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Functional studies on autoinflammatory diseases, focusing in particular on Blau syndrome

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DECLARATION

Most of the work presented in this thesis was performed between January 2011 and December 2013 in the Reumatology Unit, Department of Medicine, University of Padua.
Microarray experiments were performed during the period spent in the School of Life Sciences, University of Nottingham, UK.
Apart from the help and advices of my supervisor prof. Punzi and the Nottingham's tutors prof. Todd and dr. Tighe, the work described in this thesis is totally my own.
This thesis has not been previously submitted for any other degrees. The functional studies on Blau syndrome were previously presented in Italian and International Congresses.

Paola Galozzi
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SUMMARY

BACKGROUND

The autoinflammatory diseases are disorders characterized by recurrent inflammatory episodes in charge of different organs. There is no apparent involvement of autoantibodies nor of antigen-specific T lymphocytes, but dysregulation of the innate immune response.

The Blau syndrome (BS) is a rare autosomal dominant autoinflammatory disease clinically characterized by symmetrical arthritis, granulomatous dermatitis and recurrent uveitis. The disease is caused by single mutations in *CARD15/NOD2*, encoding the NOD2 protein that is known to regulate the defense against pathogens by activating the NF-κB signalling pathway.

In this thesis, functional analysis was carried out aimed at characterizing p.E383K mutation found in one Italian family affected by Blau syndrome. After the *in vitro* study of mutant NOD2, we investigated the activity of NF-κB and the production of pro-inflammatory cytokines both *ex vivo* and *in vitro.*

In the second part of this thesis, we assessed in detail both the signalling pathways known to be involved in inflammation and a wide range of inflammatory mediators (pro-inflammatory cytokines and chemokines) in a cohort of patients affected by different hereditary autoinflammatory diseases such as FMF and TRAPS or complex diseases like Behçet and Adult Onset Still's disease. The aim of this studies was to increase the molecular knowledge of these disorders in order to identify possible predictive or diagnostic biomarkers for each disease and to develop future preventive and therapeutic strategies.

MATERIALS AND METHODS

For *ex vivo* functional analysis on Blau syndrome, peripheral blood monocyte cells (PBMC) were collected from 2 Italian patients carrying p.E383K mutation. For the evaluation of NF-κB, PBMC were lysed and analyzed by Reverse Phase Protein Array (RPPA); while for the production of mediators of inflammation (IL1β, IL6, IL8, TNFα, IFNγ, IL12, IL17, IL22, IL23), PBMC were cultured for 7 hours in presence or absence of lipopolysaccharide LPS [100ng/ml] and muramildipeptide MDP [10μg/ml] and cytokines were quantified by ELISA and antibody microarray techniques.

For the functional analysis *in vitro* on Blau syndrome, the starting point was the creation of 3 constructs containing cDNA of human NOD2 *wild-type* and mutated p.E383K and p.R334W. These plasmids were stably transfected into HEK293 cells, cultured for 7 and 24 hours in the presence or absence of MDP [10μg/ml]. The activity of NF-κB was firstly determined indirectly by evaluating the expression of IKBα and phosphorylated form in p.E383K cellular lysates by Western blotting. RPPA also assessed NF- κB activity by analysis of the

expression pathway components compared to NOD2 *wild-type*. IL8 was assayed in the supernatants through antibody microarray technique.
In the second part of the study, serum and PBMC were collected from 40 patients with autoinflammatory diseases and 27 healthy controls. The PBMC were lysed and prepared for RPPA analysis of the major components of the NF-κB, PI3K/Akt, MAPK, JAK/STAT/c-Src and inflammasome (NALP1) signalling pathways. From the serum, mediators of inflammation (IL1β, IL6, IL8, TNFα, IFNγ, IL12, IL17, IL22, IL23) were assessed by antibody microarrays.

RESULTS
Confocal microscopy revealed that p.E383K NOD2 resides in cytoplasm of transfected HEK293 cells, as well as *wild-type* and p.R334W NOD2. The expression of p.E383K NOD2, assessed by Western blotting , do not present any variations after MDP stimulation. As regards the analysis of the NF-κB pathway, *in vitro* studies show an inactivation of this pathway in HEK293 cells presenting p.E383K NOD2, while the pathway components are upregulated *ex vivo* in comparison with healthy controls. Both i*n vitro* RPPA and indirect Western blotting analysis of total lysates from p.E383K NOD2 transfected cells present lower expression of phospho IkBα than IKBα in presence or absence of MDP stimulation. No increase expression for other components of the pathway, such as phospho NF-κB and IKKα/β, was then detected, even in nuclear extracts. Concerning cytokines in Blau syndrome, both *in vitro* and *ex vivo* studies have not identified a significant increase secretion of most of the pro-inflammatory cytokines analyzed. The dosage of IL8 *in vitro* presents low level of the chemokine in cells expressing p.E383K NOD2. The *ex vivo* results of supernatants at basal level reflect the serum results for the same cytokines analyzed. Furthermore, IL17, IL22 and IL23 release is higher in patients than in controls, while IL12 is reduced in patients. The presence of the stimulus (LPS, MDP or both) does not lead to significant increases in the levels of IL1β, IL6, IL8, TNFα and IFNγ in patients compared to controls.
The search for biomarkers in autoinflammatory diseases patients shows an up-regulation of several pro-inflammatory components of the studied pathway, when compared to healthy subjects. In Behçet disease, NF-κB, PI3K/Akt, Erk1/2 MAPK pathways are statistically up-regulated, whereas in patients with Adult Onset Still's disease all analyzed pathways appear to be activated more than in controls. In TRAPS patients, many of the components of NF-κB, PI3K/Akt, Erk1/2 and SAPK MAPK, and JAK/STAT pathways are up-regulated, while for FMF patients inflammasome and Erk1/2 and SAPK MAPK pathways result activated. In Blau syndrome components of the NF-κB, p38 MAPK and PI3K/Akt pathways are up-regulated. Cytokine analysis in different autoinflammatory diseases suggests a higher release of IL8, IL22, IL23 and IL17 in all the analyzed diseases. Furthermore, all other cytokines significantly raised in patients affected by Adult Onset Still's disease. The release of TNFα and IFNγ in TRAPS patients

is increased. In FMF patients, IL1β, IL6, IL8 and IFNγ levels result higher than in controls.

CONCLUSION

This is the first time that the mutation p.E383K in *CARD15/NOD2* associated with Blau syndrome has been functionally evaluated. The contrasting results presented by our *in vitro* and *ex vivo* data on NF-κB pathway may indicate that the activation do not depend only on NOD2 in carriers of p.E383K mutation. Further experiments need to be done to clarify these results, using also different *in vitro* models. Regarding the *ex vivo* and *in vitro* studies on cytokines levels, it seems that there is not a primary mediation of IL1β and other pro-inflammatory cytokines in Blau syndrome patients carrying p.E383K, with the exception of IL17/22/23 whose role has to be deeply investigated.

This work therefore offers an insight into the molecular pathophysiology of these autoinflammatory disease. Analyses with statistical models of the signalling molecules presenting significant raised levels will be required to generate specific diagnostic algorithms and to identify valuable biomarkers to be used as targets for specific therapeutic intervention. The cytokine profiles observed may help to distinguish different autoinflammatory diseases in terms of numbers of cytokines raised. Moreover, Th17-related cytokines (IL17/22/23), significantly raised across all diseases, suggest an important role for Th17 cells in autoinflammatory diseases. Targeting Th17 cells and their related cytokines may be an effective therapeutic approach for autoinflammatory diseases in future

SOMMARIO

SCOPO DEL LAVORO

Le malattie autoinfiammatorie sono patologie caratterizzate da episodi infiammatori recidivanti a carico di differenti organi. In esse non vi è apparente coinvolgimento di autoanticorpi nè di linfociti T antigene specifici, ma disregolazione della risposta immunitaria innata.

La Sindrome di Blau (BS) è una rara malattia autoinfiammatoria autosomica dominante caratterizzata dal punto di vista clinico da artrite simmetrica, dermatite granulomatosa e uveite ricorrente. La malattia è causata da singole mutazioni nel gene *CARD15/NOD2*, codificante la proteina NOD2 che è nota regolare il pathway di difesa da patogeni attivando la via del segnale NF-κB.

In questo studio è stata effettuata dapprima una serie di analisi funzionali volte a caratterizzare la mutazione p.E383K ritrovata in un'unica famiglia italiana affetta da sindrome di Blau. Dopo lo studio *in vitro* della proteina NOD2 mutata, si è voluta approfondire l'attività di NF-κB e la secrezione di citochine pro-infiammatorie sia *ex vivo* che *in vitro*.

Nella seconda parte della tesi, si sono volute studiare nel dettaglio sia le vie del segnale note essere implicate nell'infiammazione, sia un ampio range di mediatori dell'infiammazione (citochine pro-infiammatorie e chemochine) in una coorte di soggetti affetti da differenti malattie autoinfiammatorie sia ereditarie quali FMF e TRAPS, sia complesse quali Behçet e morbo di Still nell'adulto. Lo scopo era approfondire le conoscenze molecolari di tali patologie per identificare possibili biomarcatori predittivi o diagnostici per ciascuna malattia e sviluppare in futuro strategie terapeutiche mirate e preventive.

MATERIALI E METODI

Per l'analisi funzionale *in vitro* sulla sindrome di Blau, punto di partenza è stato la produzione di 3 costrutti contenenti cDNA umano di NOD2 *wild-type* e mutato p.E383K e p.R334W. Questi costrutti sono stati trasfettati stabilmente in cellule HEK293, poste poi in coltura per 7 e 24 ore in presenza o assenza di muramildipeptide MDP [10μg/ml]. L'attività di NF-κB è stata determinata dapprima indirettamente valutando l'espressione di IKBα e forma fosforilata nelle cellule mutagenizzate p.E383K mediante Western blotting dei lisati cellulari. In seguito mediante Reverse Phase Protein Array (RPPA) è stata valutata l'espressione dei componenti del pathway rispetto a NOD2 *wild-type*. É stata inoltre dosata l'IL8 nei surnatanti delle colture mediante tecnica antibody microarray.

Per l'analisi funzionale *ex vivo* relativa alla sindrome di Blau, sono stati analizzati 2 soggetti italiani affetti e portatori della mutazione p.E383K, da cui sono state estratte le cellule monocitarie del sangue periferico (PBMC). Per la valutazione dell'attività NF-κB, i PBMC sono stati lisati e analizzati mediante RPPA. Per la

produzione di citochine (IL1β, IL6, IL8, TNFα, IFNγ, IL12, IL17, IL22, IL23) i PBMC sono stati invece posti in coltura per 7 ore in presenza o assenza di agenti di stimolo quali lipopolisaccaride LPS [100ng/ml] e di muramildipeptide MDP [10μg/ml] e le citochine sono state dosate mediante tecnica ELISA e antibody microarray.
Nella seconda parte dello studio, sono stati presi in esame 40 pazienti affetti da malattie autoinfiammatorie (Behçet, Still nell'adulto, FMF, TRAPS e Blau in particolare) e 27 controlli sani, dai quali sono stati raccolti siero e PBMC. Quest'ultimi sono stati lisati e preparati per l'analisi RPPA dei maggiori componenti delle vie del segnale NF-κB, PI3K/Akt, MAPK, JAK/STAT/c-Src e inflammasoma (NALP1). Dal siero sono state dosate IL1β, IL6, IL8, TNFα, IFNγ, IL12, IL17, IL22, IL23 mediante antibody microarray.

RISULTATI
La microscopia confocale ha evidenziato una pronunciata presenza di p.E383K NOD2 nel citoplasma delle cellule HEK293 trasfettate, così come per NOD2 *wild-type* e mutato p.R334W. L'espressione di p.E383K NOD2, valutata mediante Western blotting, non presenta variazioni in seguito a stimolazione con MDP per 7 o 24 ore. Per quanto concerne l'analisi del pathway NF-κB, lo studio *in vitro* presenta una mancata attivazione nelle cellule HEK293 presentanti p.E383K NOD2, mentre il pathway risulta upregolato *ex vivo* rispetto ai controlli sani. *In vitro* infatti si nota un'inferiore espressione di IKBa fosforilato rispetto a IKBa in presenza o assenza di stimolazione MDP, sia nell'analisi indiretta con Western blotting sia mediante RPPA dei lisati totali delle HEK293 trasfettate con p.E383K NOD2. Non è stato rilevato poi alcun incremento di espressione per altri componenti del pathway, quali la forma fosforilata di NF-κB, IKKα/β, anche nell'estratto nucleare. Sia lo studio *in vitro* che quello *ex vivo* non hanno identificato un incremento significativo, sia a livello basale che in seguito a stimolazione, di secrezione della maggior parte delle citochine pro-infiammatorie analizzate. Il dosaggio di IL8 *in vitro* presenta bassi livelli citochinici nelle cellule esprimenti p.E383K NOD2. I risultati basali *ex vivo* da surnatante rispecchiano quanto osservato da siero per le medesime citochine analizzate. Inoltre si è notato un rilascio di IL17, IL22 e IL23 maggiore nei pazienti, unitamente ad un minor rilascio di IL12, rispetto ai controlli. La presenza di stimolo (LPS; MDP o entrambi) non ha portato a incrementi significativi dei livelli di IL1β, IL6, IL8, TNFα e IFNγ nei pazienti rispetto ai controlli.
La ricerca di biomarcatori nei pazienti affetti da malattie autoinfiammatorie ha evidenziato una up-regolazione di molti componenti pro-infiammatori dei pathwy studiati, quando comparati a soggetti sani. Nella malattia di Behçet, le vie metaboliche NF-κB, PI3K/Akt, Erk1/2 MAPK sono risultate statisticamente up-regolate, mentre nei pazienti affetti da Still dell'adulto tutti i pathway analizzati risultano essere maggiormente attivati rispetto ai controlli. Nei pazienti TRAPS, presentano livelli aumentati molti dei componenti dei pathway NF-κB, PI3K/Akt,

Erk1/2 e SAPK MAPK, e JAK/STAT, mentre per i pazienti FMF si nota l'attivazione del pathway legato all'inflammasoma e di quello Erk1/2 e SAPK MAPK. Nella sindome di Blau sono invece risultati upregolati componenti dei pathway NF-κB, PI3K/Akt e p38 MAPK.
Dall'analisi citochinica nelle differenti malattie autoinfiammatorie si evince un maggior rilascio di IL18, IL22, IL23 e IL17 in tutte le patologie analizzate. Inoltre si è notato un incremento significativo dei livelli di TNFα e IFNγ nei pazienti TRAPS, di IL1β, IL6 e IFNγ nei pazienti FMF e di tutte le citochine in analisi nei pazienti affetti da Still dell'adulto.

DISCUSSIONE

Questa è la prima volta che viene studiata a livello funzionale la mutazione p.E383K nel gene *CARD15/NOD2* associato alla sindrome di Blau. Per quanto riguarda il pathway NF-κB, i risultati contrastanti ottenuti *in vitro* ed *ex vivo*, possono indicare come l'attivazione non sia regolata solamente da NOD2 per i portatori della mutazione p.E383K. Ulteriori esperimenti saranno necessari per chiarire questi risultati, utilizzando anche differenti modelli cellulari *in vitro*. I risultati *ex vivo* e *in vitro* relativi al dosaggio citochinico non sembrano evidenziare un coinvolgimento primario di IL1β e delle altre citochine proinfiammatorie nella patogenesi della sindrome di Blau,ad eccezione di IL17/22/23, il cui ruolo dovrà essere studiato più approfonditamente.
Questo lavoro di tesi offre inoltre una panoramica della patofisiologia molecolare di differenti malattie autoinfiammatorie. Un'analisi approfondita dei componenti dei pathway individuati con elevati livelli di espressione rispetto ai controlli sani sarà necessaria per identificare validi biomarcatori da poter utilizzare come target terapeutici. I profili citochinici ottenuti possono essere d'aiuto nel discriminare le differenti patologie sulla base di quali citochine presentino dosaggi maggiori rispetto ai controlli. Inoltre, IL17 IL22 e IL23 presentano livelli significativamente più elevati in tutte le malattie analizzate, suggerendo un importante ruolo nelle autoinfiammatorie per le cellule Th17 secernenti tali citochine. Utilizzare le cellule Th17 e le citochine ad esse associate come target terapeutico potrebbe essere in futuro un nuovo approccio nella cura delle malattie autoinfiammatorie.

GENERAL INTRODUCTION

1 General overview on autoinflammatory diseases

The autoinflammatory diseases are characterized by seemingly unprovoked, recurrent episodes of fever, serositis, arthritis, and cutaneous inflammation, but the usual hallmarks of autoimmunity, namely high-titer autoantibodies and antigen-specific T cells, are usually absent [1]. The term autoinflammatory was coined to draw the distinction between this category of illnesses and the more classically recognized autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, in which the hallmarks of adaptive immunity are more evident [2]. The formal recognition and genetic understanding of autoinflammatory diseases have defined some mechanisms of self-direct inflammation independent to the adaptive immunity. Such a definition would encompass autoinflammatory mechanisms across a continuum model of immunology (a spectrum from autoimmune to autoinflammation) [3].
Although this concept originally applied to monogenic hereditary recurrent fevers, it has expanded over time to include polygenic (complex) autoinflammatory diseases. Dinarello proposed the division in classic diseases, probable and municipalities on the basis of sensitivity to inhibition of IL1 [4]. With the recognition that these inflammatory diseases without hallmarks of adaptive immunity were in fact disorders of the innate immune system, Masters and colleagues proposed a new schema based upon underlying molecular mechanisms [1]. Since the association between the autoinflammatory diseases and the innate immune system has strengthened, the "autoinflammatory disease" definition should be reassessed to include all clinical disorders marked by abnormally increased inflammation, mediated predominantly by the innate immune system, with a significant host predisposition [5]. Although these mechanistic classifications present some limits, such as the absence of naturalness and both clinic and inflammatory relevance, they are the most commonly used to discriminate the different forms of autoinflammatory diseases. Very recently, Grateau and colleagues proposed another, more clinically-oriented, definition of autoinflammatory diseases as diseases with clinical signs of inflammation, associated with elevated levels of acute-phase reactants, which are attributable to dysfunction of the innate immune system, genetically-determined or triggered by an endogenous factor. From this foundation, a clinically-based multidimensional classification of autoinflammatory diseases was described [6]
A few clinical common features have been identified between all the inherited autoinflammatory diseases, such as the recurrent nature of inflammatory episodes, the presence of fever, and the frequent involvement of skin, serous membranes,

eyes, joints, lymph nodes, gastrointestinal tract, and nervous system. Each of these syndromes may have a more or less severe inflammatory manifestations [7]. Understanding of the pathogenesis of autoinflammatory diseases has grown rapidly in the past decade owing to advances in genome research and technology. An updated classification scheme is based on the garnered molecular insights, supplanting the uncompleted clinical classification: IL1β activation disorders, NF-κB activation syndromes, protein misfolding disorders, complement regulatory diseases, disturbance in cytokine signalling, and macrophage activation syndromes [1].

Disease	Gene (Protein)	Inheritance	Clinical features	Ref
Familial autoinflammatory disorders				
Familial Mediterranean Fever (FMF)	*MEFV* (pyrin)	Autosomal recessive	Periodic fevers (lasting 3–7 days), serositis, arthritis	8,9
Tumor Necrosis Factor-associated periodic syndrome (TRAPS)	*TNFRSF1A* (TNF receptor 1)	Autosomal dominant	Periodic fevers(lasting 1-6 weeks), serositis, rash, episcleritis	2
Mevalonate kinase deficiency (MKD)	*MVK* (mevalonate kinase)	Autosomal recessive	Periodic fevers (lasting 3-7 days), non-destructive arthritis, lymphoadenopathy, vasculitic skin lesion	10,11
Cryopyrin-associated periodic syndromes (CAPS): FCAS, MWS and NOMID	*NLRP3* (NLRP3)	Autosomal dominant	Cold-induced inflammation, fevers, sterile meningitis, bone lesions	12,13
NALP12 associated Periodic syndrome	*NLRP12* (NLRP12)	Autosomal dominant	similar to CAPS	14
Blau syndrome (BS)	*CARD15/NOD2* (NOD2)	Autosomal dominant	Granulomatous dermatitis, uveitis, arthritis	15, 16
Complex disorders				
Schnitzler syndro0me	*Sporadic*	-	urticaria, intermittent fever,arthritis	17
Still disease	*Complex*	-	fever with unknown etiology, rash, arthritis, and involvement of several organ systems	18
Behçet disease	*Complex*	-	oral and genital ulcerations, folliculitis, erythema nodosum and uveitis.	19

Tab. 1: Molecular and clinical features of autoinflammatory disorders presented in this thesis

1.1 Hereditary recurrent fevers

In the last decade, the genetic cause of several familial autoinflammatory syndromes have been identified, as presented in Tab. 1. The genes that are responsible for these syndromes have been found to encode key sensors and transducers of inflammatory signal transduction pathways [3]. Some of the genes involved, such as *TNFRSF1A* were previously well known, whereas the discovery of mutations in *NLRP3, NLRP12, CARD15/NOD2* highlighted their importance in inflammatory signalling pathways.

To date, twelve are the better known hereditary monogenic disorders: familial Mediterranean fever (FMF); tumor necrosis factor receptor-associated periodic syndrome (TRAPS); mevalonate kinase deficiency (MKD); cryopyrin-associated periodic syndrome (CAPS), a group which includes familial cold urticaria syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurological cutaneous articular (CINCA); Guadeloupe Variant Periodic Fever syndrome (FCAS2); granulomatous diseses which include Blau syndrome (BS) and early-onset sarcoidosis (EOS); and, finally, the hereditary pyogenic disorders including PAPA (pyogenic arthritis, pyoderma gangrenosum, and acne) syndrome, Majeed syndrome (MS), and deficiency of the IL1 receptor antagonist (DIRA). These diseases are generally characterized by early onset but adult onset has also been described, in particular for FMF and TRAPS [20]. Type AA amyloidosis is the most serious complication of most diseases, due to excessive production of serum amyloid-A (SAA), synthesized in the liver following stimulation by certain proinflammatory cytokines, such as IL1β, and also IL6 and tumor necrosis factor-α (TNF-α).

1.1.1 Familial Mediterranean Fever - FMF

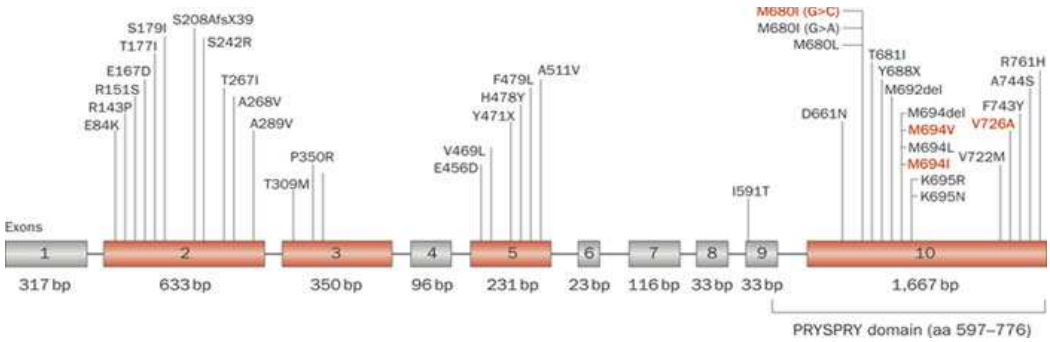


Fig. 1: Schematic representation of recessively inherited FMF-associated mutations in the *MEFV* gene [21].

FMF is inherited as a recessive disease and is characterized by short episodes of fever and inflammation involving the joints and serosal surfaces. Mutations in the *MEFV* gene, which encodes the protein pyrin, were found in 1997 to underlie most cases of this disease [8]. The mutations are present at very high frequency in several populations, in particular in the Mediterranean basin and Middle East [22].

As numerous patients with FMF clinical criteria but without genetic mutatios were reported, genetic heterogeneity was proposed [23]. Gershoni and colleagues suggested also that complex alleles can lead to a more severe form of the disease [24].

Mutations are found throughout the *MEFV* gene (http://fmf.igh.cnrs.fr/infevers), but the most severe are clustered in exon 10 (Fig. 1). Pyrin is an adaptor protein expressed in neutrophils and monocytes that contains an N-terminal eponymous pyrin domain and a domain at the C-terminal end termed the B30.2 or PRY–SPRY domain. Pyrin can assemble with ASC and pro-caspase 1 in human cells to facilitate the cleavage of pro-caspase 1. This mutation affects several cell signalling pathways affecting upregulation of caspase-1 and IL1β and inhibition or down regulation of apoptosis and NF-κB [25]. FMF is usually responsive to colchicine that is effective in preventing the attacks and the development of AA amyloidosis. However, glucocorticoid may decrease the attacks of FMF when it is used early and in high doses [26].

1.1.2 Mevalonate Kinase Deficiency - MKD

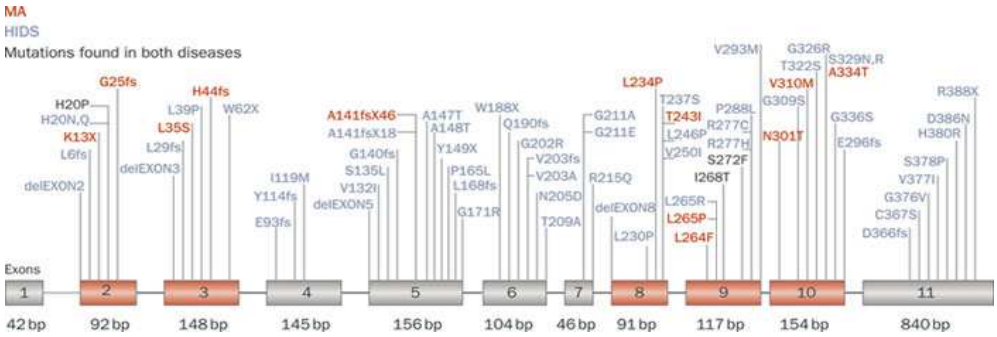


Fig. 2: Schematic representation of mutations in *MVK* that have been identified in patients presenting with MKD, in blue [21].

Mevalonate Kinase Deficiency is a recessive inherited disease, presenting an increased IgD titres, recurrent fever, macula-papular rashes and athralgia lasting for 3-7 day. This condition is caused by mutations in the mevalonate kinase (*MVK*) gene, responsible for severe mevalonic aciduria [11]. Although various ethnic and racial backgrounds can be affected by MKD, most patients have a European descent. MKD-associated mutations are broadly distributed throughout the protein sequence (Fig. 2). Mevalonate kinase is an enzyme important in cholesterol and non-sterol isoprene biosynthesis as it converts mevalonic acid to 5-phosphomevalonic acids. Recent investigation into the signalling pathways that may be affected by alteration in the MK pathway has implicated the small GTPase Rac1, PI3K and protein kinase B as key molecules that could give rise to activated caspase-1 (and IL1 and inflammation) due to isoprenoid deficiency [27].

Treatments of this disease can include anakinra (IL1 receptor antagonist), colchicine, non-steroidal anti-inflammatory drugs, and glucocorticoids. Incomplete response to etanercept has been reported [26].

1.1.3 TNF receptor-associated Periodic Syndrome (TRAPS)

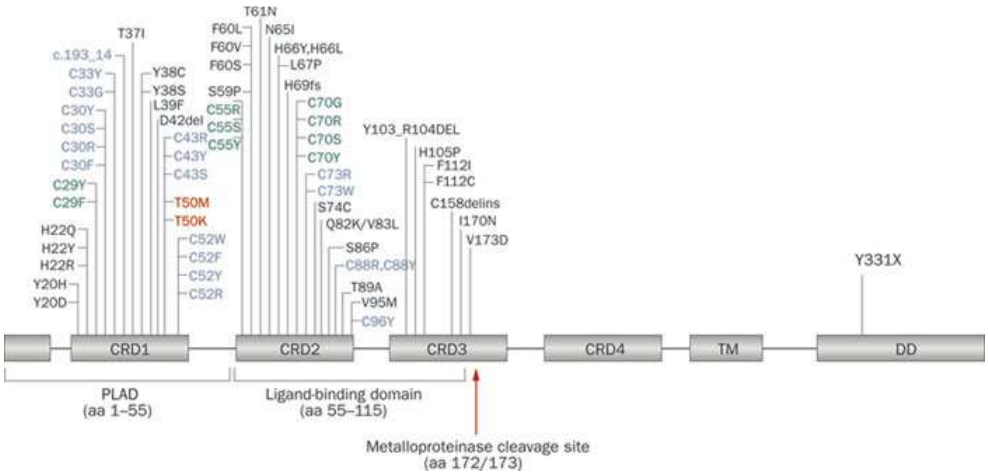


Fig. 3: Schematic representation of mutations in the TNFR1 protein that are associated with TRAPS [21].

TNF receptor-associated periodic syndrome (TRAPS) is an autosomal dominant autoinflammatory disease associated with mutations in *TNFRSF1A* gene encoding TNFR1 protein [2].

Patients experience recurrent fevers, abdominal pain, rash, and internal inflammatory manifestations such as serositis, fasciitis and episcleritis, but only 10% develop amyloidosis [28].

The majority of identified mutations in TNFR1 associated with TRAPS are missense mutations affecting the extracellular region of the receptor (http://fmf.igh.cnrs.fr/infevers/) (Fig. 3).

Mutations affecting cysteine residues that partecipate in disulphide bonds have been associated with the most severe and penetrant disease phenotype with an increased risk of amyloidosis [29].

It is still unclear the presence of hyper-inflammatory state in TRAPS patients, but there are some hypothesis. The earliest proposes a defective shedding of the mutated receptor, raised from the observation of lower serum levels of soluble TNFR1 in TRAPS patients that only slightly increased during the febrile attacks. This TNFR1 shedding defect may lead to insufficient soluble TNFR1 to neutralize TNFα, thus resulting in the continuous inflammatory stimulus that is notable in TRAPS [30]. Since this defect is not present in all patients, this hypothesis alone is not sufficient. A second hypothesis is related to protein misfolding. Intracellular aggregation of TNFR1 as misfolded protein might constitutively induce ligand independent signalling and/or induction of ER (Endoplasmic Reticulum) stress responses that may play an important role in TRAPS. Ligand independent

signalling might lead to an imbalance between cell activation and apoptosis, leading to reduced NF-κB signalling. Todd and other investigators demonstrated that TRAPS-associated mutations in the ectodomains result in the receptor displaying defective behavior, but the signalling properties of the cytoplasmic DD domain are not defective in the mutants [31]. The last hypothesis concern the rule of the NF-κB signalling. Several functional studies have noted the role of NF-κB in the transmission of the signal induced by the binding TNF-receptor. Looking at both high and low penetrance mutations associated with TRAPS, it was observed an increase in the activity of the various subunits of NF-κB and of the proinflammatory response [32].
Treatments of the inflammatory episodes of TRAPS should be glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs), in addition to TNFRSF1B: Fc fusion protein (etanercept). Other drugs such as IL1 blocking agent (anakinra) have been successfully used [26].

1.1.4 Cryopyrin-associated periodic syndrome (CAPS)

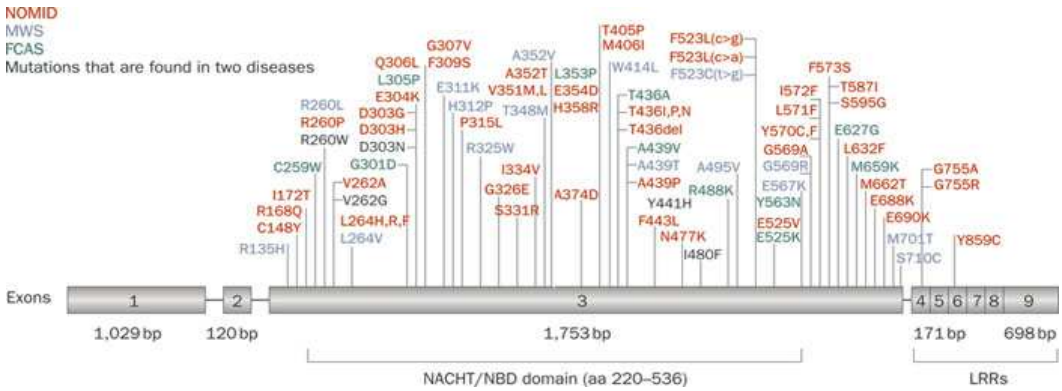


Fig. 4: Schematic representation of dominantly inherited NLRP3 mutations in patients with CAPS [21].

Cryopyrin-associated periodic syndrome (CAPS) are a group of autosomal dominant autoinflammatory diseases caused by autosomal dominant or *de novo* mutations of *NLRP3* gene that encodes cryopyrin [12]. NLRP3 belongs to the family of NLR (nucleotide binding domain and leucine-rich repeat containing proteins) that is implicated in inflammation and apoptosis [33]. C-terminal leucine-rich repeats (LRR) had been implicated in sensing bacterial components, suggesting a role in the innate immune response. The identification of *NLRP3* mutations in CAPS coincided with the realization that the NALP3 protein is a key component of the inflammasome, a multiprotein complex that can activate caspase-1 [34]. Hyper-activation of the inflammasome results in increased caspase-1 generation leading to hyper-secretion of IL1. Another proinflammatory event, activation of IL6, is mediated via NF-κB.
The clinical picture of CAPS includes:
a) Familial cold autoinflammatory syndrome (FCAS),

b) Muckle-Wells syndrome (MWS),
c) Neonatal-onset multisystem inflammatory disease (NOMID) or chronic infantile neurologic cutaneous articular (CINCA) syndrome.
These conditions are no longer considered as three sub-phenotypes with increasing severity of the heterogeneous presentation of this monogenic disease. Instead, they seem to display a continuum of symptoms [13].
Anti IL1 therapy should be the eligible one, preferring rilonacept and canakinumab to anakinra [26].

1.1.5 NALP12 associated periodic syndrome

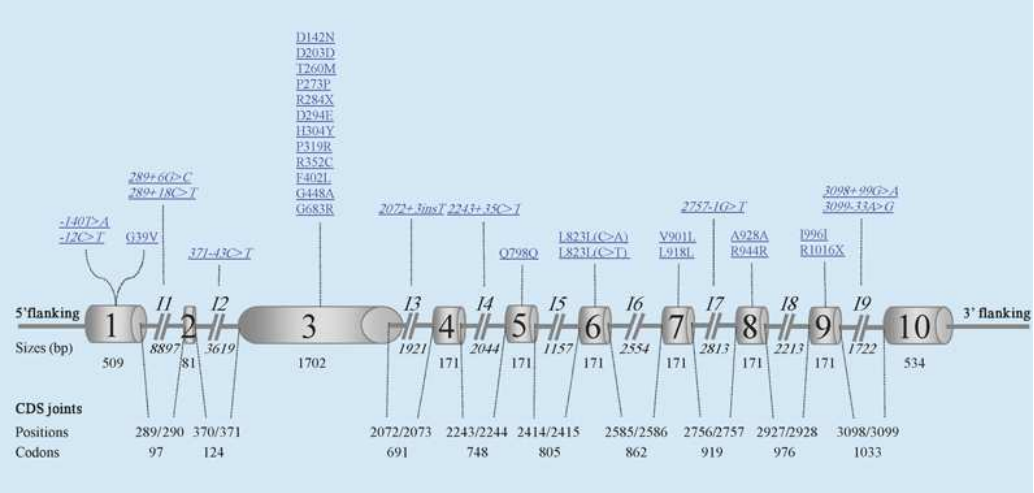


Fig. 5: Schematic representation of *NLRP12* mutations in patients with NALP12 associated periodic syndrome [http://fmf.igh.cnrs.fr/ISSAID/infevers/].

NALP12 associated periodic syndrome is an autosomal dominant disease caused by mutations in the *NLRP12* gene encoding for the protein NLRP12, which plays a crucial role in immune system mechanisms against pathogenic agents. As in the case of CAPS, the disease can be induced by generalized exposure to cold and is characterized by recurrent fever episodes lasting for 5–10 days accompanied by skin rash, headache, lymphadenopathy, mouth ulcers, and abdominal pain [14]. Treatment choice is based on the use of antihistamines, NSAIDs, and corticosteroids in less serious cases or the administration of anakinra in more serious ones [26].

1.2 Complex autoinflammatory syndromes

As prevously described, some complex diseases have been classified as autoinflammatory disorders, including vasculitic syndromes such as Behçet disease, idiopathic febrile syndrome (Still disease) and Schnitzler disease.

1.2.1 Schnitzler disease

This disease is characteristically associated with monoclonal IgM gammopathy, which may involve B-cell lineage [14]. The clinical features (urticaria, intermittent fever, arthritis and elevated acute-phase reactants) remind to the hereditary fever syndromes, in particular MWS. The biochemical data suggest an influence of proinflammatory cytokines and IL1β inhibitor is very beneficial in the treatment [35].

1.2.2 Adult Onset Still's disease (AOSD)

Adult Onset Still Disease affects people older than 16, either *de novo* or those with a history of systemic JIA (juvenil idiopatic arthritis). Disease severity varies significantly among affected subjects and, even, within the same individual. To date, the mechanisms underlying AOSD are not completely understood. High levels of interleukin IL1β, IL6, IL17, IL8, IL18, and TNF-a have been reported [36]. These findings seem highly relevant, as elevated serum IL6 and/or IL18 levels have been associated with systemic symptoms such as fever, skin rash and hepatic dysfunction, and correlated with raised serum C-reactive protein (CRP) levels. The similarities between autoinflammatory disorders and AOSD might suggest common pathogenic pathways and place AOSD as a non-familial, sporadic form. Related to the cytokines levels, IL1 inhibition is currently considered the mainstay of treatment for AOSD [37].

1.2.3 Behçet's disease (BD)

Behçet's disease is a complex chronic and relapsing inflammatory disorder that has been extensively described in young adults from Eastern Asia and Mediterranean countries. BD is considered a systemic inflammatory disease, most likely involving adaptive immunity and particularly T lymphocytes skewed towards the T helper 1 phenotype and producing the pro-inflammatory mediators IL2, IL6, IL8, IL12, IL18, TNFα and IFNγ [19]. Its essential manifestations are oral and genital ulcerations, folliculitis, erythema nodosum and uveitis. The presence of vasculitis worsens the prognosis, introducing the potential for life-threatening complications such as thrombophlebitis, arterial aneurysms and occlusion. The aetiology of BD is unknown. BD shares clinical similarities with certain well-recognised autoinflammatory disorders. Different works have demonstrated a higher frequency of MEFV mutations in patients with BD, with respect to their ethnicity [38,39]. Recently, the R92Q mutation in the TRAPS gene, which is responsible for another defect of innate immunity, was reported to have increased in patients with BD [40].

2 Blau syndrome

Blau syndrome (BS, MIM #186580) is a rare autoinflammatory granulomatous disorder described for the first time by Edward Blau in 1985 as a dominantly inherited, chronic inflammatory syndrome characterized by the clinical triad of granulomatous dermatitis, symmetric arthritis and recurrent uveitis [41]. In 1996, the BS locus was mapped in the chromosomal region 16q12.1–13, which contain one of the susceptibility genes for Crohn's disease [42]. In 2001, Miceli-Richard *et al*. identified the gene that confers susceptibility for BS, discovering three missense mutations (p.R334Q, p.R334W and p.L469F) in the region encoding the nucleotide-binding domain (NBD) of the caspase recruitment domain gene (*CARD15/NOD2*) in four French and German BS affected families [15]. The familial form of BS can be differentiated from early onset sarcoidosis (EOS), a multiorgan sporadic disease characterized by onset in the first 4 years of life, joint, skin, eye, lymph node involvement, and recurrent fevers, with possible abdominal or central nervous system involvement. In spite of the notable clinical similarities, originally BS was considered a distinct entity from EOS. Later, genetic analyses demonstrated that many patients with EOS also presented mutations in the *NOD2/CARD15* gene. For this reason, some authors have proposed that BS and EOS are, respectively, familial and sporadic forms of the same disease [43,44]. Milman *et al*. proposed to classify patients with EOS as patients with "sporadic BS" due to de novo mutations, restricting the term EOS to patients with features of sarcoidosis and without mutations in *CARD15/NOD2* [45].

2.1 Clinical aspects

The occurrence of BS is reported primarily among Caucasians although it has been also reported in Asians as well as Afro-Americans [43]. For most patients, the disease is characterized by early onset, typically at ages before 3–4 years; however, symptoms could appear after 10 years of age [45]. The most common manifestation of the disease is arthritis, often mistaken for juvenile rheumatoid arthritis (JRA) [46]. Joint manifestations usually appear such as symmetric polyarthritis, involving wrists, metacarpophalangeal (MCP), 1st metatarsophalangeal (MTP) and proximal interphalangeal (PIP) joints of hands and feet, ankles and occasionally elbows (Fig. 6a). Progression to deforming arthritis may occur, leading to severe handicap caused by flexion contractures of fingers and toes (camptodactyly) and decreased motion of large joints.
BS has been described with various skin manifestations, such as papulonodular tender brownish rash and multiple, firm, subcutaneous nodules (Fig. 6b). The appearance is variable, often symmetric, located on the trunk and/or extremities [47]. The lesions' histology consistently demonstrates non-caseating granulomas

with multinucleated giant cells [48] (Fig. 6c). ‘Comma-shaped bodies’ in epithelioid cells may be revealed by electron microscopy ,which seem to be a marker for BS [49].

The most relevant morbidity of BS is eye involvement [50]. The most common manifestations are recurrent anterior uveitis or panuveitis together with eye pain, photophobia and blurred vision. Granulomatous uveitis, often bilateral, can evolve into a cataract and band keratopathy, often requiring surgery (Fig. 6d).

Atypical cases of BS have been reported which involve organs other than the skin, joints or eyes, such as liver and kidney [51, 52].

No studies on the optimal treatment for patients with BS have been made yet, due to its rarity and the variations in the severity and evolution of its expressions. Low-dose glucocorticoids are generally satisfactory at the quiescent stage [26]. Biologic anti-cytokine agents (infliximab and anakinra) may be a promising therapeutic approach in refractory cases [53].

