at the time of operation.

Twenty children experienced spontaneous resolution at a median age of 60.2 months (range: 24-120). For these children, two different patterns of disease prior to cessation of symptoms were identified; (I) in 14 children longer intervals between febrile episodes were reported and of these 12 also experienced shorter attacks with lower levels of fever, (II) six children experienced unchanged disease pattern before a sudden spontaneous resolution.

Eight children experienced a relapse after more than six months absence of febrile episodes. The median duration of the attack-free leading up to the relapse was 20 months (quartiles: 11, 24).
5.2 Immunological aspects of PFAPA assessed by blood tests (Paper II):

22 children with PFAPA and 14 children with pneumonia were included, and clinical characteristics are given in table 2.

Table 2
Clinical characteristics of children with the periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis syndrome (PFAPA) and children with pneumonia included in paper II.

<table>
<thead>
<tr>
<th></th>
<th>PFAPA (n=22)</th>
<th>Pneumonia (n=14)</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (boys / girls)</td>
<td>14 / 8</td>
<td>7 / 7</td>
<td>0.418</td>
</tr>
<tr>
<td>Age at time of sampling</td>
<td>36.5 (23.8, 62.6)</td>
<td>34.1 (17.5, 51.1)</td>
<td>0.860</td>
</tr>
<tr>
<td>(months) †</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of fever at time</td>
<td>14.0 (11.5, 16.5)‡</td>
<td>96.0 (60.0, 135)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>of sampling (hours)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of onset (months)</td>
<td>9.5 (4.3, 12.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of febrile</td>
<td>3.8 (3.0, 5.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>episodes (days)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are given as median (quartiles) except for gender
† Age at first sample in all children
‡ n=14

Immunoglobulins
- For all children, levels of IgG, IgM and IgA were within normal limits and did not differ between children with PFAPA and controls.
- For children with PFAPA, the median level of IgD was 13.0 mg/L (quartiles: 12.9, 39.2). For children with
- Levels of IgD were within age specific normal range for all children, both PFAPA and controls [151].
Differences between febrile episodes and non-febrile periods in children with PFAPA:

- Levels of white blood cells, neutrophils and monocytes were higher during febrile episodes.
- Levels of lymphocytes and eosinophils were lower during febrile episodes (Figure 6).
- Levels of CD3+, CD4+ and CD8+ lymphocytes were lower during febrile episodes.
- Levels of CXCL10, CCL4 and IL-6 were higher during febrile episodes (Figure 6).
- Levels of sCD25 were higher during febrile episodes.

For thrombocytes, levels of CD19+ and CD56+ positive lymphocytes, levels of sCD163 and the remaining cytokines no significant differences were found.

Differences between children with PFAPA during febrile episodes and children with pneumonia during the febrile phase:

- Levels of lymphocytes and eosinophils were lower in children with PFAPA (Figure 6).
- Levels of CD3+, CD4+ and CD8+ lymphocytes were lower in children with PFAPA.
- Levels of IL-1β, IL-6, CCL2, CXCL10 and G-CSF were higher in children with PFAPA (Figure 6).
- Levels of IL-1ra, IL-2, IL-9, IL-17, CCL3, CCL5, CCL11, FGF-2, and PDGF-BB were lower children with PFAPA.

Levels of white blood cells, neutrophils, monocytes and thrombocytes, levels of CD19+ and CD56+ positive lymphocytes, levels of sCD25, sCD163 and the remaining cytokines did not differ between the groups.
Differences between children with PFAPA during the non-febrile periods and children with pneumonia after complete remission:

- Levels of IL-1β and CXCL10 were higher in children with PFAPA (Figure 6).
- Levels of IL-2, CCL5 and PDGF-BB were lower in children with PFAPA.

For all the leucocyte counts including all subsets measured by immunophenotyping, levels of sCD25, sCD163 and the remaining cytokines, the results did not differ between the groups.
Figure 6. Levels of CXCL10, Interleukin (IL) 6, CCL4 and number of lymphocytes in children with PFAPA during (f) and outside febrile episodes (nf), and in children with pneumonia (Pn) during the episode (f) and after recovery (nf). The line represents the median value. CXCL10 and IL-6 are presented with a log scale.
5.3 Immunological aspects of PFAPA studied in tonsils (Paper III)

- Microscopic examination of tonsils from children with PFAPA after hematoxylin and eosin staining showed reactive lymphoid hyperplasia dominated by well-developed germinal centers with many tingible body macrophages. The histologic findings were unspecific, and a similar morphologic appearance was also found in the tonsils from controls.
- The number of CD8+ cells in the tonsillar germinal centers was significantly lower in children with PFAPA (median number: 9 (quartiles: 5, 15)) compared to controls (18 (12, 33) (p= 0.001). For CD8 there was no significant inter-observer difference (P=0.849)
- For the other cell types no differences were found when comparing the number of cells in tonsillar germinal centers for children with PFAPA and controls.
- In a post hoc analysis, the number of CD8+ cells in tonsillar germinal centers differed between children with PFAPA with (median 14 cells; 9, 16) and without (4 cells; 3, 8) aphthous stomatitis (P=0.015).
6 Discussion

The aim of this thesis was to explore different aspects of the PFAPA syndrome in children. In the three different parts of the study we focused on clinical characteristics and incidence, immunological aspects of PFAPA assessed by studies of blood samples and tonsils of children with PFAPA and controls.

6.1 Methodological considerations

6.1.1 The PFAPA diagnosis

A correct diagnosis of PFAPA is mandatory for all the conclusions drawn from this research. In paper I, II and III, the PFAPA diagnosis was set according to the modified Marshall criteria by Thomas et al. (Table 1) [15], and additionally, PFAPA was considered a diagnosis of exclusion and was given only if no other disease could explain the symptoms. There was no international consensus defining PFAPA when our study started, and the major publications at the time included different diagnostic criteria [14-16, 23, 152]. Although efforts have been made to establish a validated classification for PFAPA [153], this has still not been achieved [154].