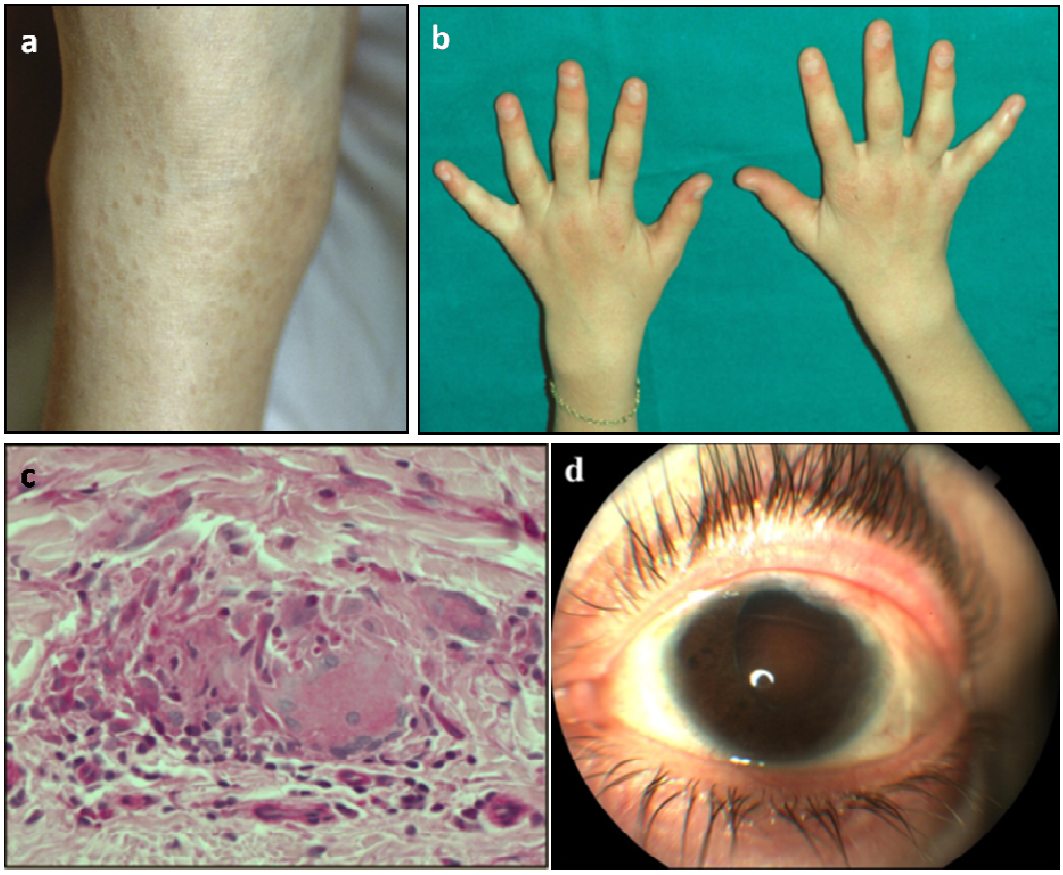


Fig. 6: Clinical and histological aspects of Blau syndrome.
a) Asymptomatic diffuse brownish papulae of some millimeter in diameter, firm subcutaneous nodules, varying in size from 5 to 30 mm in diameter, on the extensor surfaces of the legs. b) Enlargement of interphalangeal joints. c) Skin biopsy from the right forearm. Non-caseating granulomas, containing several histiocytes and multinucleated giant cells with strong PAS positivity and rare lymphocytes and eosinophils. d) Granulomatous uveitis, evolved into cataract and band keratopathy.

2.2 Genetic and functional aspects

The identification of *CARD15/NOD2* as the gene responsible for BS marked the major turning-point for BS studies [15]. Since 2001, several cohorts have been genotyped and different CARD15/NOD2 mutations have been identified in BS patients from distinct ethnic origins (Fig. 7b). The most commonly observed mutations are missense substitutions affecting the highly evolutionary conserved arginine residue at position 334 (R334W or R334Q) [15, 54-56]. These mutated alleles make codon 334 a genetic hot spot for mutations. Most of the other mutations are found only once or few times. A multiple sequence alignment of NOD2 with homologous proteins have shown that the position of BS mutations correspond to the position of pathogenic mutations in NLRP3 protein linked to CAPS [57]. This suggests a close connection between the two diseases.

CARD15/NOD2 encodes a multi-domain protein of 1040 amino acids, namely nucleotide oligomerization domain 2 (NOD2). NOD2 is a member of the NOD-like receptor family (NLR) that play an important role in innate immune defense. NOD2 expression has been mainly occurred in the cytosol of myelomonocytic, dendritic, and Paneth cells [58,59], which are major component of non-caseating granulomas. More recently NOD2 has shown to be present in the plasma membrane of intestinal epithelial cells [60]. The baseline expression of NOD2 is low, but it is known that the protein can upregulate itself, when activated by its ligands [61].

As shown in Fig. 7a, NOD2 has a typical three-domain structure composed of two N-terminal effector region consisting of caspase recruitment domain (CARD), a centrally located NACHT that is critical for activation, and nine C-terminal leucine-rich repeats (LRRs) that sense the pathogen-associated molecular patterns (PAMPs) [62].The N-terminal domain mediated signaling through its homophilic interactions with other CARD containing protein. The centrally located NACHT domain displays ATPase activity and is necessary for activation and oligomerization leading to inflammatory signaling responses [63].

NOD2 is a general detector of intracellular invasive bacterial infections, sensing small peptides such as muramyl dipeptide (MDP), a degradation product from both Gram-positive and Gram-negative bacterial peptidoglycan [64]. A crucial role for NOD2 in bacterial clearance has been demonstrated *in vitro* and *in vivo* for infection with a variety of pathogens [65, 66]. NOD2 was shown to mediate additional cellular responses to the host defense system in response to viral ssRNA [67] and to direct autophagy by recruiting one of the key components for autophagosome formation [68].

Basically, NOD2 activation leads to the activation of NF-κB that is present in the cytoplasm as an inactive form binded with inhibitory proteins known as inhibitor of κBs (IκB) (Fig. 8). NOD2 resides in the cytoplasm of resting cells in an autoinhibited conformation due to the intramolecular interactions of its LRR and CARD domains: the response to bacterial components (MDP) results in an

oligomerization via NACHT domain, thus exposing the CARD-containing effector domain and inducing the recruitment of the serine-threonine kinase RIP2 through a CARD-CARD interaction. Following its activation by NOD2, RIP2 activates the IkB-kinase-γ (IKKγ, also known as NEMO), the regulatory subunit of IKK complex, and promotes a cascade of phosphorylation. Phosphorylated IKKβ (the catalytic subunit of IKK complex) leads to a subsequently phosphorylation of the inhibitor of NF-κB IkB, thus releasing the active NF-κB which translocates to the nucleus where it initiates the transcriptional activation of proinflammatory genes [16].

Since BS is due to an increased activity of NF-κB signaling, it is generally classified as an autoinflammatory disease. However, it remains unknown how the gain-of-function mutations in NOD2 affects the pathogenesis of Blau syndrome and whether a genotype-phenotype correlation exists between the clinical manifestations and NOD2 mutations. Recent studies [69] suggest the possibility that an elevated basal NF-κB activity may affect disease progression rather than disease onset since they found that patients with mutated NOD2 and low NF-κB activity tended to experience complications (i.e. arthritis and uveitis) at a later age.

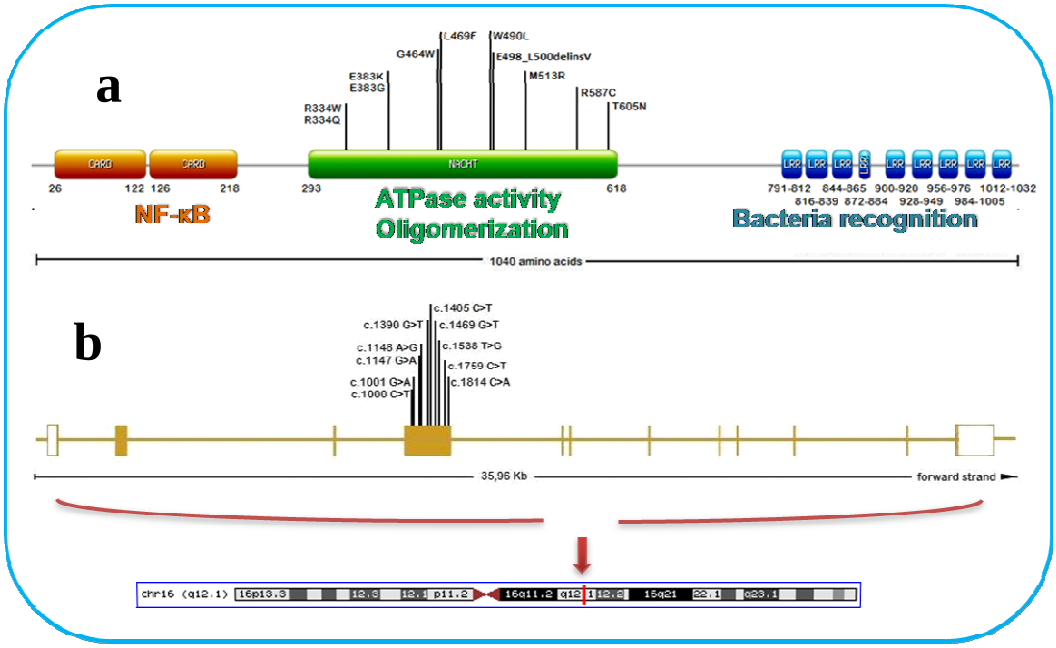


Fig. 7a-b: Genetic aspects of Blau syndrome.
a) Schematic presentation of CARD15/NOD2 gene and the nucleotide positions of BS-associated mutations (NM_022162.1). Exons are represented by boxes whereas horizontal lines stand for introns. b) Detailed representation of NOD2 displaying amino acid positions of BS-associated mutations. The numbers under the diagram are the first and last amino acids of each structural domain (data from http://www.uniprot.org/uniprot/Q9HC29).

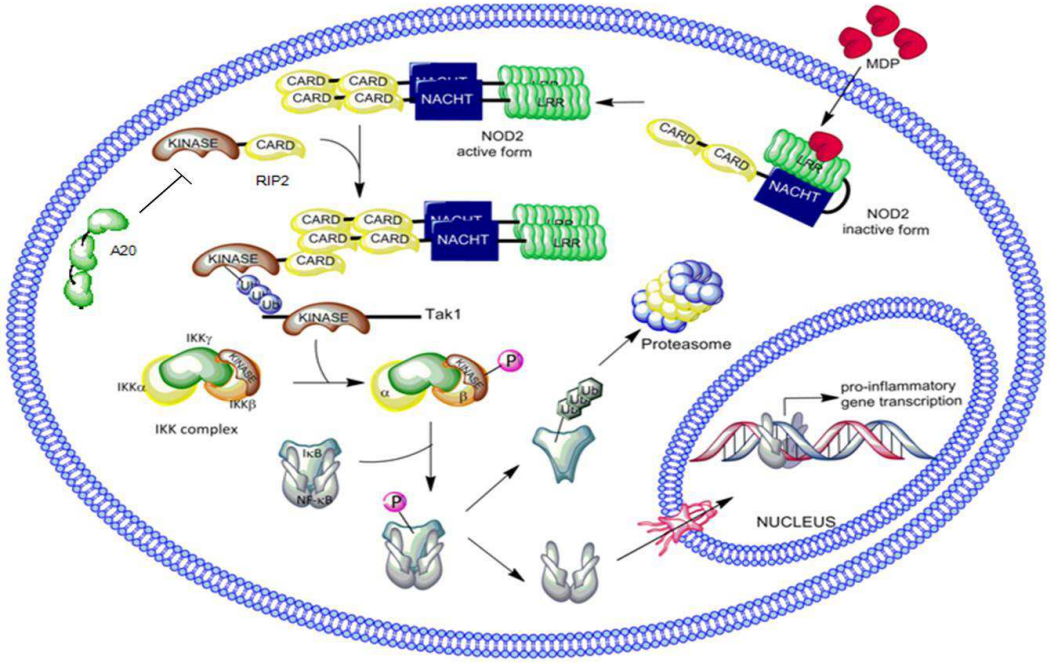


Fig. 8: Biochemical aspects of Blau syndrome.
Schematic representation of the NF-κB signalling pathway NOD2 dependent. MDP activates cytosolic NOD2 through direct interaction with the leucine-rich repeat region. Binding leads to the oligomerization of NOD2 and recruitment of the kinase RIP2 through homotypic CARD–CARD interactions. RIP2 then activates the IKK complex. This results in phosphorylation, ubiquitination and degradation of IKB, and downstream activation of associated NF-kB

3 Microarray techniques

Multiplex analytical technologies are crucial for the complex task of deciphering disease-specific biomarker patterns as they provide the opportunities for an all-inclusive approach, not previously possible using conventional technologies such as the enzyme-linked immunosorbent assay (ELISA). There are currently numerous commercially available multiplex immunoassay technologies that offer the possibility to detect multiple analytes simultaneously [70].

Protein microarray is a high-throughput method used to track the expression of proteins, to determine their function on a large scale. Protein microarray is a rapid, automated and highly sensitive technique, consuming small quantities of samples and reagents. The advantages achieved by high sensitivity, and high throughput makes protein microarrays a powerful technique in the proteomic study for discovery of new drugs, markers and detection of known proteins [71].The method consists to immobilize large numbers of proteins as a spatially organized array of spots on a coated glass slide [72]. The concept of immobilizing antibodies onto functionalized glass or plastic slide surfaces was first developed in the 1980's and realised as a multiplex alternative to the ubiquitous ELISA by the late 1990s [73, 74]. The spots may represent complex mixtures such as cell lysates, antibodies, tissue homogenates, recombinant proteins or peptides, body fluids or drugs [71, 75-78]. Density of protein spots (called features) on a printed array can easily reach 2,000 features per square centimetre. Sample volumes deposited onto the surface for any single feature would be in the 50-500 picolitre range. Both these characteristics depend upon the exact substrate material and system setup used [70].

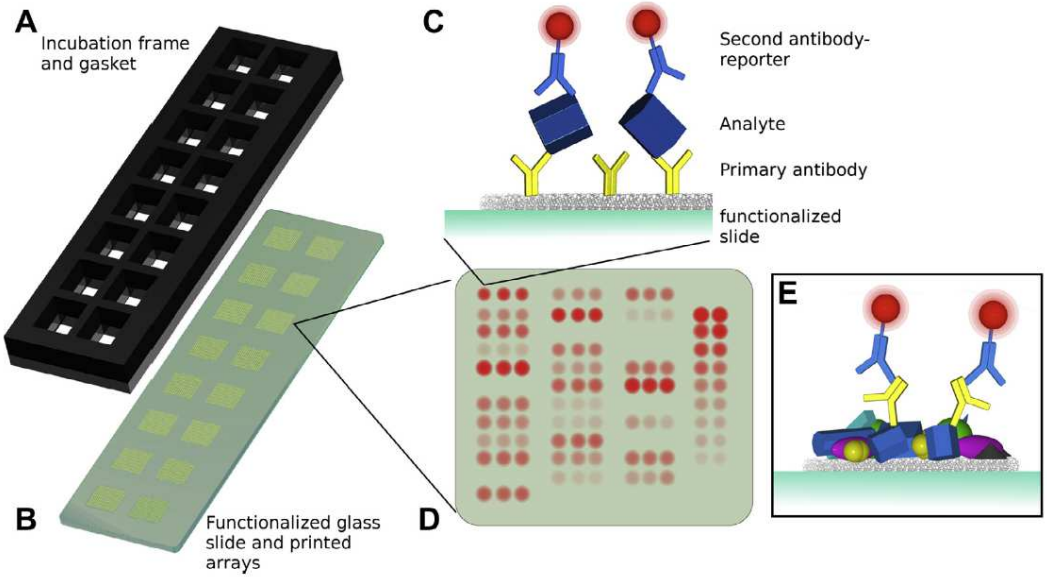


Fig. 9: Slide based multiplex arrays require individual arrays to be separated with a frame and gasket device.
A-D) Antibody microarray technique; E) Detection step for Reverse Phase Protein Array [70]

3.1 Antibody Microarray

An antibody microarray is a specific form of protein microarrays, in which a collection of capture antibodies are spotted and fixed on a solid surface such as glass, plastic or silicon chip, for the purpose of detecting antigens. The concept and methodology was first introduced by Chang in 1983 [79]. His work was focused on the employment of antibody microarrays for the detection and quantification of cells bearing certain surface antigens, particulate antigens, and soluble antigens.

Nowadays, the antibody microarray technique requires robotic printing of specific antibody sets upon an activated slide surface. Once sealed, the arrays was treated as ELISA wells, with appropriate blocking, sample exposure and washing. Detection of each target analyte was then achieved by adding a cocktail of biotinylated detection antibodies to complete the sandwich followed by amplification and streptavidin conjugated Cy5 (Fig. 9). High resolution scanning enabled signal intensity measurements for each feature, representing a separate analyzers detection. Technical replicates and some internal standards were included within the array. Different amplification methods can be used, dependent upon the testing samples. A standard curve is generated much like in a traditional ELISA, creating a dilutional series from recombinant antigen mass standards. The sensitivity of antibody microarrays is in the range of ELISA [80].

There are many advantages in antibody microarray for high throughput analysis, including the fact that it does not require direct label of proteins contained in samples fluids. It is also a high sensitive and specific assay, that allows highly sensitive detection of numerous low abundance biomarkers. Nevertheless, an obvious limitation is the requirement of two non-overlapping accessible epitopes and two validated antibodies for a target molecules [81,82]. The most common application of antibody microarrays is to profile experiments on plasma or serum samples to detect a wide range of diverse molecules, such as cytokines and chemokines [83].

3.2 Reverse Phase Protein Array

This technique was first introduced by Liotta and Petricoin in 2001 [75]. Reverse phase protein arrays (RPPA) was technically similar to the antibody microarray. Unfractionated samples (cell lysates) were printed directly onto coated glass slides and immobilized as the spot feature. The specificity of the analyte-specific antibodies was critical to ensure unbiased measurements and was verify by Western Blot. Those slides were probed with a range of antibodies specific for individual signalling molecules, both phosphorylated and non-phosphorylated. Signal detection for RPPA analysis of intracellular proteins is often performed using either a directly labeled reporter primary antibody, or primary and reporter

conjugated secondary antibody (Fig. 9c). The array is optically scanned at high resolution and the obtained data is quantified, usually as digital data [84].
There are many advantages in RPPA for high throughput analysis, including the small spot size of the individual spots (features) of 150 to 200 μm in diameter allowing the incorporation of thousands of spots per microarray. This facilitates comparisons between samples, since each sample is exposed to the same experimental conditions during analysis. RPPA is also highly sensitive due to its miniature format that provides increases in the signal density and requires extremely small amounts of sample [85]. There are some obstacles in the application of RPPA, related to the requirement of high affinity antibodies and to the state of the proteins to analyse. To overcome these obstacles, all antibodies should be tested for specificity and sensitivity and all the proteins have to be denatured [86,87].
An important application of RPPA is to investigate cellular signalling pathways in normal and diseased conditions. RPPA can be used to visualize the state of signal transduction pathways, providing data on post-translational modifications such as phosphorylation, degradation, protein cleavage and ubiquitination [88, 89]. RPPA data can be useful to identified novel diagnostic, prognostic and predictive biomarkers on a wide range of diseases, in order to clinically stratify different patients groups, so as to develop personalized, preventive or therapeutic strategies.

3.3 Microarray requirements

The optimization of assay conditions is one of the major challenge for microarray technology.

Printing surface
The demand for even higher densities as well as the need for decreased sample consumption and quantification led to the application of glass slides as solid supports with coated surfaces.
The ideal printing surface should provide high sample binding capacity, good spot morphology, minimal autofluorescence or colour background and compatibility with available detection methods. There are a variety of commercially available activated glass or coated slides, such as nitrocellulose, amino-, aldehyde-silane and epoxy-silane glass slides [90]. Microporous surfaces and membranes such as nitrocellulose, nylon and polyvinylidine difluoride (PVDF) have high binding capacity and nitrocellulose coated glass slides are commonly used surfaces for protein arrays [91].
In this study nitrocellulose slides were used for RPPA, whereas poly-l-lysine for antibody microarray. Advantages for using nitrocellulose slides are the high affinity binding property, the commercial availability and the compatibility with

different methods of detection. The auto-fluorescence of nitrocellulose is the main disadvantage. Glass slides with poly-l-lysine are instead easy to prepare, handle and robust. They present very good signal-to-noise ratios and high homogeneity, even though a detection limit at 2000 amol/spot [90].

Printing condition

Prior to printing samples on the slides there are some parameters that should be considered including the type of pin used for depositing the sample onto the surface, the capabilities of the printing robot, sample volume, viscosity and the numbers of arrays and slide surface to be used. Dependent on the arraying robot capabilities and substrate design, a spot array design can be tailored according to the number of samples [92]. Temperature, humidity and dust/debris are critical conditions during printing, since they can easily affect array quality of printing. Increased temperature and decreased humidity may cause evaporation of the sample, whereas dust on the substratum causes interference with detection methods.

Blocking Buffer

The blocking buffer is important in decreasing the detection background and provide good signal to noise ratios. Furthermore, the use of blocking conditions in RPPA that differ from those used for Western blot validation of the primary antibodies can alter the data output [92].

Amplification

Amplification of microarray signals enhances the signal especially if the target analyte has low abundance. There are different methods of amplification that have been used, especially in antibody microarray. Tyramide signal amplification (TSA) is based on the horseradish peroxidase (HRP)-catalysed deposition of reactive biotinylated tyramine at sites of immunoreactivity, which binds to protein molecules at the sites of HRP activity. Biotinylated tyramine reacts with peroxidase in the presence of hydrogen peroxide and forms a reactive group with a short half-life, which is thought to bind covalently to proteins at electron-rich amino acid residues. This results in an increased amount of biotin at sites of immunoreactivity, which can be detected by streptavidin-based methods, leading to a significant improvement on the sensitivity of signals [94,95]. The use of TSA is sensitive and specific. Another amplification technique employs the 3DNA dendrimers technology from Genisphere (Hatfield, PA, USA) [96]. These fluorescently labeled dendrimers are conjugated to Streptavidin, allowing for detection of biotinylated targets. The signal amplification is significant, due to the 40 fluorescent dyes per Streptavidin molecule.

Detection methods

In this study, we have not tested different detection methods. It was already confirmed from previous work (data unpublished; MBTI group, Nottingham) that infrared should be the most convenient, sensitive and eligible method to detect specific proteins.

There are different methods for detecting the target analyte on microarray slides, such as Immunofluorescence, Colorimetric and Near-Infrared (NIR).

Due to health, safety issues and long detection times the use of radioactive detection methods in microarray is very limited.

Chemiluminescent detection method is based on the similar procedure used for western blotting, in which the antigen is identified with primary antibody followed by secondary antibody conjugated with either alkaline phosphatase (AP) or HRP. Detection is achieved through the cleavage of a chemiluminescent agent and the reaction product produces luminescence in proportion to the amount of protein. Even if this method is sensitive and rapid, the low feature resolution is an important drawback.

Another technique is based on colorimetric detection. Chromogens are molecules that act as substrates for an enzymatic reaction to generate a coloured substance. The most common chromogen used in microarray is diaminobenzidine (DAB) with HRP labelled antibodies. The brown DAB precipitate is stable and produces signals with relatively low background. However, there are several disadvantages, such as the mutagenicity of DAB, the limited lifetime of the reaction and the low dynamic range of chromogenic imaging [97, 98].

Fluorescence in microarray was firstly described in 1990 [99]. Fluorescent molecules absorb photons from an external light source, causing excitation of electrons within the molecule andemission of light at a different wavelength to the excitation one. Light emission can be quantified with great sensitivity using CCD detection systems.

Fluorescence gives high dynamic range relative to colorimetric methods, from violet (405nm) through green (532nm), yellow (594nm) and red (635nm) regions. Combining each antibody with a dye presenting a unique excitation and emission spectra can allow the detection of multiple signals in a single assay. The disadvantages of visible wavelength fluorescence-based detection include problems of non-specific auto-fluorescence from proteins and other components that cause significant reduction in signal-to-noise ratios.

Recently, the use of near-infrared (NIR; 680-800nm) fluorescence dyes has proved to dramatically increase signal-to-noise ratios with minimal auto-fluorescence, with reduction of the background noise [100]. It is however advantageous when using nitrocellulose and fluorescent reporter dyes. The use of two dyes on the same printed spot to detect two target has a very high sensitivity.

4 Signalling pathways studied in this thesis

Additional mechanisms linking innate immune-mediated inflammation with a variety of cellular processes, including protein misfolding, oxidative stress and mitochondrial dysfunction, have been recognised to play a role in the pathogenesis of some monogenic autoinflammatory conditions [101]. Those processes can later activate other inflammatory pathways with host defence function, such as MAPK, JNK, and NF-κB pathways. Along these pathways, inflammasomes are more than ever believed to play a central role in the recognition of exogenous and endogenous danger signals in autoinflammatory diseases [102]. Fig. 10 and 11 present schematic diagrams of the pathways, known to be involved in autoinflammatory diseases and analyzed in this thesis.

4.1 NF-κB pathway

NF-κB is a transcription factor that controls the expression of many genes involved in immune responses, cell proliferation, cell differentiation, cell apoptosis, inflammation, and oncogenesis. In mammals, NF-κB is considered to be the master regulator of both innate and adaptive immunity, inflammatory responses, cell survival and organ development [103].
NF-κB is a member of the Rel family proteins (that can bind to specific DNA sequences) which includes five related members that share the so-called Rel homology domain (RHD), about 300 amino acids long that is located toward the N-terminus of the protein and is responsible for dimerization, DNA binding and interaction with IkB (the cytoplasmic inhibitor of NF-κB).
Expression of NF-κB is regulated by diverse stimuli including pro-inflammatory cytokines such as tumour necrosis factor (TNFα) and interleukin-1, bacterial products including lipopolysaccharide (LPS), various viral proteins such as human T-cell leukemia virus type 1 (HTLV-1) and physical stress, for instance ultraviolet radiation and reactive oxygen stimuli (ROS) [104]. During resting conditions, NF-κB is present in the cytoplasm as an inactive form bonded with inhibitory proteins IkBα (inhibitor of IkBs family, which includes seven members all characterized by ankyrin repeats that mask the NF-κB nuclear localization domain and inhibit both its targeting to the nucleus and its DNA-binding activity [105]. All IkB proteins have two Serine (S) residues within their N-terminal domain. In response to stimulation, phosphorylation of these conserved S residues by IkBkinase (IKK) leads to the immediate polyubiquitination of IkB proteins by ubiquitin ligase E3 complex, rapid degradation of ubiquitinated IkB proteins by the 26S proteasome [106] and release of NF-κB from inhibition, allowing its nuclear translocation. IkB Kinase (IKK) is a 700-900 kDa complex, containing two catalytic subunits, IKKα and IKKβ and a regulatory subunit NEMO (NF-κB essential modulator). IKKα and IKKβ are Serine/Threonine kinases with protein

interaction motifs including a leucine zipper (LZ) and a helix loop helix (HLH) domain in their C-terminal domains [107]. HSP-90/Cdc37, a chaperone, may also associate with the IKK complex, whereas many upstream kinases may regulate the IKK activity, such as MEKK1 or TGFβ-Activated Kinase 1 (TAK1).
Once in the nucleus, NF-κB binds specific promoters that ensure transcription of the target genes encoding numerous pro-inflammatory cytokines, chemokines, adhesion molecules, growth factors, immune receptors and prosurvival proteins [108].
There are two main pathways for activation of NF-κB in response to stimulation. The canonical pathway in response to cytokines such as TNFα, microbial products and stress, depends on activation of IKK complex and results in nuclear translocation of NF-κB [109]. The non-canonical or alternative pathway depends on activation of IKK complex that results in processing of p100 NF-κB protein and release of p52. This pathway occurs mainly in B cells [109].
The known mechanism underlying NF-κB activation by NOD2 is related to a changing conformation of NOD2 in response to bacterial components. The conformational change results in activation of NOD2, downstream engagement of receptor interacting protein-2 (RIP2) and subsequent activation of NF-κB [110]. RIP2 is an adaptor protein and is a CARD-containing Serine/Threonine kinase that physically associates with the CARD domains of NOD2; these homotypic CARD–CARD interactions are important for the activation of NF-κB [111]. Recent results proved the essential role of RIP2 in the modulation of both innate and adaptive immunity triggered by NOD2 ligands [112] and, in RIP2-deficient mice, it was established that RIP2 played a critical role not only in NF-κB, but also in mitogen-activated protein kinase signalling (MAPK) [113]. However, NOD2-dependent but RIP2-independent signalling also exists: Travassos and colleagues demonstrated that in RIP2-deficient cells a NOD2 dependent induction of autophagy occurs, and this involves the direct recruitment of the autophagy protein ATG16L1 to the activated NOD2 complex [68].
Negative feedback for NF-κB is necessary to prevent chronic inflammation caused by continues activation of NF-κB. One of the regulatory molecules is IkBα, which is a target gene of the active NF-κB transcription factor; another one is A20, that negatively activates NF-κB by binding to key regulators such as NEMO or NOD2 in active form and causes de-ubiquitination [114].

4.2 PI3K/AKT pathway

PI3K is a heterodimeric lipid enzyme that belongs to a large family of PI3K related kinases capable of phosphorylation of phosphoinositides (rare lipids). PI3K activates intracellular signalling proteins that play a crucial role in cell survival, differentiation, proliferation and migration pathways in different cell types including cells of innate and adaptive immunity [115].

PI3K presents a catalytic and regulatory subunit [116]. The catalytic p110 subunit has Serine/Threonine protein kinase activity as well as phosphoinositide kinase activity [117].The regulatory p85 subunit consists of multiple domains including Proline rich domains and SH domains for binding [118].

PI3K activation requires two steps: the assembly of p85/p110 heterodimer and the interaction with the activator proteins, that may plasma membrane receptors (receptors of platelet, epidermal or insulin-like growth factor) or non-receptor such as cytokines and cell attachment to the extracellular matrix such as p60 Src [119].

Activated PI3K phosphorylates phosphoinositides (PtdIns), generating Phosphoinositide (PtdIns) phosphates (PtdIns 3,4-P2or PtdIns 3,4,5-P3). These rare lipids help to recruit pleckstrin homology (PH) domain containing proteins such as Akt and PDK1 (phosphoinositide dependent kinase 1) to the cell membrane. PI3K is associated with membrane receptor complexes that bring PI3K into the proximity of Jak2 and c-Src. This will allow Jak2 and c-Src (non receptor tryrosine kinases) to activate PI3K and its downstream target, Akt [120]. PTEN is a lipid phosphatase that inhibits the ability of PI3K to phosphorylate Akt [121].

Akt (also known as protein kinase B, PKB) is a Serine/Threonine protein kinase and is an important regulator of cell survival and proliferation. Akt activation is achieved by site-specific phosphorylation at Threonine 308 and Serine 473, after PI3K activation.

However, PI3K independent activation of Akt has been reported: cAMP-elevating agents such as prostaglandin-E1, 8-bromo-c-AMP, forskolin and chlorophenylthio-cAMP were demonstrated to activate Akt through Protein Kinase A (PKA) [122].

Akt can bealso activated by Ca2+/calmodulin-dependent kinase, cellular stress and heatshock protein especially as Hsp-27 [123].

Upon activation, Akt is recruited to the plasma membrane through the pleckstrin homology (PH) domains, and then recruits many downstream substrates that promote cell survival or inhibit apoptosis. These substrates include Bad, GSK-3β, transcription factors, Caspase-9 and IKK [124].

There are different mechanisms of anti-apoptotic effects of the PI3K/Akt pathway. In some cells NF-κB is the main target of PI3K/Akt [125].However, there are survival mechanisms other than NF-κB that can activate several survival targets, including components of cell death machinery (such as BAD, cFLIP) [126].

The PI3K/Akt signalling pathway is involved in regulating the inflammatory response. The role of the PI3K/Akt pathway in inflammation-mediated diseases such as rheumatoid arthritis, multiple sclerosis, asthma, chronic obstructive pulmonary disease, psoriasis, and atherosclerosis has been reported [127, 128]. PI3K is predominantly expressed in leukocytes and is critical to the inflammatory response and the function of leukocytes [129]. The role of Akt in inflammation is

still under investigation. However, Di Lorenzo and colleagues demonstrated the critical role of Akt1 in acute inflammation by vascular permeability that leads to oedema and leukocyte extravasations [130].

4.3 MAPK pathway

Mitogen activated protein kinases (MAPK) are Serine/Threonine protein kinases that are activated by various extracellular stimuli as proinflammatory cytokines, heat shock, mitogen, osmotic stress and other stimuli. MAPK regulate different cell activities including gene expression, cell survival, cell proliferation, and apoptosis.

Several groups of MAPK family have been identified, including extracellular regulatory kinase (ERK1/2/5, also known as classical MAPK), p38MAPK and c-Jun N-terminal kinase/stress activated protein kinases (JNK1/2/3/SAPKs). Activation of each group requires dual phosphorylation of Threonine and Tyrosine within the activation loop of the MAPK through a cascade composed of MAPK, MAPK kinases (MEKs) and MAPKKK. MEKs are specific for each isoform of MAPK family. For instance ERK1/ERK2 are activated by MEK1 and MEK2, JNK is activated by MEK4 and MEK7, and p38 MAPK is activated by MEK3 and MEK6 [131].

One of the most important and most studied MAPK pathway is ERK1/2.

There are many stimuli that can activate the ERK1/2 MAPK pathway such as growth factors, serum, and phorbol esters and to a lesser extent, cytokines, osmotic stress, ligands of theheterotrimeric G protein-coupled receptors, and microtubule disorganization [132]. Activation of ERK1/ERK2 starts at the plasma membrane where cell surface receptors transmit signals that allow subsequential binding between Ras, Raf-1 kinase (a member of MEKK family) and protein 14-3-3 [133]. That enables Raf activation by membrane kinases such as protein kinase C (PKC) family, Src. After Ras/14-3-3/Raf-1 activation, the signal is transmitted to the next step in this cascade, MEKs (MEK 1 and 2) that can be activated mainly by Raf-1 involving serine phosphorylation [134]. Activated MEKs in turn initiate dual phosphorylation of ERK1/ERK2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop. This dual phosphorylation allows nuclear translocation of activated ERK1/2 [135].

ERK1/2 can phosphorylate large number of nuclear, cytosolic and structural regulatory proteins that control several cellular processes mainly cell transcription, proliferation, cytoskeletal rearrangment and translation [136]. For example, ERK1/2 can indirectly regulate some kinases responsible for controlling transcription by phosphorylation of regulatory molecules such as NF-κB, c-AMP or glycogen synthase kinase 3 (GSK 3) [137].

p38 MAPK was firstly studied in 1993 [138]. Different extracellular stimuli including pro-inflammatory cytokines, TNFα, interleukin-1, protein synthesis

inhibitors, and chemically stressful stimuli can all activate p38 MAPK pathway. The upstream activators of p38 MAPK are two specific MKK family members, MEK3 and MEK6 [139]. Recently, another upstream molecule named MEK5 (also called TAK1) may serve as an upstream kinase of MEK3 and 6 [140]. Activation of the p38 results from phosphorylation of a conserved Thr-Gly-Tyr (TGY) motif in their activation loop by the MKK3/6. Active p38 MAPK targets several downstream protein kinases including: MAP kinase-activated protein kinase 2 and 3 (MAPKAPK2 and 3) and MSK1/2 [141]. MAPKAPK2 phosphorylates and activates heat-shock protein27 (HSP-27), while MSK1/2 can phosphorylate and activate transcription factors that regulate survival, such as CREB. Cellular response to p38 MAPK activation changes depending on the cell type and the stimulus and p38 MAPK has been reported to promote both cell survival and cell death [142]. There are important associations between expression of p38 MAPK and inflammation as p38 MAPK is activated mainly by numerous inflammatory stimuli and there after affects directly or indirectly the transcription of genes encoding inflammatory molecules [143].

JNK family was firstly isolated in 1990 [144]. There are three main forms of JNK: JNK1,JNK2, and SAPK that are widely expressed in all cells. Stress factor stimuli activate JNKs, such as cytokines, UV irradiation, growth factor deprivation, DNA-damaging agents, and, to a lesser extent, some G protein coupled receptors, serum, and growth factors [138]. Upon stimulation, signals are transmitted to several MAPKKs that phosphorylate MEK4 and MEK7 which in turn causes dual phosphorylation on Tyrosine and Threonine residues within a conserved Thr-Pro-Tyr (TPY) motif. Between the transcription factors that are phosphorylated by active JNKs, c-Jun is the most well known. Serine phosphorylation of c-Jun by JNK leads to increased c-Jun-dependent transcription [145]. Other transcription factors that also can be phosphorylated by active JNKs are: ATF-2, NF-ATc1, HSF-1, and STAT3 [145].

4.4 JAK2/STAT3/c-Src pathway

JAKs (Janus family of tyrosine kinases) are a family of large non-receptor tyrosine kinase proteins, including Jak1, Jak2, Jak3 and Tyk2 [146]. They are widely expressed, except Jak3 restricted to myeloid and lymphoid cells. Stimulation of JAKs results in STAT transcription factor activity.

STATs (signal transducers and activators of transcription) comprise a family of seven structurally and functionally related proteins: Stat1,Stat2, Stat3, Stat4, Stat5a and Stat5b, Stat6f. Activated STATs play a critical role in regulating innate and acquired host immune responses [147].

Src family is non-receptor protein Tyrosine kinase that was first discovered in 1979 [148].

Some cytokine receptors lack catalytic kinase activity and depend on JAK family of tyrosine kinases to provide this activity. It has been shown that JAKs are constitutively associated with a Proline-rich, membrane proximal domain of these receptors [149]. JAK pathway activation initiates by binding of the cytokine to the corresponding receptor, which leads in turn to conformational changes in the cytoplasmic side bringing JAKs into the proximity of each other. Activated JAKs act as docking sites for STATs and other regulatory molecules. Recruitment of STAT to the receptor initiates its phosphorylation and activation. Activated STATs separate from the receptor, dimerize and translocate to the nucleus [147].
SHP-2 protein has been found to negatively regulate JAKs activity by dephosphorylation [150]. Activated STATs target specific genes, SOCS (suppressors of cytokine signalling), which antagonise STATs activity and establishing an important and classic negative feedback mechanism [151]. Another down regulation or inhibition mechanism of STAT pathway is modification of STATs protein as dephosphorylation, methylation or formation of truncated isoforms of STATs [152].
C-Src is activated by dephosphorylation of Tyrosine527 by SHP-1 phosphatase tyrosin kinase [153].

4.5 INFLAMMASOME pathway

The structure of the inflammasome has first been described by Tschopp and co-workers in 2002 [154]. The macromolecular complex includes a NOD-like receptor (NLR) associated with ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)) and procaspase 1 [155]. According to the structure of the NLR involved, 4 types of inflammasomes have been described to date. The NLRP1-inflammasome may be activated by a toxin derived from anthrax, the NLRC4-inflammasome by bacterial flagellin and an inflammasome of the PYHIN (pyrin and HIN domain-containing protein) family, termed AIM2-inflammasome (“absent in melanoma 2”) by binding to viral and bacterial double-stranded DNA from intracellular pathogens [156,157].
External and endogenous “dangers” are recognized by these “danger sensors” and are able to induce an inflammatory reaction. In addition to PAMPs and DAMPs, the inflammasome may interact with and be stimulated by proteins such as pyrin, proline–serine–threonine phosphatase-interacting protein 1 (PSTPIP1) and mevalonate kinase (MK) [158].
The most studied inflammasome is the NLRP3-inflammasome. It may also be regarded as the most important, since it can be activated by a large variety of PAMPs and DAMPs. In addition activation may be achieved by reactive oxygenspecies (ROS), proteases released into the cytosol by lysosomal damage caused by amyloid-β, silica, cathepsin B or cholesterol crystals, or potassium efflux from the cell [159-160].

The NLRP3 protein has a tripartite domain organization: an amino-terminal death-fold domain (a pyrin domain), a central NACHT nucleotide-binding domain with ATPase activity and a role in the oligomerization of the protein, and carboxy-terminal leucine-rich repeats (LRRs) with regulatory functions and involved in ligand interaction [33]. The death-fold domains of the NLR protein interact with those of ASC and/or caspase. ASC is an adaptor protein encoded by PYCARD and consists of two death-fold domains: one pyrin domain and one caspase activation and recruitment domain (CARD). ASC interacts with the upstream inflammasome sensor molecules via the pyrin domain. This interaction triggers the assembly of ASC into a large protein consisting mainly of multimers of ASC dimers. Using its CARD, ASC brings monomers of pro-caspase 1 into close proximity [162].

Upon activation of the inflammasome, procaspase 1 will be activated to caspase 1 which in turn cleaves inactive pro-interleukin-1β to its active form IL1β. IL1β is, besides TNFα, one of the major mediators of fever and inflammation in humans. IL1β has also been called the “prototypic alarm cytokine” [162], recognizing its pivotal role in innate immunity. Once released, IL1β leads to dimerization of the IL1 receptor accessory protein (IL1RAcP) with the IL1-receptor type 1 (IL1R1). This causes a cascade of downstream signals which finally result in the activation of NF-κB and the production and release of other inflammatory cytokines. The IL1 receptor antagonist (IL1-RA) may prevent recruitment of IL1RAcP and has an important role in regulating the process [163, 164].

As a consequence of inflammasome activation, a large variety of cytokines are produced and released by cells of the innate immune system (monocytes, macrophages, dendritic cells) [165]. They include the IL1 family (IL1, IL18, IL33), the TNF family (TNFα, LTα), the IL6 family (IL6,IL11), the IL17 family (IL17A, IL25), and type 1 IFNs (IFNα, IFNβ) [165].

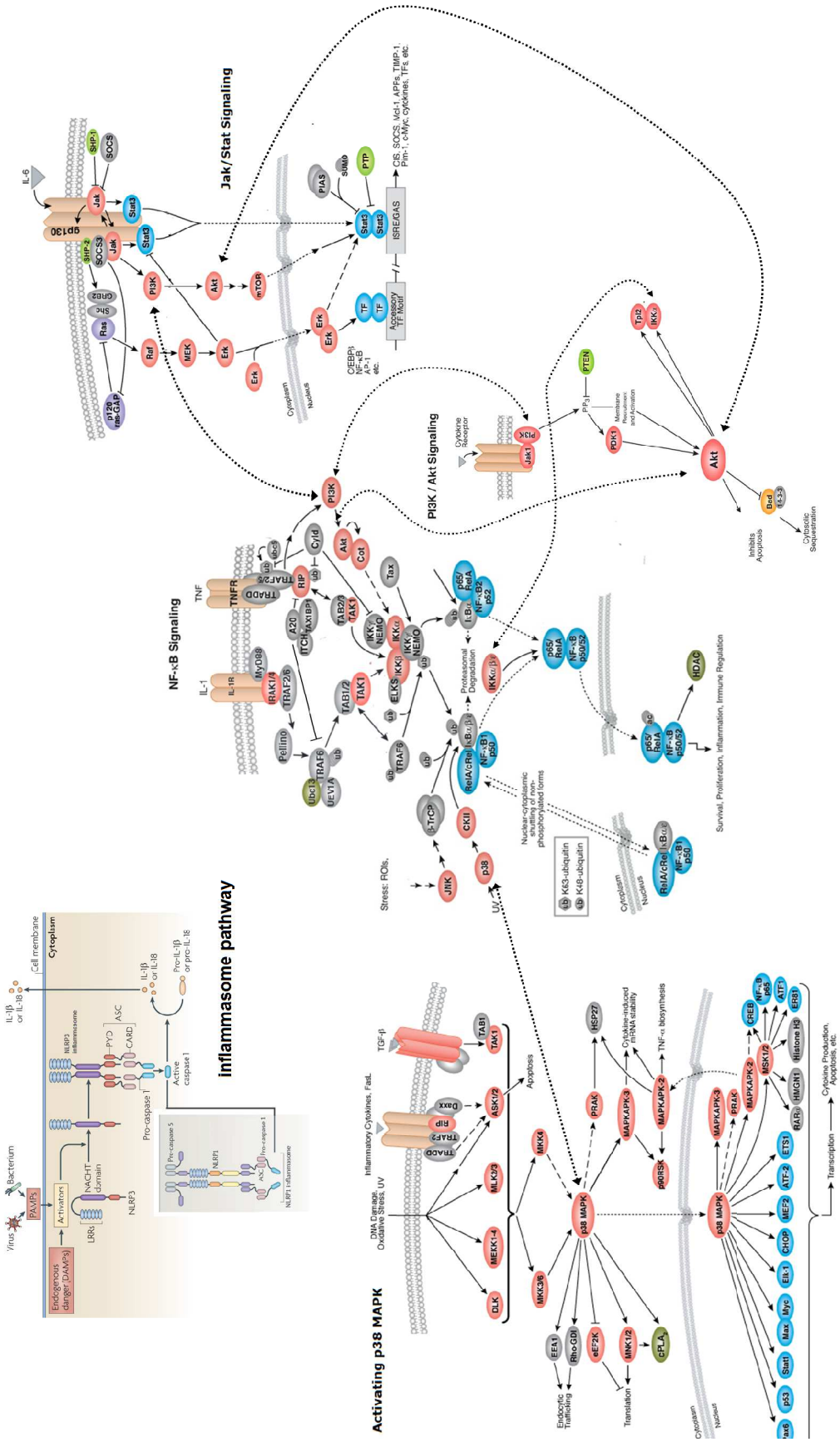


Fig. 10: Signalling pathway analyzed in this thesis work:
Inflammasome pathway (modified from [166]), NF-κB, JAK/STAT, PI3K/Akt and MAPK pathways (Cell Signaling Technology®).

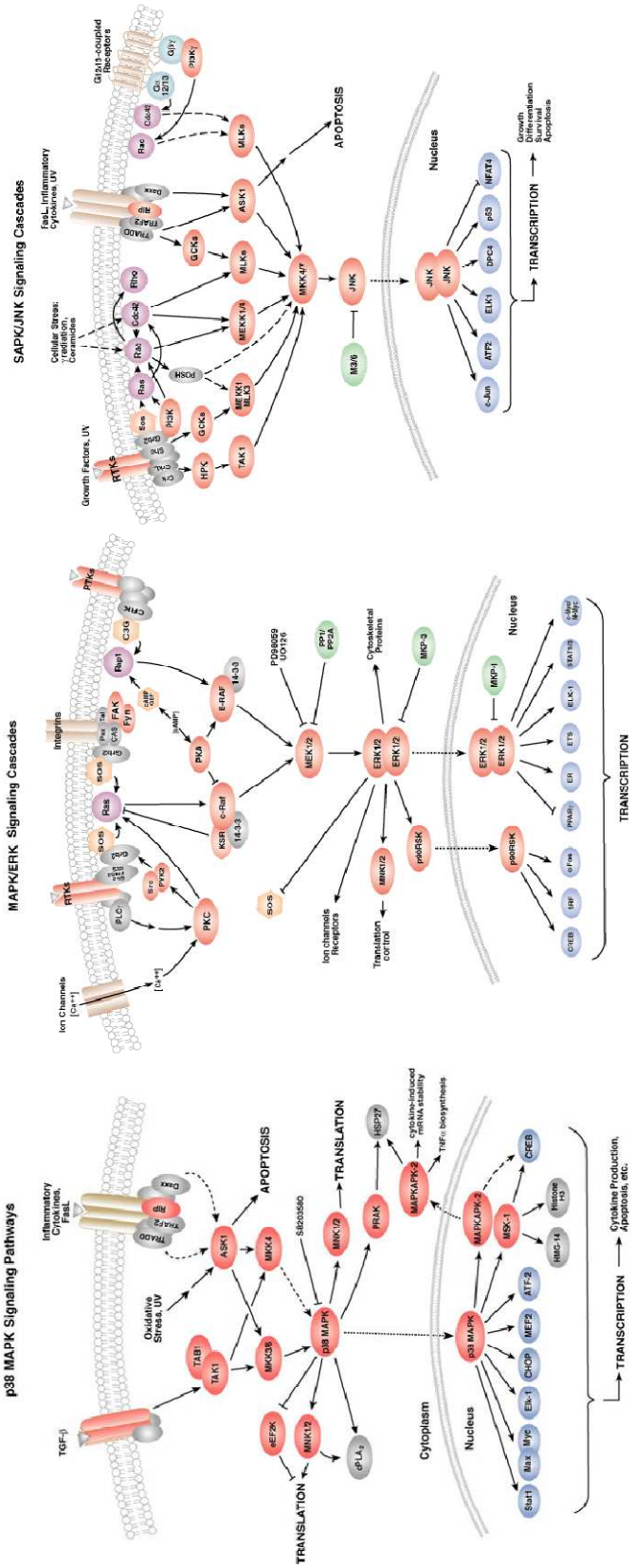


Fig. 11: p38, Erk and SAPK/JNK MAPK Signalling pathways (Cell Signaling Technology®).

5 Pro-inflammatory cytokines discussed in this thesis

The disease-based discovery of the molecular basis for autoinflammatory disorders has led not only to a rapidly growing number of clinically and genetically identifiable diseases, but has unmantled key inflammatory pathways such as the potent role of the interleukin IL1 and other pro-inflammatory cytokines.

5.1 Interleukin 1 (IL1)

The IL1 family plays an important role in inflammation and host defence. Up to 11 members of this family have been identified to date [167]. Of those, only five have been thoroughly studied: IL1α, IL1β, IL18, IL1RA and the recently reported IL33 [168]. The remaining six (IL1F5; IL1F6; IL1F7; IL1F8; IL1F9; IL1F10) have been shown to be expressed in various cell types or tissues but their functions remain to be determined.

IL1α and IL1β are pro-inflammatory cytokines. Both are synthesized as precursor molecules (pro-IL1α and pro-IL1β) by many different cell types. Pro-IL1α is biologically active and needs to be cleaved by calpain to generate the smaller mature protein. By contrast, pro-IL1β is biologically inactive and requires enzymatic cleavage by caspase-1 in order to become active. IL1α is primarily bound to the membrane whereas IL1β is secreted and thus represents the predominant extracellular form of IL1 [169,170]. Because IL1β is the most powerful endogenous fever-inducing molecule (pyrogen) known, there are multiple checkpoints to control its production and effects, including both the regulation of inflammasome activation and the control of its end-organ activity through IL1Ra. Although the major sources of IL1β are blood monocytes, tissue macrophages, and dendritic cells [171], it should be noted that leukocytes producing this cytokine are found in immunologically privileged organs, such as the kidney, heart, skeletal muscle, and brain [172]. Interleukin 1 is involved in the pathogenesis of numerous diseases with an inflammatory component [173]. In particular, as presented by Agostini and colleagues, IL1β has a prominent role in autoinflammatory diseases activated by NALP3 inflammasome [174]. The observation that metabolic substrates that accumulate in target tissues can stimulate the NLRP3 inflammasome to release IL1β has led to studies of the use of IL1 blocking agents in some diseases. Despite the success of IL1 blocking therapy, the use in a wider spectrum of autoinflammatory conditions has uncovered disease subsets that are not responsive to IL1 blockade.

5.2 Interleukin 6 (IL6)

The IL6 family is composed of IL6, IL11, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC). Members of this family display pro- but also anti-inflammatory effects and play a central role in hemopoiesis as well as in innate and adaptive immune responses.

Activation of IL6 signalling is mediated through the IL6/sIL6R complex, a process known as “trans-signalization” and a unique example of a soluble cytokine receptor displaying agonistic effects. IL6 is secreted by many cell types, including B and T lymphocytes, monocytes, fibroblasts, keratinocytes, endothelial cells, mesenchymal cells and certain types of tumor cells. IL6 induces the differentiation of B lymphocytes into plasma cells as well as the proliferation of T lymphocytes, differentiation of cytotoxic T cells and IL2 production. IL6 also induces the differentiation of macrophages and megakaryocytes [175].

Recently, IL6 has been described to participate in the differentiation of a novel T cell subset, Th17, which displays pro-inflammatory functions [176]. IL23 is responsible for the expansion of Th17 previously differentiated, while IL6 and TGF-β are responsible for the differentiation of Th17 from their naïve precursors [177].

IL6 is likely to be involved in the pathogenesis of inflammatory diseases. It is also found at high level in serum of periodic fever patients [178] and also in complex disorders such as Still's and Behçet diseases [179, 36].

5.3 Interleukin 8 (IL8)

Interleukin 8 is a member of the CXC chemokine family and was initially identified as a neutrophil chemotactic and activating factor [180]. Since then, a variety of other proinflammatory activities have been attributed to IL8, including immune cell activation and promotion of angiogenesis [181,182]. IL8 can be produced by a variety of cell types involved in inflammation, including monocytes and endothelial cells. Activation of cells occurs after binding to the IL8 receptors, CXCR1 and CXCR2, expressed on neutrophils, monocytes, endothelial cells, astrocytes, and microglia [183]. IL8 expression is regulated by different mechanisms, including NF-κB pathway [184].

IL8 is implicated in chronic inflammation, as suggested for inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis [185,186]. IL8 levels are increased in the serum of diverse autoinflammatory diseases affected subjects, in particular it can be a marker of activity in Behçet patients [187].

5.4 Tumour Necrosis Factor α (TNFα)

Among all the members of the tumour necrosis factor family, TNFα and TNFβ are the most pleotropic cytokines. Although they share biological activities and homology, they are structurally different as TNFα presents cysteine residues [188]. TNFα is produced by activated immune cells including macrophages, monocytes, lymphocytes such as T-cells and natural killer cells (NK) and other cells including fibroblasts, smooth muscle cells and epithelial cells. TNFα is synthesised as a homotrimer type II transmembrane pro-TNFα that is cleaved by a metalloproteinase to release C-terminal soluble TNFα [189].
TNFα controls and orchestrates the immune responses at several levels [190]. It has also a protective role against many infections and an important function in organogenesis [191, 192].The pathological effects of TNFα includes involvement in cancers, inflammation, autoimmune diseases [193].

5.5 Interferons (IFNs)

The IFNs were originally discovered as agents that interfere with viral replication. They are classified into type I and type II according to receptor specificity and sequence homology.
IFNγ is the sole type II IFN. It is structurally unrelated to type I IFNs, binds to a different receptor, and is encoded by a separate chromosomal locus. It is secreted by B cells, NKT cells, and professional antigen-presenting cells (APCs) [194,195]. IFNγ production by professional APCs (monocyte/macrophage, dendritic cells) acting locally may be important in cell self-activation and activation of nearby cells. IFNγ secretion by NK cells is likely to be important in early host defence against infection. IFNγ production is controlled by cytokines, such as IL12 and IL18. These cytokines serve as a bridge to link infection with IFNγ production in the innate immune response [196]. IFNγ is known to heighten immune surveillance and immune system function during infection [197].

5.6 Interleukin 12, 17, 22 and 23 (IL12, IL17, IL22 and IL22)

IL12 is produced primarily by monocytes, macrophages, dendritic cells, and B cells. The major functions of IL12 include induction of IFNγ production from natural killer (NK) cells and T cells, enhancement of cytotoxity of NK and cytotoxic T cells, and differentiation of naïve T cells into Th1 effectors, suggesting a key role for IL12 in the development of cell-mediated immunity [198]. Although IL12 is important for host defence, over expression of IL12 can cause persistent inflammation leading to autoimmune disorders such as multiple sclerosis.
The recent discovery of IL23 has led to a re-evaluation of interleukin 12 biology, as they share a common p40 subunit. IL23 is a cytokine that drives autoimmune diseases, including psoriasis and inflammatory bowel diseases [199,200]. IL23 is

secreted by human DCs exposed to gram-negative bacteria. The main distinguishing feature of IL23 is its ability to induce CD4+ Th17 cells to produce IL17 [201]. The IL23-IL17 pathway plays an important role in terms of the induction of inflammatory cytokines contributing to autoimmunity as well as protective responses against infection.

IL17 is a potent proinflammatory cytokine that amplifies ongoing inflammation by inducing expression of TNFα, IL1β, and IL6 in epithelial and endothelial cells as well as other cell types such as keratinocytes, synoviocytes, fibroblasts, and macrophages.

Interestingly, a recent report brings to light a potential role for Th17 cells in the autoinflammatory disorder adult-onset Still's disease (AOSD) [202].

Th17 cells produce also IL22, a cytokine that is important for the modulation of tissue responses during inflammation. Through activation of STAT3 signalling pathway, IL22 induces proliferative and anti-apoptotic pathways, as well as anti-microbial molecules that help prevent tissue damage and aid in its repair. IL22 plays also an important role in inflammation, including chronic inflammatory diseases and infectious diseases [203].

AIM OF THIS WORK

The autoinflammatory diseases are a heterogeneus group of inflammatory syndromes caused by primary dysfunction of the innate immune system, without evidence of adaptive immune dysregulation. They comprise both hereditary (such as FMF, MKD, TRAPS, CAPS and Blau syndrome) and multifactorial (Still's and Behçet's diseases) disorders. Despite major advances in genetics and pathophysiology of these diseases, the signalling pathways and the inflammatory mediators related to autoinflammatory disorders are far to be deeply investigated.

There are two main aims in this project:

1. Functional characterization of the Blau syndrome-associated mutation (p.E383K), recently found in the affected members of an Italian family, by studying *in vitro* its pathogenic potential from NOD2-transfected HEK293 cells. To confirm the preliminary *in vitro* results, additional *ex vivo* studies were performed using protein microarray techniques.
2. Characterization at molecular level of a wide range of hereditary and multifactorial autoinflammatory diseases, in order to identify possible predictive or diagnostic biomarkers for each disease and to develop preventive and therapeutic strategies. We evaluated both signalling intermediates and inflammatory mediators in pathways known to be involved in inflammatory responses by means of protein microarray based systems

MATERIALS AND METHODS

1 Genetic and functional studies on Blau syndrome

1.1 Clinical Evaluation

The study involved subjects belonging to an Italian family, affected by BS and followed by the Rheumatic Unit, University of Padua for the past 25 years (Fig. 12a) [204]. Clinical diagnosis for BS was based on physical features, such as chronic bilateral uveitis, symmetrical arthritis involving fingers wrists and feet, and skin manifestations, consisting of widespread papules. Skin biopsies have revealed non-caseating granulomas with multinucleated giant cells which, at electron microscopy, revealed "comma-shaped bodies" in epithelioid cells, thought to be a marker for BS (Fig. 12b). Each patient underwent all routine laboratory investigations, including serum lipids, rheumatoid factor and antinuclear antibodies, that were within normal limits. The proband showed asymptomatic, diffuse, miliary brownish papule and firm subcutaneous nodules, varying in size from 5 to 30 mm diameter, on the dorsa of hands and feet and on the extensor leg surfaces. No specific treatment was prescribed to the probandexceptfor NSAIDs, which were to be taken when necessary for a few days' time for pain control. Her daughter, instead, followed a low doses of oral steroids treatment which yielded a good response [205].
Blood sampling for DNA, PBMC cytokine assays and functional analysis were performed under informed consent, according to the pertinent Italian legislation.

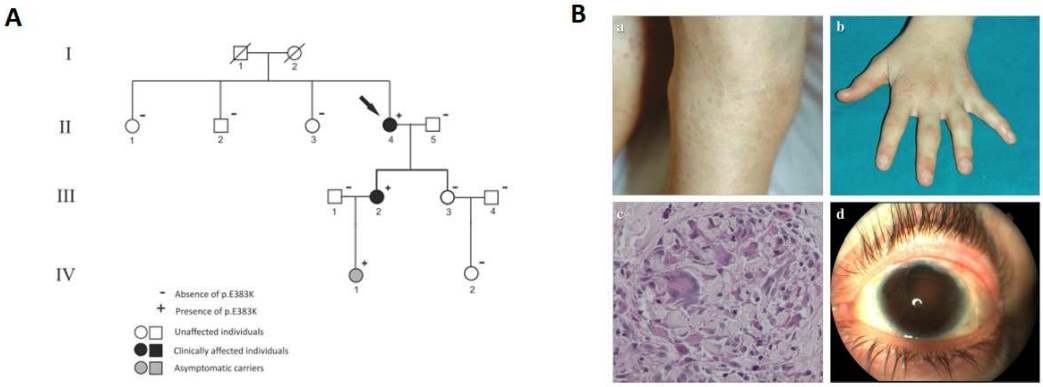


Fig. 12: Blau syndrome family.
A) Pedigree structure of the proband and her family. Filled-in symbols indicate clinically affected family members, both proband (subject II-4) and her daughter (subject III-2). Shaded symbol represents proband's granddaughter (subject IV-1), an asyntomatic carrier of the p.E383K mutation. Absence (-) or presence (+) of p.E383K mutation is indicated.
B) Clinical and histological aspects of Blau syndrome. a) Asymptomatic diffuse brownish papulae, firm subcutaneous nodules, on the extensor surfaces of the legs. b) Enlargement of interphalangeal joints. c) Skin biopsy from the right forearm. Non-caseating granulomas, containing several histiocytes and multinucleated giant cells with strong PAS positivity and rare lymphocytes and eosinophils. d) Granulomatous uveitis.

1.2 Screening for mutations

Genomic DNA was extracted from blood samples and its concentration is determined with Nanodrop (ND 1000 Spectrophotomer). All PCR primers used in this thesis were designed by PRIMER3 (http://frodo.wi.mit.edu) and produced by Invitrogen. The primers obtained from PRIMER3 were chosen after analysis with BLAT (http://genome.ucsc.edu) to avoid both SNPs presence within their sequences and annealing in additional wrong regions.

1.2.1 DHPLC analysis

PCR Amplification

The analysis of *CARD15/NOD2* gene in 3 BS patients were performed firstly by denaturing high-performance liquid chromatography (DHPLC). Samples were amplified in a final volume of 50µl containing approximately 100ng of genomic DNA, 10X PCR buffer II (Applied Biosystems), 25mM $MgCl_2$ (Applied Biosystems), 6pmoli of each primer (Invitrogen), 5mM deoxinucleotide triphosphates (Invitrogen) , and 2.5U of DNA TAQ GOLD polymerase (Applied Biosystem).

PCR conditions for all the DNA fragments were as follows: an initial denaturation cycle of 95°C for 7 minutes followed by 35 cycles composed by a denaturation step (95°C) for 30 seconds, an annealing step for 30 seconds, and an extension step (72°C) for 45 seconds. A final extension of 72°C for 7 minutes completes the amplification. The amplification product was verified using 2% agarose gel, and visualized using Gel Red™ Nucleic Acid Gel Stain (Biotium) and ultraviolet light. As the length of fragment suitable for DHPLC analysis is less than 400bp, this long exon was split in shorter amplicons. All primer sequences and PCR conditions used are reported in the below table (Tab. 2).

AMPLICON	Tm	Base pair	T°c DHPLC+TIME SHIFT	FORWARD 5' 3'	REVERSE 5'3'
4a	60°C	550bp	61.5°C 63.5°C	CGCTGGCTCTCCTATCCCTT	TTGACCAACATCAGGCCAACAG
4b	60°C	487bp	61°C 63.1°C + 0.5	CTGCAGGGCAAGACTTCCA	AGGGCTGAGGTCTCTTGGA
4c	60°C	524bp	62.4°C 63.2°C	TCCGCACCGAGTTCAACCTC	CGCGGCAAAGAAGCACTGGA
4d	60°C	490bp	62.2°C	GCCAAGAGCGTGCATGCCAT	CACCAGACCCAGCACATAGG
4e	60°C	494bp	63.9°C 62.5°C + 1	ACCTTCAGATCACAGCAGCC	GCTCCCCCATACCTGAAC

Tab. 2: Condition for PCR amplification and DHPLC analysis of *CARD15/NOD2* gene for BS-associated mutation.

DHPLC Analysis

Denaturing high-performance liquid chromatography (DHPLC) compares two or more DNA sequences as a mixture of denatured and reannealed PCR amplicons, revealing the presence of a mutation by the differential retention of homo- and

heteroduplex DNA on reversed-phase chromatography supports under partial heat denaturation [206].

DHPLC analysis was performed on a Transgenomic Wave DNA Fragment Analysis System (Model 3500HT; Transgenomic) with a DNASep column (Transgenomic). PCR fragments were denatured for 5min at 95°C and then left to re-anneal slowly at room temperature to promote the formation of heteroduplex. Separation was performed at a flow rate of 0.9ml/min for Mutation Detection application (8min). The acetonitrile gradient was adjusted to elute the amplicon at half run, around 4min for Mutation Detection application and 1.5min for Rapid DNA application. The gradient was obtained by mixing Buffer A (0.1M TEAA, pH 7.0) and Buffer B (0.1M TEAA, pH 7.0, 25% acetonitrile). Buffer B increase was 2% per min (with flow rate 0.9ml/min) and 5% per min (with flow rate 1.5ml/min). Column temperatures were calculated by NAVIGATOR software (Transgenomic). Whenever fragments showed different melting domains, additional analyzing temperatures were used, to optimize resolution. These temperatures should give the 75-80% of double strand DNA within the fragments. DHPLC conditions used to analyze CARD15/NOD2 gene are listed in the Tab. 2 presented above.

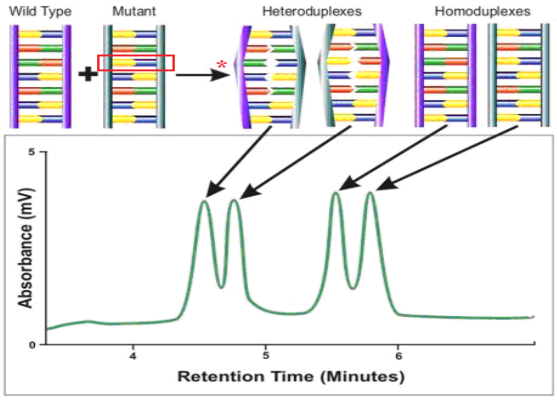


Fig. 13: DHPLC scheme.
When two DNA molecules, differing by as little as a single mismatch, are heated and cooled together, two homoduplex and two heteroduplex species are formed. The chromatographic separation of homo- and heteroduplex is the basis of the DHPLC technique.

1.2.2 DNA Sequencing

Whenever a putative mutation was detected with DHPLC technique, a DNA sequencing was performing to confirm it. Primers and dNTPs unconsumed during the PCR amplification can interfere with the sequencing method. The purification was provided by usage of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (EXO-SAP). After mixing together 5µl of PCR amplification and 0.5µl of each enzyme, the solution has to be incubate firstly at 37°C for 15min to activate and then at 80°C for 15 min to inactivate the reaction. The sequencing reaction requires 3ng of purified DNA per 100bp and 10pmol of primer for sequencing (forward o reverse). PCR amplicons were sequenced at the

BMR genomics by an ABI 3730XL DNA sequencer (Applied Biosystems). CHROMAS software (Version 2.33; Technelysium) and the LASERGENE software package (SeqMan II, DNASTAR) were used to edit, assemble and translate sequences.

1.3 Cytokines release from PBMC

Collection of PBMC

Single paired samples of blood were collected from the two adult Blau syndrome patients described previously and three healthy controls. PBMC were isolated by Ficoll-density gradient separation, as the protocol below.

- Thaw blood sample (collected in EDTA tube) and transferred to a sterile 50ml tube.
- Add PBS+2%FCS at 1:2 ratio, then gentle mixing the tube.
- Layer 3ml of Histopaque-1077 at the bottom of a new tube and then carefully layer the blood sample using a Pasteur pipette. Do not mix the blood and Histopaque.
- Spin at 900×g for 30min at room temperature.
- Using a Pasteur pipette carefully remove the white 'buffy coat layer' and transfer it to a new universal container. This layer contains PBMCs.
- Wash the cells in PBS+2%FCS (make up total volume to approximately 15ml). Centrifuge cells at 400×g for 10 minutes at room temperature.
- Pour off supernatant very gently and resuspend cells in 8ml of an hemolysis solution (8,26g NH4Cl, 1g KHCO3, 0,037g EDTANa4) for 10min, shaking sometimes.
- Add PBS+2%FCS until the final volume of 15ml and then centrifuge at 400×g for 10 minutes at room temperature.
- Pour off the supernatant and wash the pellet with PBS. Centrifuge cells at 400×g for 10 minutes at room temperature.
- Pour off supernatant gently and resuspend cells in 10ml of RPMI complete media (10%FCS+1%Glutamine).
- Count the cells using Bürker chamber and diluting the cells in Trypan Blue. To obtain the concentration of the suspension, use the formula:

$$cell/ml = mean\ of\ readings \times titration\ factor \times 10^3$$

Cell culture and stimulation of PBMC

Fresh PBMC were seeded in 12-well plate inRPMI media containing 1%Glutamine and 10%FCS in an atmosphere of 95/5% air/CO_2 at 37°C at a density of $2 \cdot 10^6$cell/2ml. The cells were cultured for 7 and 24 hours either without stimulation or in the presence of muramyldipeptide [10µg/ml] (Sigma-Aldrich), purified lipopolysaccharide from *E.Coli* 055:B5 [100ng/ml] (Sigma-Aldrich) or a combination of both. All experiments were made in duplicate.

Muramyldipeptide (MDP) is a synthetic immunoreactive peptide consisting of N-acetyl muramic acid attached to a short amino acid chain of L-Ala-D-isoGln [207]. Lipopolysaccharide (LPS) is a TLR -4 agonist and the major component of the outer membrane of Gram-negative bacteria.

ELISA analysis

At the expected times, the cell culture were proceeded as follow:

- Collect the surnatant of each well in different marked eppendorf.
- Centrifuge at 400×g for 10 minutes at room temperature to pellet accidental cellular materials.
- Collect the surnatant and store at -80°C.
- In a specific tube, each surnatant was diluted at 1:50 in a total volume of 1ml BSA4% solution and store at -20°C until cytokine assays were performed

Cytokines levels were measured through enzyme-linked immunosorbent assay (ELISA) by Hospital Laboratories. ELISA is a simple technique that allows rapid screening or quantification of a large number of samples for the presence of an analyte or the antibody recognizing it. ELISA, however, remains popular because of its ease of performance and automation, accuracy, and the ready availability of inexpensive reagents. One limitation of the ELISA technique is that it provides information on the presence of an analyte but no information on its biochemical properties, such as molecular weight or its spatial distribution in a tissue.

1.4 *CARD15/NOD2* constructs for *in vitro* experiments

To directly evaluate the pathogenic potentials of the mutation identified in the Italian BS patients (p.E383K), the starting point is to create a construct with the *wild-type* and mutated *CARD15/NOD2* cDNA cloned inside. The *CARD15/NOD2 wild-type* construct was already available in the lab, presenting the cDNA cloning into pCMV-Tag2c vector. I did a mutagenesis step to introduce p.E383K in the *wild-type* construct. Having a construct carrying another BS-associated mutation should be very useful, especially as a positive control for the further experiments. p.R334W mutation is one of the most frequent and deeply studied BS-associated mutation. Due to the hardness of mutagenesis in the region full of tandem repeats , I used a different way to obtain the p.R334W mutated construct. I have started from a different construct pcDNA3-R334W, presenting the mutated p.R334W *CARD15/NOD2* cDNA.

A couple of restriction enzymes (BamHI and XhoI) are used to cut this construct to obtain the linear strand of p.R334W *CARD15/NOD2* cDNA. The same restriction enzymes cut the pCMV-Tag2c-*CARD15/NOD2 wild-type* construct to get the vector pCMV-Tag2c with compatible ends for ligation with the previously

obtained cDNA. DNA ligase catalyzes the joining using the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides in the sticky-ended configuration [208]. This allows the "pasting" together of p.R334W *CARD15/NOD2* cDNA insert and receptive pCMV-Tag2c vector. So, those constructs are ready for further transfection experiments.

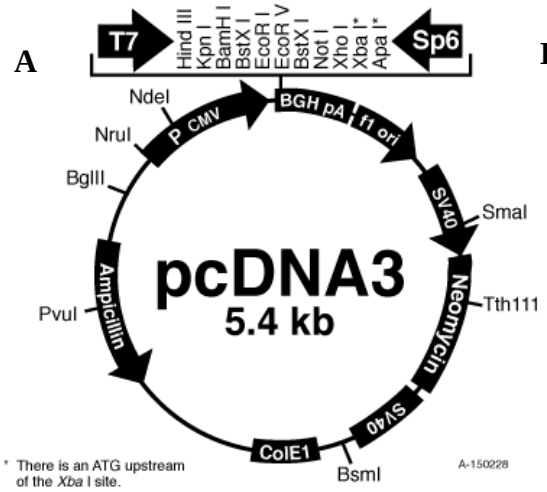
1.4.1 Vectors

pcDNA3

In the lab p.R334W *CARD15/NOD2* cDNA was available kindly provided by Dr G Núñez (Ann Arbor, MI, USA) cloning into pcDNA3 vector. This plasmid offers the following features, as shows in Fig. 14:

- Cytomegalovirus (CMV) enhancer-promoter for high-level expression
- Large multiple cloning site with 11 unique restriction enzyme sites and either forward (+) or reverse (-) orientations
- Bovine Growth Hormone (BGH) polyadenylation signal and transcription termination sequence for enhanced mRNA stability
- Ampicillin resistance gene and pUC origin for selection and maintenance in *E. coli*

A



B

Comments for pcDNA3:
5446 nucleotides

CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH poly A: bases 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
Neomycin ORF: bases 2151-2945
SV40 poly A: bases 3000-3372
ColE1 origin: bases 3632-4305
Ampicillin ORF: bases 4450-5310

C

putative transcriptional start — T7 promoter

824 GCTAACTAGA GAACCCACTG CTTACTGGCT TATCGAAATT AATACGACTC ACTATAGGGA

Hind III — *Kpn* I — *Bam*H I — *Bst* XI — *Eco* R I

884 GACCCAAGCT TGGTACCGAG CTCGGATCCA CTAGTAACGG CCGCCAGTGT GCTGGAATTC

Eco R V — *Bst* X I — *Not* I — *Xho* I — *Xba* I — *Apa* I

944 TGCAGATATC CATCACACTG GCGGCCGCTC GAGCATGCAT CTAGAGGGCC CTATTCTATA

Sp6 promoter

1004 GTGTCACCTA AATGCTAGAG CTCGCTGATC AGCCTCGACT GTGCCTTCTA GTTGCCAGCC

Fig. 14: pcDNA3 plasmid.
A) pcDNA3 plasmid circle map (Life Technology); **B)** List of features and their position; **C)** Multiple cloning site region with position of restriction enzymes.

pCMV-Tag2c

The expression vector used for this thesis work is pCMV-Tag2c (4.3 kb), kindly provided by Dr T Kufer with *CARD15/NOD2 wild-type* cDNA (GenBank NM_022162.1) cloning inside. pCMV-Tag2c is an N-terminal FLAG® mammalian expression vector (Fig. 15). The FLAG epitope is a small, highly immunoreactive tag, composed of 8 amino acid residues (DYKDDDDK) and is not likely to interfere with the function of the target protein. A Kozak consensus at N-terminal sequence provides optimal expression of the fusion protein. The neomycin-resistance gene is under control of both the prokaryotic β-lactamase promoter to provide kanamycin resistance in bacteria and the SV40 early promoter to provide G418 resistance in mammalian cells. The multiple cloning site (MCS) of the plasmid allows for a variety of cloning strategies.

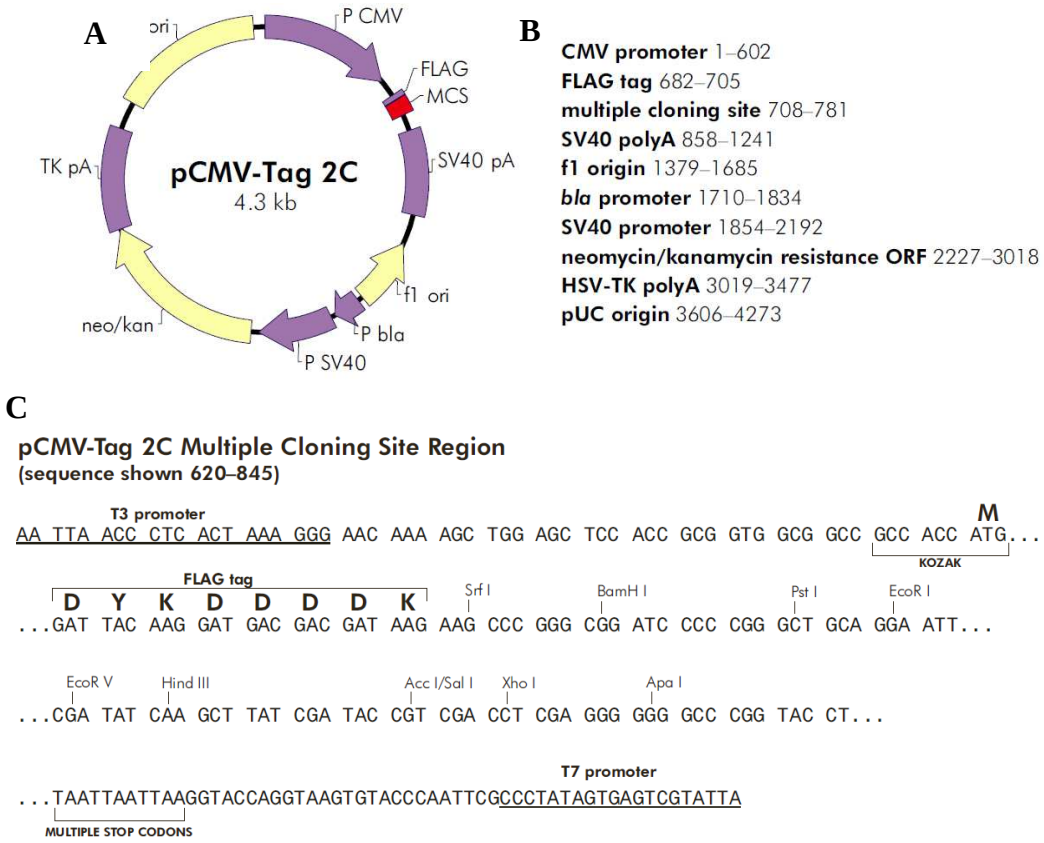


Fig. 15: pCMV-Tag2c plasmid.
A) pCMV-Tag2c plasmid circle map (Sigma); **B)** List of features and their position; **C)** Multiple cloning site region with position of restriction enzymes and FLAG® tag.

1.4.2 One step direct Mutagenesis for p.E383K*CARD15/NOD2*construct

Site-directed mutagenesis is an effective technique for introducing mutations or short deletions into a gene of interest. The important starting step is the design of the mutagenic primers.

Mutagenic primers design

The mutagenic oligonucleotide primers were designed using the following guidelines:

- The mutagenic primers for point substitutions or insertion must contain the desired mutation, whereas the mutagenic primers for deletions must skip the sequence corresponding to the lost oligonucleotides.
- Primers should be between 25 and 45 bases in length, with a melting temperature (*T*m) of ≥78°C. Primers longer than 45 bases may be used, but using longer primers increase the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction. The following formula is commonly used for estimating the *T*m of primers:

  $$T_m = 81.5 + 0.41(\%GC) - {}^{675}/_{N} - \%mismatch\ ,$$

  where *N* is the primer length in bases.

  For calculating *T*m for primers intended to introduce insertions or deletions, there is a modified formula to use:

  $$T_m = 81.5 + 0.41(\%GC) - {}^{675}/_{N}\ ,$$

  where *N* does not include the bases which are being inserted or deleted.
- The desired mutation should be in the middle of the primer with 10-15 bases of correct sequence on both sides.
- The primer optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.
- Primers for insertion or deletions must be purified by polyacrylamide gel electrophoresis (PAGE). Failure to purify the primers results in a significant decrease in mutation efficiency.

One step direct mutagenesis

Site-directed mutagenesis was performed on *CARD15/NOD2 wild-type* - pCMV Tag2c construct, in order to reproduce the mutation p.E383K (c.1147G>A) presents in our BS patients.

The following mutagenic primers were used separately and with different annealing condition (60°C-62°C). This allowed to choose the better primer and temperature for a higher mutagenesis yield.

```
E383K-F: CCTTTGATGGCTTTGACAAGTTCAAGTTCAGGTTC;
E383K-R: GAACCTGAACTTGAACTTGTCAAAGCCATCAAAGG
```

Set up PCR reaction:

Template DNA (327ng/µl stock)	1.1µl
Primer F or R (1µM)	5µl
dNTPs (10mM)	1.5µl
10X Pfu Buffer	2.5µl
Pfupolymerase (Stratagene, 2.5U/µl)	0.5µl
Sterile water	15µl
Final volume	**25µl**

PCR conditions:

Denaturation:	95°C	for 3min
Then 23 cycles of:	62°C	for 30sec
	62°C	for 90sec
	68°C	for 18min

1µl of Dpn I, a methylation-sensitive restriction enzyme, was added to the PCR product and then was incubated for 1-2 hours at 37°C to get rid to the *wild-type* methylated host strand.

1.4.3 Construction of p.R334W *CARD15/NOD2* pCMV-Tag2c plasmid

Digestion

The cDNA of human p.R334W *CARD15/NOD2*was kindly provided by Dr G Núñez (Ann Arbor, MI, USA) cloning into pcDNA3 vector. The mammalian vector pCMV-Tag2c was kindly provided by Dr T Kufer with *CARD15/NOD2 wild-type* cDNA cloning inside. It is necessary to digest both constructs in order to obtain the vector pCMV-Tag2c with compatible ends with the p.R334W *CARD15/NOD2* cDNA. BamHI and XhoI (NEB, UK) are the more suitable couple of restriction enzymes in this case for a double digestion.

Set up restriction reaction:

***CARD15/NOD2 wild-type*- pCMVTag2c**		**p.R334W *CARD15/NOD2* - pcDNA3**	
Construct	15 µg	Construct	15 µg
Buffer3 10X	3 µl	Buffer3 10x	3.5µl
XhoI	3µl	XhoI	3µl
BSA 100X	0.3 µl	BSA 100X	0.35 µl
water	6.7 µl	water	4.15 µl
Final volume	30 µl	Final volume	35µl

- **Digestion O/N at37°C**
- **Stop restriction at 65°C for 20min**
- **Add 3 µl of BamHI**
- **Digestion at 37°C for 4 hours**

Expected bands from digestion of *CARD15/NOD2 wild type*- pCMVTag2c		**Expected bands from digestion of p.R334W *CARD15/NOD2* - pcDNA3**	
4300bp	Linearized vector	5.400 bp	Linearized vector
3200bp	insert	3200 bp	Insert

Purification

A particular good purification of the digestion products is necessary to obtain the higher amount of linearized pCMVTag2c vector and p.R334W *CARD15/NOD2* cDNA insert.

The Wizard® SV Gel and PCR Clean-Up System (Promega) is a membrane-based method designed to extract and purify DNA fragment up to 10kb from standard agarose gels. It is based on the ability of DNA to bind to silica membrane in the presence of chaotropic salts.

Ligation

T4 DNA Ligase (Promega) catalyzes the joining of pCMVTag2c linearized vector and p.R334W *CARD15/NOD2* cDNA between the 5′-phosphateand the 3′-hydroxyl groups of adjacent nucleotides in a cohesive-ended configuration. The following formula is commonly used for estimating the optimal quantity of cDNA to be used:

$$\frac{ng\ of\ vector \times kb\ size\ of\ insert}{kb\ size\ of\ vector} \times molar\ ratio\ of\ {}^{insert}/_{vector} = ng\ of\ insert$$

Set up ligation reaction:

Reagents	**Quantity**	**Reagents**	**Quantity**
Vector pCMVTag2c	180ng	Vector pCMVTag2c	180 ng
p.R334W *CARD15/NOD2* insert	134 ng	p.R334W *CARD15/NOD2* insert	402 ng
Ligase 10X Buffer	1 µl	Ligase 10X Buffer	1 µl
T4 DNA ligase	1.2 µl	T4 DNA ligase	1.2 µl
Nuclease-free Water	5.75 µl	Nuclease-free Water	4.15 µl
Final volume	10 µl	Final volume	10 µl
Ratio insert/vector = 1:1		**Ratio insert/vector = 3:1**	

To increase the efficiency rate of the ligation reaction, I have tested different conditions. I used two molar ration of insert:vector (1:1 and 3:1) and two incubation temperature (4°C and 16°C O/N).

1.4.4 One shot chemical transformation and preparing cultures of bacteria

All the constructs prepared above were cloned in Top10 chemically competent *E.Coli* (Life Technology). To maximise the transfected efficiency, a key parameter is the purity of plasmid DNA. The constructs have to be deprived of phenols, ethanol, proteins and detergents.
The protocol consists of these following steps:

- Thaw on ice one 50µl vial of One Shot® TOP10 cells (Life Technology) for each transformation.
- Add 2µl of plasmid DNA directly into the aliquot of bacteria and mix gently without pipetting.
- Incubate on ice for 30min and then heat-shock the cells for 30sec at 42°C without shaking. The cells are highly sensitive to mechanical lysis caused by pipetting.
- Immediately transfer the tubes to ice and add 250µl of SOC medium previously warmed at room temperature. SOC is a rich medium composed by 2% Tryptone, 0.5% Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose
- Cap the tube tightly and shake the tube (200rpm) at 37°C for 1 hour
- Incubate at 37°C for 30minutes two plates (LB plates containing 50µg/µl ampicillin) for each transformation
- Spread 50µl and 200µl from each transformation on 2 pre-warmed plates. Spreading the plates with different volume ensured that at least one plate would have well-spaced colonies. The bacteria selection medium was LB (Luria-Bertani: 1%Tryptone, 0.5% Yeast extract, 1% NaCl) added with 1.5% Select-Agar and containing 50µg/µl Kanamycin.
- Incubate at 37°C O/N

1.4.5 Purification of plasmid

Wizard® Plus SV Minipreps DNA Purification System (Promega)
This miniprep procedure provides a simple, reliable method for rapid isolation of any plasmid DNA from *E.coli* hosts, most efficiently when the plasmid is less than 20 kb in size. An overnight colture is harvested with centrifugation and subjected to a modified alkaline-SDS lysis procedure followed by adsorption of the plasmid DNA onto silica in the presence of high salts. Contaminants are then removed by a spin wash step. Finally, the bound plasmid DNA is eluted in water or Tris-EDTA buffer.
The recovered plasmid DNA, predominately in its supercoleid form, is checked in a 1% agarose gel.

PureYield™ Plasmid Midiprep System (Promega)
The system provides a rapid method for purification using a silica-membrane column. The bacterial cells are lysed by a set of buffers based on the NaOH/SDS lysis method. After equilibration of the column together with the corresponding column filter, the entire lysate is loaded by gravity flow and simultaneously cleared by the equilibrated filter. Plasmid DNA is bound to the silica resin and after a washing step is eluted, precipitated and dissolved in a suitable buffer for further use. From 50ml of an overnight recombinant *E.coli* culture is possible to isolate more than 50µg of high copy plasmid DNA.
The recovered plasmid DNA has to be checked in a 1% agarose gel.

1.4.6 Sequencing of the final constructs

Each plasmids were verified for unwanted variations both in the insert sequence and in the functional region of the plasmid (CMV promoter and FLAG). The three final constructs have been sequenced using the primers listed below.
The primer CARD15_vect1R verified the accuracy of the FLAG, the promoter CMV and BamHI restriction site. The primer CARD15_vect9F identified the accuracy of the XhoI, whereas the primer CARD15_vect4F showed the two mutations inserted in *CARD15/NOD2*.
For the sequencing, we used the service provided by the BMR genomics and prepared 200ng of each plasmid DNA and 1µl of each primers.