During the study period, the specificity of the diagnostic criteria for PFAPA has been questioned. A research community at the Giannina Gaslini Institute in Genoa, Italy, has published several papers related to this issue [155-158]. They found that some patients fulfilling the clinical PFAPA criteria were carriers of either diagnostic mutations for monogenic periodic fever syndromes (MKD, TRAPS and FMF) or low-penetrance or incomplete genotypes [155, 157]. Consequently, they concluded that the clinical criteria for PFAPA do not exclude children with monogenic periodic fever syndromes [155, 157]. They also introduced a clinical score (the Gaslini diagnostic score), to predict when genetic testing is recommended. Other studies have also demonstrated diagnostic mutations, low penetration mutations or incomplete genotypes for monogenic periodic fever syndromes in some children fulfilling the clinical criteria for PFAPA [154, 159-162], and one study concluded that carriage of one MEFV mutation may attenuate disease severity in PFAPA [163].
The Gaslini diagnostic score was not used in this study. If the score had been applied, more children would have been tested genetically, and we cannot exclude that some children would have received another diagnosis explaining their periodic fever. However, the Norwegian and the southern European population are quite different, and the Gaslini score may not be suitable in Norway.

TRAPS is an autosomal dominant disease caused by different mutations in the TNFRSF1A gene [164]. The R92Q mutation of the TNFRSF1A gene is a low-penetrance mutation associated with the TRAPS phenotype, but with a frequency of 1-4% in the general population [165, 166]. It is not known if that an R92Q mutation alone is sufficient to cause disease, and maybe additional factors are required [167, 168]. Children carrying the R92Q mutation may have clinical characteristics resembling PFAPA, and generally they have a milder TRAPS phenotype compared to children with other TNFRSF1A mutations [148, 169].

In 41 of 46 children included in paper I, and in all children included in paper II and III, a thorough clinical examination was performed during at least one typical febrile PFAPA episode. For the five remaining children, the febrile episodes were thoroughly documented by the child’s GP, communicated either directly or by review of their journals, before the diagnosis was made.

No rashes, hepatosplenomegaly, conjunctivitis, periorbital edema, apparent muscle aches or signs of serositis other than the presence of mild to moderate stomach ache were found on examinations. No trigger factors (as for instance childhood vaccinations) for febrile episodes were reported. No children were of recent Dutch, French or Irish ancestry. However, there has been a widespread maritime activity between Norway and other European countries since the Viking Age, and it is not unlikely that presumed ethnic Norwegian families may have foreign ancestors.

One boy whose parents were from Iraq was included in paper I and II. During the febrile episodes, he had occasional mild complaints of abdominal pain in addition to exudative pharyngitis, aphthous stomatitis and cervical adenitis, but otherwise he showed no sign of other disease manifestations indicative of FMF. He was examined during many febrile episodes. Another boy who had parents from Lebanon was included in paper I. He had significant exudative pharyngitis during the febrile episodes
episodes, and no sign of serositis or other disease manifestations indicative of FMF symptoms. These children did not have family history of FMF or recurrent fever. They were both followed until spontaneous resolution, and they had been symptom-free for several years by the end of the studies. Genetic testing for FMF was not performed for these two children as FMF was considered unlikely. However, in hindsight it may be debated if FMF could be ruled out entirely on clinical grounds in these children. Fever alone may be a symptom of FMF and a family history is not always present [136]. Evaluation of the response to a test trail of colchicine and genetic testing for FMF could have been performed before these children were included in the study.

Although, FMF to our knowledge never has been diagnosed in the ethnic Scandinavian population, it is an important differential diagnosis in periodic fever due to the increasing number of immigrants. In 2013, Wekell et al. showed that the prevalence of FMF among immigrants in Sweden was in the same range as in their country of origin, and that the time from the first symptom to diagnosis was four years [140].

Until recently, genetic testing for TRAPS, MKD and CINCA has not been readily available in Norway, and these tests were not performed on a routine basis. Children with MKD may exhibit mevalonate in the urine during febrile episodes, and this analysis could have been performed in all children. However, to our knowledge this test has never been validated with regards to excluding MKD, but a positive test would have indicated a need for further genetic testing [143, 170]. If a child showed symptoms indicative of a monogenetic periodic fever syndrome, genetic testing was performed at a foreign laboratory.

Only children a) fulfilling the diagnostic criteria b) with symptoms consistent with PFAPA, and c) without symptoms suggestive of another disease were included in this study. Based on the aforementioned literature, children with monogenic periodic fever syndromes may not always be distinguished from PFAPA on clinical grounds. We cannot with absolute certainty exclude that such patients were included, although we believe that this risk was low.
6.1.2 Collection of clinical data

The data describing the clinical characteristics of the children in this study were obtained from the patient’s history, examinations during the afebrile period as well as the febrile episodes and from communication with the child’s GP. A questionnaire was handed out at the time of inclusion and a follow-up telephone-interview was conducted with one of the parents.

The questionnaire (appendix I), was used in order to collect structured data from the parents of all children, and the information obtained complemented the information collected during the visits at the hospital. The questions focused primarily on onset, frequency of episodes, symptoms and the use of antibiotics prior to diagnosis. The parents were also encouraged to keep fever-calendars.

The telephone interview (appendix II) focused on the child’s current health situation, family history, changes in the fever pattern, and if applicable on how and when febrile episodes ended. Some of the children had been in remission for several years when the interview was conducted, and the relatively long time that had elapsed may have caused a recall bias.

This multimodal approach for obtaining data was chosen in order to provide a thorough clinical workup of all the children, to ensure good quality of information and to avoid over-diagnosing PFAPA.

6.1.3 Preparation and implementation of laboratory analyzes

Blood samples (Paper II)

In order to ensure the immediate, cooling, centrifugation, separation and freezing of the blood samples, only JF or a designated nurse at the Pediatric ward had responsibility for these procedures. Samples were stored at -80 °C for up to four years. It has been demonstrated that samples for cytokine analysis, even at this temperature, may deteriorate with time [171]. Proteases present in samples may degrade cytokines. Fast freezing and the avoidance of thaw-freeze cycles are necessary to prevent deterioration of samples [172]. All cytokine analyses were performed the first time the samples were thawed, but one thaw-freeze cycle occurred before sCD163 and sCD25 were analyzed. An instant freezing technique using liquid nitrogen was not used, but
the samples were cooled during centrifugation immediately after venipuncture, and then frozen after separation. A shorter storage time and performing all the analyses the first time the samples were thawed could have improved the accuracy of the results. However, the study had to go on for several years in order to gain a moderate sample size, and the decision to perform measurements of soluble receptors was made after the cytokines had been analyzed.

There are many pitfalls when performing analyzes using enzyme-linked immunosorbent assay (ELISA) and multiplex based immunoassays. The 27-plex cytokine panel and the ELISA based tests used for analyzing sCD163 and sCD25 were complex to perform. Small human inaccuracies may have serious consequences for the results. Two laboratory technicians with experience in performing these analyses supervised JF in all laboratory work which was specific for this study and outside the routine. The cytokine analyses were performed in duplex and sCD163 and sCD25 samples were analyzed singly. A higher accuracy could have been achieved if all analyses were performed in triplex with exclusion of outliers.