SEQUENCING PRIMERs

INSERT SEQUENCE		**FUNCTIONAL REGIONS OF pCMVTag2c VECTOR**
CARD15_vect1F	CARD15_vect6F	CARD15_vect1R
CACGATGAGGAGGAAAGAGC	TGCTGCTACGTGTTCTCAGC	TGGAAGCCCTCGTAGTCCTC
CARD15_vect1R	CARD15_vect7F	CARD15_vect9F
TGGAAGCCCTCGTAGTCCTC	CAGATCACAGCAGCCTTCCT	TCCGAGGCAACACCTCCTTG
CARD15_vect2F	CARD15_vect8F	E7901CMV-F
TACTTGGGCCTGTCAGAAGC	GAGTGTGCTGCCCTGGCCTT	GCCATTGCATACGTTGTATC
CARD15_vect3F	CARD15_vect9F	
GCTGCCACATGCAAGAAGTA	TCCGAGGCAACACCTCCTTG	
CARD15_vect4F	CARD15_vect10F	
CAGTGGCAAGAGCACGCTCC	CTGAAGTTGTCCAATAACTG	
CARD15_vect5F		
CATCCGCACCGAGTTCAACCT		

1.5 *In vitro* experiments on transfected HEK293 cells

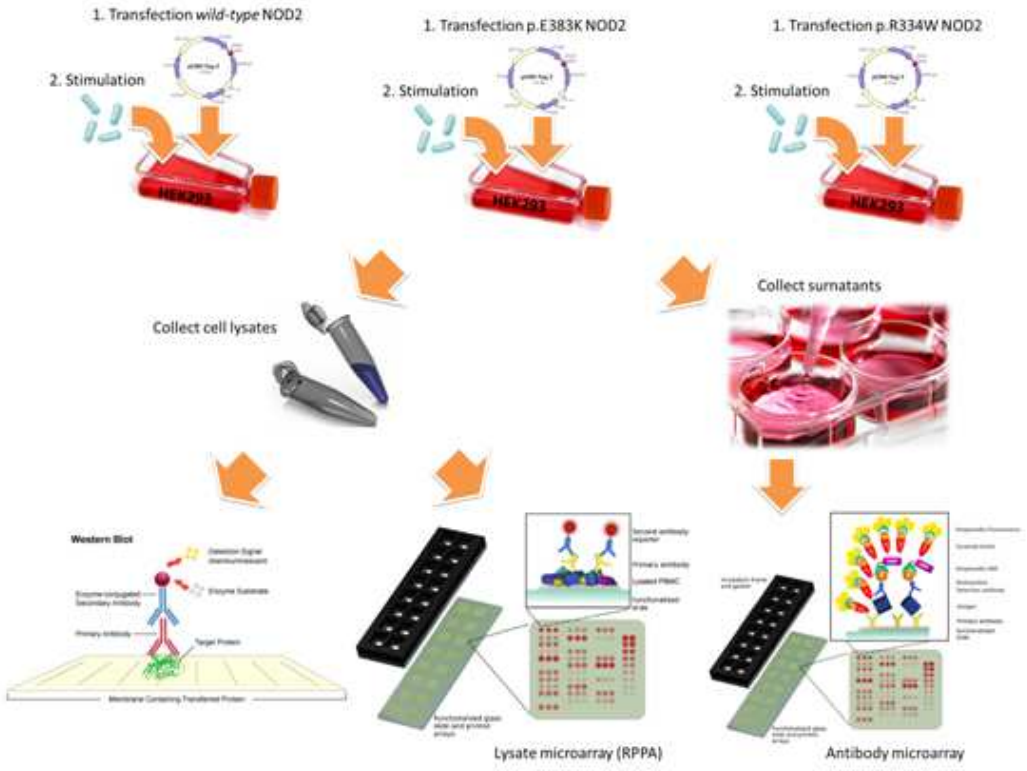


Fig. 16: Main procedures in *in vitro* studies on transfected cells.
Both microarray techniques are explained in the microarray section.

1.5.1 HEK293 cell cultures

The HEK293 cell line, derived from human embryonic kidney cells, has been extensively used as an expression tool for recombinant proteins. Although of epithelial origin, its biochemical machinery is capable of carrying out most of the post-translational folding and processing required to generate functional, mature protein [209]. The principal characteristics of these cells are their quick and easy reproduction and maintenance, amenability and high efficiency of transfection using a wide variety of methods. These, and other attributes, also mean that biochemical and cell biological evaluations of expressed proteins can be performed in concert with functional analyses.
HEK293 cells grow adherent in T75 flasks at 37°C in an atmosphere of 95% air/5% CO_2 and remain biochemically active at 50-70% confluency. The cells are expected to double approximately every 12 hours. This cell line requires complete DMEM High glucose 90% medium (Life Technology) supplemented with Gentamycin (final concentration 0.1%), 10%FCS and 2% L-glutamine.

1.5.2 Transfection

The ability to generate HEK cell phenotypes by the introduction of exogenous nucleic acid depends on a range of transfection techniques. *Lipofectamine™ 2000* (Life Technologies) represents one of the more recent cationic lipid encapsulation reagents which deliver DNA to the cells by fusion and incorporation of lipid microsomes into the cells' lipid bilayer.
After the transfection, the cells are able to express human *wild-type* or mutated (p.R334W or p.E383K) *CARD15/NOD2* gene. These exogenous proteins should be recognized by the N-terminal FLAG sequence.

Preparing cultures
HEK293 cells were seeded in 6-well plates incomplete DMEM without Gentamycin. The density of cells must be sufficient to achieve 70% confluence at the time of transfection.

Transfection technique
Each construct was transfected in triplicate, increasing the likelihood of positive transfected cells.
The following protocol is to intend for single well:

- Prepare for each construct two dilutions: one with 0.4µg/µl of plasmid DNA in a final volume of 250µl of DMEM reduced serum medium, the other with 10µl of Lipofectamine™ 2000 in 240µl of DMEM reduced serum medium.
- Add the diluted plasmid DNA to the diluted Lipofectamine™ 2000, mix and incubate for 20min at room temperature
- In the meantime, discard the medium in 6-well plate and add DMEM reduced serum medium
- Add the mixing DNA/Lipofectamine drop by drop and incubate at 37°C for 6hours
- Discard the medium and add complete DMEM medium to increase the proliferative growth.
- After 48hours post-transfection, the positive HEK293 cells were undergone to selection with Geneticin G418 [0.8 mg/ml]
- The three transfected cell lines are maintained in selection, let expanding in T75 flasks and froze.

1.5.3 Localization of NOD2

The aim of this experiment is to localize in the transfected HEK293 cells the *wild-type* and the two mutated NOD2 protein.

Preparing cultures

Each transfected cell cultures were seeded in 6-well plates in duplicate and allowed to growth in ethanol flame-cleaned coverslips coated with poly-l-lysine solution (Sigma Aldrich). The working density of cells was around 70% confluence. Before proceeding with the immunofluorescence assay, it is necessary to fix and permeabilize the cells to ensure free access of the antibody to its antigen. Perfect fixation would immobilize the antigens, while retaining authentic cellular and subcellular architecture and permitting unhindered access of antibodies to all cells and subcellular compartments. Fixation with Methanol/Acetone would also permeabilize the cells, removing lipids and creating holes in membrane.

- Discard the medium and wash each well three times with PBS
- Add 2ml of Methanol/Acetone 1:1 and incubate at -20°C for 20min
- Discard the fixative solution, cover and store at -20°C

Immunostaining

Each transfected and fixated cell cultures were undergone to indirect immunofluorescence, using a specific primary antibody against N-terminal FLAG sequence of exogenous NOD2.

- Hydrate each well with PBS for 10min at room temperature
- To avoid aspecific binding, each coverglass is treated with PBS+1%BSA for 1hour at room temperature
- Wash twice with PBS for 5min each
- Pour drop by drop 100µl of primary antibody ANTI-FLAG (Sigma Aldrich) at 1:300 in PBS+1%BSA and incubate O/N at 4°C in humid atmosphere.
- Wash 3 times with PBS for 5min each at room temperature
- Pour drop by drop 100µl of secondary antibody *Cy3-conjugated Anti-Rabbit* (Listarfish) at 1:400 in PBS+1%BSA and incubate at room temperature for 1hour in the dark.
- Wash 3 times with PBS for 5min each at room temperature
- Mounting each coverslip in a ethanol flame-cleaned slide with mounting solution containg DAPI at 1:10,000.
- Store the slides at 4°C in the dark

Confocal Imaging

Slides were inspected and photographed using first an optical fluorescence microscope (Leika 5000) and then a confocal fluorescent microscope (Radiance 2000, BioRad) using multitracking (line switching) for two-colours imaging (40X). Image acquisition was performed with Laser Sharp Scanning software (Bio-Rad Laboratories). The fluorescence microscope was used to visualize fluorescent tagged proteins and blue-fluorescent nuclei, whereas confocal fluorescent microscopy was used to accentuate the 3D nature of the samples.

1.5.4 Proteins expression

The aim of these experiments is to evaluate the expression of *wild-type* and mutated (p.R334W and p.E383K) NOD2 proteins after exposition to relevant pathogen-associated molecular patterns (PAMPs). Another goal is to investigate the NF-κB pathway activation through indirect evaluation of phosphorylation of NF-κB inhibitor, IKBα.

Stimulation and lysis of cells

A selection of PAMPs were used as stimuli to trigger the inflammatory response associated to NOD2. The pathogen-associated molecular patterns (PAMPs) are a diverse set of microbial molecules which share a number of different recognizable biochemical features that alert the organism to intruding pathogens [210]. In literature, NOD2 is described as a general detector of intracellular invasive bacterial infections, sensing small peptides such as muramyl dipeptide (MDP), a degradation product from both Gram-positive and Gram-negative bacterial peptidoglycan [64].

- The three transfected HEK293 cell lines are seeded in duplicate at 10^5 cell/ml in 6 well-plate and grown in complete DMEM medium.
- One day before stimulation, the culture medium was replaced by starvation medium, i.e. serum free DMEM medium. The goal of starvation is to stop the cellular proliferation in order to synchronize cell cycle and reduce unwanted signalling effects.

The experimental condition used was the same to test different hypotesis:

- different concentrations of MDP, to test the sensitivity of the cells;
- different concentrations of MDP, to observe the consequences of NF-κB signalling pathway.

For the "sensitivity" experiment and also cytokines detection:

- Add drop by drop different concentrations MDP [0ng/ml, 1µg/ml, 10µg/ml, 50µg/ml, 100µg/ml]
- Stimulate for 7hours or 24hours at 37°C
- At the proper time, collect the surnatants, lyse the cells and store at -80°C

For the NF- κB pathway activation experiment:

- Stimulate with MDP [0ng/ml, 1µg/ml, 10µg/ml] and collect surnatants and lysated cells after 7 or 24hours

The lysis protocol followed these steps:

- Wash the cells twice with ice-cold PBS
- Add 200µl of Lysis Buffer, composed by 1X RIPA buffer an 1X Phosphatase and protease inhibitor (Thermo Scientific) and 5U/ml Benzonase (Sigma Aldrich).
- Keep the plate on ice for 20min shaking frequently
- Scrape each well and collect the lysates in pre-chilled tubes
- Centrifuge at 16,000×*g* for 10min at 4°C
- Store the surnatants at -80°C

BCA assay

To assess yields in whole cell lysates, the BCA assay (Thermo Scientific) measures the absorbance at 562nm of total protein compared to a protein standard. The method relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the protein present in the lysated samples.

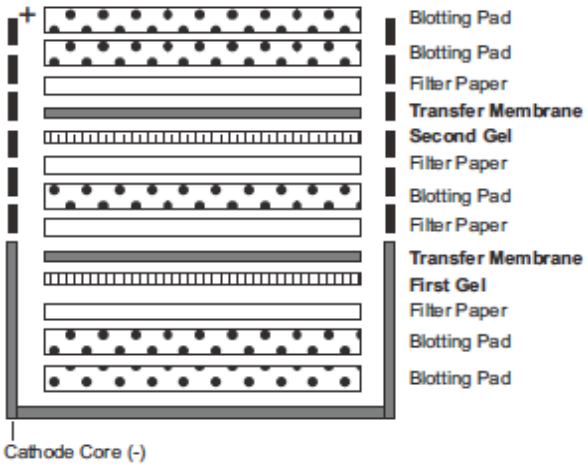
Protein detection (electrophoresis and Western blotting)

Separation of proteins was achieved through an SDS-PAGE gel according to the Laemmli method [211], whereas detection of the proteins of interest was achieved by ECL Western blotting. This technique is a light emitting non-radioactive method performed by the oxidation of luminol by the HRP in the presence of phenols.

- Prepare samples adding 1.3µg/µl of lysates in 20µl of 1X SDS loading buffer (SDS and 1X β-mercaptoethanol)
- Add Blue Bromophenol powder and heatthe samples at 95°C for 5min
- Place the gel into Western tank. The wells facing inwards with blank on other side if only running one gel.
- Load 30µl into each well of the 4-20% precast Tris glycine gel (Novex, Life Technology)
- Load 5µl in the first well of Spectra™ Multicolour Broad Range Protein Ladder (Thermo Scientist) to check the transfer from gel to membrane
- Load 5µl in the last well of MagicMark™ XP Western Protein Standard (Life Technologies) specifically designed for ECL detection
- Gels were run at 150V for 90min in 1X running buffer (Tris/Glycine/SDS buffer, BioRad) using a XCellSureLockTM Mini-Cell and Novex Power Ease 500 Power Supply (Life Technologies)

After electrophoresis, proteins were transfected to nitrocellulose membrane by wet transfer and detected by Chemiluminescence with the specific primary and secondary HRP labelled antibodies listed in Tab. 3.

- Soak blotting papers, sponges and nitrocellulose membrane in 1X transfer buffer (Tris/Glycine buffer, 20% Methanol)
- Prepare the blotter as the follow diagram:



- Protein transfection took place under 125mA for 2hours
- Block non-specific binding site soaking the membranes in 25ml of blocking buffer (5% non-fat dried milk, 0.1% v/v Tween20 in TBS) for 1hour at room temperature on a shaker
- Rinse the membranes 3 times for 5min each in 15ml TBS-Tween20
- Incubate the membranes with 10ml primary antibodies diluted 1:1,000 in 5%BSA/TBS/Tween20 O/N at 4°C with shaking.
- Wash the membranes 3 times for 5min each in 15ml TBS-Tween20
- Incubate the membranes with 10ml secondary Horseradish peroxidase conjugate antibodies (1:2,000) for 1hour at room temperature on shaking
- Wash the membranes for 5min in 15ml TBS-Tween20
- Incubate the membranes with 4ml Enhanced Chemiluminescence (ECL) Detection Reagent (GE healthcare) for 1min
- In a film case, overlay the enveloped membranes with a sheet of Kodak film for different exposure time (5sec to 30min)
- Soak the film in Developer solution (Sigma Aldrich), fix in Fixer solution (Sigma Aldrich), wash in water and dry
- ImageJ software was used to measure the densities of the resulting bands.

PRIMARY ANTIBODIES			**SECONDARY HRP CONJUGATED ANTIBODIES**		
ANTI-NOD2	1:250	eBioscience	Anti-rabbit HRP conjugated	1:2,000	Dako
ANTI-IKBα	1:1,000	Cell Signaling	Anti-mouse HRP conjugated	1:2,000	Dako
ANTI-P IKBα	1:1,000	Cell Signaling			

Tab. 3: List of primary and secondary antibodies used for Western Blotting.
The ANTI-NOD2 was a mouse antibody, whereas ANTI-IKBα and ANTI-P IKBα were rabbit antibodies.

Following ECL detection, it is possible to complete remove the primary and secondary antibodies from the membranes to sequential reprobing of membrane several times.

- Submerge the membrane in Stripping buffer (100mM β-Mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH 6.7) and incubate at 50°C for30 min with occasional agitation.
- Wash the membrane twice for 10min each in TBS-Tween20 at room temperature.
- Block the membrane with Blocking buffer (5% non-fat dried milk in TBS-Tween20) for 1 hour at room temperature.
- Repeat the immuno detection protocol, adding primary and secondary antibody as above

2 Microarray experiments

2.1 Samples

The microarray-based proteomic technologies described in this chapter have employed different kind of samples: lysated peripheral blood mononuclear cells (PBMCs) and sera from subjects affected by autoinflammatory diseases; lysates and surnatants from *wild-type* and mutated NOD2-transfected cells.
Concerning the transfected cells, most of the samples were described in the previous section. In addition, the transfected HEK293 cells underwent to another lysis experiment. The NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Thermo Scientific) enabled stepwise separation and preparation of cytoplasmic and nuclear extracts from cultured cells. Nuclear extracts are generally preferred to whole cell lysates for gene regulation studies.
After stimulation of *wild-type* and mutated (p.R334W and p.E383K) cells with MDP [10µg/ml] for 7hours, the cultures were trypsinized and cellular pellet was collected as dry as possible. Following the company protocol's steps, the addition of Cytoplasmic Extraction Reagents allows to obtain the cytoplasmic fraction in surnatants. Further processing of obtained pellet with ice-cold Nuclear Extractio Reagent concentrates the nuclear fraction in surnatant. Both extracts are then stored at -80°C.
Besides the transfected cells, the other samples used for the microarray experiments were PBMCs and sera from 40 patients affected by different autoinflammatory diseases such as FMF, TRAPS, MVK, Blau syndrome, Still's disease, Behçet disease, CAPS. All patients underwent genetic screening for mutations on the genes involved in periodic fevers, inflammasome-related diseases and Blau syndrome, using standardised techniques. In addition to the patients, 27 samples from healthy controls subjects were collected.

Tab. 4 summarizes all observed mutations and polymorphisms, therapy and other information useful to depict each patient.
Single paired samples of blood were collected from patients and healthy controls on approval of the Hospital of Padova Ethics Committee for Clinical Research. Sera were obtained by centrifugation of blood samples in a single-clot tube at 400×*g* for 10min. PBMCs were isolated by Ficoll-density gradient separation, as describe previously but then the cells were lysated and stored as in the follow protocol to be ready for microarray assays.

- Centrifuge the PBMCs at 400×*g* for 10min at room temperature.
- Pour off the supernatant and wash the pellet with PBS. Centrifuge cells at 400×g for 10 minutes at room temperature.
- Pour off the supernatant and add 100µl of lysis buffer (RIPA Buffer, 1X Protease Phosphatase Inhibitors 100X EDTA Free, 5U/ml Benzonase).
- Incubate the tube in ice and vortex for 15sec every 5min, for a total of 30min.
- Centrifuge the tubes at 10,000×*g* for 10min at 4°C.
- Aliquot 30µl of lysated in eppendorf tubes and add 10µl of 4XSDS solution (0.25% M TrisHCl ph 6.8, 30% Glycerol, 8% SDS and 10% β-mercaptoethanol)
- Boil at 95°C for 5min then spin the tubes.
- Store the tubes at -80°C.

Tab. 4: Database of 40 enrolled patients (A) and healthy controls (B) used for microarray experiments presented in this thesis.
A. Classification of patients. Subjects from P1 to P29 refer to UOC Reumatologia-University of Padua, whereas subjects from P30 to P40 belong to the Research Centre of Autoimmune and autoinflammatory diseases-Siena

N.	SEX	AGE	DISEASE	GENE	MUTATIONS	POLYMORPHISMS	TREATMENTS
P1	M	19/12/1969	Behçet	MVK	/	c.78+61 A>G, c.510C>T, c.632-18A>G, c.885+24G>A	Cyclosporine 200mg/day, Colchicine 1g/day
P2	M	22/07/1988	Still	MVK	S52N (hetero)		Kineret 100mg/day
P3	F	15/10/1974	Still	MVK	/	c.371+8C>T, c.632-18A>G	Colchicine 1g/day
P4	F	27/04/1980	Still	MVK	/	c.371+8C>T, c.632-18A>G	Methotrexate
P5	M	02/05/1942	TRAPS	TNFRSF1A	S59P (hetero)		Methyprednisolone 4mg
P6	F	04/01/1982	FMF	MEFV	E148Q (hetero)		Colchicine until 2 years ago
P7	F	25/04/1967	Behçet	MVK, TNFRSF1A	/	MVK: c78+61A>G, c632-18 A>G, c.885+24 G>A TNFRSF1A: c.473-33 C>T, c.625+10 A>G	Colchicine
P8	F	15/11/1980	Behçet	negative for MVK			Kineret
P9	F	29/04/1953	Blau	NOD2	E383K (hetero)		Humira 40mg for 14 days
P10	F	30/03/1978	Blau	NOD2	E383K (hetero)		None
P11	F	19/09/1964	FMF	MEFV,MVK, TNFRSF1A	MEFV: E148Q, M680I MVK: S52N	MEFV: R314R, E474E, D510D, Q474Q MVK: D170D, c.632-18A>G, c.885+24G>A TNFRSF1A: c.473-33C>T, c.625+10C>T	Colchicine 1mg/day
P12	M	14/10/1974	FMF	MEFV, TNFRSF1A	MEFV: R202Q	MEFV: G138G, A165A, E474E, D510D, Q474Q TNFRSF1A: c.473-33C>T	Colchicine
P13	M	04/04/1972	Still	TNFRSF1A	/	c.625+10C>T	None
P14	M	01/11/1951	Still	TNFRSF1A	/	c.625+10C>T	prednisolone 2,5 mg/day

P15	F	01/06/1985	BEHÇET	MVK	/	D170D, C.632-18A>G, C.885+24G>A	STEROIDS ON DEMAND
P16	F	20/07/1993	FMF	MEFV, MVK	/	MEFV: R314R MVK: D170D, c.632-18A>G, c.885+24G>A	None
P17	F	01/07/1966	TRAPS	MEFV, TNFRSF1A, MVK	R92Q (hetero)	MEFV: D102D, G138G, A165A TNFRSF1A: c.625+10A>G MVK: c.885+24G>A	Infliximab
P18	F	16/09/1975	Schnitzel	No screening			Kineret
P19	F	13/08/1967	Still	No screening			Kineret
P20	F	04/06/1968	Still	No screening			Kineret
P21	F	31/05/1978	FMF	MEFV, TNFRSF1A	MEFV: E148Q, P369S(hetero)	TNFRSF1A: c.625+10A>G	Colchicine
P22	M	10/04/1990	TRAPS	MVK, TNFRSF1A	/	MEFV: R314R; TRAPS: c.625+10A>G, c.626-32G>T	None
P23	F	15/11/1980	Behçet	negative for MVK			Kineret
P24	M	21/08/1985	TRAPS+ Behçet	MEFV, TNFRSF1A, MVK	/	MEFV: D102D, G138G,A165A, R202Q, R314R TNFRSF1A: c.625+10A>G MVK: c.885+24G>A	Colchicine 1g/day
P25	F	01/06/1985	Behçet	MVK		D170D, c.632-18A>G, c.885+24G>A	Steroids on demand
P26	M	02/05/1955	Behçet +FMF	MVK, TNFRSF1A	/	MEFV: D102D, G138G,A165A, R202Q, R314R TNFRSF1A: c.625+10A>G	Colchicine
P27	F	25/04/1967	Behçet	MVK, TNFR1A	/	MVK: c78+61A>G, c632-18 A>G, c.885+24 G>A TNFRSF1A: c.473-33 C>T,c.625+10 A>G	Colchicine
P28	F	26/06/1987	Behçet	No screening			None
P29	F	16/01/1948	Still	MEFV, TNFRSF1A, MVK	/	MEFV: D102D,R314R,D723D TNFRSF1A: c.473-33C>T MVK: D170D, c.632-18A>G, c.885+24G>A	Anti-IL1ra

P30	M	28/06/1998	MKD	MVK	V377I (HOMO)	N.P.	UNKNOWN
P31	M	10/03/1976	FMF	MEFV	M694I (hetero)	n.p.	None
P32	F	06/04/1976	TRAPS	TNFRSF1A	V95M (hetero)	n.p.	Canakinunab 150mg/8weeks
P33	M	31/07/1978	TRAPS	TNFRSF1A	delY103-R104	n.p.	Etanercept 50mg/week
P34	M	02/10/1974	NAPS12	NLRP12	G448A (hetero)	n.p.	None
P35	M	07/12/1980	TRAPS	TNFRSF1A	T50M (hetero)		None
P36	F	31/07/1958	TRAPS	TNFRSF1A	R104Q (hetero)	n.p.	Anakinra 100mg/day
P37	M	03/12/1971	TRAPS	TNFRSF1A	T50M (hetero)	n.p.	Anakinra 100mg/day
P38	M	31/07/1978	TRAPS	TNFRSF1A	R92Q (hetero)	n.p.	None
P39	F	01/01/1994	TRAPS	TNFRSF1A	D12E (hetero)	n.p.	Anakinra 100mg/day
P40	F	17/08/1956	TRAPS	TNFRSF1A	R92Q (hetero)	n.p.	Anakinra 100mg/day

B. Classification of healthy controls.

N.	SEX	AGE	N.	SEX	AGE	N.	SEX	AGE	N.	SEX	AGE
1	F	25	8	F	47	15	F	39	H4	M	35
2	F	41	9	F	55	16	M	41	H5	M	39
3	F	30	10	F	32	17	F	33	H6	M	33
4	F	32	11	M	24	18	F	29	H7	F	55
5	M	30	12	F	53	H1	M	15	H8	M	42
6	M	46	13	M	34	H2	M	37	H9	M	30
7	F	46	14	F	32	H3	F	37			

2.2 Reverse Phase Protein Array

Reverse Phase Protein Array (RPPA) technique was optimized and used to study different signalling pathways in transfected HEK293 and in PBMC of patients in comparison to healthy controls. Fig. 17 shows the principles of this assay.

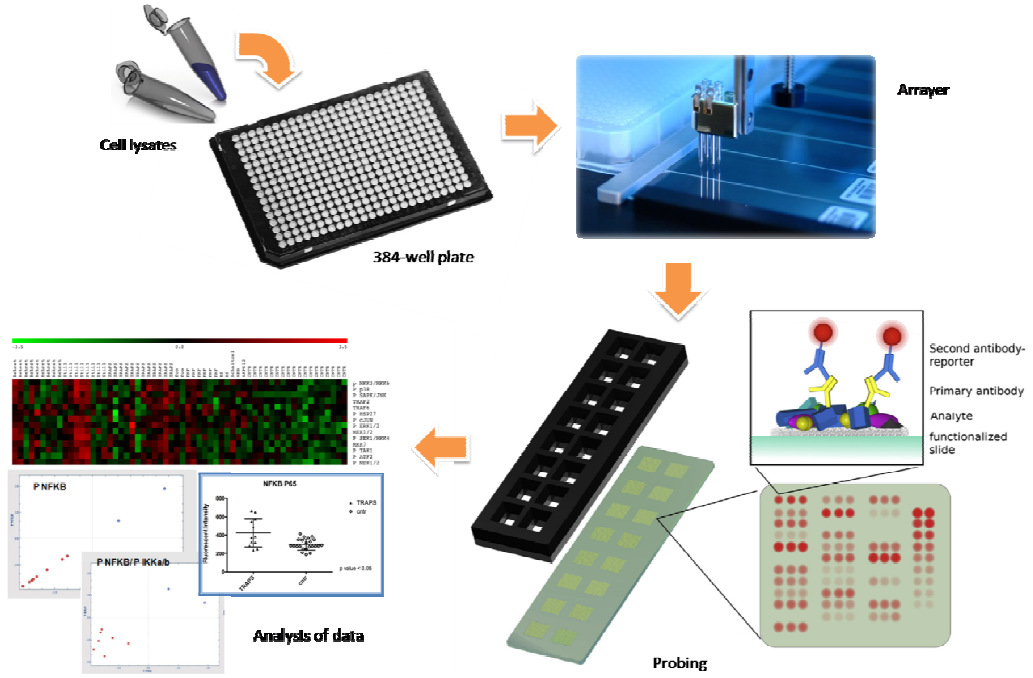


Fig. 17: Principles of RPPA microarray.
Lysated cells were prepared in 384-well plates in SDS sample and spotted on the nitrocellulose slide by a MicroGrid II robot. The slides were probed with Immunohistochemical staining and protein expressions were calculated using reverse phase protein analizer (RPPanalizer). The data were graphically presented as heatmaps, scatter plot or cluster density maps.

2.2.1 Protocol

The cells lysates were loaded into a 384-well plate (Genetix, UK) and serially diluted 3 times in 1X SDS buffer (4XSDS diluted in lysis buffer, previously described).
Normalization of the protein of interest was achieved through the detection of an housekeeping protein. β-actin is expected to be expressed at a constant level, unaffected by the experimental conditions. Using the near-infrared detection technique, it was possible to incubate antibody against β-actin from different species together with the target specific antibodies and detect these at a different wavelength. Using this approach, the normalizer signal and the target specific signal were generated from the same spot (Fig. 18). All infrared dye (IRD) antibodies were purchased from LICOR Bioscience.

Printing sample

Samples were spotted onto nitrocellulose-coated glass slides (FASTSlides, Whatman, Schleicher&Schuell) with a microarrayer (MicroGridII, BioRobotics Inc.) using silicon pins. Before starting up printing the slides, the arrayer was set as following:

- Replace the main wash station with clean distilled water.
- Clean the silicon pin heating in the oxidizing flame and wash twice in the wash station
- Edit the array pattern, the pitch between spots and the format
- Adjust the target height at 0.5mm (distance the pin moves towards the slide) and the dwell time at 0.5sec (time the pin pauses on the slide).
- Optimise the humidity of the arrayer to 60% to prevent samples evaporation from the plate and the pin

Probing slides

Make sure all reagents and chemicals up to room temperature

- Place the printed slides in slide holders, ensuring each nitrocellulose pad fit in the holder.
- Block the slides adding in each pad 200µl of I-Block buffer prepared dissolving 50mg I-block powder (Applied Biosystem) in 25ml of PBS-0.01%Tween 20
- Cover the slides to prevent evaporation and leave O/N at 4°C with constant rocking.
- Prepare 200µl dilution of each antibody (see Antibodies list in Tab. 5) in antibody Diluent (Dako) with 1:1,000 mouse monoclonal anti-Actin antibody (Sigma Aldrich)
- Discard the I-block buffer and wash 5 times for 3min each with 1X TBS-0.01%Tween20 with constant rocking
- Add each antibody dilution in the correct pad and cover with foil to avoid evaporation
- Leave the slides O/N at 4°C with constant rocking
- Prepare dilution of infrared secondary antibodies anti-rabbit and anti-mouse 1:5,000 in 1X TBS-0.01%Tween20
- Wash the slides 5 times for 3min each with 1X TBS-0.01%Tween20 with constant rocking
- Add 200µl of the diluted secondary antibodies in each pad
- Incubate for 30min at room temperature with constant rocking and foil cover
- Wash the slides 5 times for 3min each with 1X TBS-0.01%Tween20 with constant rocking
- Disassemble the slide holders and rinse the slides with ultra pure water
- Dry the slides by centrifugation at 500×*g* for 5min
- Scan the slides with an Near-Infrared Odyssey scanner (LI-COR, Biosciences) with high resolution (21µm) in both channels 700nm and

800nm, for detection of anti-mouse and anti-rabbit secondary antibodies respectively.

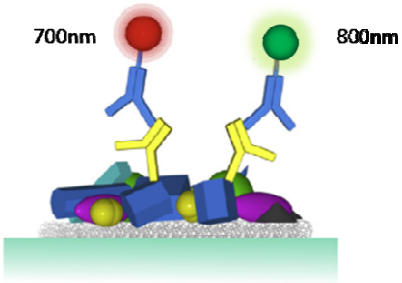


Fig. 18: Near Infrared detection of signals.
The arrayed slide was incubated with primary antibody followed by secondary antibodies labelled with infrared dye against β-actin (red colour at 700nm) or target proteins (green colour at 800nm).

Analysis of microarray data

The RPPA data analysis consists of calculating the concentration of each samples, after background correction and normalization, using signal intensities data gained from a image software. Axon GenePix Pro-6 Microarray Image Analysis software was the bioinformatics tool used to determine the raw signal intensities of the spots generating gpr files. RPPanalyzer, was the module designed for the R statistical language that was developed by Mannsperger and colleagues [212] to read RPPA data and to perform the basic analysis on them. It is open-source and freely available as an R-package on the CRAN platform (http://cran.r-project.org/).

- After scanning the slides with LICOR Odyssey, save the image as a16-bit gray scale TIFF file
- Import in GenePix Pro software each scanned image and the gal file saved from the arrayer. This file gave specific information about the location, size, and name of each spot on the slide
- Combine together those files to obtain the gpr files. These tab delimited text files contain the localization, an identification variable of the samples on the array and a fluorescence intensity that represents the raw expression values.
- Prepare two additional sources of information. The sample description file holds all pheno-data of the individual samples, describing the corresponding position in the source well plate. The slide description file, containing the description of the array features and the information of the antibodies used, represents the feature data of the experiment and provides the key to link the corresponding GPR files.
- Import data in R, open-sorce platform for statistical and bioinformatics method exchange [213],and generate a list (R-object) of four components. The first element contains a matrix of foreground signals (pixel intensities per spot) and the second element corresponds to local background. The

third and fourth elements contain the feature and phenotypic information respectively.

- Correct the background using the subtract method.
- Calculate the relative protein concentration by calculating the median of the replicate spots at the highest concentration if the sample dilution series showed linear correlation with the total protein concentration [214].
- Normalize the data to correct differences of total protein concentration on the spot which resulted from preparation and spotting of the samples. The level of each antibody signal was adjusted to the housekeeping protein signal (β-actin) from the same spot. That means every individual spot had its own normaliser signal, providing the most accurate data.

The data, exported in excel files, could be visualized and undergone to other statistical analysis. Their significance was tested using non-parametric analysis and graphed with GraphPad Prisms5 software. P value was considered significant if it is less than 0.05. Column and scatter graphs were used to visualize the distribution of the data points in order to assess differential expression of proteins in comparison to a control group. Heatmaps were another tool to visualize the protein expression and show structures of the data with the use of a hierarchical clustering. To obtain heatmaps, the normalized data need extra manipulations, such as be transformed in log2 and adjusted for technical variability inside an array using the dye effect correction. To create and analyse heatmaps, in this thesis I used both MeV software and J-Express Pro software. MeV software is a tool for data analysis that provides an intuitive graphical interface for clustering, classification and statistical tests. J-Express Pro is the best choice for clustering analysis, in particular supervised cluster analysis and self-organizing maps.

2.2.2 Antibodies

Testing primary antibodies:

In reverse phase protein arrays, the primary antibodies used for the detection of specific antigen in the printed lysates should be tested before use, to ensure that each antibody provides a defined single specific signal band. In this study the primary antibodies were tested by Western Blot technique.

- Mix together lysates of different cell lines under different treatment conditions
- Prepare loading samples adding mixing lysates in 1X SDS loading buffer (SDS and 1X β-mercaptoethanol) at 1:4 dilution
- Add Blue Bromophenol powder and heatthe loading samples at 95°C for 5min
- Load 200µl into in the single well of a precast 12% Tris Glycine Gel (Novex, Life Technologies)

- Run to separate the proteins and transfer to a nitrocellulose membrane as describe previously (1.5.3)
- Block non-specific binding site soaking the membranes in 25ml of blocking buffer (5% non-fat dried milk, 0.1% v/v Tween20 in TBS) for 1hour at room temperature on a shaker
- Rinse the membranes 3 times for 5min each in 15ml TBS-Tween20
- Insert the membrane in a blotter (Miniblotter, Immunetics)
- Add 200µl primary antibodies in each separate strip chambers created by the blotter.
- Dilute the primary antibodies 1:1,000 in 5%BSA/TBS/Tween20 O/N at 4°C with shaking.
- Wash the membranes 3 times for 5min each in 15ml TBS-Tween20
- Detection of the bands by Chemiluminescence (ECL) technique as described before.

Tab. 5: List of Primary and Secondary Antibodies used in RPPA experiments.
(*) All the listed Primary antibodies were produced in Rabbit except IL1β produced in Mouse. The Primary antibodies were purchased from Cell Signaling Technology® (next page).

MAPK PATHWAY	
Antibody	**Dilution**
p MKK3/MKK6 (Ser189/Ser207)	1;100
p P38 MAPK (Thr180/Tyr182)	1;1,000
p SAPK/JNK (Thr183/Tyr185)	1;1,000
TRAF2	1;500
TRAF6	1;500
p HSP27 (Ser82)	1;50
p c-JUN (Ser63)	1;200
p ERK1/2 (Thr202/Tyr204)	1;2,000
MEK1/2	1;250
p MEK1/2 (Ser217/Ser221)	1:250
p SEK1/MKK4 (Ser257/Thr261)	1;250
MKK7	1;100
p TAK1 (Ser412)	1;100
p ATF2 (Thr71)	1:250

SRC/JAK/STAT3 PATHWAY	
Antibody	**Dilution**
p STAT3 (Tyr705)	1;100
p c-SRC (Tyr416)	1;10,000
SOCS3	1;500
SHIP2	1;2,000
p RIP2 (Ser176)	1;500
p JAK2 (Tyr1007)	1;1,000

NF-KB PATHWAY	
Antibody	**Dilution**
NF-κB	1:250
p NF-κB p65 (Ser536)	1:100
IKBα	1:100
p IKBα (Ser32)	1:500
IKKα	1:250
IKKβ	1:250
p IKKα/β (Ser176/Ser177)	1:250
IKKγ	1:250
p IRAK1 (Thr209)	1:3,000
A20/TNFAIP3	1:150

INFLAMMASOME PATHWAY and MyD88	
Antibody	**Dilution**
IL1β (*)	1;100
p STAT1	1;250
p STAT4	1;100
ASC (TMS1)	1:150
CASPASE1	1;250
CLEAVED CASPASE1	1;50
NALP1	1;150
BCL-XL	1;250
BCL-2	1;250
p BCL-2	1;100
MyD88	1:50

PI3/AKT PATHWAY	
Antibody	**Dilution**
p eNOS (Ser1177)	1;500
p BAD (Ser136)	1;500
p AKT (Thr308)	1;50
p AKT (Ser473)	1;25
PI3K p100α	1;250
PI3K p85	1;250
p PDK1 (Ser259)	1;1,000
p GSK 3β (Ser9)	1;500
p c-RAF (Ser259)	1;1,000
p PTEN (Ser380)	1;1,000

HOUSEKEEPING ANTIBODIES	
Antibody	**Dilution**
Actin (Mouse)	1:1,000
Actin (Rabbit)	1:1,000

SECONDARY IRD ANTIBODIES	
Antibody	**Dilution**
Infrared Mouse IgG	1:5,,
Infrared Rabbit IgG	1:5,000

2.3 Antibody Array

The antibody microarray is an array-based system to measure cytokines and other serum proteins levels from transfected cells surnatants and sera from both autoinflammatory diseases patients and healthy controls.
This technique is summarized in Fig. 19.

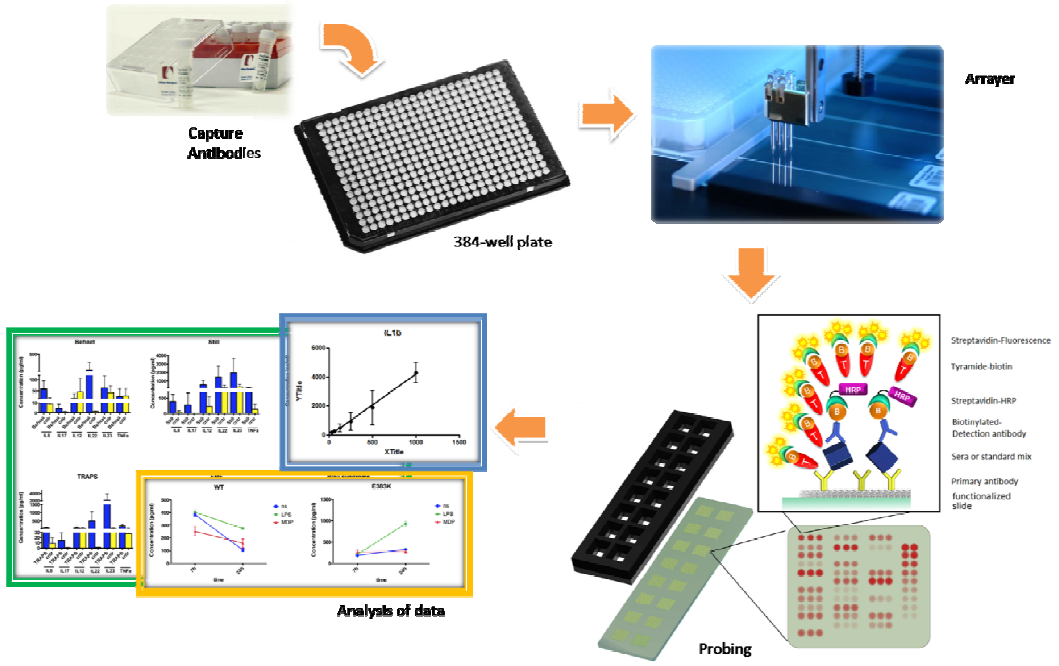


Fig. 19: Principles of antibody microarray.
Capture antibodies dilutions were prepared in 384-well plates and spotted on the poly-l-lysine slide by a MicroGrid II robot. A cocktail of recombinant protein standards are added to the slides, along with serum, to enable quantification. A cocktail of biotinylated detection antibodies are added to each block followed by amplification using Bio-Rad Amplification Reagent. Streptavidin-conjugated cy5 is finally added and read serum levels of cytokines are calculated. The data were graphically presented as column or time course plots-

2.3.1 Protocol

The following protocol varies related to the samples that have to be tested. Serum needs amplification of the microarray signals enhancing the signal detection, whereas it is not necessary for surnatants. There are different methods of amplification that can be used. we have tested either a kit based on dendrimers an fluorescent dyes (Ultra Amp™, Genisphere) or a common used Tyramide amplification (Bio-Rad Amplification Reagent), presented in Fig. 20. To avoid non specific background especially due to samples containing abundant quantities of biotin-binding protein , Avidin/Biotin blocking kit (Invitrogen) was tested before detection with Streptavidin Secondary Antibodies.

Printing step

Be sure that Capture Antibodies and Printing Buffer were at room temperature

- Add 100µg/ml of each Capture antibody in 384well plate, diluting them in Printing Buffer (50mM Trehalose, 1X PBS, 0.01% Tween-20) as shown in Tab. 6
- Spot Capture antibodies onto poly-l-lysine coated glass slides (Sigma Aldrich) with a microarrayer (MicroGrid II, BioRobotics Inc.) using silicon pins.
- Before starting up printing the slides, set up the arrayer as previous described for RPPA.