**Tonsils (Paper III)**

Evaluating tissue using immunohistochemistry is a complex process, and all steps including sampling, preservation, preparation and staining of the tissue may have impact on the final result. A poorly stained specimen may provide a false negative result, and excessive staining may provide a false positive result.

In order to minimalize the risk for technical errors we chose CD markers of interest that were widely used by our laboratory and known to perform well. Samples were handled as a part of the standard routines and skilled technicians performed all tasks right up to the completion of the final slide.

Only a small fraction of the available tonsil tissue was studied. A more accurate result may have been achieved if a higher number of germinal centers were studied, but the laborious method limited the possibilities of a more extensive study. As germinal centers were selected at random, some variation was expected. The threshold for re-evaluating a given slide was set at 20% inter-observer discrepancy. The discrepancy was mainly a problem for the most frequent (CD3+ and CD4+) cells,
and the threshold for re-evaluation could have been set lower for the other cell types. For CD8+ cells, which were significantly lower in tonsillar germinal centers of children with PFAPA, no significant inter-observer difference was found. T cell areas varied in cell density, and due to their shape a standard field of vision defined these areas poorly. When evaluating T cell areas accurate and consistent results were not achieved, and comparisons were therefore abandoned. A digital analysis of scanned slides would have made it possible to study larger areas more accurately, and probably it would also have been possible to assess T cell areas. This type of analysis was not available during the study period.

6.1.4 Control groups

Children with PFAPA may have significantly elevated levels of CRP during febrile episodes [25], and controls with a significant bacterial infection were sought (Paper II). Children with pneumonia were chosen as a control group, and in order to substantiate a bacterial etiology a minimum CRP of at least 150 mg/L was set as a criterion in addition to a radiological verified consolidation.

Samples from children with PFAPA were taken early after the onset of a febrile episode, and always within the first 24 hours in order to detect the initial cytokine pattern. Children with pneumonia had been sick with fever for a significantly longer period when their blood tests were taken. In another paper, we showed that the children with PFAPA also had significantly lower levels of CRP and procalcitonin than the controls during the febrile phase [173]. The differences in timing of blood samples during the febrile phase between children with PFAPA and controls may limit the information gained from comparison of these two groups. This problem was considered when designing the study, but as children with short duration of significant fever and inflammation are not readily available as controls (because they don’t see a doctor this early) this design was selected. These differences must be considered when interpreting the results.

The second blood sample from the children with pneumonia was taken at least four weeks after the resolution of their infection. A symptom-free interval of at least one month was considered sufficient with regards to immunologic resolution from the
infection and a return to baseline values. However, healthy children could have been included as a second control group for the children with PFAPA during the symptom-free period.

For paper III, a control group of children with tonsillar hypertrophy was included. Tonsils from healthy children are not available, and hypertrophic tonsils may differ from to healthy tonsils in respect to the aspects studied in paper III.

The tonsils from the children with PFAPA were removed during the afebrile period, and some factors related to the etiology may only be present during the febrile episodes. However, removing tonsils during febrile episodes would increase the risk of complications and is therefore unacceptable.

6.1.5 Statistical considerations

Multiple testing

With cytokines, hematologic parameters, soluble receptors and immunoglobulins a total of 44 variables were included in the statistical calculations for paper II. This may create a problem with multiple testing.

When deciding upon a test panel of cytokines, both the multiplex kit composition and provider agreements determined the process. We planned for analyses of important pro-inflammatory cytokines and all cytokines of interest identified in earlier studies. In order to achieve this, a kit analyzing many cytokines was chosen, but then some cytokines of lesser interest were also included.

When multiple tests are performed, the risk of a type I error (falsely rejecting the null hypothesis) increases [174, 175]. The problem with multiple testing could have been addressed differently, either by reducing the number of tests or correcting for multiple testing. The purpose of using the Bonferroni’s correction is to maintain the risk of making a type I error at about 5%. However, when a relative large number of tests are preformed, the full Bonferroni’s correction is regarded as conservative, as the calculated significance level is lower than needed to achieve a maintained significance level. Additionally, there is also an increased risk of making a type II error (falsely retaining the null hypothesis) because the level of significance for each test is lowered [174].
In our study, correlation between several of the different variables could be expected, and a full Bonferroni’s correction seemed too conservative when presenting the results. The reader was alerted of the risk of type I errors, and exact P-levels were provided in order to make assessment of the findings possible.

**Power**

The statistical power of a study is a measure of the probability of correctly identifying a difference (of interest) between two groups if such a difference truly exists in the populations they represent. In an underpowered study, the risk of making a type II error increases. Higher statistical power may be achieved by increasing the sample size, but it also depends on the chosen level of statistical significance and the magnitude of the difference of interest [175].

Sample size calculations could have been performed before the study was launched in order to evaluate the statistical power of the study. However, efforts were made to include all children with active PFAPA during the study period. As the number of children with PFAPA was limited by the number of children available, sample size calculations were not performed. More children with PFAPA could have been included with a longer inclusion period, or by extending the study area by collaboration with others. More children could also have been recruited for the control groups. As a result of the moderate sample size, the study may have failed to identify minor differences between the groups. However, minor albeit statistical significant differences may not have been clinically relevant. We expected that eventual differences related to etiology of PFAPA explored in paper II and III would be of such a magnitude that they would be detectable even with a limited sample size.
6.2 Epidemiology and clinical characteristics (Paper I)

6.2.1 Setting and incidence

In order for the study to be genuinely population based, all children with true PFAPA within the catchment area must have been referred to hospital and a correct diagnosis set. A true population based study guaranteed to include all subjects with a disease is a utopia, but considering the nature of the PFAPA syndrome, it is likely that the children eventually are referred to specialist care.

Throughout the study period children with recurrent fever were evaluated systematically. All patients referred to the outpatient clinic at the Pediatric Department due to recurrent fever were evaluated by one of the authors. For some children, PFAPA was considered during the first contact if they were admitted on suspicion of a serious infection with pharyngitis, cervical adenitis or aphthous stomatitis and negative diagnostic workup for bacterial and viral etiology. Parents and GPs were then informed about the syndrome and encouraged to contact us if similar episodes occurred.