Probing slides

Make sure all reagents and chemicals up to room temperature

The following protocol is for sera samples. It described Avidin/Biotin block (within square brackets) and **Ultra Amp™ amplification** (within curly brackets):

- Block the slides with I-Block (dissolving 50mg I-block powder in 25ml of PBS-0.01%Tween 20) adding 100µl into each pad and incubate on the shaker at room temperature for 1hour.
- Prepare the Standard Antibodies Master Mix, mixing all the cytokines to be tested at top concentration of 1,000pg/ml each in 400µl of Reagent Diluent
- Prepare a serial dilution from the Master Mix in Reagent Diluent as: 1,000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.5pg/ml, 7.8pg/ml and 0pg/ml (Blank)
- Wash the slide 3 times with PBS-0.01%Tween20 for 3min each with constant rocking
- Add 100µl of Standard Antibodies dilution and sera one in each pad and incubate 1hour with constant rocking
- Wash the slides 3 times with PBS-0.01%Tween20 for 3min each with constant rocking

{ [
- Apply 2 drops of Avidin in each pad and incubate for 10min at room temperature
- Wash 6 times for 1min each with PBS-0.01%Tween20
- Apply 2 drops of Biotin in each pad and incubate for 10min at room temperature
- Wash 6 times for 1min each with PBS-0.01%Tween20

] }

- Prepare the Detection Antibodies, mixing all the detection Antibodies in Reagent diluent as shown in Tab. 6
- Add 100µl of Detection Antibodies Mix to each pad and incubate 1hour with constant rocking

- Prepare a Ultra Amp™ stock solution at 1:1,000 in BSA1X in PBS addicted with DNA Block
- Wash the slides 3 times with PBS-0.01%Tween20 for 3min each with constant rocking
- Add 100µl of Ultra Amp™ solution and incubate at the dark for 15min with constant rocking
- Rinse the slides with ultra pure water
- Spin for 5min at 1,200×*g* at room temperature
- Scan at 635nm with Axon 4200AL scanner

The **Tyramide amplification** is based on the horseradish pexoridase (HRP) catalysed deposition of reactive biotinylated tyramine at site of immunoreactivity [95]. The kit used in this thesis was from BioRad.

- After the detection step with Detection Antibodies mix (as reported in the protocol above), wash the slides 3 times with PBS-0.01%Tween20 for 3min each with constant rocking
- Add 100µl of Streptavidin Biorad HRP at 1:1,000 for 15min with constant rocking
- Wash the slides 3 times with PBS-0.01%Tween20
- Add 100µl of BioRaD AMPLIFICATION UNIT mix (Reagent2X, Reagent4X and ultra pure water at the ratio 4:1:4)
- Wash the slides 3 times with 20%DMSO/PBS-0.01%Tween20
- Wash the slides 3 times with PBS-0.01%Tween20
- Add 100µl of Streptavidin Cy5 prepared as 1:1,000 in 3%BSA
- Incubate for 15min with constant rocking in the dark
- Rinse the slides with ultra pure water
- Spin for 5min at 1,200×*g* at room temperature
- Scan at 635nm with Axon 4200AL scanner

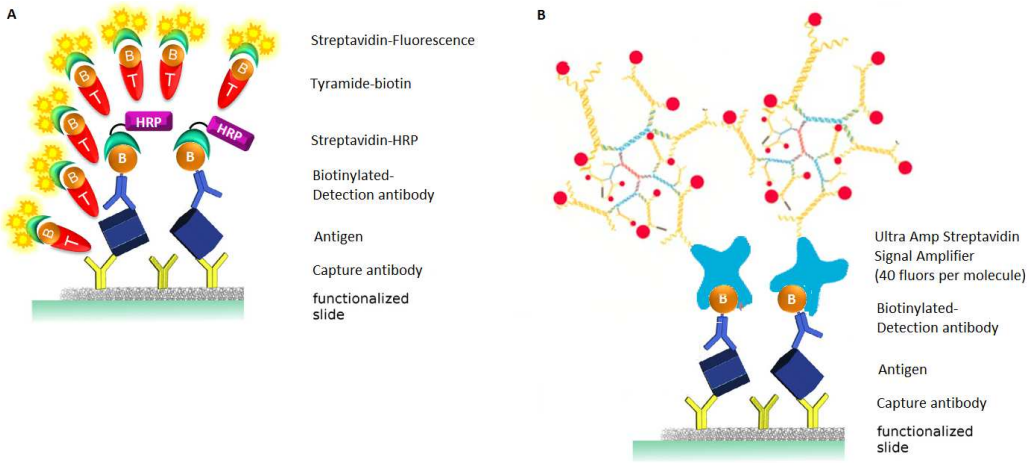


Fig. 20: Tyramide amplification and Genisphere Ultra Amp amplification.

For surnatants samples, the protocol was similar to the previous one without the amplification steps. The detection was achieved trough Standard Streptavidin Cy5 Fluorescent Conjugated Antibodies.

- Block the slides with I-Block adding 100µl to each pad and incubate on the shaker at room temperature for 1hour
- Prepare the Standard Antibodies as a mix containing the cytokines at top concentration (1,000pg/ml) and serial dilute it in Reagent Diluent.
- Do not dilute the surnatants
- Wash the slide 3 times with PBS-0.01%Tween20 for 3min each on the shaker.
- Add 100µl of Standard Antibodies and samples dilutions and incubate them on the shaker 1hour.
- Wash the slide 3 times with PBS-0.01%Tween20
- Dilute the Detection Antibodies in Reagent Diluent and add 100µl to each pad
- Incubate the slides on the shaker 1hour
- Wash the slides 3 times with PBS-0.01%Tween20
- Add 100µl of Streptavidin Cy5 to each pad prepared as 1:1,000 in 3% BSA.
- Incubate for 15min on the shaker in the dark
- Rinse the slides with ultra pure water
- Spin for 5min at 1,200×*g* at room temperature
- Scan at 635nm with Axon 4200AL scanner

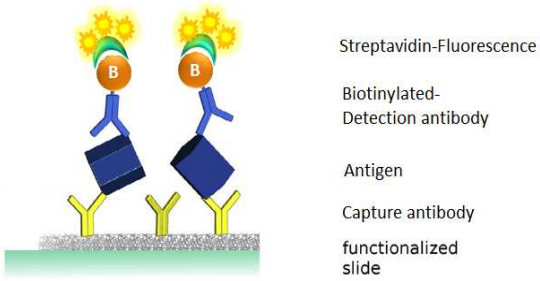


Fig. 21: Cy5 fluorescence detection at 635nm.

Analysis of data

The antibody microarray data analysis consists of calculate the concentration of each cytokine for all the samples using a standard curve and the intensity's data gained from the image software. Axon GenePix Pro-6 Microarray Image Analysis software was the bioinformatics tool used to determine the raw signal intensities of the spots generating gpr files. Means and Standard Deviation of the samples were evaluated using GraphPad Prism 5 (GraphPad, San Diego, USA). The standard data plots a linear regression line through at least 6 points whereas the samples interpolates on that regression line and measure their concentration.

- After scanning with Axon 4200AL, save the image as a 16-bit gray scale TIFF file
- Import in GenePix Pro software each scanned image and the gal file saved from the arrayer. This file constructs an array of blocks to match the size and positioning of printed features gaving specific information about the location, size, and name of each citokine printed on the slide
- Combine together those files to obtain the gpr files. These tab delimited text files contain the localization and an identification variable of the samples on the array and a fluorescence intensity that represents the raw expression values.
- Create an excel file reporting the median of the raw expression values without the background signal for each samples and the standards
- Calculate the average and the standard deviation for each cytokines
- Import the average and standard deviation data from standards in GraphPad Prism 5.0 software
- Plot a linear regression to obtain a standard curve for each cytokine
- Imported the samples average and standard deviation data and interpolate the intensity values on the appropriate standard curve.

The data could be visualized and undergone to other statistical analysis. Their significance was tested using non-parametric tests and graphed with GraphPad Prisms5 software. Column and scatter graphs were used to visualize the distribution of the data points in order to assess differential level of cytokines in comparison to a control group.

2.3.2 Antibodies

In antibody microarray, the set of antibodies used for capture and detect each specific analyte was purchased from the DuoSet ELISA kit (R&D). Each kit contains the basic components required for the development of sandwich ELISAs to measure a specific cytokine: capture antibody, biotinylated detection antibody and a protein standard.
In the following Tab. 6, all the antibodies used in these assays are listed, correlated with the working concentrations.

A

CYTOKINE	CAPTURE ANTIBODY	DETECTION ANTIBODY	PROTEIN STANDARD
IL1β	C_i 720 µg/ml C_f 100 µg/ml	C_i 36 µg/ml C_f 200ng/ml	C_i 90 ng/ml C_f 1,000pg/ml
IL6	C_i 350 µg/ml C_f 100 µg/ml	C_i 9 µg/ml C_f 50ng/ml	C_i 120 ng/ml C_f 1,000 pg/ml
IL8	C_i 720 µg/ml C_f 100 µg/ml	C_i 3.6 µg/ml C_f 20ng/ml	C_i 100 ng/ml C_f 1,000 pg/ml
TNFα	C_i 720 µg/ml C_f 100 µg/ml	C_i 90 µg/ml C_f 500ng/ml	C_i 370 ng/ml C_f 1,000 pg/ml
IFNγ	C_i 720 µg/ml C_f 100 µg/ml	C_i 9 µg/ml C_f 50ng/ml	C_i 55ng/ml C_f 1,000 pg/ml
IL17	C_i 720 µg/ml C_f 100 µg/ml	C_i 27 µg/ml C_f 150ng/ml	C_i 25ng/ml C_f 1,000 pg/ml
IL12	C_i 720 µg/ml C_f 100 µg/ml	C_i 18 µg/ml C_f 100ng/ml	C_i 210 ng/ml C_f 1,000 pg/ml
IL22	C_i 360 µg/ml C_f 100 µg/ml	C_i 45 µg/ml C_f 250ng/ml	C_i 340 ng/ml C_f 1,000 pg/ml
IL23	C_i 1,080 µg/ml C_f 100 µg/ml	C_i 72 µg/ml C_f 400ng/ml	C_i 530 ng/ml C_f 1,000 pg/ml

B

ANTIBODY	DILUTION	COMPANY
Streptavidin Cy5	1:1,000	Life Technology
Streptavidin HRP	1:1,000	BioRad

Tab. 6: List of antibodies for microarray assay.
A) List of antibodies and concentrations used in Antibody Microarray assays. All of them were purchased from R&D. C_i stands for initial concentration, whereas C_f for final concentration.
B) Streptavidin antibodies used for amplification or biotinylated complex detection.

RESULTS

1 Genetic and functional studies on Blau syndrome

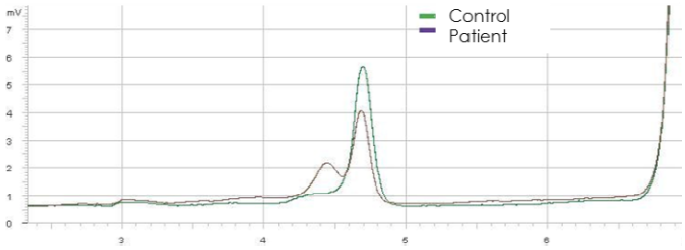
1.1 Mutation screening of *CARD15/NOD2* gene

The caspase recruitment domain gene *CARD15/NOD2* has been mapped in the chromosomal region 16q12.1–13 [42]. The identification of *CARD15/NOD2* as the gene responsible for Blau syndrome was the major breakthrough for BS research [215]. Up to date, 11 Blau-associated genetic mutations have been identified within this gene, almost in heterozygous state. Two of these mutations (p.R334Q and p.R334W) account for more than 50% of the mutated alleles [216], making codon 334 a genetic hot spot for mutations (Fig. 21). The genetic sequence of *CARD15/NOD2*, composed of 11 exons, was also divided into fragments in order to easily amplified and analyze the gene. The exon 4 was for example split into 5 amplicons, due to its length. Since all the mutations known to be correlated with BS are located in exon 4, only this sequence underwent to the genetic screening.
In 2005, van Duist and colleagues have found a new mutation p.E383K in the only Italian family affected by BS [56]. We have repeated the screening for BS mutations in this family, including the new members using denaturing high-performance liquid chromatography (DHPLC) and subsequent direct DNA sequencing.

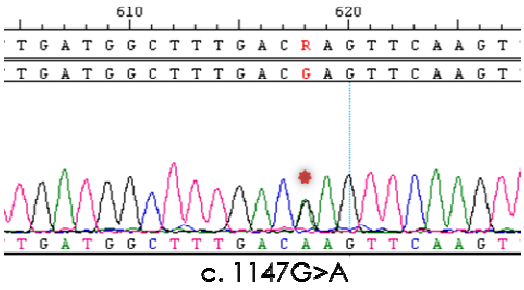
EXON 4 MUTATION: c.1147 G>A → p.E383K

DHPLC analysis and sequencing showed only a nucleotide variation in all the affected subjects (Fig. 22A and B). The mutation detected was the already found c.1147G>A leading to a missense variation Glu383Lys in exon 4. All other family members screened for such mutations resulted negative for *CARD15/NOD2* mutations. Sequence alignment of the involved 4^{th} exon fragment of CARD15/NOD2 with corresponding sequences of different mammals shows that p.E383K mutation occurred in a conserved sequence.

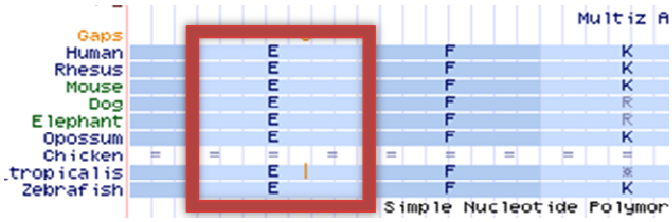
A



B



C



D

E383K	
Location in the gene	exon 4
Usual protein name (complete nomenclature)	E383K (p.Glu383Lys)
Sequence change	c.1147G>A
rs Number	rs10489477
Sequence	cDNA: TTTCATCCCTTTCACGAGTTCAACTTCACCT
Alteration	Substitution
N base(s)	1
Base substituded	G>A
Consequence	Unknown
Functional tests	Yes
N Controls	200
Technique(s) used	Sequencing dHPLC
Change/define RFLP	No
Disease related symptoms in this patient	Symptomatic
Associated phenotype in this patient a variant observed in symptomatic subjects does not imply its causal role.	Blau syndrome
Country of origin / Ancestry	Italy / Unknown

Fig. 22: c.1147 G>A → p.E383K.
A) DHPLC elution profiles of *CARD15/NOD2* exon 4 amplicon: two peaks were observed for the patient (in violet), compared with the single peak of the control (in green).This abnormal elution profile was due to a heterozygous substitution (c.1147G>A), as shown by DNA sequencing (B). C) The aminoacid involved in this mutation (Glu383) is known to be a residue conserved among different species. D) From Infevers database, the genetic and clinical information about this variation (http://fmf.igh.cnrs.fr/ISSAID/infevers/index.php).

1.2 Functional analysis of mutant p.E383K NOD2 protein

To evaluate pathogenic potentials of the NOD2 missense mutation p.E383K, full-length *wild-type* cDNA was directionally cloned in eukaryotic expression vector to obtain a fusion protein with FLAG. Furthermore, we used NOD2 carrying p.R334W mutation as a positive control, since this variation is one of the most frequent and deeply studied in Blau syndrome (Fig. 23A). Mutated proteins carrying p.E383K were obtained by site directed mutagenesis of the *wild-type* construct. p.R334W mutated proteins derived instead from cut and paste technique between the *wild-type* construct and the mutated coding sequence (Fig. 23B).

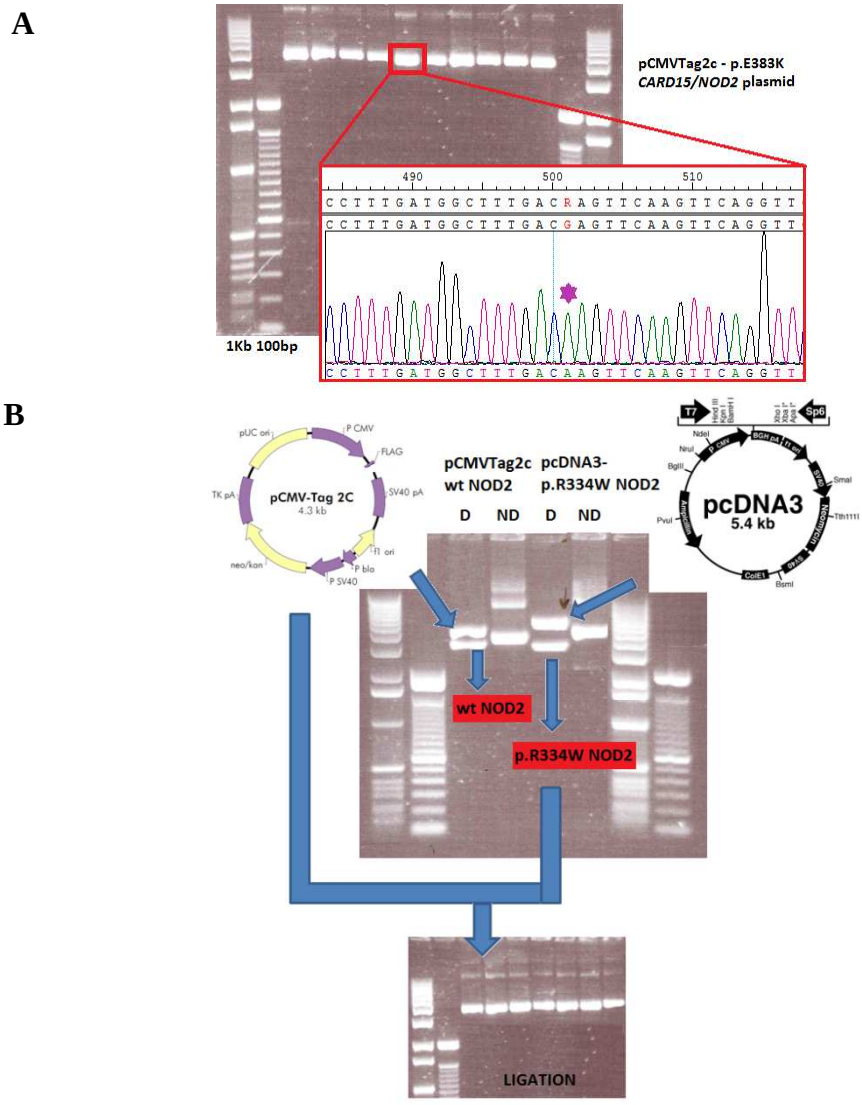


Fig. 23: NOD2 expressions vectors.
A) Electrophoresis test in agarose gel 1% (w/v) of the p.E383K mutated pCMVTag2c-NOD2 *wild-type* plasmid. The direct sequencing has confirmed the presence of the inserted mutation (starred nucleotide). B) Electrophoresis separation in agarose gel 1% (w/v) of the digested plasmids, pCMVTag2c-NOD2 *wild-type* and pcDNA3-R334W NOD2.Important for the subsequent ligation reaction are the highlighted bands related to pCMVTag2c linearized vector (4.3 kb) and p.R334W NOD2 cDNA (3.2 kb).

The starting point for create these plasmids is the optimization of mutagenesis conditions and ligation reaction. Concerning the mutagenesis, two primers (forward and reverse) and a range of *annealing* temperature were tested separately for obtaining p.E383K mutation in NOD2. Base on the results of those tests, the use of primer forward and annealing temperature of 62°C gave the highest yield of mutagenesis. Concerning the ligation reaction between p.R334W mutated NOD2 and expression vector pCMVTag2c, different conditions were tested to increase the efficiency of the reaction. Setting-up a ligation reaction with molar ration 3:1 gave in this case the major yield and number of positive clones.

1.2.1 Localization

Constructs were transfected in the human embryonic kidney cell lines HEK293, extensively used as an expression tool for recombinant proteins since it was generated over 25 years ago. Although of epithelial origin, its biochemical machinery is capable of carrying out most of the post-translational folding and processing required to generate functional, mature protein from a wide spectrum of both mammalian and non-mammalian nucleic acids [209].
On these transfected cells, indirectly immunofluorescence assay was performed to display the cellular localization of human *wild-type* and mutant NOD2, choosing the appropriate antibodies (anti-FLAG primary and anti-mouse secondary conjugated with rhodamine).
Indirect immunofluorescence analysis in transfected HEK293 cells revealed pronounced cytoplasmic localization of the *wild-type* fusion protein (Fig. 24A). In addition to the protein signal, it is possible to discriminate the nuclei detected in blue with DAPI. Protein carrying mutations p.E383K and p.R334W were predominantly localized in the cytoplasm, although a lower amount of rhodamine signal was detectable in the membrane of p.R334W mutated cells (Fig. 24B and C). These results are comparable to the one referred to *wild-type* protein, suggesting that there is no evidence of altered localization of NOD2 in relation with the different carried mutations.
Moreover, immunostaining results showed a reduced fluorescence signal between *wild-type* and mutated NOD2, suggesting a lower expression of mutated proteins in transfected cells.

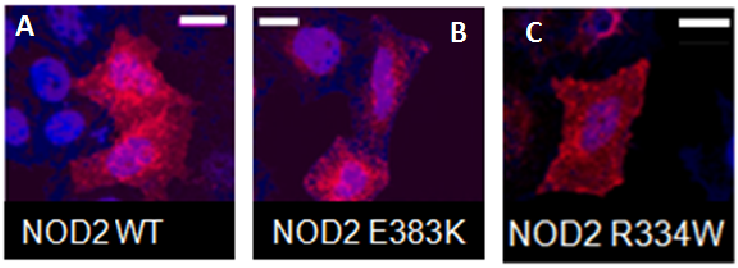


Fig. 24: Transfection studies in HEK293 cells.
Wild-type NOD2 is localised at the cytoplasm (A),as well as p.E383K and p.R334W mainly detected in the cytoplasm (B and C).

1.2.2 NOD2 expression

In order to detect the expression of the exogenous protein NOD2, both *wild-type* and mutated, Western blotting analysis was performed in the lysates obtained from in the transfected HEK293 cells.

First of all, the expression of NOD2 in HEK293 cells has been compared with the expression of the *wild-type* exogenous NOD2 in transfected HEK293 cells, to assess the influence of endogenous protein in further experiments.
Following the protocol previously explained in Materials and Methods, the HEK293 cell lines both transfected and untransfected were lysated and proceeded to be ready for Western blotting.
As shown in Fig. 25A, the contribution of endogenous NOD2 protein can be considered negligible compared to the expression of exogenous *wild-type* protein in absence of stimulation.
We also made this endogenous/exogenous protein comparison after stimulation with bacterial wall components, like the peptidoglycan muramyl dipeptide (MDP).
In the absence of the receptor for LPS, we did not evaluate this TLR agonist *in vitro*, but only *ex vivo* in PBMC from patients. Fig. 25A showed also that MDP did not enhance the endogenous NOD2 expression, whose level seemed to be several times lower than exogenous *wild-type* NOD2.

Furthermore, different concentrations of MDP were tested in *wild-type* NOD2 transfected HEK293 cells, to assess the sensitivity of these cells and to choose the better stimulus concentration. MDP at 0µg/ml, 1µg/ml and 10µg/ml stimulated the *wild-type* NOD2 transfected cells for 7 and 24 hours, as described previously. Western blotting results were presented in fig. 25B. After 7 hous, unstimulated cells seemed to express more *wild-type* protein, compared to the 1µg/ml MDP stimulated cells. The expression level at 10µg/ml MDP stimulation are definitively higher than at 1µg/ml but slightly lower than at 0µg/ml stimulation. The same trend was observed for the 24hours stimulation.
Higher concentrations of MDP [50µg/ml and 100µg/ml] were tested as well, but the cell cultures died before the 7 hours have passed. This result showed that concentrations above the 10µg/ml were not compatible with regular cellular growth. Then, 10µg/ml was the concentration choosed for the experiments performed with NOD2 transfected HEK293 cells.

From the analysis of the *wild-type* NOD2 expression, the following step was to evaluate the expression of the two mutant forms p.E383K and p.R334W in transfected HEK293 cells. As presented in the materials and methods, these cells were cultured for 7 and 24 hours in the presence or absence of stimulation MDP and then lysed for western blotting.

As shown in Fig. 25C, the signal related to the expression of p.E383K NOD2 after 24 hours did not seem to vary with or without stimulation. This result was comparable for the control mutation p.R334W. In figure 25C the results after 7 hours were not shown as they presented an identical trend to the one reported for 24 hours, although with lower levels of expression.
Moreover, comparing the expression levels of *wild-type* and mutated NOD2, we could not spot any differences in the expression trend shown pre/after stimulation.

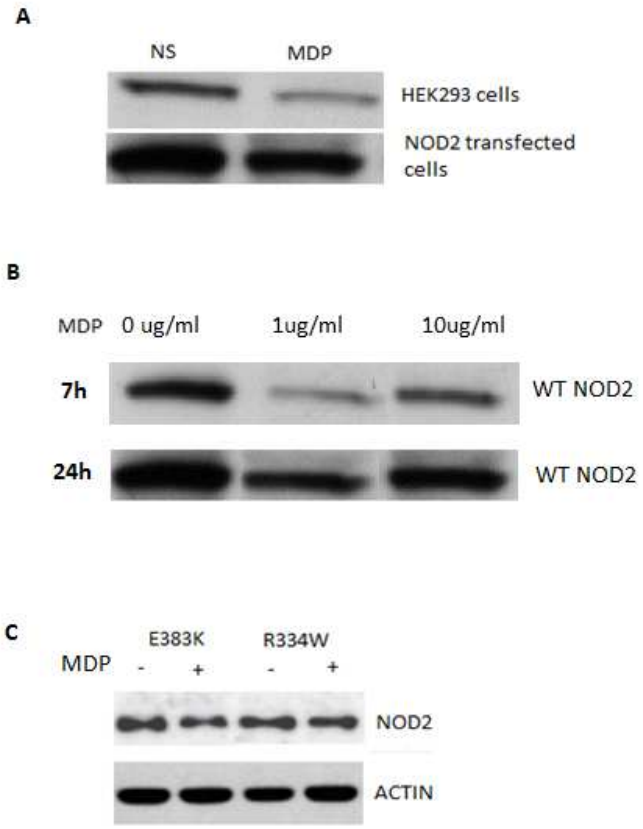


Fig. 25: NOD2 expression.
A) Comparison of endogenous NOD2 (upper lane) and exogenous *wild-type* NOD2 (bottom lane) with or without MDP stimulation [10µg/ml]. B) Different MDP concentrations [0-1µg/ml-10µg/ml] after 7 and 24 hours stimulation of *wild-type* NOD2 transfected cells. C) Mutated NOD2 (p.E383K and p.R334W) expression in presence or absence of 10µg/ml MDP stimulation after 24hours.

1.3 Pathway NF-κB studied from patients PBMC and NOD2 transfected HEK293 cell lines

In order to carry on the evaluation of the pathogenic potential of p.E383K mutation in Blau syndrome, we examined both *in vitro* and *ex vivo* the ability of this mutation to activate the signalling pathway NF-κB known to be associated with this autoinflammatory disease. Foregoing *in vitro* studies have reported that

HEK293 cells transfected with a NOD2 construct engineered to contain a Blau syndrome mutation demonstrated elevated basal activity of an NF-κB reporter compared with cells transfected with a *wild-type* NOD2 construct and higher amounts of NF-κB reporter activation in response to muramyldipeptide [217,218]. From these *in vitro* observations, a "gain of function" hypothesis has been proposed for BS mutations, which predicts that patients with Blau syndrome would spontaneously release more cytokines that can be transcriptionally up-regulated by NF-κB activity (such as IL1β).
The follow study may help to have a complete view in the NF-κB pathway involved in Blau syndrome.

1.3.1 *IN VITRO* analysis

The starting point for the *in vitro* studies were HEK293 cells stably transfected with constructs engineered to contain the *CARD15/NOD2* gene in the *wild-type* and mutated forms p.E383K and p.R334W. These cells were cultured in the presence or absence of stimulation mediated by MDP and lysated after 7 and 24 hours, as described in materials and methods. NF-κB activity has been evaluated *in vitro* through two different technique: Western blotting and Reverse Phase Protein Array (RPPA).

Western blotting

The Western blot technique is an immunochemical technique that allows to identify the presence of a particular protein in a mixture of proteins separated though electrophoresis, by means of the recognition by specific antibodies. Although the technique is not strictly quantitative , it is possible to evaluate the protein expression relative to a constitutively active protein (e.g. β-actin). By measuring the density of the protein band of interest and normalizing to β-actin, we obtained values of relative density that allowed to a more closely evaluation of the bands observed in the blot membrane.
With this technique, we studied indirectly the activity of NF-κB *in vitro* in HEK293 cells expressing *wild-type* and mutated NOD2. Remembering that NF-κB is activated by phosphorylation and degradation of IKBα induced by NOD2 and translocates to the nucleus where it promotes the transcription of proinflammatory genes, we evaluated the expression of IKBα and its phosphorylated form. Therefore, if IKBα would be mostly present in the phosphorylated form, this may mean an activation of NF-κB and subsequent cascade activation of the inflammatory response mediated by the pathway.
Starting materials were the whole lysates of HEK293 cells transfected with *wild-type* and p.E383K mutated NOD2, obtained after stimulation for 7 and 24 hours with MDP [10µg/ml].

In Fig. 26 was presented the blotting results at 7 (upper) and 24 (middle) hours and relative profile plot with relative density data (bottom).
Observing the graph on the left, under unstimulation condition in the *wild-type* NOD2 transfected cell the expression of IKBα were higher than the phosphorylated protein, whereas MDP stimulation seemed to provoke a slightly increase of phospho IKBα, as expected from literature data. The graph on the right concerning p.E383K NOD2 showed a similar trend in the two protein expression in presence or absence of stimulation. IKBα were higher expressed than the phosphorylated protein, implying a failure to activate NF-κB pathway.

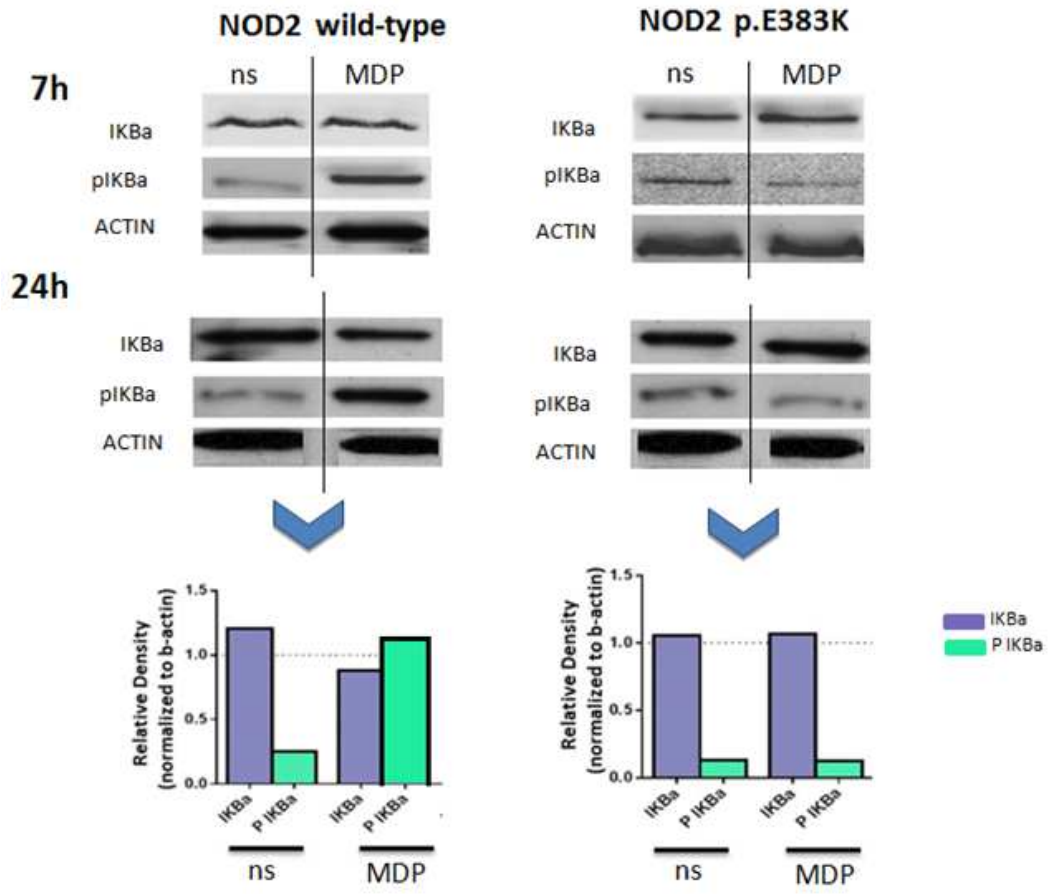


Fig. 26: Western blot analysis of IKBα and phospho IKB α expression in HEK293 cells presenting *wild-type* and p.E383K mutated NOD2.
Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The graphs under the western blotting images represent relative density of the bands (IKBα in violet and phospho IKBα in green) normalized to β-actin and are referred to 24h stimulation data. 7h stimulation data were not plotted since the trend is similar to the one at 24h.

Reverse Phase Protein Array (RPPA)

To confirm and deeply investigate the results obtained from Western blot, we performed a protein microarray, a high-throughput assay more sensitive and less samples wasting than Western Blot.

The whole lysates of HEK293 cells transfected with *wild-type* and p.E383K mutated NOD2, obtained after stimulation for 7hours were used for a first analysis of NF- κB pathway activation.

Reverse phase protein array technique was applied to measure the level of several component of the NF-κB signalling pathway, such as IKBα, IKKα/β and their phosphorylation forms. In these experiments the expression level of the analysed proteins was normalized to actin as a house keeping protein.

IKBα and phospho IKB α levels was presented in Fig. 27.

In absence of stimulation, *wild-type* NOD2 cells did not show any difference in the two proteins levels, as seen for p.E383K NOD2 cells. After stimulation with 10µg/ml of MDP, in the *wild-type* NOD2 cells there were a significant increase of phospho IKBα compared to IKBα level and also compared to the unstimulated one ($p<0.05$). The mutated NOD2, instead, did not show any increase in proteins levels, that were comparable to the unstimulated ones.

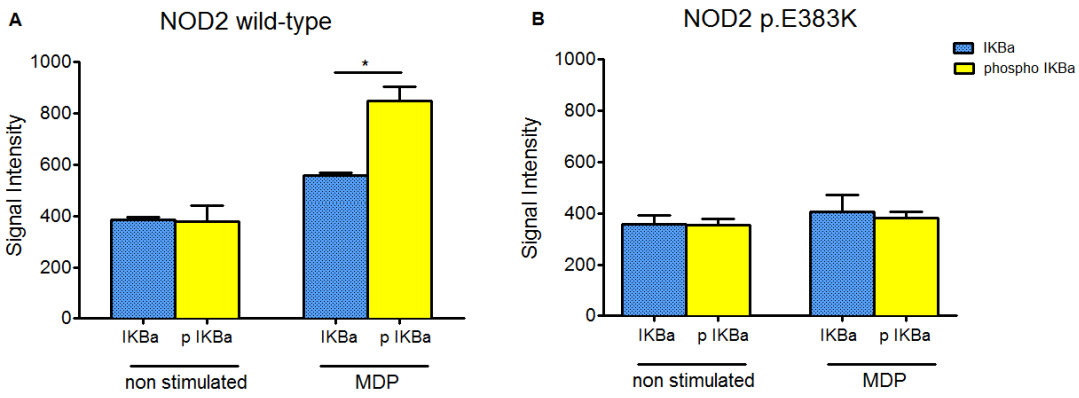


Fig. 27: Comparison between IKBα and phospho IKBα in whole lysates obtained from A) *wild-type* NOD2 and B) p.E383K NOD2 transfected HEK293 cells
*$p<0.05$, as statistical significant level.

IKKα and IKKβ levels was presented in Fig. 28.

In *wild-type* NOD2 cells, slight but consistently higher level of IKKα and IKKβ were showed after stimulation with 10µg/ml of MDP ($p<0.05$). Moreover, in absence of stimulation, we could see a slightly higher level of IKKα and IKKβ in *wild-type* cells compared to p.E383K NOD2 cells, even if the increase was not statistically significant. In presence of MDP stimulation, these comparison was definitively slight but statistically consistent ($p<0.05$).

In p.E383K mutated NOD2 cells, no differences between stimulated and unstimulated levels of both IKKα and IKKβ were shown.

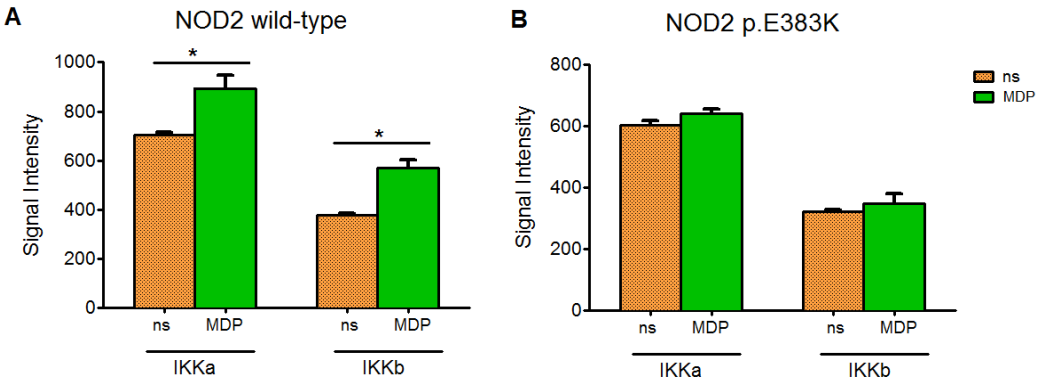


Fig. 28: Comparison between IKK*α* and IKK*β* levels in whole lysates obtained from A) *wild-type* NOD2 and B) p.E383K NOD2 transfected HEK293 cells.
Ns indicates absence of stimulation, whereas MDP stands for stimulation with [10µg/ml] of muramyl dipeptide. *p<0.05 as statistical significant level

Activation of the NF-κB transcription factor in HEK293 transfected cells with mutant NOD2 (p.E383K and p.R334W) was investigated and compared to that with the *wild-type* transfectant. Nuclear extracts are generally preferred to whole cell lysates for this protein regulation studies, since NF-κB is known to move to the nucleus to control transcription of pro-inflammatory genes.
To study NF-κB behaviour, transfected HEK293 cells were stimulated with MDP [10µg/ml] for 7hours and then nuclear cytoplasmic separation was performed using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Thermo Fisher Scientific) as described in the methods.
The expression of NF-κB p65 and the phosphorylated form was determined together with other NF-κB pathway components, such as IKBα, IKKα/β, and their phosphorylated form in the nuclear fractions of *wild-type* and mutated cells. Even for this analysis, technique of choice was lysate microarray (RPPA) and all the results were normalized to the levels of the house keeping protein.
The heatmap in Fig. 29 summarizes the activity of NF- κB pathway in the nuclear fraction, while Fig. 30 showed the RPPA data analyzed for each NF-κB pathway components.

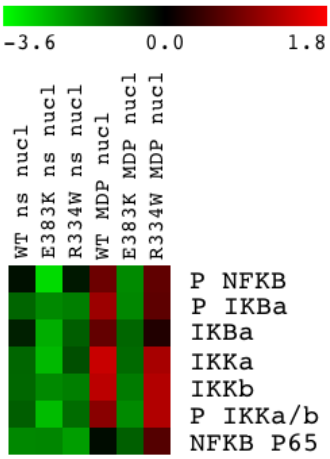


Fig. 29: Heatmap of NF-κB signalling pathway in nuclear fraction of NOD2 transfected HEK293 cell lines.
The multiple different proteins are outlined on the horizontal axis, and the lysates phenotype is on the vertical axis. Red and green colours indicate highand low protein expression, respectively.

As regards the mutation p.R334W, the data obtained for the basal activity of NF-κB are in line with those reported in the literature.
The mutation in fact revealed a ~2-fold increase in basal level of NF-κB compared to *wild-type* allele ($p<0.05$). The NF-κB activity of R334W was further enhanced by addition of MDP ($p<0.01$). This mutation also leaded to a slight increase of expression of the phosphorylated form of NF-κB to the basal level by comparing it to the *wild-type* ($p<0.05$). We could note that the stimulation with MDP further increased the expression of phospho NF-KB both in *wild-type* and in p.R334W ($p <0.01$). Active phospho NF-KB level in p.R334W cells is similar to the one in *wild-type* cells ($p>0.05$). The results showed also similar level of phospho IKBα in p.R334W cells compared to *wild-type* cells, but a statistical enhance of both levels after MDP stimulation ($p<0.05$). A similar trend was shown by the other observed pathway components.
Regarding the mutation p.E383K at baseline, it was showed no statistically significant difference in the level of NF-κB compared to *wild-type*, not even an increase in production as a result of stimulation with MDP. The phosphorylated form of NF-κB instead showed a significant decrease ($p <0.01$) compared to *wild-type* at the basal level. After stimulation with MDP, we noted a clear decrease compared to *wild-type* ($p<0.01$). IKBα, IKKα, IKKβ and their phosphorylated forms showed instead a similar level to *wild-type* in absence of stimulation, except for IKBα ($p<0.05$). Even with MDP stimulation, the proteins did not show enhanced expressions, except for IKBα ($p<0.05$). These levels appeared to be statistically lower than *wild-type* ($p<0.05$ for IKBα, phospho IKBα, IKKβ and phospho IKKα/β; $p<0.01$ for IKKα).

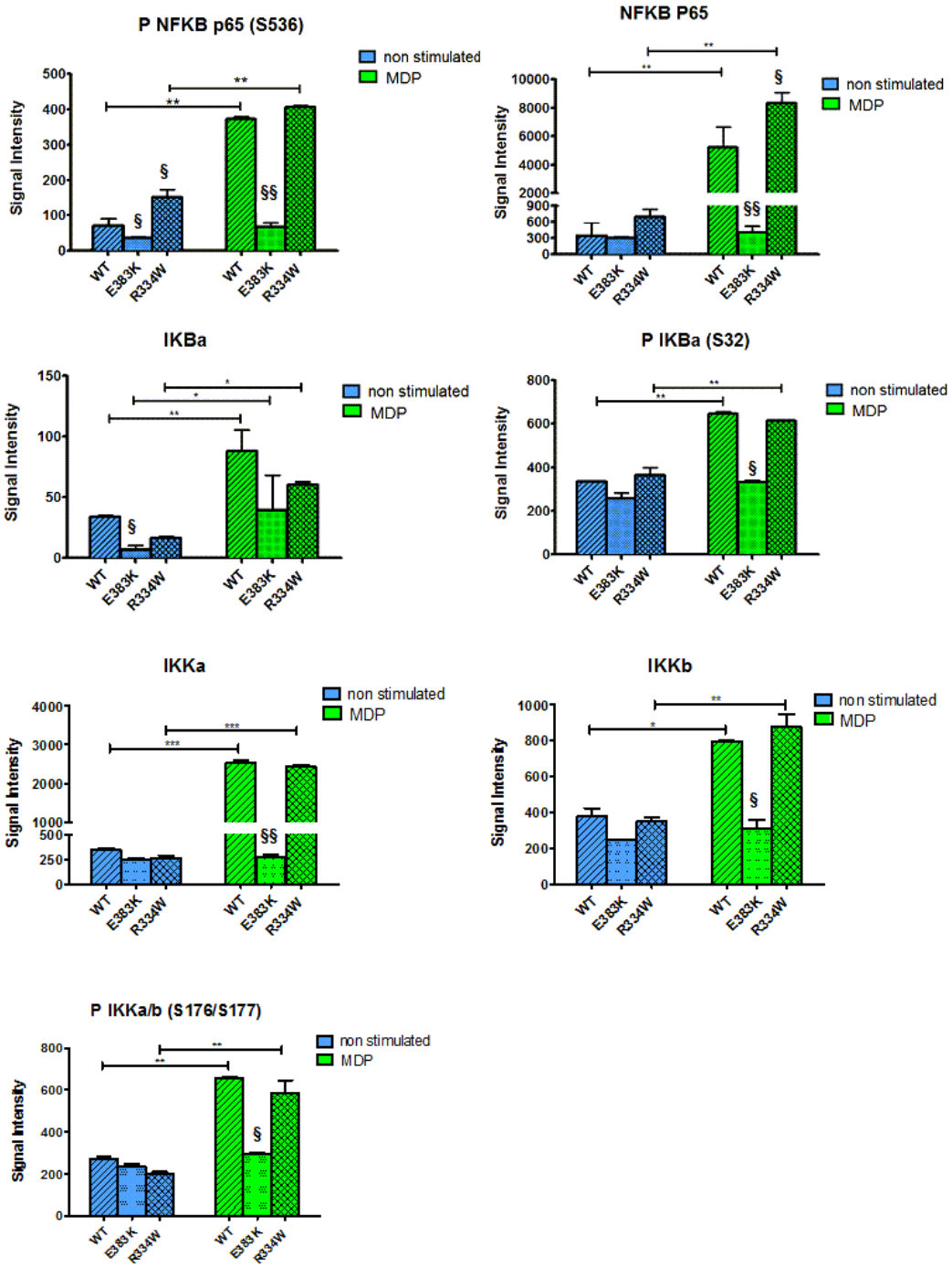


Fig. 30: Comparison of RPPA data for NF-κB, IKBα, IKK*α*/β and their phosphorylated forms obtained from nuclear fraction of lysated *wild-type* and mutated NOD2 transfected HEK293 cells. In blue unstimulated data; in green data after 7h stimulation with MDP.
*p<0.05, **p<0.01, ***p<0.005 as significant statistical values in comparison between ns (non stimulated) and MDP.
§p<0.05, §§p<0.01 as statistical significant values in comparison *wild-type*/ mutated proteins.