The majority of the children diagnosed with PFAPA had received five or more treatments with antibiotics prior to referral, and the time between onset and diagnosis was long for some of the children. Some children with PFAPA living within the study area may not have been identified, and factors like mild disease, short total duration of the syndrome and older age of onset may have reduced the likelihood of referral. As discussed above, there is also a risk of over-diagnosing PFAPA, and if children were falsely diagnosed with the syndrome this would have biased both the incidence and the description of clinical characteristics.

Except from a preliminary rapport from our study group [24], this is to our knowledge the first incidence of children with PFAPA being published. We believe that this is a conservative estimate, because presumably the risk of not having identified every child with PFAPA is greater than the risk of over-diagnosis.

Children who were diagnosed with PFAPA prior to 2004 and followed at the hospital’s outpatient clinic were included in the study. Several children who were diagnosed with PFAPA in 2004 had onset of symptoms in 2003, and as the probability
of identifying children with disease onset in 2003 was regarded equal to the following years, these children were included for the calculation of incidence.

No of the other children evaluated for recurrent fever with onset during the first five years of life were diagnosed with other periodic fever syndromes than PFAPA during the study period. However, one child was diagnosed with CINCA during the study period. This child was not included in the study because a pattern of periodic fever was not present, and PFAPA was never considered as a differential diagnosis.

6.2.2 Clinical characteristics

There are some variations between studies, but in general the occurrence of cervical adenitis and pharyngitis is quite similar and higher than the occurrence of aphthous stomatitis (Table 3). The occurrence of cardinal symptoms and duration and frequency of febrile episodes described in our study are comparable to other international studies. Surprisingly, we found that the examination during the febrile episodes sometimes revealed clinical signs that the parents were unaware of. Differences in approach may to some extent explain the low occurrence of symptoms in some studies.

Boys are overrepresented in most studies on PFAPA, and in the current study the percentage of boys was among the highest in the literature (Table 3). The reason for the male predominance in PFAPA is unknown, and apparently there is no gender bias in MKD, TRAPS and CINCA [145, 168, 176]. However, in FMF a male predominance at ratios of 3:2 has been shown, and males may have an increased risk of amyloidosis [136, 177].

The median age of onset found in this study is the lowest reported in the literature so far. High awareness of periodic fever during the study period, early recognition of PFAPA, a thorough medical history and a questionnaire focused on the time of onset may have improved the accuracy of this information in this study. Although disease onset before the age of five years is listed as a criterion in the modified Marshall criteria (Table 1) [15], this is not consistent for all studies. The inclusion of adolescents may to some extent explain the higher age of onset found in some studies. We are not aware of any children at our clinic during the study period
with onset of PFAPA after the age of five, and no children were excluded due to this criterion. PFAPA with onset in older children and adults has been described [14, 16, 154, 178-180].

Although PFAPA is mainly a disease of early childhood and the majority of patients described fulfill the age criterion, there is in our opinion no apparent rationale for excluding patients older than five years from the diagnosis. In the largest cohort presented so far, including a total of 301 PFAPA patients, Hofer et al. found disease onset under the age of 5 in 90% of the cases [154]. In the patients with older age at onset, abdominal and osteoarticular pain was found more frequently.

Recurrent fever in a first degree relative was reported for eight children. The febrile episodes were usually combined with sore throat or other symptoms indicative of an upper airway infection. In seven of these family members, the episodes ceased after tonsillectomy. We were unable to substantiate a definite PFAPA diagnose in any of these family members, although PFAPA appears to be the most likely diagnose in some of the cases. For eight more children tonsillectomy had been performed on a first degree relative, but recurrent fever was not the primary indication for surgery.

Since 2009, familial occurrence of PFAPA has been documented in several small reports including up to four patients [181-184], and also in three larger studies [154, 185, 186].
Table 3

The number of patients, the percentage of boys, occurrence of cardinal symptoms and age of onset in major publications on PFAPA

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Boys (Percentage)</th>
<th>Cervical adenitis</th>
<th>Pharyngitis</th>
<th>Aphthous stomatitis</th>
<th>Age of onset (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Padeh et al. (1999)</td>
<td>28</td>
<td>71</td>
<td>100</td>
<td>100</td>
<td>68</td>
<td>50</td>
</tr>
<tr>
<td>Thomas et al. (1999)</td>
<td>94</td>
<td>55</td>
<td>77</td>
<td>65</td>
<td>67</td>
<td>34</td>
</tr>
<tr>
<td>Tasher et al. (2006)</td>
<td>54</td>
<td>61</td>
<td>61</td>
<td>96</td>
<td>39</td>
<td>23</td>
</tr>
<tr>
<td>Garavello et al. (2009)</td>
<td>39</td>
<td>41</td>
<td>85</td>
<td>97</td>
<td>59</td>
<td>35 &amp; 37*</td>
</tr>
<tr>
<td>Brown et al. (2010)†</td>
<td>10</td>
<td>50</td>
<td>90</td>
<td>100</td>
<td>90</td>
<td>23</td>
</tr>
<tr>
<td>Dagan et al. (2010)</td>
<td>57</td>
<td>58</td>
<td>44</td>
<td>n.s.</td>
<td>33</td>
<td>31</td>
</tr>
<tr>
<td>Fedrer et al. (2010)†‡</td>
<td>105</td>
<td>62</td>
<td>62</td>
<td>85</td>
<td>38</td>
<td>40</td>
</tr>
<tr>
<td>Berkun et al. (2011)</td>
<td>124</td>
<td>57</td>
<td>n.s.</td>
<td>100</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>Førsølv et al. (2011)</td>
<td>46</td>
<td>70</td>
<td>93</td>
<td>83</td>
<td>46</td>
<td>11</td>
</tr>
<tr>
<td>Pelagatti et al. (2011)†</td>
<td>64</td>
<td>59</td>
<td>86</td>
<td>83</td>
<td>63</td>
<td>19</td>
</tr>
<tr>
<td>Stojanov et al. (2011)‡</td>
<td>21</td>
<td>62</td>
<td>100</td>
<td>86</td>
<td>62</td>
<td>13</td>
</tr>
<tr>
<td>Kolly et al. (2012)†</td>
<td>15</td>
<td>73</td>
<td>80</td>
<td>100</td>
<td>60</td>
<td>22</td>
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<tr>
<td>Krol et al. (2013)</td>
<td>125</td>
<td>50</td>
<td>78</td>
<td>91</td>
<td>41</td>
<td>23</td>
</tr>
<tr>
<td>Kyvsgaard et al. (2012)Ψ</td>
<td>31</td>
<td>68</td>
<td>98</td>
<td>84</td>
<td>55</td>
<td>33</td>
</tr>
<tr>
<td>Licameli et al. (2012)</td>
<td>124</td>
<td>60</td>
<td>84</td>
<td>79</td>
<td>44</td>
<td>n.s.</td>
</tr>
<tr>
<td>Taniuchi et al. (2013)</td>
<td>20</td>
<td>45</td>
<td>n.s.</td>
<td>100</td>
<td>60</td>
<td>34 &amp; 59*</td>
</tr>
<tr>
<td>Hofer et al. (2014)†</td>
<td>301</td>
<td>53</td>
<td>78</td>
<td>90</td>
<td>57</td>
<td>20</td>
</tr>
<tr>
<td>Vigo et al. (2014)</td>
<td>275</td>
<td>60</td>
<td>48</td>
<td>70</td>
<td>30</td>
<td>28</td>
</tr>
</tbody>
</table>

PFAPA: Periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis
n: Number of patients
The share of boys and the occurrence of cardinal symptoms are given as percent.
Age of onset is given as months and may be either a median or mean value depending on the publication
n.s.: not stated
* The age of onset is given for two different subgroups
† & ‡ There may be overlap between these study populations
Ψ The occurrence of cardinal symptoms was extrapolated from a figure
6.2.3 Outcome

Resolution of PFAPA is not clearly defined in the literature. In two studies referring to the duration of PFAPA no apparent definition of resolution is given [14, 180], and in two other studies resolution was defined as absence of febrile episodes for one year [15, 187, 188]. The median or mean duration of PFAPA in these studies ranged from 1.4 to 8.0 years. In 2011, Wurster et al. published a unique study on long term follow-up in PFAPA [187]. The original register included 94 patients [15], 34 patients were lost from follow-up, and one had died. 50 of 59 subjects had ceased having febrile episodes after a mean total duration of 6.3 years. The remaining nine subjects had at follow-up experienced febrile episodes for a mean of 18.1 years.

In the current study resolution of PFAPA was defined as no episodes of periodic fever during the past six months. The reported overall duration of the PFAPA syndrome may depend significantly on the study design and on the prevalence of tonsillectomy, and this may to some extent explain the variation between different studies. In our study we provided information on total duration of PFAPA for all 37 children who had experienced resolution collectively and the age of resolution for children either spontaneous or after tonsillectomy separately. The total duration of PFAPA could have been given for both groups separately in order to state the duration of PFAPA from onset to spontaneous resolution clearly. However, the children referred to tonsillectomy in our study were selected (in terms of disease duration and intensity), and it is possible that the duration of PFAPA observed in the children who experienced spontaneous resolution may not have been representative for all children with PFAPA.

In this study (Paper I and III), all 21 children who underwent tonsillectomy experienced prompt resolution of PFAPA, but for one girl (Paper III) the episodes relapsed. For one boy (Paper I), only a partial tonsillectomy was performed due to his low age, and noteworthy he also experienced termination of febrile episodes. However, this study was not designed to evaluate the effect of tonsillectomy in PFAPA.
In addition to the children reported in the present study (Paper I and III), outcomes after 294 tonsillectomies with or without adenoidectomy for children with PFAPA are reported in the literature [14, 16, 19-22, 96, 162, 180, 186-196]. Surgery was curative for 254 children, partly helpful for 11 children and ineffective for 26 children, three children were lost from follow-up. Additionally, two randomized controlled trials have explored the effect of tonsillectomy or adenotonsillectomy on PFAPA [23, 197]. As featured in the introduction, the first study concluded that tonsillectomy was helpful in PFAPA, but this result has been questioned due to a vague definition of PFAPA [23]. However, a similar conclusion was drawn in the latter study, where Garavello et al. found complete resolution in 63% of the children in the surgery group (adenotonsillectomy) and in 5% of the children in the control group (P< 0.001) [197]. Due to the relatively few patients included in randomized controlled trials and the benign prognosis in the absence of surgery, it is still debated whether tonsillectomy with or without adenoidectomy is justified as a treatment option in PFAPA [186, 198].

### 6.3 Immunological aspects of PFAPA assessed by blood tests

#### 6.3.1 Immunoglobulin D and hematologic parameters

While planning this study, elevated levels of IgD were regarded as a marker for MKD (formerly denoted Hyper IgD syndrome) [142]. However, it has later been shown that elevated levels of IgD is not always present in in children with MKD, and elevated levels of IgD is also a poor predictor of MKD even in children with relevant symptoms [143]. Elevated levels of IgD have been proposed to be a frequent epiphenomenon in PFAPA in two studies [14, 199], and in one study, three children with PFAPA showed more class-switched IgD⁺ IgM⁻ plasmablasts and more IgD armed mucosal basophils compared to healthy controls [56]. However, our finding with normal levels of IgD in all except one child with PFAPA (Paper I and II) is in agreement with other studies [15, 162, 180].

According to the diagnostic criteria, cyclic neutropenia should be excluded in all children before setting the PFAPA diagnosis. We measured neutrophils twice
weekly between two subsequent attacks. Cyclic neutropenia may also be excluded by a mutation analysis of the \textit{ELANE} gene. To our knowledge, cyclic neutropenia has never been diagnosed in children evaluated for PFAPA, but as it is a severe disease it is important to exclude this diagnosis.

An increase in white blood cell count and neutrophil count during febrile episodes of PFAPA has been described in many earlier publications [14, 15, 18, 19, 101, 160, 200, 201]. Consistent with our results, three previous studies described decreased levels of lymphocytes [160, 200, 201], and one of these also described decreased levels of both CD4+ and CD8+ lymphocytes and eosinophil count and increased levels of monocytes during febrile episodes of PFAPA compared to the afebrile period [201]. The observed increase in levels of white blood cells is due mainly to an increase in levels of neutrophils consistent with a left shift, and may be explained by the inflammation seen during febrile episodes of PFAPA. This is an unspecific finding also seen during infections and other types of acute phase reactions [202].