1.3.2 *EX VIVO* analysis

NF-κB signalling pathway has been studied in detail in lysated PBMC samples from Italian Blau syndrome patients and all data were compared to healthy controls, matched by similar age and sex.
Patients PBMC were purified and proceeded as described previously in materials and methods. Reverse phase protein array technique was applied to measure the level of several component of the NF-κB signalling pathway, such as NF-κB, IKB α, IKKα/β and their phosphorylation forms. In these experiments the expression level of the analysed proteins was normalized to β-actin as a house keeping protein.
Fig. 31 summarizes the activity of NF- κB pathway in both patients and healthy controls.

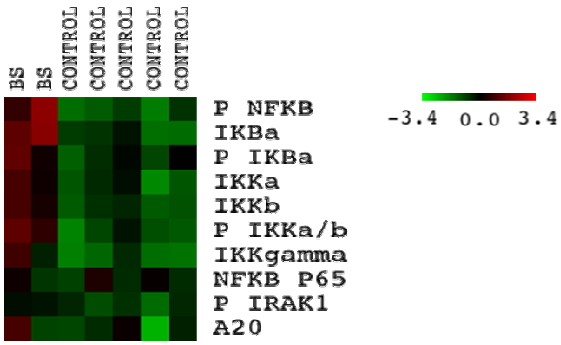


Fig. 31: Heatmap of NF-κB signalling pathway in Blau syndrome.
The multiple different proteins are outlined on the horizontal axis, and the lysates phenotype is on the vertical axis. Red and green colours indicate high and low protein expression, respectively. From the figure the levels of different regulatory molecules of NF-κB are down-regulated in healthy controls.

The lysates microarray data showed higher level of phospho NF-κB, IKBα, phospho IKBα, IKKα, IKKβ and phospho IKKα/β in Blau syndrome patients than in healthy controls (p value <0.05 or less), as shown in Fig. 32.

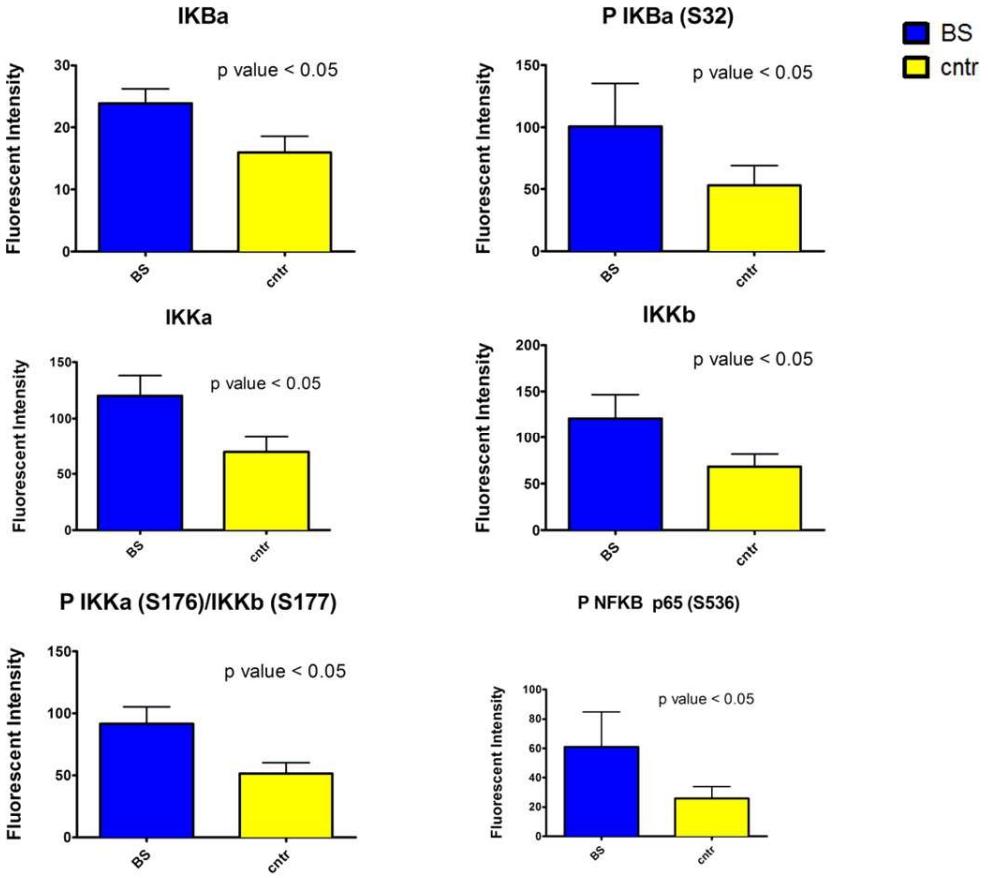


Fig. 32: NF-κB signalling pathway in Blau syndrome.
Comparison of protien expression levels between 2 Blau syndrome patients and healthy controls PBMC lysates. Statistical significance at p value <0.05.

1.4 Cytokines release from patients PBMC and NOD2 transfected HEK293 cell lines

To define the biological effects of pE383K BS-associated mutation, we examined both *in vitro* and *ex vivo* the ability of this mutation to produce cytokines in the absence or presence of activating stimuli. This study using Blau syndrome patients cells and NOD2 transfected cells may help to have a better understanding of cytokines and inflammatory markers involved in Blau syndrome.

1.4.1 *IN VITRO* analysis

The starting point for the *in vitro* studies were HEK293 cells stably transfected with the home-made constructs containing the *CARD15/NOD2* gene in the *wild-type* and mutated forms p.E383K and p.R334W. These cells were cultured in the presence or absence of stimulation mediated by MDP and the supernatants were collected after 7 and 24 hours.

It has been chosen to use the technique antibody microarray to evaluate the production of cytokines from supernatants. The method and the specific amplification was widely explained in materials and methods.
Since HEK293 cells produce only a limited set of cytokines and markers of inflammation, it was evaluated only the production of IL8, chemokine known to induce an inflammatory response to external stimuli.
Analyzing the data obtained after 7 hours, we noted that in the absence of stimulation (ns) the levels of IL8 in cells expressing *wild-type* NOD2 were statistically higher ($p < 0.05$) than those presented by p.E383K mutated cells (Fig. 33). Stimulation with MDP showed a similar level in the *wild-type* NOD2 cells, while there was a high increase of IL8 levels in cells expressing p.R334W NOD2 ($p<0.05$). Concerning the mutation p.E383K, there was not differences of concentration in the presence or absence of the stimulus.
A similar trend for all the three cell lines was noted for the secretion of IL8 after 24 hours.

Since it is known that exposure to MDP causes an impared response to subsequent MDP administration (called MDP tolerance) in NOD2 *wild-type* [218], we aimed to determine whether this condition was induced also by p.E383K mutated NOD2.Transfected cells were treated with MDP for 4 hours, then washed and restimulated with MDP for 24hours. Consistent with the literature data, NOD2 *wild-type* showed a tolerant state in response to subsequent MDP treatment, resulting in the reduced release of IL8 upon the second MDP stimulation ($p<0.01$) (Fig. 34). Concerning the p.E383K mutated NOD2, the results showed that pretreatment with MDP did not inhibit proinflammatory response to subsequent MDP treatment. IL8 concentration seemed to be slightly increased after the second MDP stimulation, but the difference was not statistically significant.

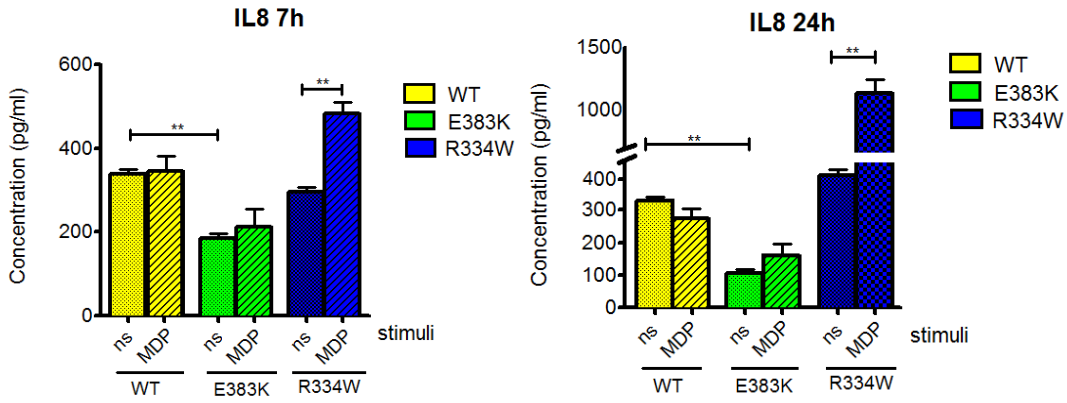


Fig. 33: Production of IL8 by supernatants of NOD2 transfected cells, both *wild-type* and mutated. Stimulation: none (ns) and muramyl dipeptide (MDP [10µg/ml]) for 7 or 24 hour.
** $p<0.05$ as statistical significant value

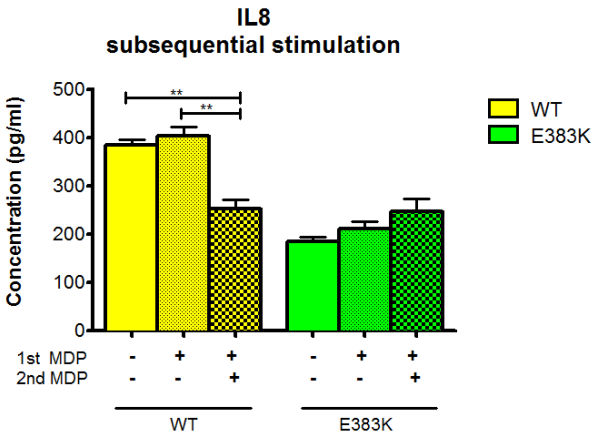


Fig. 34: Production of IL8 by supernatants of NOD2 *wild-type* and p.E383K transfected cells, after a subsequent treatment with muramyl dipeptide (MDP [10µg/ml]).
The cells were pretreated with MDP for 4h, washed and restimulated with MDP for 24h.
**p<0.05 as statistical significant value

1.4.2 *EX VIVO* analysis

Cytokines and other markers of inflammation production has been evaluated *ex vivo* by means of two different technique: ELISA and antibody Microarray. In addition to assessing the sensitivity and accuracy of the two different techniques, this choice was dictated by the availability of pairs capture/detection antibodies for the microarray technique.

ELISA technique

Enzyme-linked immunosorbent assay (ELISA) allowed rapid quantification of the levels of several cytokines and other inflammatory markers from PBMC surnatants obtained from Blau syndrome patients and healthy controls. These cells were cultured in presence or absence of inflammation stimuli, LPS [100ng/ml] and MDP [10µg/ml], for 7 hours.
The classical pro-inflammatory cytokines (IL1β, IL6, TNFα), chemokines (IL8) and interferon-gamma (IFNγ) were tested in the surnatants collected from patients and controls PBMC.
The results are presented in the graphs in Fig. 35 and 36. In absence of stimulation, no statistical differences in cytokine levels were detected between patients and controls, except for IL8 that was secreted at higher titre by healthy controls ($p<0.05$) (Fig. 35A).
In the presence of stimulation with the TLR agonist (LPS), the release of cytokines by Blau patients PBMC was not elevated compared to that of healthy controls. The concentration values were indeed very similar or even statistically significant lower, such as for IL6 ($p<0.05$) (Fig. 35B). Despite this results, the cytokines levels after LPS stimulation in patients showed a significant increase compared to the non stimulated ones, as expected (Fig. 36A). IL6, IL8 and TNFα

concentrations were highly release after LPS stimulation ($p<0.005$, $p<0.005$ and $p<0.05$ respectively), with the exception of IL1β and IFNγ.
Even the NOD2 agonist (MDP) did not seem to stimulate a high cytokine release in patients compared to healthy controls. IL8 was the only marker that showed a significant decrease in concentration in Blau syndrome patients ($p<0.005$) (Fig. 35C). Evaluating only Blau syndrome patients (Fig. 36A), it was notable that there was an increase of production of only IL8 ($p <0.05$), while levels of all other cytokines were similar to the non-stimulated ones or statistically lower to LPS stimulated ones ($p <0.005$ for IL6 and TNFα).
This study failed to identify an increase in cytokine production in patients following double stimulation, but this could be seen in controls (Fig. 35D). IL1β, IL6 and TNFα showed higher concentration in controls ($p<0.005$ for all the cytokines). In patients, a synergistic effect was detected in IL6, IL8, and TNFα production when the MDP were supported with the NLR agonist ($p<0.005$, $p<0.05$, $p<0.05$ respectively) (Fig. 36B).

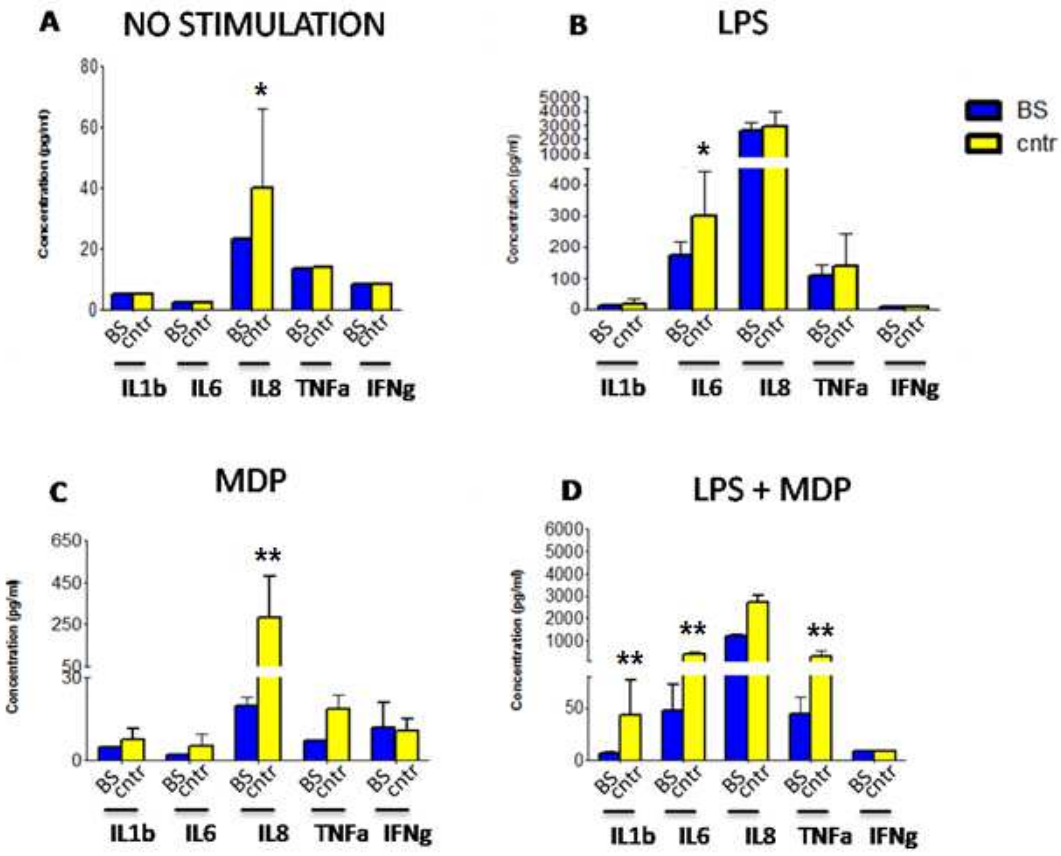


Fig. 35: Production of cytokines by supernatant of PBMC obtained from Blau syndrome patients, in presence or absence of stimulation after 7 hours.
LPS [100ng/ml] and MDP [10µg/ml] are used as individually or associated stimuli. The data shown are means of data from duplicate experiments and the error bars indicate standard deviation. *p<0.05; **p<0.005: p values less than 0.05 are considered statistical significant.

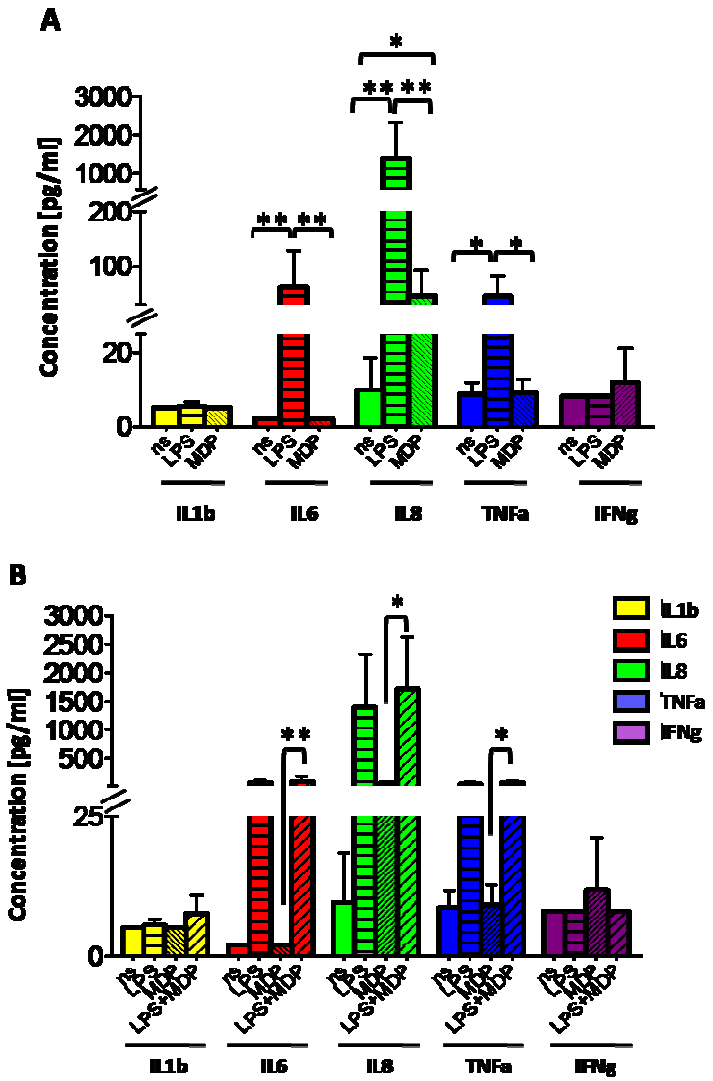


Fig. 36: Production of cytokines levels in Blau syndrome patients in presence or absence of stimulation after 7 hours.
The data shown are means of data from duplicate experiments and the error bars indicate standard deviation. A) Comparison between individual stimuli and absence of stimulation. B) Comparison between double stimulation and MDP stimulus.
*p<0.05;**p<0.005: p values <0.05 are statistical significant

Antibody microarray

Cytokines and other markers of inflammation production has been evaluated through antibody Microarray technique from serum samples obtained by Italian patients affected by different autoinflammatory diseases. Once measured the cytokines level as described in materials and methods, all patients data were compared to Italian healthy controls data.
The range of cytokines in analysis was expanded to all compounds that are known to play an important role in inflammation. Other than only testing the typical pro-inflammatory cytokines, such as IL1β IL6 TNFα, the serum samples are evaluated for the production of novel inflammation-associated cytokines, such as IL22, IL23

and IL17. This study using 9 cytokines and inflammatory markers may help to have a better understanding of inflammatory processes involved in Blau syndrome.

Comparison between concentrations of cytokines and inflammatory markers in Blau syndrome patients and healthy controls are shown in Fig. 37 The most notably difference was detected for IL22 and IL23, highly expressed in several different inflammatory condition and presenting pro-inflammatory properties. The concentration of IL22 in patients was >80-fold ($p<0.005$) higher than in controls, while IL23 concentration was 13-fold ($p<0.005$) higher. Statistically significant differences between patients and controls were also detected for IL17 and IL12, even though for the latter cytokine the level in patients are 3-times ($p<0.05$) lower than in controls. Concerning IL17, the concentration in patients was found to be 13-fold higher than in controls ($p<0.05$).
The concentration of all other cytokines was not statistically different from controls.

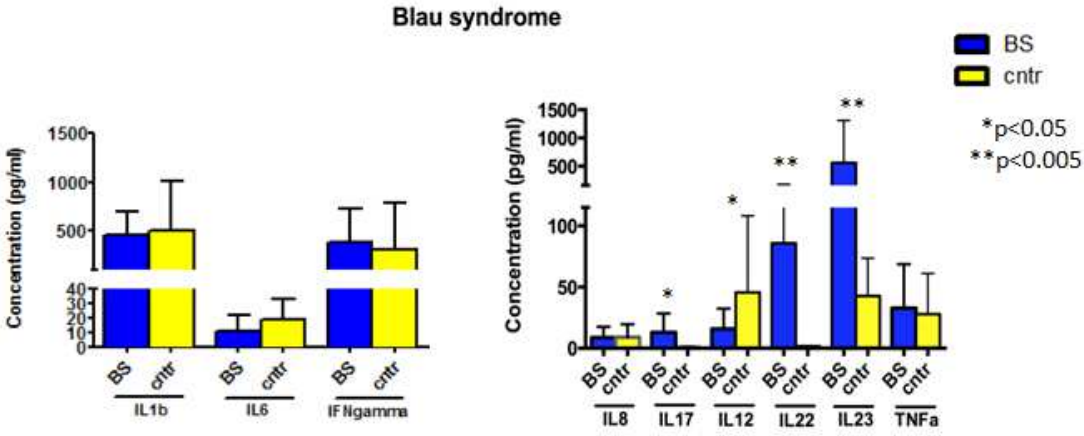


Fig. 37: Graphs of cytokines and marker of inflammation concentrations in serum from Blau syndrome patients compared to healthy controls.
The data shown are means of data from triplicate experiments and the error bars indicate standard deviation.
* $p<0.05$; ** $p<0.005$: p values less than 0.05 are statistical significant.

2 Evaluation of possible biomarkers in autoinflammatory diseases

Biomarkers are defined as "characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention". They provide an objective measure to assess disease severity, progression, risk and survival. Since identification of biomarkers patterns is a sizeable problem, the use of an array-based system to measure cytokines/other serum proteins and signalling intermediates would represent an enormous advance. Reverse phase protein array (RPPA) and antibody microarray are the techniques used in this thesis work. These proteomic technologies are currently employing by the MBTI Research Group at Nottingham University, where they have been developed and optimised.

2.1 Optimisation of assays

The optimisation of assay conditions is another major challenge for microarray technology.

The provision of optimal binding conditions is a crucial feature of the antibody microarray support. The demand for even higher densities as well as the need for decreased sample consumption and quantification led to the application of glass slides as solid supports with coated surfaces. The optimal substrate was defined as one that bound the greatest amount of antibody with minimal background. For the antibody microarray experiments presented in this thesis, poly-l-lysine enhanced surface slides gave the greatest degree of binding along with a minimal background. Due to the low abundance of some cytokines, a main issue is to provide a good signal through a correct amplification. The best results were achieved using simple Tyramide amplification rather than Genisphere or Avidin/Biotin blocking, as shown in Fig. 38. It is clear that the Genisphere amplification method (Fig. 38A) generates a signal directly from the sample, with a presence of detectable background. The addiction of Avidin/Biotin blocking steps showed surprisingly low signals and higher background (Fig. 38B), even if the Avidin/Biotin blockage meant to decrease the aspecific signals. Tyramide amplification, provided by Cy5 dye, seems to give virtually undetectable background staining and higher samples signals (Fig. 38C).

In RPPA, printing of lysates is a complicated process and requires printing buffer that keeps protein denatured, reduced and soluble at room temperature. Also, the blocking buffer is important in decreasing the detection background and provide good signal to noise ratios. The highest specific signal intensity was obtained when I-Block was used as the blocking buffer and SDS is added to the printing buffer, as shown in Fig. 39.

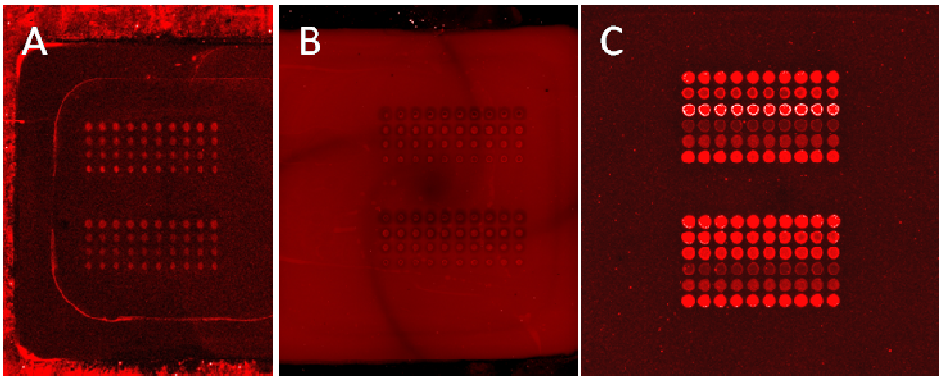


Fig. 38: Amplification assessment.
Each capture antibody was printed twice and 10 times in a raw onto poly-l-lysine slides as described. A cocktail of recombinant standards were added, along with sera. Biotinylated detection antibodies were added, followed by different amplification methods.
A) Genisphere Ultra Amp amplification, B) Avidin/biotin blocking solution and C) Tyramide amplification.

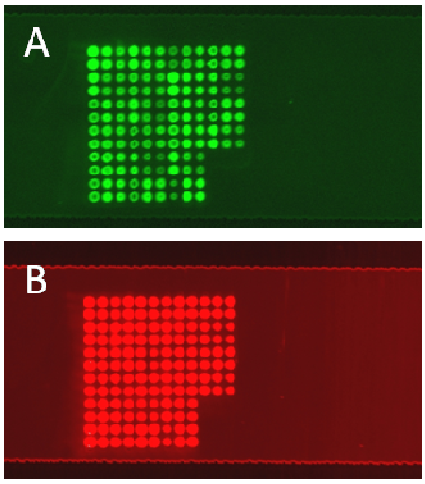


Fig. 39: Background assessment.
Cell lysates were prepared and diluted (2 fold 2 times); doubling dilution series was printed onto nitrocellulose-coated slides as described. Individual arrays were then probed with anti β-actin antibody and all arrays were then visualized by applying 680 nm near-infrared dye.
A) green signals from tested molecules; B) red signals from actin.

Testing the specificity of primary antibodies for RPPA

Western blotting is a valuable technique to test the specificity of primary antibodies before using in microarray experiment. The detection of a single defined product (often one band) indicates highly specific and sensitive primary antibodies.
All antibodies, listed in Tab. 5 and used for studying signalling pathways associated with autoinflammatory, were tested using a mixed pool of activated lysated cells. The results, shown in Fig. 40, 41, 42,43 and 44, presented single bands of the tested antibodies at the molecular weights expected for each target.

NF-κB pathway

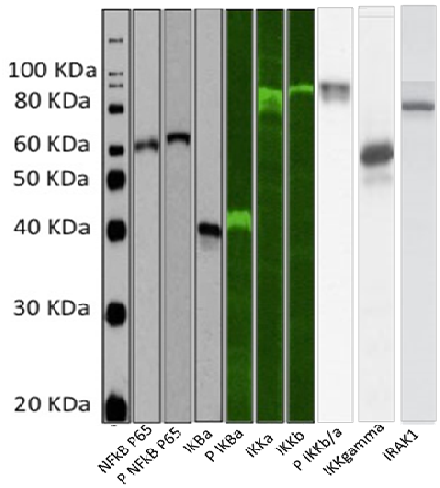


Fig. 40: Western blotting for testing antibodies used in NF-κB study.
This figure shows western blotting results of testing antibodies specific for the NF-κB pathway components including a protein marker on the left. The three "green" antibodies were detected by near infrared detection method.

PI3K/Akt pathway

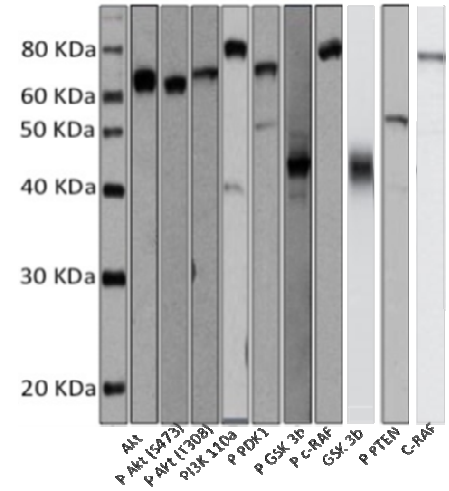


Fig. 41: Western blotting for testing antibodies used in PI3K/Akt study.
This figure shows western blotting strips for antibodies to PI3K/Akt pathway components including a protein marker (on left). Some antibodies, such as PI3K and phospho-PDK1 show weak secondary bands but these are at relatively low level compared to the known target band in each case

MAPK pathway

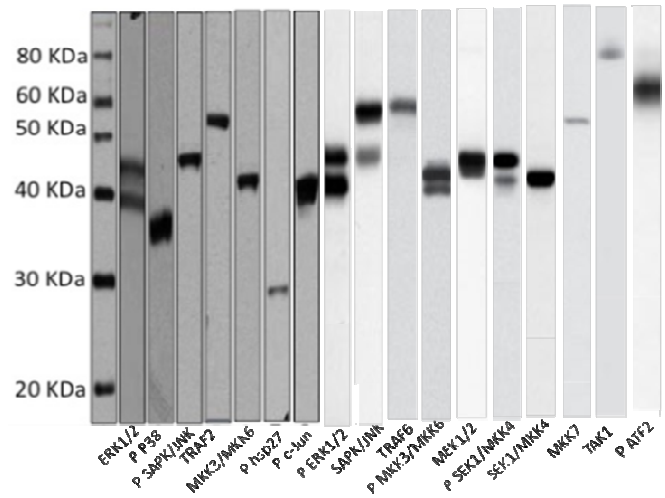


Fig. 42: Western blotting for testing antibodies used in MAPK study.
This figure shows western blotting strips for antibodies to MAPK pathway components including a protein marker (left). ERK1/2, MKK3/MKK6 and P SEK1/MKK4 reveal the characteristic 2 bands.

STAT3/JNK/c-Src pathway

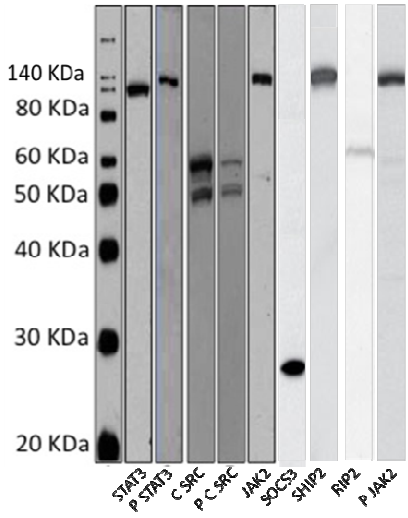


Fig. 43: Western blotting for testing antibodies used in STAT3/Jak2/c-Src study.
This figure shows western blotting strips for antibodies to STAT3/Jak2/c-Src pathway components including a protein marker (left)

Inflammasome pathway and MyD88

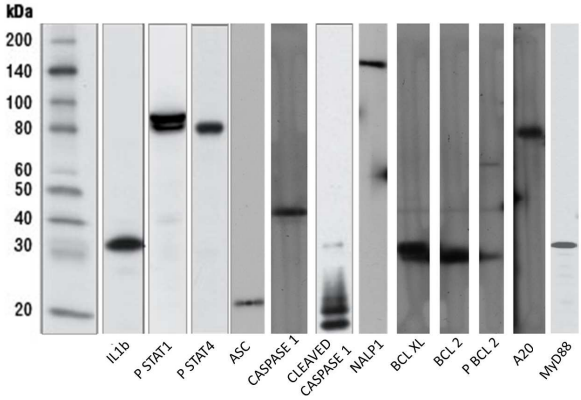


Fig. 44: Western blotting for testing antibodies used in inflammasome study and MyD88.
This figure shows western blotting strips for antibodies to inflammasome pathway components including a protein marker (left) and MyD88 protein (right)

2.2 NF-κB pathway in autoinflammatory diseases

NF-κB signalling pathway has been studied in detail in lysated PBMC samples from Italian patients affected by different autoinflammatory diseases. All data were compared to healthy controls, matched by similar age and sex.
As described in the introduction, NF-κB activation in turn activates IKK complex that phosphorylates IkBα, which normally binds to NF-κB complex and inhibits its action. Phosphorylated IkBα is ubiquitinated and addressed to proteasomal degradation, while NF-κB can translocated to the nucleus. In the nucleus, NF-κB mediates inflammatory gene transcription, cell survival and cell proliferation.
Patients PBMC were purified and proceeded as described previously in materials and methods. Reverse phase protein array technique was applied to measure the level of several component of the NF-κB signalling pathway, such as NF-κB, IKBα, IKKα/β and their phosphorylation forms. In these experiments the expression level of the analysed proteins was normalized to β-actin as a house keeping protein.
Fig. 45 summarizes the activity of NF-κB pathway in both patients and healthy controls.

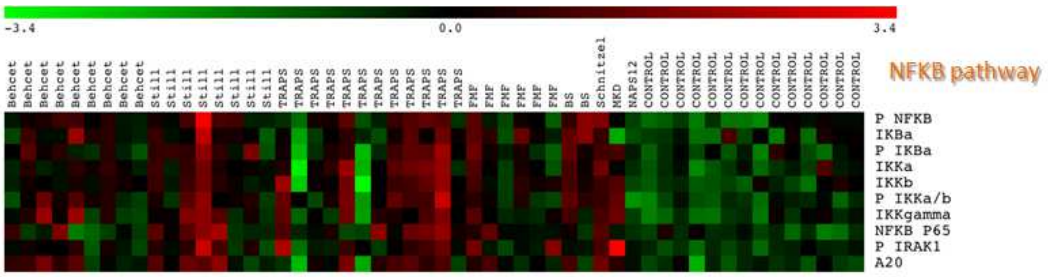


Fig. 45: Heatmap of NF-κB signalling pathway.
The multiple different proteins are outlined on the horizontal axis, and the lysates phenotype is on the vertical axis. Red and green colours indicate high and low protein expression, respectively.
From the figure the levels of different regulatory molecules of NF-κB are down-regulated in healthy controls and in some sporadic patients.

2.2.1 Behçet disease

The lysates microarray data showed higher level of phospho NF-κB, IKKα, IKKβ, phospho IKKα/β, IKKγ and A20 in Behçet patients than in healthy controls (p value <0.05) (Fig. 46). The other proteins implied in NF-κB pathway have not any statistically significant difference from the controls.

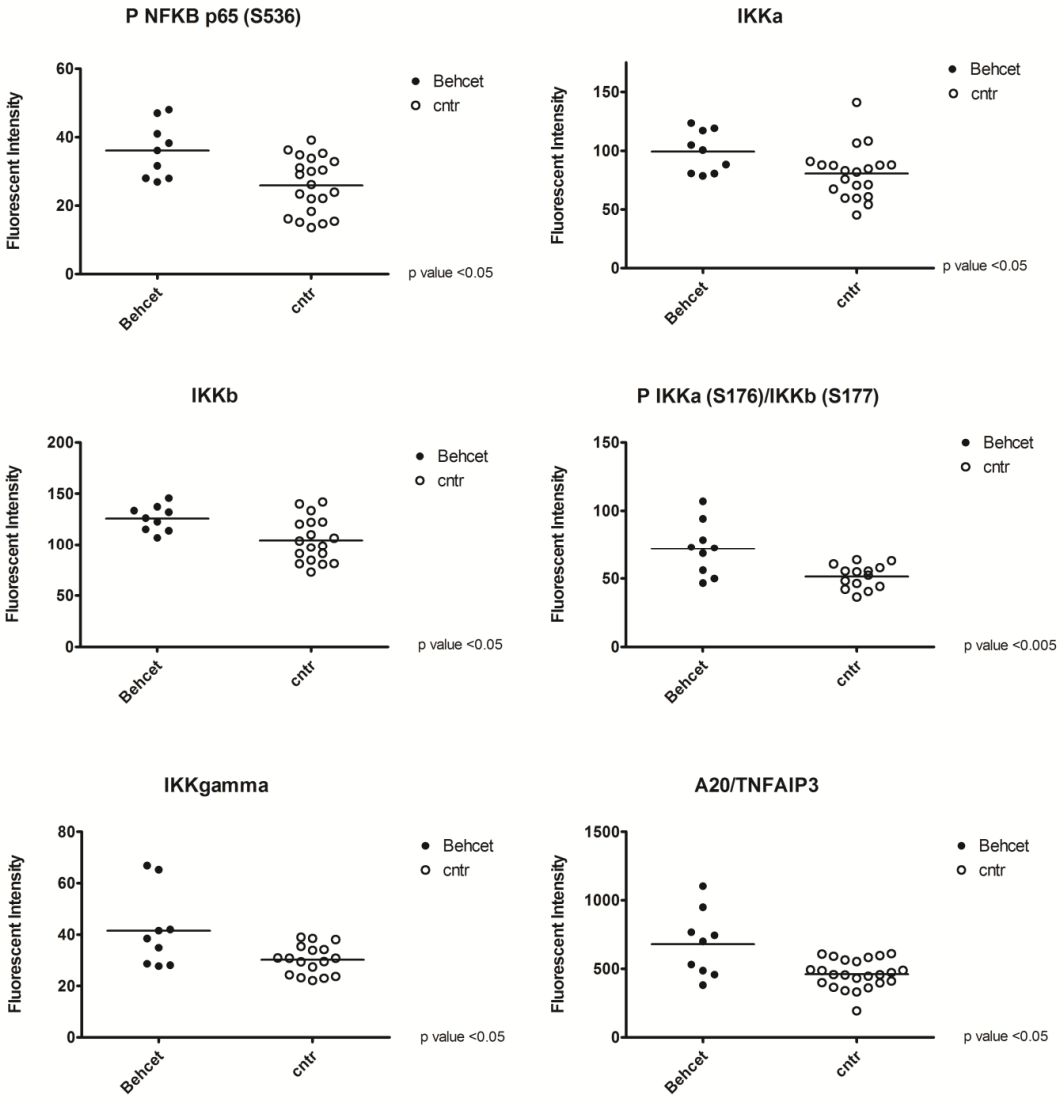


Fig. 46: NF-κB signalling pathway in Behçet disease.
Statistically significant comparisons between expression levels of phospho NF-κB, IKKα, IKKβ, phospho IKKα/β, IKKγ and A20 measured in patients and healthy controls.

2.2.2 Adult onset Still's disease

In PBMC lysates obtained from Still patients and controls, the level of the expression of NF-κB, phospho NF-κB, phospho IKBα, IKKα/β and the phosphorylated form, IKKγ and A20 was statistically significant as p values were <0.05 or less (Fig. 47).

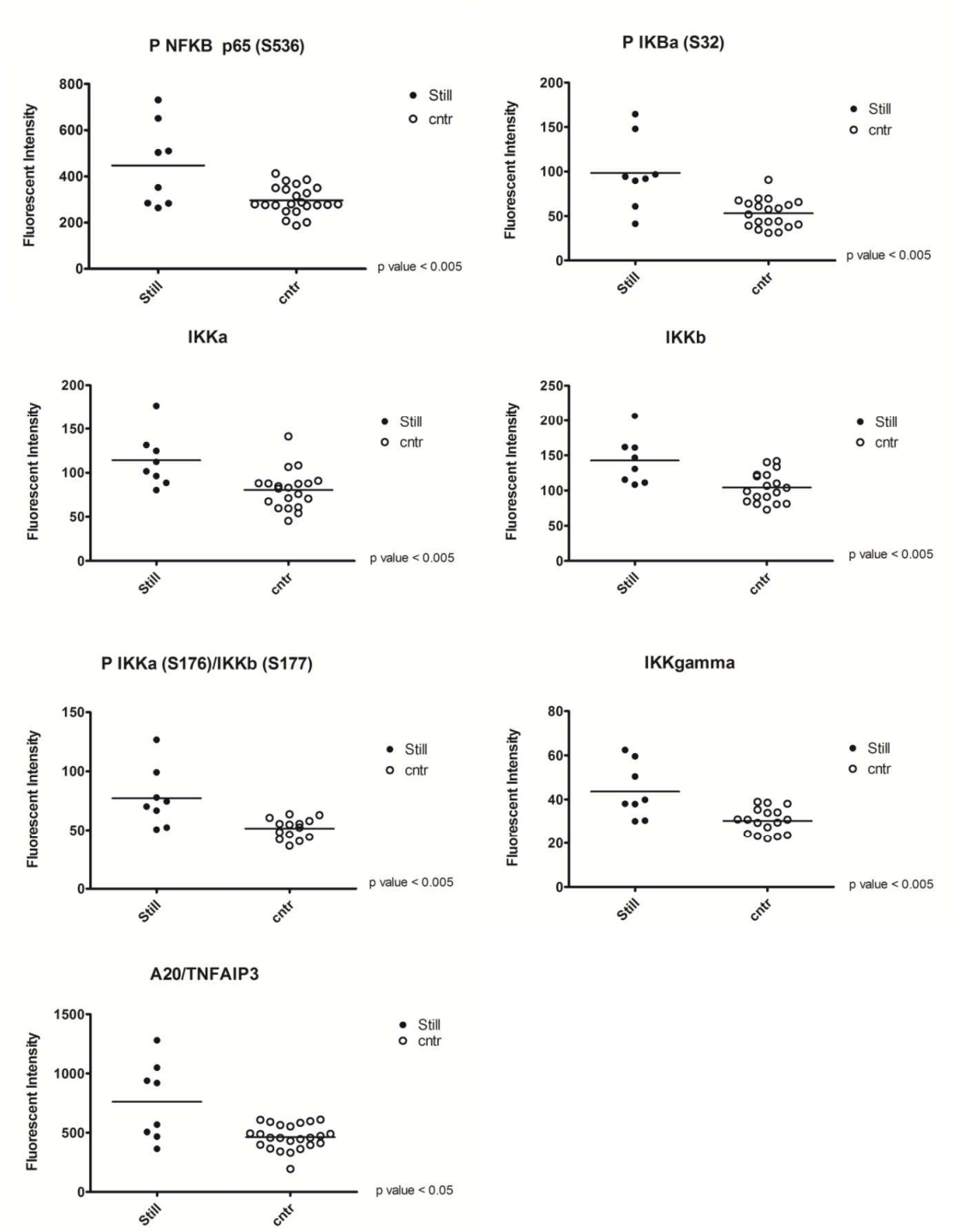


Fig. 47: NF-κB signalling pathway in AOSD.
Statistically significant comparisons of NF-κB, phospho NF-κB, phospho IKBα, IKKα/β, phospho IKKα/β, IKKγ and A20 levels between patients and controls.