The decrease in levels of circulating CD4+ and CD8+ lymphocytes may be due to attraction of these cells to peripheral tissues. Interestingly, the observed levels of eosinophils and lymphocytes with subpopulations of CD3+, CD4+ and CD8+ cells were lower in children with PFAPA during the febrile episodes than in children with pneumonia during the febrile phase, while the other hematologic parameters including levels of white blood cells and neutrophils did not differ. In another study, the observed lymphocyte count was lower during febrile episodes of PFAPA than during episodes of hereditary periodic fever [201]. Together, this may indicate that the observed decrease in levels of lymphocytes and eosinophils is a feature linked to the etiology of PFAPA. However, Sundqvist et al. compared levels of leucocytes in children with PFAPA during febrile episodes and in five controls with short duration of fever and abdominal pain, and found no differences in white blood cell count, neutrophil count, monocyte count, lymphocyte count or eosinophil count between the two groups [203].
6.3.2 Cytokines, chemokines and soluble receptors

When this study started only one paper addressing the levels of inflammatory proteins in PFAPA had been published [18], but parallel to our studies, several other papers have been published on this topic [160, 200, 201, 204, 205]. The ability to compare these studies is limited by differences in the panel of tests, timing of the analyses and the control groups.

An increase of IL-6 during febrile episodes is the most consistent finding in all analysis of cytokines in PFAPA [18, 160, 200, 201, 204, 205]. IL-6, a pro-inflammatory cytokine, is mainly, but not exclusively produced by innate immune cells such as monocytes and macrophages [58, 74]. Many of the features observed during the febrile episodes of PFAPA may be attributed to IL-6. IL-6 mediates T cell activation, induces differentiation of B cells into plasma cells, induce pyrexia and stimulates hepatocyte production of acute phase proteins such as CRP and serum amyloid A [58, 74, 206]. The elevated level of IL-6 during febrile episodes of PFAPA shown in paper II corresponds well with the observed median maximum CRP level shown in paper I. We found that levels of the acute phase parameter CRP may be significantly increased, and we consider this a typical and important finding during febrile episodes of PFAPA. In benign viral infections CRP levels are usually low [207], and a CRP-value measured on day two or three of a febrile episode may help distinguishing recurrent viral infections from PFAPA. The CRP value presented in paper I is somewhat higher than in other studies [18, 154, 160, 200, 201, 208], but this may be due to differences in the selection and timing of the analyses.

Consistent with our results, increased levels of CCL-4 and CXCL10 during PFAPA related febrile episodes has been found previously in one and three studies respectively [160, 200, 201]. However, we were the first to report increased levels of CXCL10 during the afebrile period compared to healthy controls.

CCL-4 (formerly denoted Macrophage Inflammatory protein 1β) is mainly but not exclusively produced by activated macrophages [209]. CCL-4 acts pro-inflammatory primarily through chemotaxis towards (other) macrophages, dendritic cells and T cells. Noteworthy, glucocorticoids are effective in reducing IL-6 and CCL-4 release [206, 209], which may explain the abrupt effect from a single dose of a
systemic glucocorticoid widely documented in PFAPA [14, 99]. However, this response is not unique to PFAPA, and may also be present in monogenic periodic fever syndromes [132, 148].

CXCL10 (formerly denoted: interferon-gamma-inducible-protein-10 or IP-10) is a small chemokine also acting as a pro-inflammatory agonist through chemotaxis and activation of immune cells including T cells, NK cells, monocytes, macrophages and dendritic cells [210]. CXCL10 is one of three ligands that activate the CXCR3 chemokine receptor which is highly expressed on T\textsubscript{H1} cells, effector CD8+ cells, NK and NKT cells (Figure 3) [211]. CXCL10 is produced by a variety of cells including monocytes, keratinocytes, T cells, neutrophils and stromal cells when stimulated by INF-\textgamma, the key cytokine in T\textsubscript{H1} immune responses [212, 213]. Although T\textsubscript{H1} cells are regarded as the major producers of INF-\textgamma, other cell types including stimulated APCs may also produce this cytokine [69]. Increased levels of INF-\textgamma during febrile episodes of PFAPA have been found in two studies [18, 205], and consistent with our study, two other studies did not confirm this finding [200, 201]. It has been shown that CXCL10 release may be induced by TNF-\textalpha in mice in absence of INF-\textgamma [214], and by TLR4 activation by lipopolysaccharide in human monocytes and pericytes (figure 4) [215, 216].

Detection of increased levels of CXCL10 during the afebrile period may indicate that CXCL10 has a more central role in the etiology of PFAPA than earlier recognized, and that pathways involving this chemokine to some extent are active between episodes. As this finding has not been confirmed in other studies, the result must be interpreted with caution [160, 201, 205]. Interestingly, Stojanov et al. showed elevated levels of CXCL10 in four of 17 children with PFAPA between febrile episodes, but the overall result was not different from the afebrile controls [201]. Very recently, Dytrych et al. showed significantly higher expression levels of CXCL10, CXCL9 and CCL19 genes in tonsils from children with PFAPA who underwent tonsillectomy during the afebrile period compared to tonsils from controls [217].

The pattern of cytokines we observed suggests that innate immune cells in the monocyte-macrophage system are involved in the pathogenesis of PFAPA, and consequently supporting that PFAPA belongs to the AIDs. However, neither IL-6,
CCL4 nor CXCL10 have unique cellular origin and innate and adaptive immune responses overlap. In the conclusion of paper 2 we state that: “We have shown increased levels of cytokines during the febrile attacks of PFAPA, indicating an innate immune response as the initial step. Further, we found a decrease in subsets of T cells, suggesting a subsequent adaptive immune response with activation and redistribution of these cells to local tissue.” However, it may be argued that these observations were made at the same time, and it is not possible to determine if the release of the aforementioned cytokines preceded the decrease in T lymphocytes.

The levels of IL-1β were higher in the children with PFAPA both during febrile episodes and in the afebrile period than in children with pneumonia and after full recovery respectively. However, the levels of IL-1β did not increase during febrile episodes in children with PFAPA, and the levels measured both during febrile episodes and in the afebrile period were close to the detection limit. The comprehensive study by Stojanov et al., published in 2011, explored the etiology of PFAPA through different modalities including gene expression profiling [201]. Based on their results, they hypothesized that IL-1β activation may initiate the febrile episodes of PFAPA, and they showed a prompt effect from IL-1 receptor blockade in five children. IL-1β was also suggested as a key cytokine in PFAPA by Kolly et al., showing increased serum levels of caspase-1, an enzyme crucial for activation of IL-1 [160]. Further, they found increased secretion of IL-1β from stimulated peripheral blood mononuclear cells and monocytes during febrile episodes compared to the afebrile period.

The IL-2 receptor is expressed on T_{Reg} cells of thymic origin, and upon activation they may shed the receptor in large amounts [218]. Soluble IL-2 receptor or sCD25 has been shown to perform well as a biomarker of sepsis and also to correlate with the severity of other infections [219, 220]. Stojanov et al. showed a significant decrease of CD4+CD25+ cells during febrile episodes of PFAPA, indicating that shedding of CD25 occurs [201]. We found a significant increase in levels of sCD25 in children with PFAPA during febrile episodes, but no difference between febrile children with PFAPA and children with pneumonia. This may suggest that activation
of T_{Reg} cells was present in both groups; and that sCD25 as a biomarker does not discriminate well between PFAPA and pneumonia.

CD163 is a hemoglobin scavenger receptor, and a specific marker of macrophages and monocytes [221, 222]. Soluble CD163 is a biomarker for acute and chronic inflammation related to an increased macrophage activity [222]. We found no change in levels of sCD163 during febrile episodes of PFAPA, suggesting that sCD163 is not suitable as a biomarker for PFAPA related febrile episodes. We also assessed the number of CD163+ cells in tonsillar germinal centers without finding any difference between children with PFAPA and controls (Paper III).

Cytokines are unstable molecules, and although their plasma concentrations easily raise more than thousand fold during inflammation, they generally have short half-lives of minutes up to a few hours [223]. The detection of a given cytokine in serum during the course of an infection or another type of inflammatory condition therefore relies on sustained release. Cytokines act in cascades and during infections or other inflammatory processes a network of pro-inflammatory cytokines is set into action [60]. Due to the differences in timing of analyses, the information gained from comparing levels of cytokines in blood samples taken during the febrile episodes in children with PFAPA and children with pneumonia was limited. However, we think that both the differences in levels of lymphocytes and the observed higher levels of IL-6 and CXCL10 in children with PFAPA during febrile episodes compared to children with pneumonia is of interest.

### 6.3.3 Other perspectives

During the progress of this thesis other studies have explored different immunological aspects that may contribute to the understanding of the etiology of PFAPA.

Neutrophil CD64 is a known biomarker of sepsis [224]. Yamazaki et al. showed that CD64 is expressed to a greater extent on both neutrophils and monocytes during febrile attacks of PFAPA than in controls during febrile episodes of CAPS and TRAPS and during infections [205]. Neutrophil function in PFAPA was explored in one study,
and three neutrophil functions namely apoptosis, priming and generation of an intracellular oxidative burst were altered in PFAPA [203].

Two studies have demonstrated a correlation between PFAPA and vitamin D deficiency [225, 226], and a beneficial response after vitamin D supplementation in terms of milder and/or less frequent episodes was observed [226]. It is of note that vitamin D deficiency may play a role in different types of inflammatory disorders [227], and vitamin D supplementation may counteract the release of CXCL10 [228].

6.4 Immunological aspects of PFAPA studied in tonsils

The observed effect of surgery in PFAPA raises important questions about the etiology of the disease, and may complicate the understanding of PFAPA as an autoinflammatory disease. A dysregulation of the immune system on genetic or developmental basis should not be confined only to a small fraction (the tonsils and adenoid) of the immune system. On the other hand, an effect from tonsillectomy may indicate that microbiological contact with the immune system in this location may be an initial event in febrile attacks in PFAPA. It is possible that children with PFAPA, experience bursts of inflammation in response to such a trigger, due to a dysregulated immune system.

Prior to our study, histologic evaluation of tonsils from 28 children with PFAPA was published, and all studies described unspecific lymphoid hyperplasia or chronic tonsillitis with preservation of the architecture [19, 22, 192, 217]. These findings are consistent with the present study, and we also found that the histologic appearance (after hematoxylin and eosin staining) was similar in tonsils from children with PFAPA and controls with tonsillar hypertrophy.

Valenzuela et al. reported similar gene expression for pro-inflammatory, effector and regulatory cytokines in tonsils from children with PFAPA and controls, except for IL-4 gene expression which was lower in children with PFAPA [195]. This may suggest that inhibition of a T_{H}2 immune response is present in PFAPA. Brown et al. suggested that T cell regulation may be altered in PFAPA, as they found decreased levels of IL-7 and IL-17 during and in between febrile episodes in children with
PFAPA compared to healthy controls [200]. We found (Paper II) lower levels of IL-17 in children during febrile episodes of PFAPA compared to children with pneumonia, but no differences for IL-7.

The decreased number of CD8+ cells in tonsillar germinal centers found in children with PFAPA compared to controls may indicate that children with PFAPA have altered affinity of these cells for this compartment. Very recently, Dytrych et al. found increased proportion of CD8+ cells and decreased proportion of CD19+ cells in dissociated tonsil tissue from children with PFAPA compared to controls. As their study of entire tonsil specimens shows opposite results in respect to CD8+ cells compared to our findings in tonsil germinal centers, this may suggest that the CD8+ cells found in tonsil germinal centers are of a distinct subtype.

The role and function of CD8+ cells in tonsillar germinal centers was not clarified in our study, and there is paucity of information on these cells in the literature. However, in 2007 a subset of human CD8+ cells locating to tonsillar germinal centers were described [229]. These CD8+ cells had a functional profile suggesting that they were antigen experienced early effector memory T cells, and they also expressed CXCR5, the receptor for the CXCL13 chemokine. CXCL13 is produced primarily by follicular dendritic cells, and is crucial for the attraction of cells such as B-cells and CXCR5+CD4+ helper T cells to the B cell follicles [229, 230].

In a murine model, Kim et al. have shown that a regulatory-like CD8+ cell suppressed germinal center T follicular helper cells [231]. It is not known if such regulatory CD8+ cells take part in the regulation of germinal center activation in humans [232], nor if they play a part in the etiology of PFAPA.

In a post hoc analysis we found lower significantly higher levels of CD8+ cells in tonsillar germinal centers from the children with PFAPA who had aphthous stomatitis and a full cluster of cardinal symptoms, compared to the children who only had pharyngitis and cervical adenitis. Aphthous stomatitis is generally the least common cardinal symptom in PFAPA (table 3), and our finding may indicate that differences in the pathophysiology exists between children with PFAPA with and without this symptom.
7 Conclusion

In this thesis an incidence of PFAPA was estimated to be to 2.3 per 10 000 children up to 5 years of age. In South Rogaland, onset of PFAPA was frequent during the first year of life, earlier than previously described. Consistent with earlier studies a male predominance was found. Cervical adenitis and pharyngitis were the most frequent cardinal symptoms, and aphthous stomatitis was present in less than half of the children. In the 37 children followed until resolution, either spontaneous or after tonsillectomy, the median total duration was slightly more than three years. All children who underwent tonsillectomy experienced prompt resolution.