2.2.3 TRAPS

The data obtained from TRAPS patients and controls showed a statistically significant increased level of NF-κB and phosphorylated form, phospho IKBα, IKKγ, IKKα/β in patients compared to controls (p value <0.05). Also the phosphorylated form of IKKα/β and IRAK1 presented higher expression in patients (p value <0.005) (Fig.48).

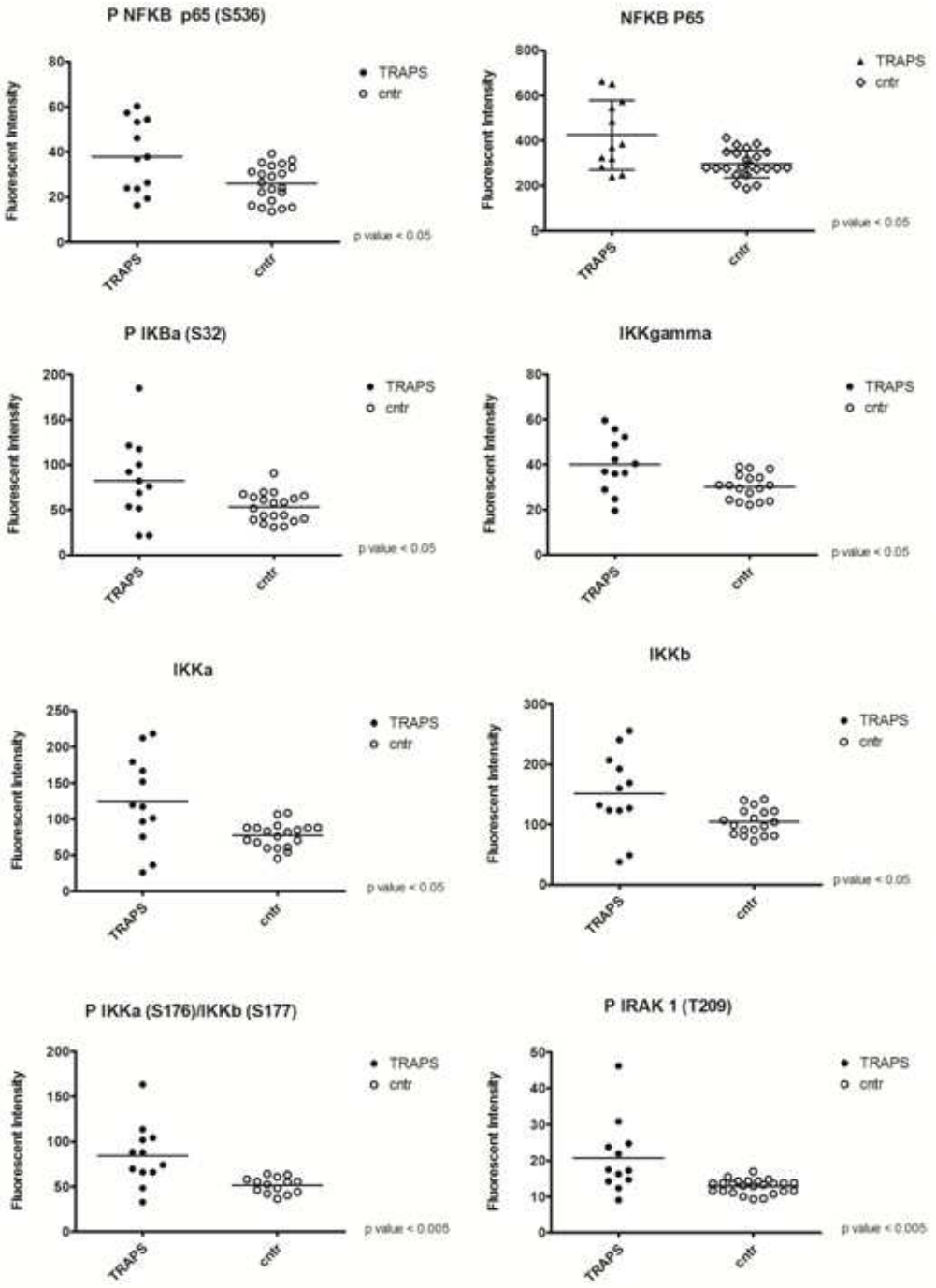


Fig. 48: NF-κB signalling pathway in TRAPS.
Comparisons of NF-κB, phospho NF-κB, phospho IKBα, IKKγ, IKKα/β, phospho IKKα/β and phospho IRAK1 levels between patients and controls are statistically significant.

2.2.4 FMF

Data from FMF patients showed higher expression (p value < 0.05) of only IKKβ and phospho IRAK1 compared to healthy controls (Fig. 49).

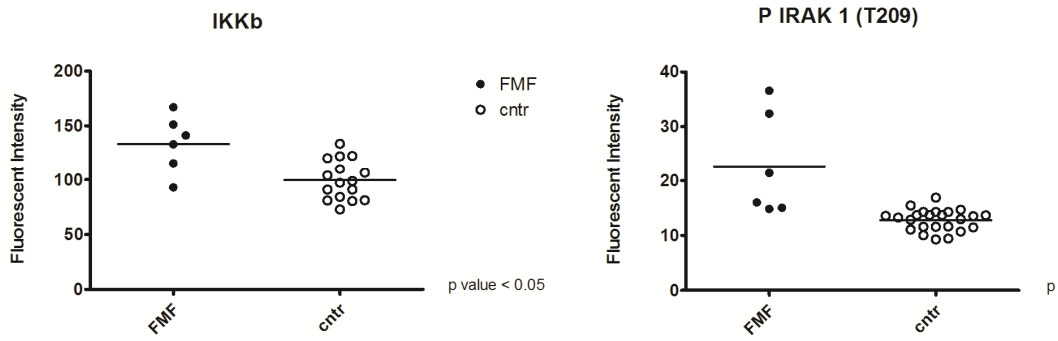


Fig. 49: NF-κB signalling pathway in FMF.
Comparison of IKKβ and phospho IRAK1 levels is significant between patients and healthy controls.

2.2.5 Blau syndrome

The NF-κB signalling pathway was extensively described previously.

2.2.6 Other autoinflammatory diseases

Since the number of patients does not allow accurate statistical analysis, for Schnitzel, MKD and NALPS12 diseases patients we may only study the heatmap and see if the expression of pathway components are higher or lower compared to healthy controls.
The patient affected of Schnitzel disease seemed to show an activation of the NF-κB pathway, while the NALPS12 patient data was comparable to the controls. For the MKD patient, it is possible to notice increased expression of the IKK complex, an over-expression of P IRAK1 and a strong sub-expression of IKBα.

2.3 PI3K/Akt pathway in autoinflammatory diseases

PI3K/Akt signalling pathway has been studied in detail in lysated PBMC samples from Italian patients affected by different autoinflammatory diseases. Patients data were then compared to healthy controls.
As previously described, PI3K is activated by both receptor and non-receptor tyrosine kinases (Src). Active PI3Kactivates many downstream targets including Akt, ERK and JNK. Akt is phosphorylated byPDK1 and this will in turn stimulate different downstream signalling that inhibits apoptosis.
Patients PBMC were proceeded as described previously. RPPA technique was applied to measure the level of several component of the PI3K/Akt signalling pathway, in both phosphorylated and not phosphorylated form. In these experiments the expression level of the analysed proteins was normalized to β-actin as house keeping protein.
The heatmap shows the activity of PI3K/Akt pathway in both patients and healthy controls (Fig. 50).

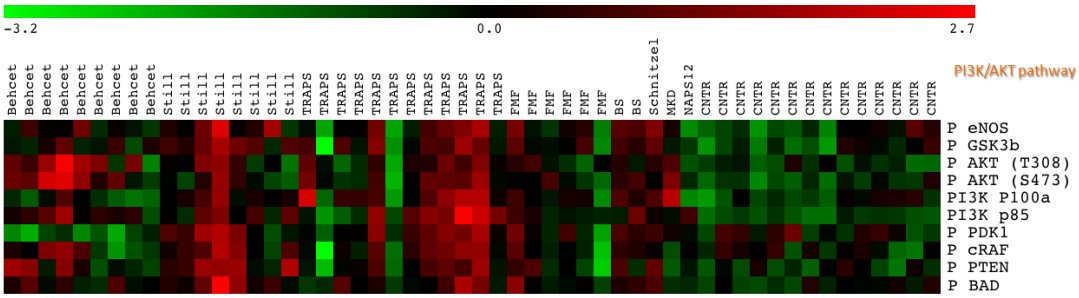


Fig. 50: Heatmap of PI3K/Akt signalling pathway.
The multiple different proteins are outlined on the horizontal axis, and the lysate phenotype is on the vertical axis. Red and green colours indicate highand low protein expression, respectively.
From the figure the levels of different regulatory molecules of the pathway are down-regulated in healthy controls and in some sporadic patients.

2.3.1 Behçet disease

The data obtained from lysates microarray (Fig. 51) showed only for phospho AKT and PI3K p85 a statistically significant increase of expression, compared to controls (p values <0.05 or less).

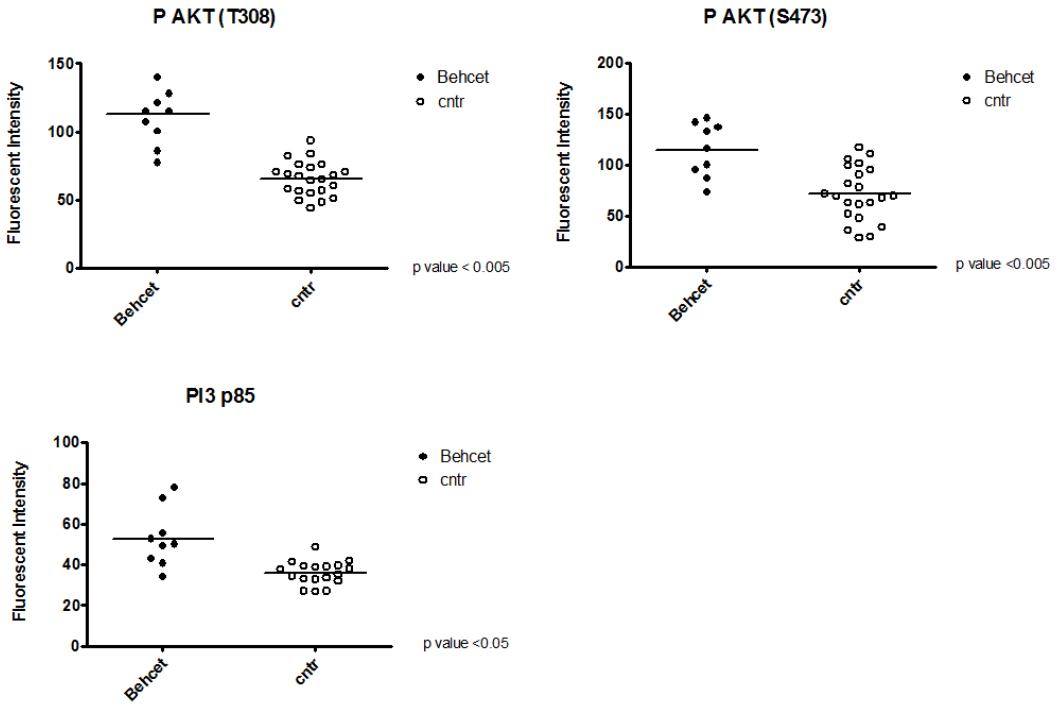


Fig. 51: PI3K/Akt signalling pathway in Behçet disease.
Significant comparison of phospho Akt and PI3K p85 levels between patients and controls.

2.3.2 Adult Onset Still's disease

Fig. 52 showed higher level of phosphorylated forms of AKT (T308), eNOS, PTEN, cRAF, and GSK-3β and the two subunit p85 and p100 of PI3 protein in Still patients than in healthy controls (p value <0.05 or less).

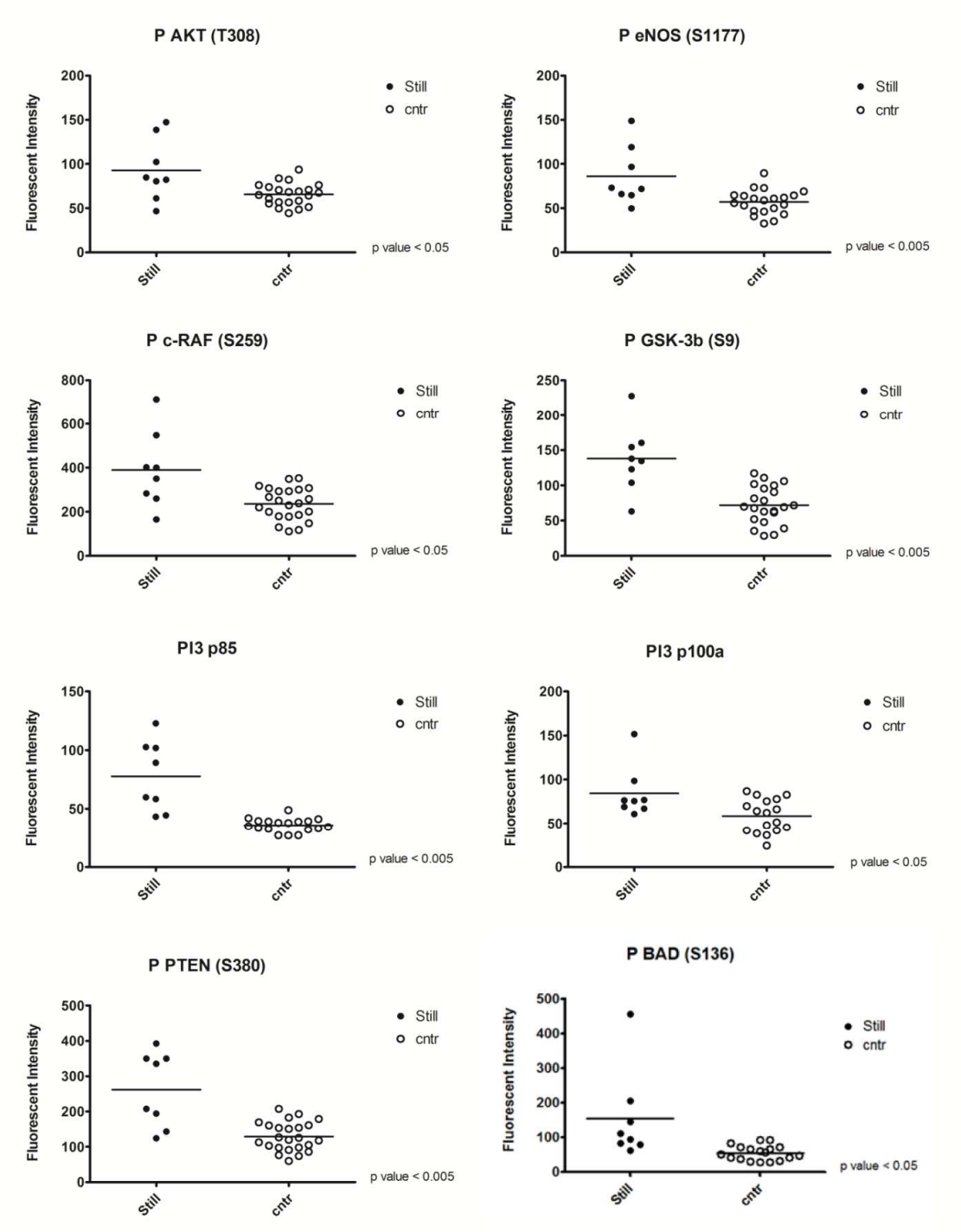


Fig. 52: PI3K/Akt signalling pathway in AOSD.
Statically significant comparison between patients and controls of phospho Akt, phospho eNOS, phospho c-RAF, phospho GSK-3β, PI3 p85, PI3 p110, phospho PTEN and phospho BAD levels

2.3.3 TRAPS

The data obtained from lysates microarray showed for phospho AKT (T308 and S473), PI3p100 and PI3p85, and phospho GSK-3β a statistically significant increase of expression, compared to controls (Fig. 53). P values are less than 0.05 for phospho GSK-3β and lower for other components.

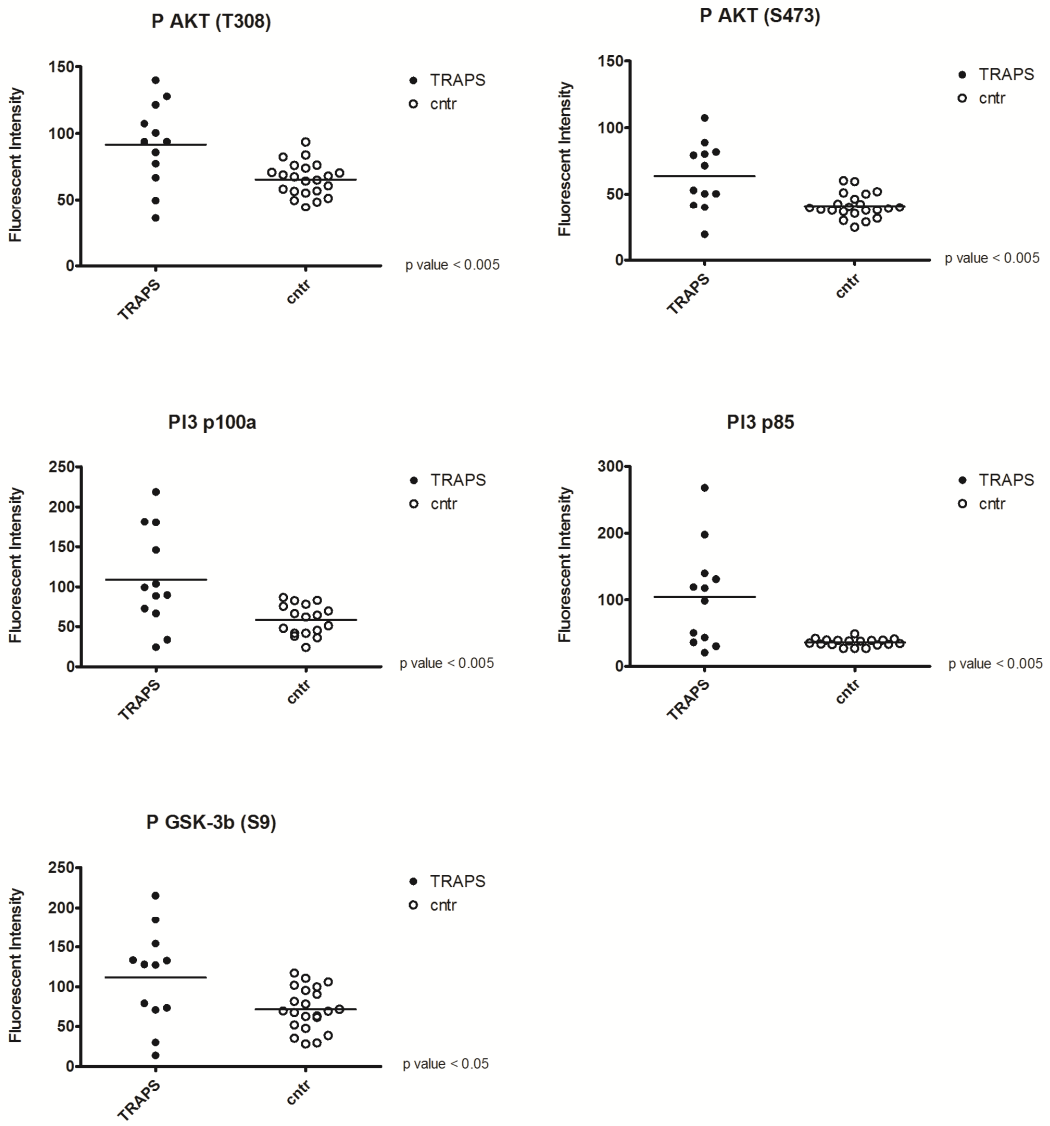


Fig. 53: PI3K/Akt signalling pathway in TRAPS.
Significant comparison of phospho Akt, PI3 p85, PI3K p100 and phospho GSK-3β levels between patients and controls.

2.3.4 FMF

The microarray data showed significant higher expression only for PI3 p85 compared to healthy controls (p value <0.005) (Fig. 54). For the inflammasome pathway components, no statistically significant comparison was found.

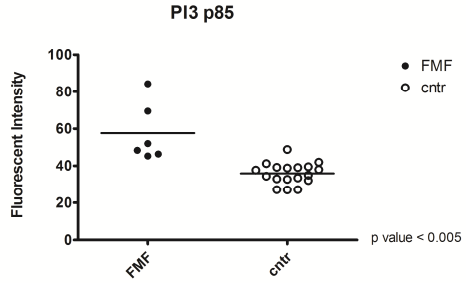


Fig. 54: PI3K/Akt signalling pathway in FMF. Comparison of PI3K p85 level between patients and controls is statistically significant.

2.3.5 Blau syndrome

In PBMC lysates obtained from Blau syndrome patients and controls, PI3p85 and the phosphorylated forms of eNOS, GSK-3β, AKT (S473), and BAD are statistically more expressed than in controls (p values <0.05) (Fig. 55).

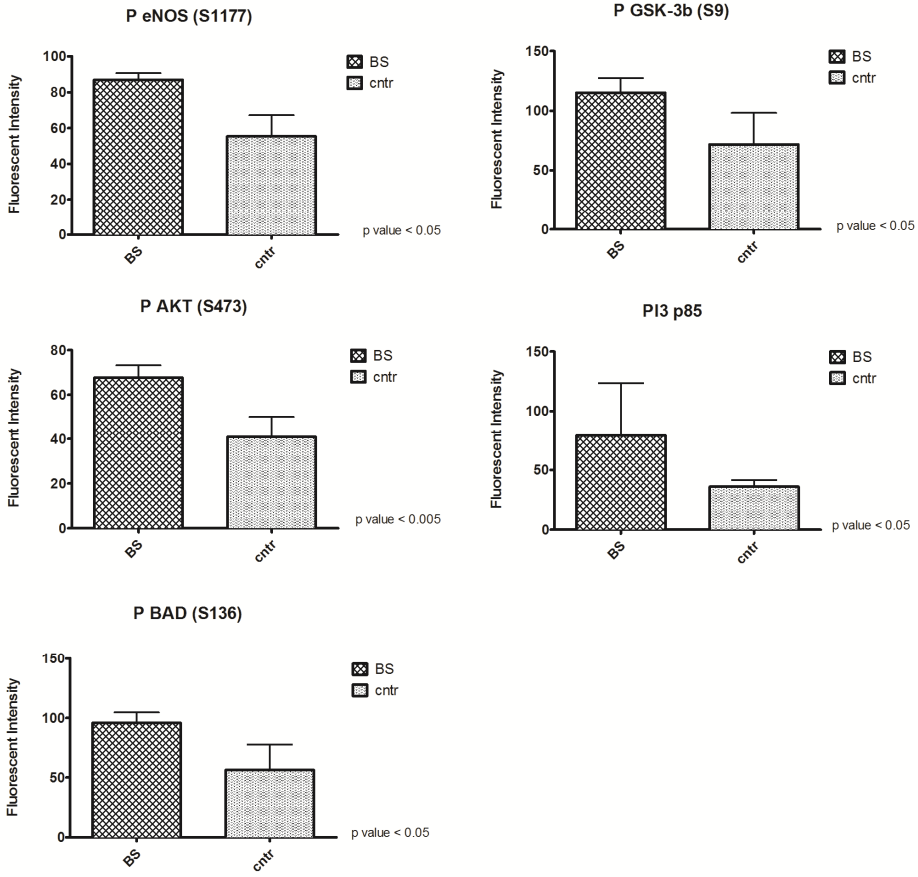


Fig. 55: PI3K/Akt signalling pathway in Blau syndrome.
Comparisons of phospho eNOS, phospho GSK-33β, phospho Akt, PI3K p85 and phospho BAD levels between patients and controls are statistically significant.

2.3.6 Other autoinflammatory diseases

Evaluating the heatmap for the remaining individual autoinflammatory patients, both Schnitzel and MKD patient showed an increased expression of PI3K/Akt pathway components, except for P cRAF and P PTEN in MKD patient. NALP12 patient, instead, showed a similar expression to healthy controls.

2.4 MAPK pathway in autoinflammatory diseases

MAPK signalling pathway has been studied in detail in lysated PBMC samples from Italian autoinflammatory diseases and controls.
MAPK is stimulated by different external stimuli, such as TNFα and IL1.TRAF2 is also an important activator of this pathway. The external stimulus starts a downstream signalling cascade that could activate ASK1,MKK3, MKK4 and MKK6. The downstream signalling ends in activation of HSP-27 and transcription of different pro-inflammatory molecules such as ATF2 and ELK1.
Patients PBMC were purified and proceeded as described previously. Reverse phase protein array technique was applied to measure the level of several component of the MAPK signalling pathway, most of them phosphorylated. In these experiments the expression level of the analysed proteins was normalized to β-actin as a house keeping protein. Patients data were then statistically compared with healthy controls data.
The heatmap presented in Fig. 56 summarizes the activity of PI3K/Akt pathway in both patients and healthy controls.

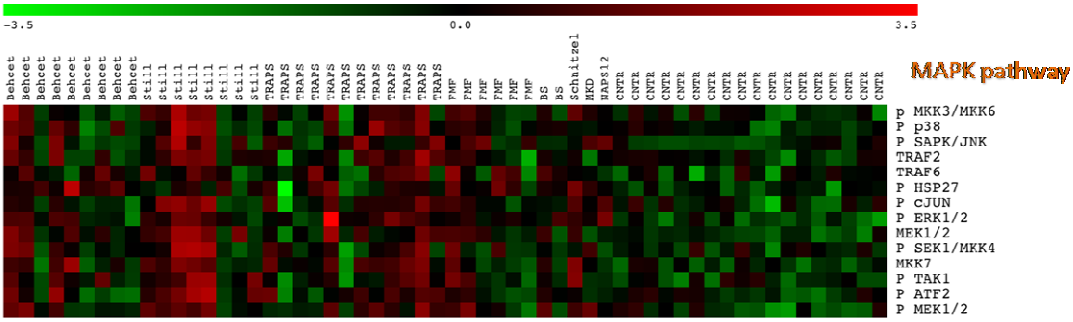


Fig. 56: Heatmap of MPK signalling pathway.
The multiple different proteins are outlined on the horizontal axis, and the lysate phenotype is on the vertical axis. Red and green colours indicate highand low protein expression, respectively.
From the figure the levels of different regulatory molecules of the pathway are down-regulated in healthy controls and in some sporadic patients.

2.4.1 Behçet disease

In PBMC lysates obtained from Behçet patients and controls, the level of the expression of phospho ERK1/2, MEK1/2 and phospho HSP27 was statistically significant as p values were <0.05 or less (Fig. 57)

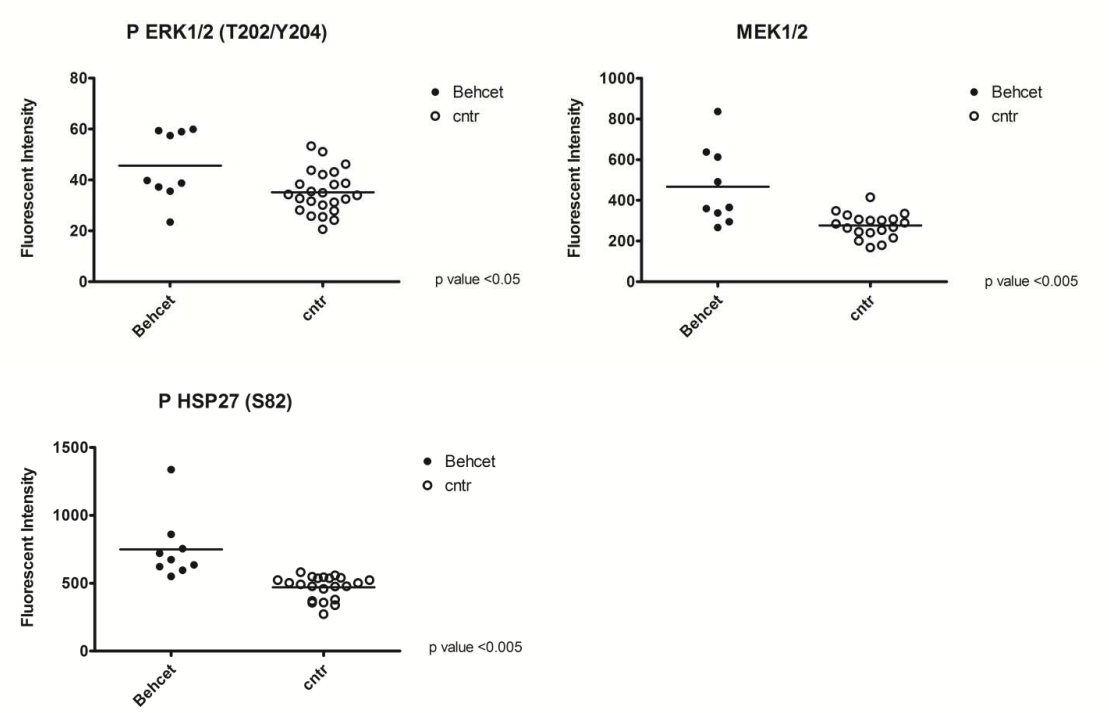


Fig. 57: MAPK signalling pathway in Behçet disease.
Comparison of phospho Erk, MEK1/2 and phospho HSP27 levels between patients and controls are statistically significant.

2.4.2 Adult Onset Still's disease

The data obtained from lysates microarray showed only for a phospho MKK3/MKK6, phospho SAPK/JNK, TRAF2, phospho ERK1/2, MEK1/2, MKK7, phospho TAK1 and phospho ATF2 a statistically significant increase of expression, compared to controls, with p values less than 0.05 (Fig. 58).

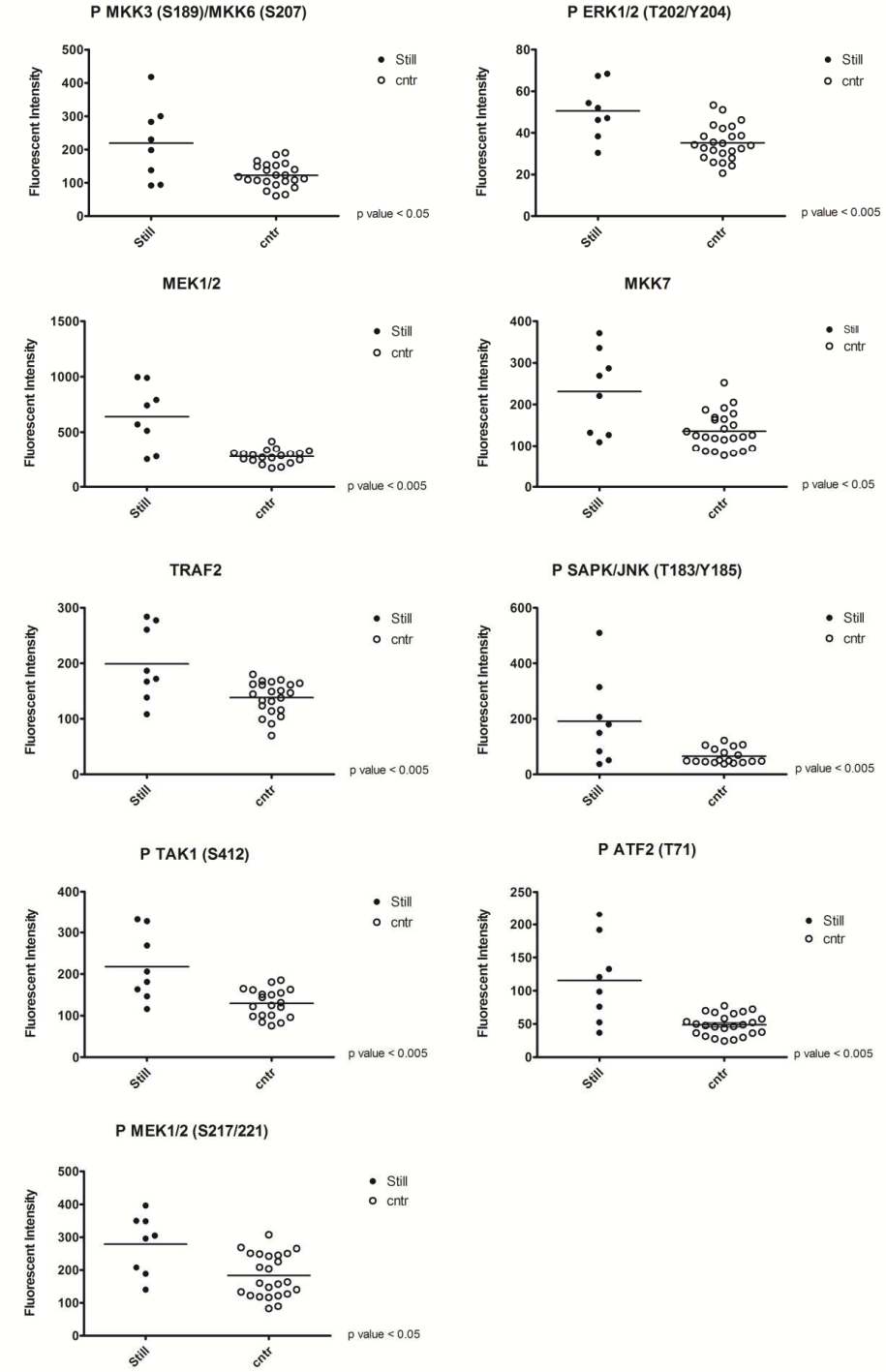


Fig. 58: MAPK signalling pathway in AOSD.
Significant comparisons of phospho MKK3/6, phospho Erk, MEK1/2, MKK7, TRAF2, phospho SAPK/JNK, phospho TAK1, phospho ATF2 and phospho MEK1/2 levels between patients and controls.

2.4.3 TRAPS

In PBMC lysates obtained from TRAPS patients and controls, the level of the expression of TRAF2, MEK1/2 and phosphorylated form, phospho SAPK/JNK, MKK7, phospho ERK1/2 and phosho ATF2 was statistically significant as p values were <0.05 or lower (Fig. 59).

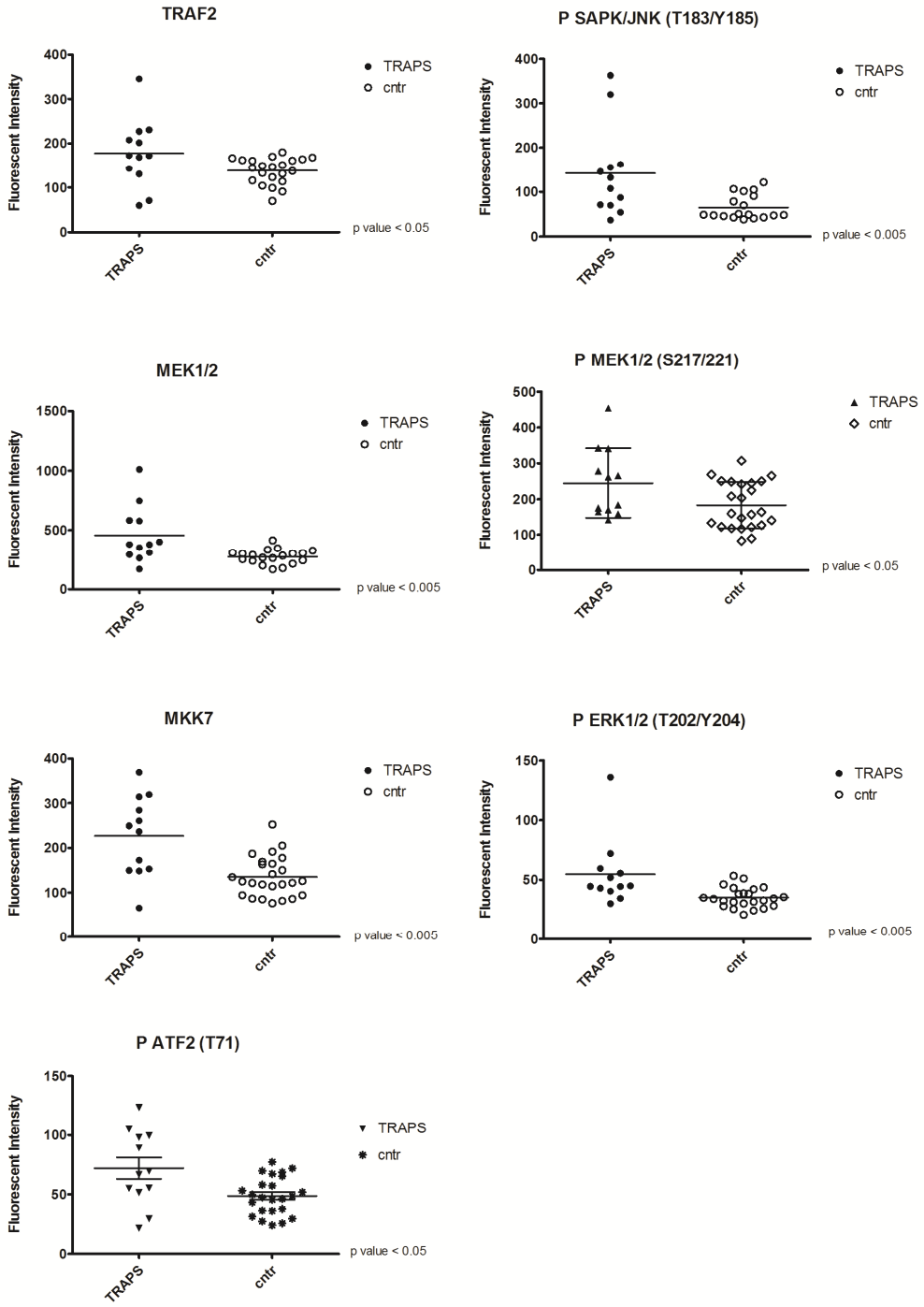


Fig. 59: MAPK signalling pathway in TRAPS.
Statistically significant comparisons of TRAF2, phospho SAPK/JNK, MEK1/2, phospho MEK1/2, MKK7, phospho Erk and phospho ATF2 levels between patients and healthy controls.

2.4.4 FMF

Fig. 60 showed higher expression of MEK 1/2 and the phosphorylated forms of SAPK/JNK, ERK1/2 and HSP27 compared to healthy controls. Significant P values are <0.05 or less.

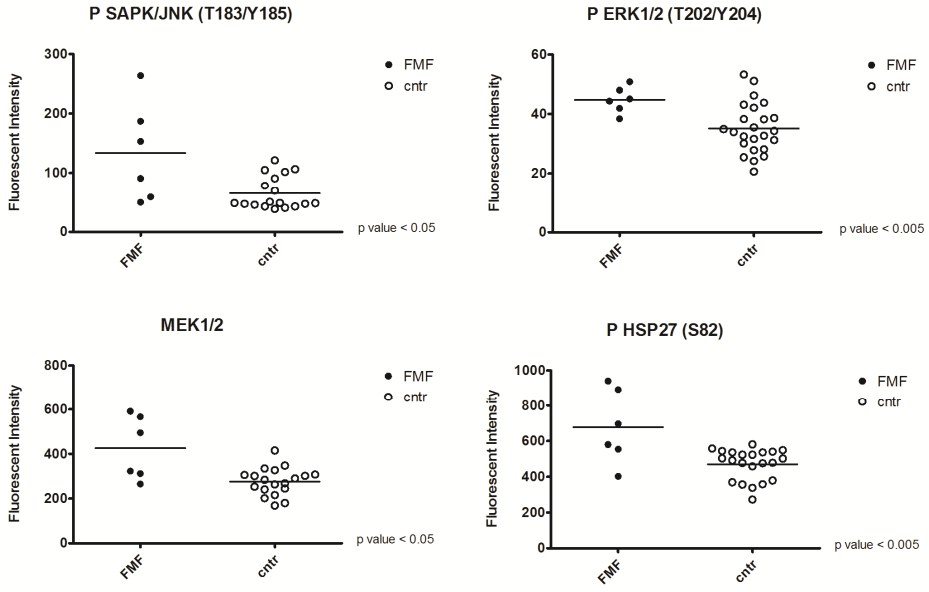


Fig. 60: MAPK signalling pathway in FMF.
Significant comparisons of phospho SAPK/JNK, phospho Erk, phospho HSP27 and MEK1/2 levels between patients and controls.

2.4.5 Blau syndrome

The data obtained from Blau syndrome patients and controls showed that the expression level of phospho HSP27 and phospho P38 was statistically significant (P values <0.05) (Fig. 61).

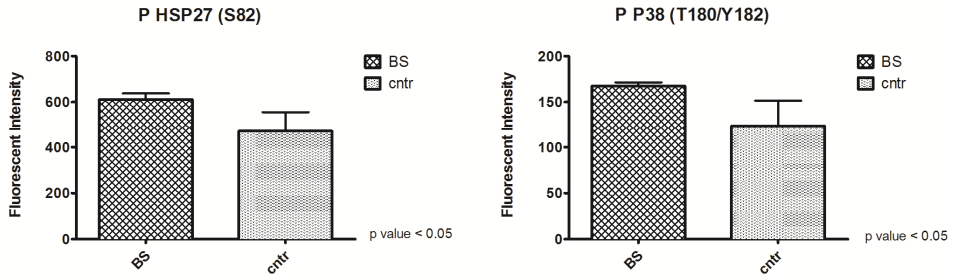


Fig. 61: MAPK signalling pathway in Blau syndrome.
Significant comparisons of phospho HSP27 and phospho P38 levels between patients and controls.

2.4.6 Other autoinflammatory diseases

Observing the heatmap, MKK7, phospho TAK1 and phospho HSP27 levels seemed to be increased in Schnitzel patient. MKD patient showed an higher expression of phospho SAPK/JNK and phospho MEK1/2, while NALP12 patient

showed a slightly higher expression of phosphorylated forms of SAPK/JNK, cJun and ERK1/2.

2.5 JAK/STAT/c-Src pathway in autoinflammatory diseases

JAK/STAT/cSrc signalling pathway has been studied in detail in lysated PBMC samples from Italian patients affected by different autoinflammatory diseases. Patients PBMC were purified and proceeded as described previously. RPPA technique was applied tomeasure the level of several component of the signalling pathway, such as STAT3, JAK2 and phosphorylated Src. In these experiments the expression level of the analysed proteins was normalized to actin as a house keeping protein. The data obtained from patients were then compared to healthy controls.