During febrile episodes the children with PFAPA had significantly increased levels of the cytokines IL-6, CCL4 and CXCL10 in blood, indicating activation of the innate immune system. CXCL10 was also elevated during remission when compared to afebrile controls, suggesting a persistent activation of the immune system in these children. During the febrile episodes there was a relative decline in levels of lymphocytes, both CD4+ and CD8+ cells and eosinophils compared to the afebrile period in children with PFAPA. Further, the levels of these cells were significantly lower in the children with PFAPA during febrile episodes than in febrile controls with pneumonia. Except for one child (paper I) with elevated IgD levels and a normal MKD mutation analysis, levels of IgG, IgM, IgA and IgD were within age specific normal ranges for all children and did not differ between children with PFAPA and controls.

Palatine tonsils from children with PFAPA showed reactive lymphoid tissue and the morphologic appearance was similar to the control group. The tonsillar germinal centers had reduced number of CD8+ cells in children with PFAPA compared to controls, but no differences were found for the other cell types studied. Reduced number of CD8+ cells in tonsillar germinal centers may be a feature linked to the etiology of PFAPA.
8 Future Perspectives:

Despite considerable research activity over the past three decades many aspects of PFAPA is still unresolved. Several studies including ours have substantiated that a dysregulation of the immune system is present in children with PFAPA, but further research is still needed in order to clarify the etiology and to reveal eventual predisposing factors.

The tonsils from children with PFAPA should be studied further in order to understand why and how tonsillectomy changes the clinical course of the syndrome. In this work a more thoroughly characterization of the CD8+ cells in tonsillar germinal centers would be included, and this may be achieved by combining different CD markers and then analyze images of tonsil slides digitally.

Over the years we have identified many children with PFAPA in our region, and as we now have a fairly large patient cohort different types of studies may be performed. PFAPA may be a polygenic disorder, and mapping of mutations or gene variants in children with PFAPA may pinpoint risk-factors or develop diagnostic tests for the syndrome. We are planning to launch a study addressing these issues in collaboration with the Center for Medical Genetics and Molecular Medicine at Haukeland University Hospital.

While working on this thesis we have experienced that inconsistent definitions of PFAPA complicates the comparison of different studies. Hopefully, a biological marker for the syndrome will be found, but until then an international consensus on the diagnostic criteria for PFAPA is needed to improve the diagnostic accuracy.

PFAPA is an important differential diagnosis in children with periodic fever, and pediatricians working with these children have an important role in making the syndrome known among GPs and other health care workers. This may shorten the time from onset to diagnosis in these children, and avoid unnecessary use of antibiotics and parental concern.
9 Reference list


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10 Errata

Paper I:
Two errors occurred in the late editorial process after the article was accepted by Acta Paediatrica. The errors were not present in the first, accepted and online available edition of the article. Due to a very short deadline for proofreading of the final and printed edition these errors were unfortunately not discovered.

In the abstract it is stated that:

_Nearly 37 children were followed until resolution._

The correct sentence should have been:

_Thirty-seven children were followed until resolution._

In the results section a result is repeated and one result is lost:

_The median age at the time of resolution was 52.1 months (quartiles: 40.3, 71.4). The median age at the time of resolution for all children was 52.1 months (quartiles: 40.3, 71.4)._  

The correct sentences should have been:

_The median age at the time of resolution was 52.1 months (quartiles: 40.3, 71.4). The median total duration of the PFAPA syndrome from onset to resolution was 40.5 months (quartiles: 28.2, 55.4)._  

Additional error in paper I:

In the discussion:

“multiple-centered studies” should have read “multiple-center studies”

Paper II:

In the methods section:

“making theme prone to” should have read “making them prone to”

In the results section:

“an in between” should have read “and in between”

The p-value for CXCL10 in table 1 (Pn-nf vs PFAPA-nf) should have read < 0.001 (not 0.000).
11 Appendix

I: Questionnaire used at diagnosis (Paper I)

Spørreskjema ved periodisk febersyndrom

1. Angi alder for når de regelmessige episodene med feber startet:

2. I perioden med mest regelmessig feber, ca hvor ofte kom feberepisodene:

3. Angi ca. alder for når feberepisodene avtok vesentlig i hyppighet:
   Ev sett x om feberepisodene pågår fortsatt:

4. Angi ca hvor mange dager en gjennomsnittlig feberepisode varer: ________

5. Sett X ved symptomer eller funn som opptrer eller opptrådte regelmessig ved feberepisodene (sett så mange X som er aktuelt):
   - Feber over 39 grader:    Vesentlig redusert allmenntilstand:
   - Forstørrede lymfeknuter på hals:   Sår i munnen:
   - Sår eller vond hals:    Påvist halsinfeksjon hos lege:
   - Hodepine:      Magesmerter:
   - Kvalme:
   - Annet:

6. Angi ca hvor mange antibiotikakurer barnet fikk før diagnosen periodisk
   febersyndrom ble gitt:  0-1:    2-4:     5-10:    mer enn 10:

7. Før diagnosen periodisk febersyndrom ble gitt, hvilke diagnose(r) ble vanligvis gitt
   ved eventuelle legebesøk:

8. Er det andre i nær familie som har hatt tilsvarende symptomer (ja/nei):

   Dersom ja, beskriv (eventuelt på baksiden):

   Eventuelt andre kommentarer (bruk eventuelt baksiden ved behov):
II: Outline of questions from the follow-up interview (Paper I)

Mal for telefonintervju

- Pasient nummer:
- Dato for intervju:
- Barnets alder nå (MND):
- Har barnet fremdeles feberepisoder?
- Hvis ja:
  - Har mønsteret (intervall, varighet av episoder, symptomer ved episoder) endret seg fra tidligere?
  - Intervall nå (tid mellom episoder):
  - Intensitet nå (lengde av episoder, symptomer, feber):

- Hvis febere episodene har opphørt:
  - Når opphørte episodene?
  - Endret mønsteret seg i perioden før opphør?
  - Medvirket noe til at barnet ble friskt?

- Annet:
  - Har barnet fått fjernet mandlene? Når?
  - Påvirket inngrepet febere episodene?
  - Har foreldre eller søsken fjernet mandlene?
  - Har det vært en lengre periode med opphør av symptomer og deretter oppstart på ny?
  - Hvor lenge varte oppholdet?
  - Har barnet fått noen andre sykdommer/diagnoser?
  - Er det andre i familien som har hatt hyppige febere episodere? Hvem? Når i livet?