A variety of ligands and their receptors stimulate the JAK/STAT/Src pathway. Intracellular activation occurs when ligand binding induces the multimerization of receptor subunits. For signal propagation through either homodimers or heteromultimers, the cytoplasmic domains of two receptor subunits must be associated with JAK. The activated JAK subsequently phosphorylates additional targets, including the major substrate, STATs. Once phosphorylated, STAT moves into the nucleus and starts transcriptions of many inflammatory cytokines.

The heatmap presented in Fig. 62 summarizes the activity of PI3K/Akt pathway in both patients and healthy controls.

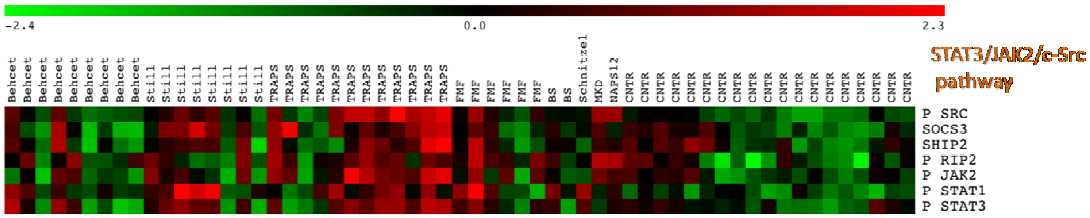


Fig. 62: Heatmap of JAK/STAT/c-Src signalling pathway.
The multiple different proteins are outlined on the horizontal axis, and the lysates phenotype is on the vertical axis. Red and green colours indicate high and low protein expression, respectively.
From the figure the levels of different regulatorymolecules of the pathway are down-regulated in most of the healthy controls and in some sporadic Behçet, Still, TRAPS and FMF patients.

2.5.1 Behçet disease

The data obtained from lysates microarray (Fig. 63) showed only for phospho STAT1 a statistically significant increase of expression, compared to controls (p value <0.05). For the other pathway components, the expression varies along patients.

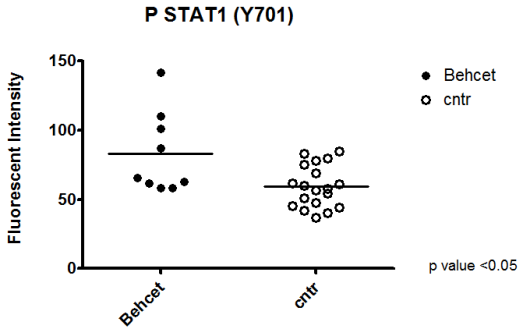


Fig. 63: JNK/STAT/c-Src signalling pathway in Behçet disease.
Comparison of phospho STAT1 between patients and controls is statistically significant.

2.5.2 Adult Onset Still's disease

In PBMC lysates obtained from Still disease patients and controls, the level of the expression of phospho Src, SOCS3, phospho STAT1 and STAT3 was statistically significant as p values were <0.05 or lower (Fig. 64).

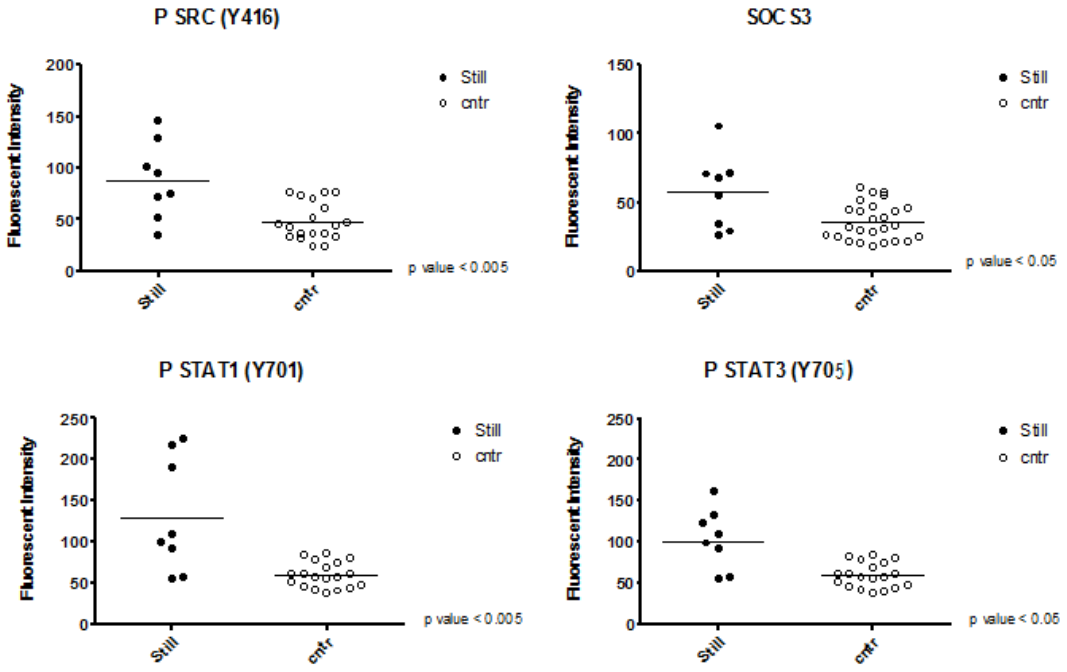


Fig. 64: JNK/STAT/c-Src signalling pathway in AOSD.
Significant comparisons of phospho Src, SOCS3, phospho STAT1 and phospho STAT3 levels between patients and controls.

2.5.3 TRAPS

The lysate microarray data, shown in Fig. 65, presented higher level of SOCS3 and phosphorylated form of Src, STAT1, JAK2, RIP2, STAT3 in TRAPS patients than in healthy controls (p value <0.05).

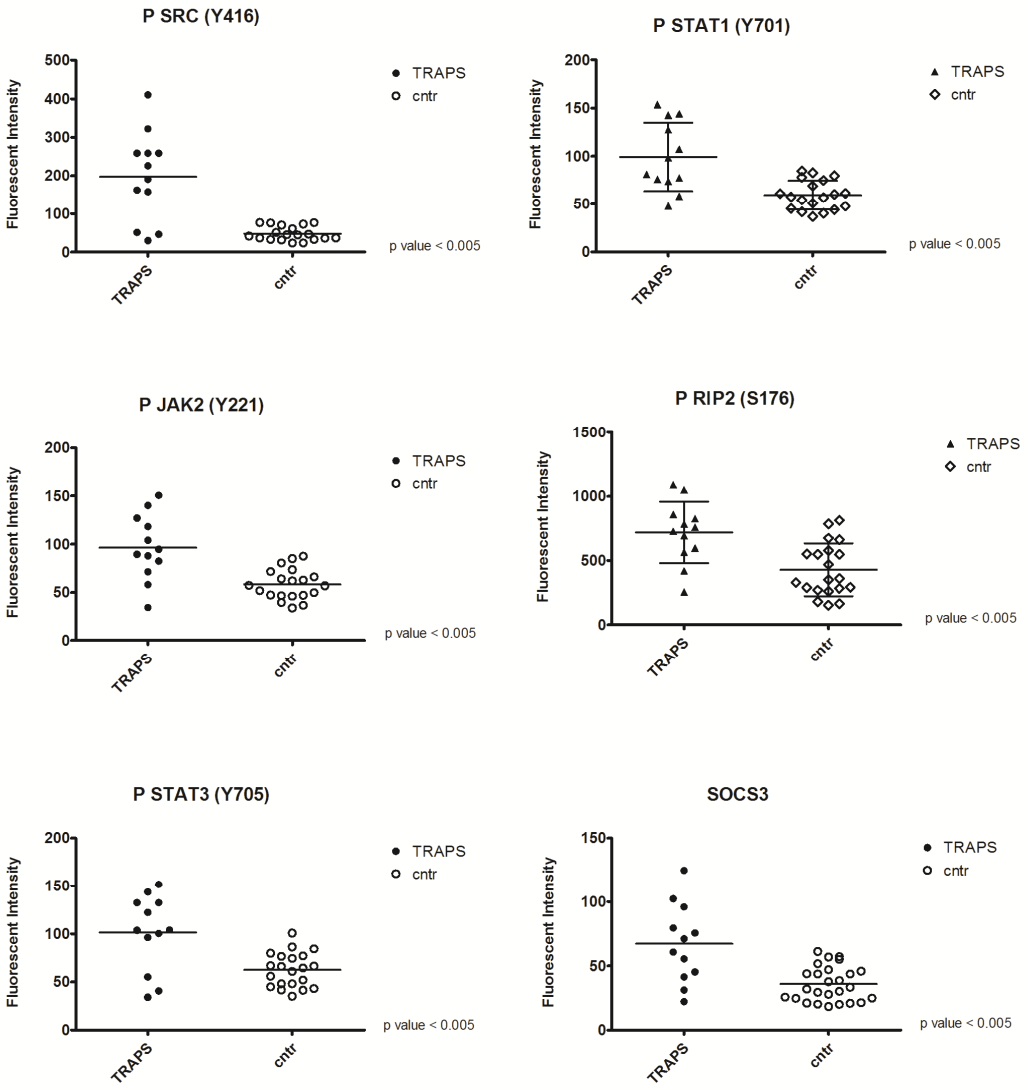


Fig. 65: JNK/STAT/c-Src signalling pathway in TRAPS.
Statistically significant comparisons of phospho Src, phospho STAT1, phospho JAK2, phopsho RIP2, phospho STAT3 and SOCS3 between patients and healthy controls.

2.5.4 FMF

FMF patients showed a higher level of phosphrylated form of Src and RIP2 compared to healthy controls (p values <0.005) (Fig. 66).

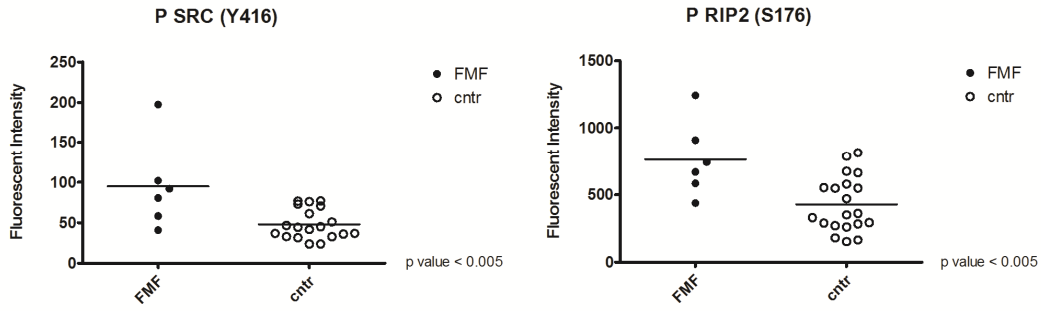


Fig. 66: JNK/STAT/c-Src signalling pathway in FMF.
Significant comparisons of phospho Src and phospho RIP2 between patients and controls.

2.5.5 Blau syndrome

In BS lysates, only phospho STAT1 showed an higher level (p value <0.05) compared to healthy controls data (Fig. 67).

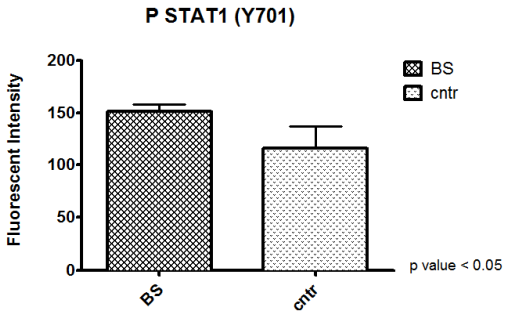


Fig. 67: JNK/STAT/c-Src signalling pathway in Blau syndrome.
Comparison of phospho STAT1 between patients and controls is statistically significant.

2.5.6 Other autoinflammatory diseases

Observing the heatmap, MKD and NALP12 patients showed a similar up-regulation of the JNK/STAT/c-Src pathway components, whereas in Schnitzel patient this pathway seemed not to be activated.

2.6 INFLAMMASOME pathway in autoinflammatory diseases and MyD88 protein evaluation

In presence of stimuli, such as PAMPs and DAMPs, a protein from NLR family (NLRP1, NLRP3 or NLRC4) were able to assemble and oligomerize into a common structure with adaptor proteins like ASC. Once active, the inflammasome binds to pro-caspase1 via its own CARD domain or via the CARD domain of the adaptor protein ASC which it binds to during inflammasome formation. In its full form, the inflammasome induces the autocatalytic cleavage of pro-caspase1 into p20 and p10 subunits. Once active, caspase1 can carry out a variety of processes in response to the initial inflammatory signal, such as production of active IL1. The Bcl-2 family members Bcl-2 and Bcl-XL, have been shown to specifically interact with NLRP1 to inhibit ATP binding and subsequent oligomerization.

The inflammasome signalling pathway has been studied in detail in lysated PBMC samples from Italian patients affected by different autoinflammatory diseases. Patients PBMC were proceeded as described in materials and methods. Reverse phase protein array technique measured the expression levels of several component of this signalling pathway, that were later normalized to β-actin as house keeping protein. The data obtained from patients were then compared to healthy controls data.

We also evaluated MyD88 expression level in autoinflammatory diseases patients and controls.

The heatmap presented in Fig. 68 summarizes the activity of the signalling mediators in both patients and healthy controls.

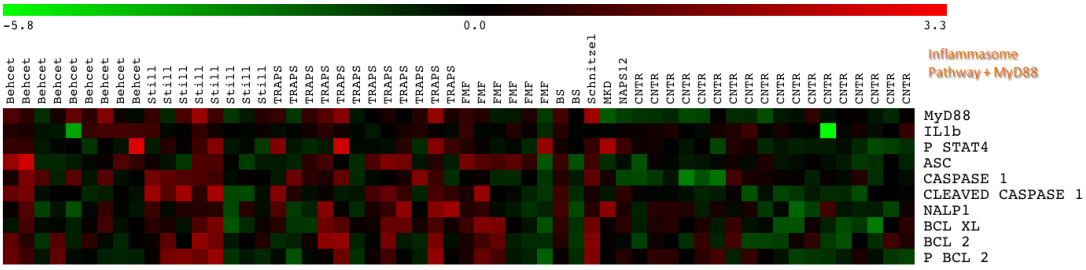


Fig. 68: Heatmap of Inflammasome signalling pathway.
The multiple different proteins are outlined on the horizontal axis, and the lysates phenotype is on the vertical axis. Red and green colours indicate high and low protein expression, respectively.
From the figure the levels of different regulatory molecules of the pathway are down-regulated in healthy controls and in some sporadic patients.

2.6.1 Behçet disease

The lysates microarray data showed a statistical increased expression only for MyD88 in Behçet patients than in healthy controls (p value <0.05) (Fig. 69). Concerning the inflammasome pathway components, the variability between patients seems to be too high for a statistical analysis.

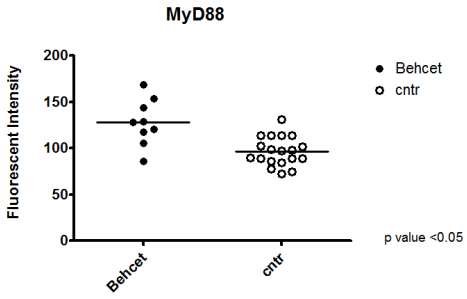


Fig. 69: Significant comparison of MyD88 level in Behçet patients versus healthy controls.

2.6.2 Adult Onset Still's disease

In PBMC lysates, the level of the expression of MyD88, phospho BAD, CASPASE 1 and cleaved form, BCL-2 and the phosphorylated form was statistically significant as p values were <0.05 or less (Fig. 70).

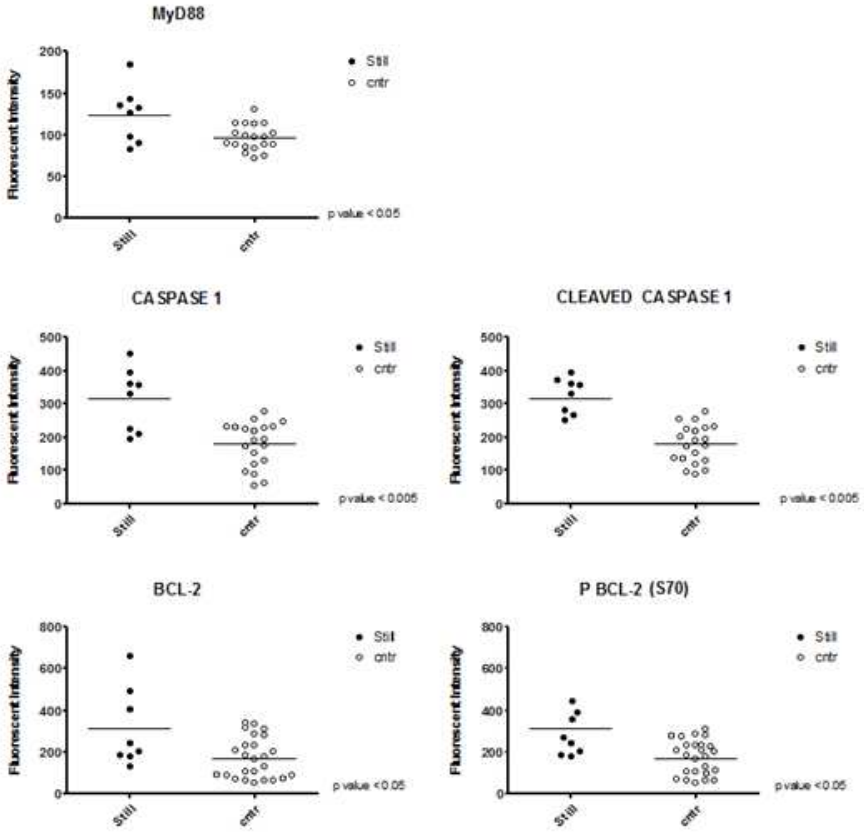


Fig. 70: Inflammasome signalling pathway in AOSD.
Significant comparisons of caspase1, cleaved caspase1, BCL-2 and phospho BCL-2 between patients and controls. Also MyD88 level is statistically significant.

2.6.3 TRAPS

The data obtained from lysates microarray showed only for MyD88, cleaved caspase 1 and BCL-XL a statistically significant increase of expression, compared to controls ($p<0.005$) (Fig. 71).

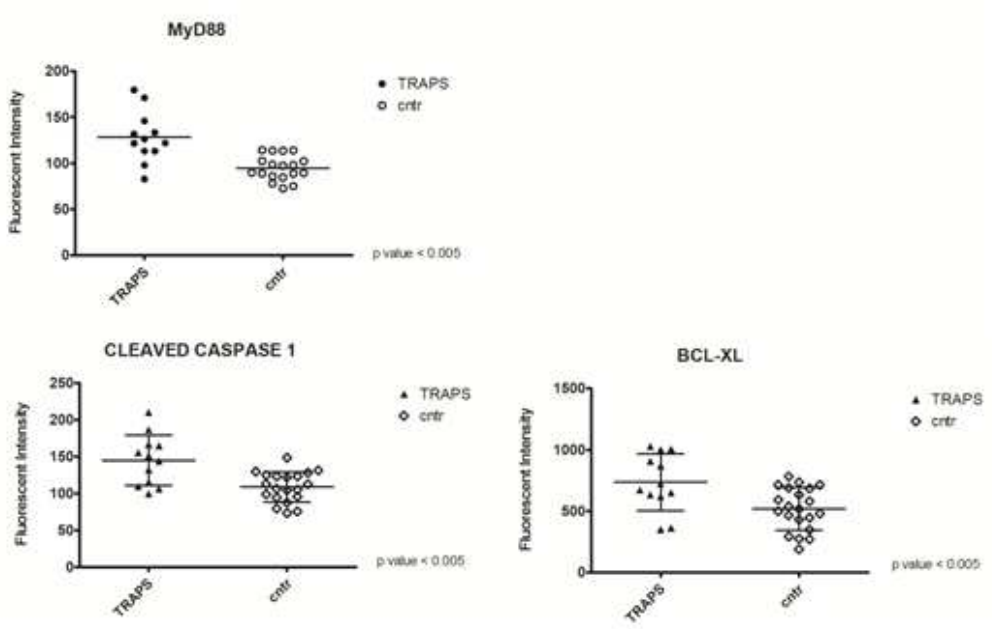


Fig. 71: Inflammasome signalling pathway and MyD88 level in TRAPS.
Statically significant comparisons of cleaved caspase1 and BCL-XL between patients and controls.

2.6.4 FMF

The microarray data showed higher level of ASC, NALP1 and BCL-XL in FMF patients than in healthy controls (p value <0.05 or less) (Fig. 72).

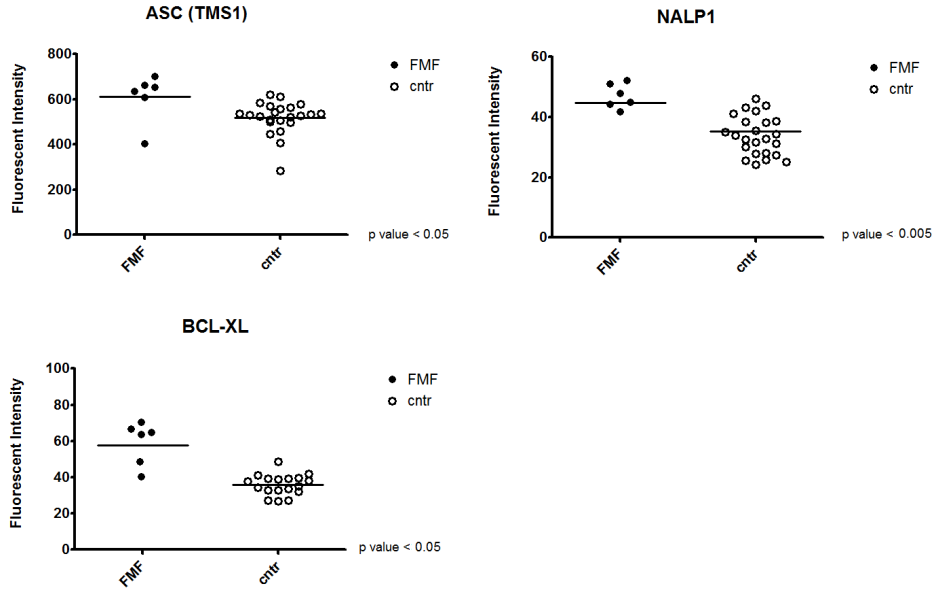


Fig. 72: Inflammasome signalling pathway in FMF.
Significant comparisons of ASC, NALP1 and BCL-XL between patients and healthy controls.

2.6.5 Blau syndrome

Data from BS patients did not show any statistically significant difference in the expression of inflammasome components, compared to healthy controls. Even MyD88 level was not significantly higher in BS patients than in controls.

2.6.6 Other autoinflammatory diseases

Evaluating the data from the heatmap, Schnitzel patient showed a higher expression of all the inflammasome components compared to healthy controls, while NALP12 patient had a profile similar to the controls. MKD patient showed an increased expression only for STAT4 and NALP1.

2.7 Cytokines in autoinflammatory diseases

Cytokines and other markers of inflammation production has been evaluated through antibody Microarray technique from serum samples obtained by Italian patients affected by different autoinflammatory diseases. Once measured the cytokines level as described in materials and methods, all data were compared to Italian healthy controls presenting similar age and sex.

We focused on a wide range of cytokines and compounds that are known to play an important role in inflammation, IL1β in primis. We expanded the analysis from typical pro-inflammatory cytokines, such as IL1β IL6 TNFα, to novel inflammation-associated pro-inflammatory cytokines, such as IL22, IL23 and IL17. This study using 9 cytokines and inflammatory markers may help to have a better understanding of inflammatory processes involved in the autoinflammatory diseases.

2.7.1 Behçet disease

Concentrations of systemic cytokines and markers of inflammation in Behçet disease are shown in Fig. 73. The most notably difference was detected for IL22, a cytokine that plays an important role in inflammation, including chronic inflammatory diseases and infectious diseases. In patients, the concentration of IL22 was more than 10-fold ($p<0.005$) higher than in controls. Statistically significant differences between patients and controls were also detected for IL8, IL17 and IL12, even though for the latter cytokine the level in patients are 2-times ($p<0.05$) lower than in controls. Concerning IL8, the concentration in patients was found to be 5-fold higher than in controls ($p<0.01$). The same trend was observed for IL17 ($p<0.05$).

The concentration of all other markers was not statistically different from controls.

For IL23, the patients cytokine level was slightly increased, even if the value was not significant.

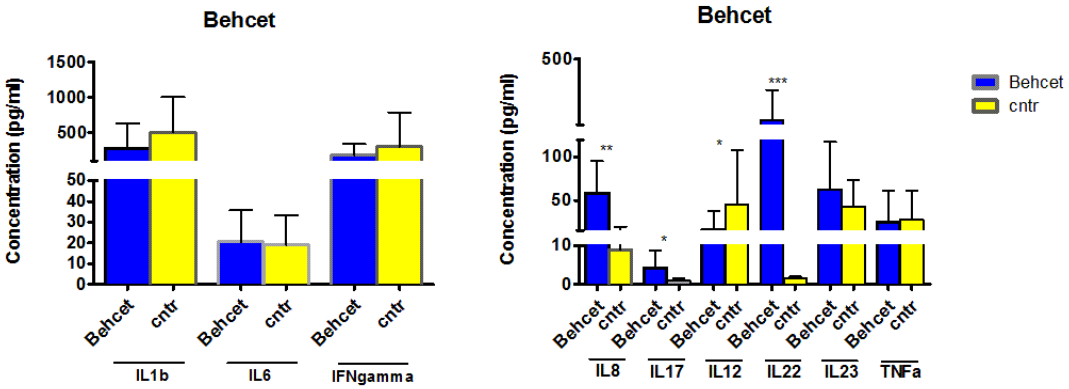


Fig. 73: Graphs of cytokines and marker of inflammation concentrations in serum from Behçet patients compared to healthy controls.
The presented data are means of data from triplicate experiments and the error bars indicate standard deviation.
* p<0.05; ** p<0.01; ***p<0.005, as statistical significance is for p values less than 0.05

2.7.2 Adult Onset Still's disease

Fig. 74 showed the concentration levels of cytokines and inflammatory markers in AOSD disease. The results showed that all the analyzed cytokines and markers presented a statistically significant difference between patients and controls. In patients, the concentration of IL1β was 10-fold higher than in controls ($p<0.05$) and the same trend was also observed for IL6 and IFNγ. Extremely high IL22 and IL23 concentrations were detected in patients, with the eight-fold ($p<0.05$) and six-fold ($p<0.05$) increase over the values obtained for control subjects, respectively. Increased concentration levels were also observed for IL8, IL12 and TNFα, presenting respectively 10-fold, 12-fold and 6-fold higher values than controls. An extremely higher increase (50-fold, $p<0.01$) was found for IL17 concentration level in patients, compared to controls.

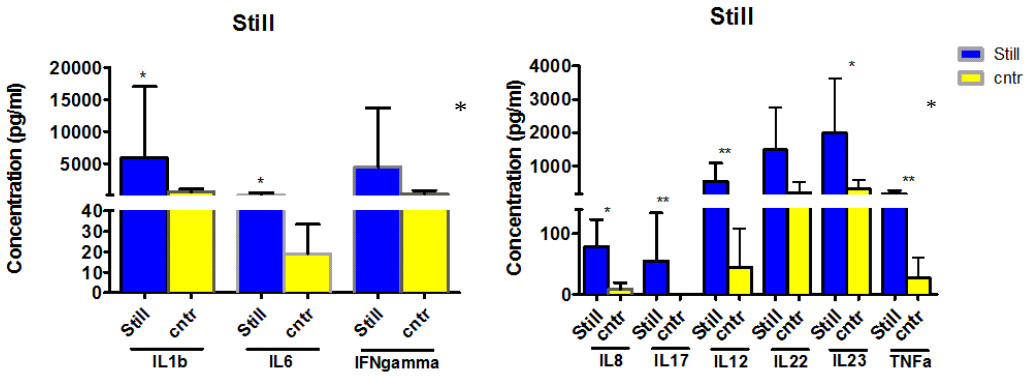


Fig. 74: Graphs of cytokines and marker of inflammation concentrations in serum from Still disease patients compared to healthy controls.
The data shown are means of data from triplicate experiments and the error bars indicate standard deviation. * p<0.05; ** p<0.01: p values less than 0.05 are considered statistical significant

2.7.3 TRAPS

Comparison between concentrations of cytokines and inflammatory markers in TRAPS patients and healthy controls are shown in Fig. 75 The most notably difference was detected for IL23, member of the IL12 family of cytokines with pro-inflammatory properties. It is a key participant in central regulation of the cellular mechanisms involved in inflammation. The concentration of IL23 in patients was more than 50-fold ($p<0.01$) higher than in controls. Statistically significant differences between patients and controls were detected for all the analyzed markers, except for IL1β, L6 and IL12. IL6 showed a slightly increased but not significant concentration level in patients.

The level of IFNγ was found to be 4-fold higher in patients compared to controls ($p<0.05$). Increased concentration levels were also observed for IL8, IL17 and TNFα, presenting respectively 7-fold, 14-fold and 6-fold higher values than controls with p values <0.05 or less. An extremely higher increase (>200-fold, $p<0.005$) was found for IL22 concentration level in patients, compared to controls.

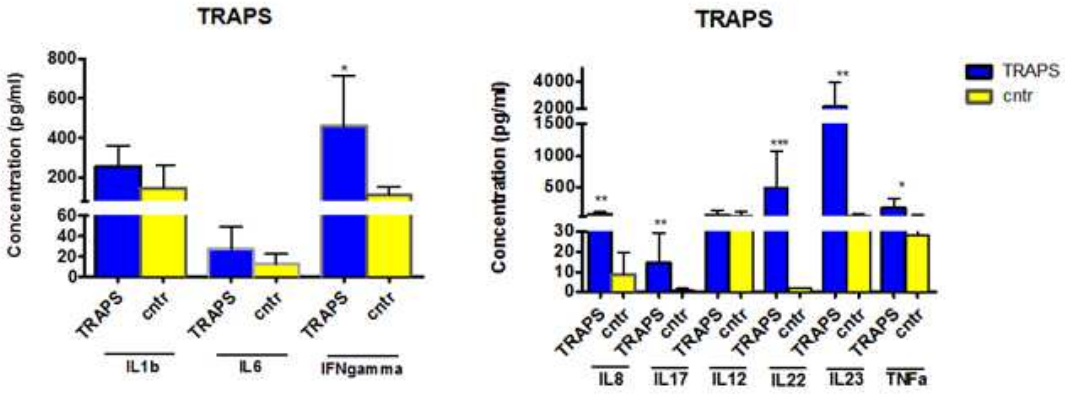


Fig. 75: Graphs of cytokines and marker of inflammation concentrations in serum from TRAPS patients compared to healthy controls.
The data are means of triplicate experiments results and the error bars indicate standard deviation.
* $p<0.05$; ** $p<0.01$; ***$p<0.005$. Statistical significance is for p values less than 0.05

2.7.4 FMF

Concentrations of systemic cytokines and markers of inflammation in FMF are shown in Fig. 76. Interesting cytokines with an high concentration in FMF patients were IL22 and IL23, highly expressed in several different inflammatory condition and related to each other since IL22 is an effector cytokine downstream of IL23. The concentration of IL22 in patients was 15-fold ($p<0.01$) higher than in controls, while IL23 concentration was 5-fold ($p<0.01$) higher. The level of IL1β was found to be 19-fold higher in patients compared to controls ($p<0.05$). The same trend was observed for IFNγ ($p<0.05$). Incredibly high increase of concentration was found in patients for IL6 and IL17 with 40-fold and >60-fold increase respectively over the values obtained for control subjects. Increased

concentration level was also observed for IL8 presenting a 12-fold higher values than controls ($p < 0.05$).
The concentration of IL12 and TNFα was not statistically different from controls, even if both cytokines showed a slightly higher concentration value than controls.

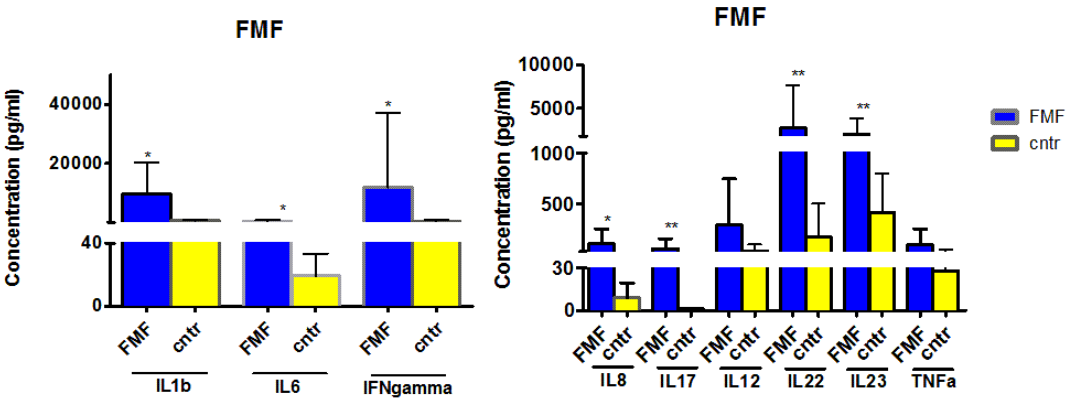


Fig. 76: Graphs of cytokines and marker of inflammation concentrations in serum from FMF patients compared to healthy controls.
The data are means of data from triplicate experiments and the error bars indicate standard deviation. * $p<0.05$; ** $p<0.01$; p values less than 0.05 are considered statistical significant.

DISCUSSION

The autoinflammatory diseases are a heterogeneous group of inflammatory syndromes caused by primary dysfunction of the innate immune system, without evidence of adaptive immune deregulation. While initially focused on hereditary recurrent fevers (HRFs), autoinflammatory diseases now encompass a wide spectrum of disorders ranging from rare monogenic (such as Blau syndrome) to more frequent multifactorial diseases (as Behçet and Still's diseases). Different genome-wide association studies have begun to elucidate the molecular basis of autoinflammatory diseases. Most of these studies underline the prominence of the IL1β-activating inflammasome and its regulation in a large number of diseases. More recently, additional mechanisms linking innate immune-mediated inflammation with a variety of cellular processes, including protein misfolding, oxidative stress and mitochondrial dysfunction, have been recognized to play a role in the pathogenesis of some monogenic autoinflammatory conditions. Those processes can later activate other inflammatory pathways with host defense function, such as MAPK, JNK and NF-κB pathways. Even though important aspects of the pathogenesis of autoinflammatory diseases have been clarified, all the inflammatory-related signalling pathways and cytokines possibly involved are far to be deeply investigated.

1 Functional studies on p.E383K Blau syndrome-associated mutation

The *CARD15/NOD2* gene encodes a cellular recognition molecule (NOD2) mediating NF-κB activation in response to the muramyldipeptide component of bacterial peptidoglycan. A distinct group of mutations in this gene causes, in heterozygous state, the rare autoinflammatory disease, named Blau syndrome.
Up to date, 11 mutations have been identified to be associated at this disease and those referred to codon 334 were more frequently presented (p.R334Q/W).
In 2005, our group has identified the mutation p.E383K in an Italian family affected by Blau syndrome [56].
This mutation is located in the central NACHT domain and changes Glutamate E383 to Lysine. Up to date, no functional studies are presented in literature concerning this mutation. Its pathogenicity is strongly supported by several, although indirect, pieces of evidence. As shown in Fig.12 and Fig.22, p.E383K clearly cosegregates with the disease in a dominant manner and the glutamate E383 is known to be a conserved residue among different species.
As reported in literature, NOD2 expression has mainly occurred in the cytosol of myelomonocytic cells [219], dendritic cells [58] and Paneth cells [59]. More

recently NOD2 was shown to be associated with the plasma membrane of intestinal epithelial cells [60].
In vitro immunofluorescent studies on transfected HEK293 cells demonstrated that both missense mutations studied (p.E383K and p.R334W) did not affect the normal cytosolic cellular localisation of NOD2, suggesting that there is no evidence that the protein localization is not related to the carried mutation. Interesting would be studying also the localization of p.E383K mutated NOD2 in patients cells.
In vitro expression study by means of Western blotting techniques showed that *wild-type* NOD2 expression on transfected cells did not increase after stimulation with muramyl dipeptide (MDP), a degradation product from bacterial peptidoglycan. The expression of both mutated NOD2 proteins showed a similar trend. To better understand these data, a further study at transcriptional level has to be performed, maybe using also patients monocytes.

1.1 NF-κB pathway activation

To define the biological effect of p.E383K mutation in Blau syndrome, we examined the NF-κB activation both *in vitro* and *ex vivo*.
HEK293 cells transfected with human recombinant NOD2 either *wild-type* or p.E383K mutant form were used as a possible *in vitro* model for studying the activation of this pathway. Firstly, indirect test of NF-κB activation was performed, detecting the expression of NF-κB inhibitor (IKBα) and its phosphorylated form by Western blotting. A higher expression of phospho IKBα, revealing an activation of NF-κB, was seen only for *wild-type* NOD2 after stimulation with MDP. This result was confirmed also by microarray data on IKBα and phospho IKBα obtained from whole lysated transfected cells. We also observed an increase on IKK complex levels in *wild-type* NOD2 after stimulation. This result could suggest that increased IKB kinase (IKK) levels were associated with a more active IKBα, implying a higher level of its phosphorylated form. Taking together all these findings, we could conclude that NF-κB activity was increased only in *wild-type* NOD2 cells after stimulation.
A wider overview in NF-κB pathway *in vitro* was obtained by microarray analysis of nuclear extracts of both *wild-type* and mutated (p.E383K and p.R334W) NOD2. At basal level, RPPA data showed increased level of NF-κB and its phosphorylated form in p.R334W mutant compared to *wild-type*, while a similar or even decreased level of NF-κB and phospho NF-κB was seen in p.E383K mutant compared to *wild-type* protein. Addition of MDP to p.R334W NOD2 cells showed a similar or slightly higher level of NF-κB and phospho NF-κB compared to *wild-type* NOD2 cells. No increased levels of other pathway components were observed for p.E383K NOD2 cells. All these data suggested that at basal level p.E383K NOD2 cells did not present an increase of NF-κB activity, compared to

the *wild-type*. p.R334W mutated NOD2, instead, significantly increased the basal NF-κB activity compared to the *wild-type*, as similarly presented in previous studies in literature [43, 217]. In contrast, addition of MDP to p.R334W mutant further elevated the NF-κB activity up to almost the same level as the case of the *wild-type* NOD2. This data is comparable to the one referred to the same mutation reported by Kanazawa and colleagues [43]. In p.E383K mutant, NF-κB persisted to present a reduced activity compared to *wild-type* NOD2 after MDP stimulation.
To verify the results concerning p.E383K mutation, we evaluated the pathway by means of lysate microarray *ex vivo*, using patients PBMC carrying this missense variation. The expression levels of phopho IKBα and phospho NF-κB, as well as IKK protein complex, were higher in patients than in controls, implying pathway activation. An increase on IKK levels were associated with a more active IKBα, as shown in the results. These *ex vivo* findings are opposite to the ones previously observed *in vitro*. It is possible that *in vitro* p.E383K mutants may need enhancer factors at genetic or protein levels, probably presented in patients cells, to increase the inflammatory response. From these results, HEK293 cells did not seem to be a good *in vitro* model for p.E383K mutation in Blau syndrome. Future studies could focus on the evaluation of different other cell lines for creating a better *in vitro* model. Given that NOD2 is naturally expressed in myelomonocytic cells, THP-1 cells would be a good option [220]. THP-1 is a human monocytic cell line, able to differentiate in macrophages and perfect to study pro-inflammatory cytokines secretion and IL1β in primis, although these cells are known to be difficult to transfect. It would be interesting also to perform further *ex vivo* studies on different cell lineages other than PBMC, since NOD2 has also been detected in epithelial and vascular endothelial cells [59, 221]. Moreover, an *ex vivo* analysis of the pathway activation after pathogen stimulation should be performed.
Following on from the previously presented results, we could also suggest that it was not only the HEK293 cell line that leads to the lack of NF-κB activity of p.E383K NOD2, but may be also the properties of this mutation itself. Since p.R334W NOD2 was shown to enhance the pathway in HEK293 cells, this perhaps suggests some differences in the behaviour of both mutants. This aspect could be worth analyzed in future.

1.2 Cytokines profile

We further investigated whether the presence of p.E383K mutation in NOD2 would upregulate the pro-inflammatory cytokines production both *in vitro* and *ex vivo*.
In vitro analysis on transfected cells evaluated only the production of IL8, since HEK293 cells secretes a limited set of inflammatory cytokines. The basal level of IL8 produced by p.E383K mutated cells were significantly lower than in *wild-type* cells. Stimulation with MDP leaded also to a less IL8 level in p.E383K compared

to *wild-type*. Observing these results, we can suppose that the lack of increase of pro-inflammatory cytokines in p.E383K NOD2 cells could be linked to the failed activation of the NF-κB pathway in these cells. Results from p.R334W mutated cells seemed to support this hypothesis. Even if there was not a significantly higher level of IL8 in absence of stimulation, in p.R334W NOD2 transfected cells IL8 production were slightly increased after stimulation with MDP compared to *wild-type*.

Ex vivo data from patients PBMC and serum showed a similar trend for IL1β, IL6, IL8,TNFα and IFNγ production compared to healthy controls.

PBMC and serum from Blau syndrome patients did not show higher basal production of pro-inflammatory cytokines, even if the concentration values were generally higher in serum samples. LPS or MDP stimulation in PBMC showed a similar or lower levels of these cytokines than in healthy controls. Furthermore, no synergistic cytokine release was observed in patients compared to controls when muramyldipeptide was used in combination with TLR agonist.

All these observations were unexpected since BS is known to have an autoinflammatory nature due to its "gain-of-function" associated mutations. This hypothesis predicted that patients with Blau syndrome would spontaneously release more cytokines that could be transcriptionally up-regulated by NF-κB activity (such as IL1β). However, recent works in literature also observed no evidence of excess pro-inflammatory cytokines such as IL1β and TNFα in PBMC from Blau syndrome patients compared to healthy controls [222]. In addition to this, Son and collegues confirmed *ex vivo* that BS is not associated with increased basal levels of TNFα [223].

Focusing on IL1β, its responses in PBMCs from subjects with Blau syndrome appeared to be slightly attenuated when compared with the responses in PBMCs from the control group, in presence or absence of stimulation. This suggests that an exaggerated NOD2 response leading to IL1β release is not a direct mechanism explaining the pathophysiology of Blau syndrome. IL1β blocking therapy seems not to be an eligible treatment for BS. In literature some authors report the use of biologic anti-cytokine agents such as infliximab, a TNFα inhibitor and anakinra, the IL1 receptor antagonist, but the results are variable, particularly with regard to ocular morbidity [53, 224].

Levels of the other cytokine measured in PBMC with NOD2 mutations were also not elevated. In patients serum we also evaluated the release of IL17, IL12, IL22 and IL23 at basal condition. The high levels of IL17, IL22 and IL23 in BS patients suggested that they might play a significant role of in the pathogenesis of the disease. In literature, those cytokines are extensively studied in inflammatory conditions. IL17 is known to be an important proinflammatory cytokine upregulated in chronic inflammation [225]. IL23 has been shown to be necessary for the development and maintenance of certain inflammatory disease [226]. IL22 has also a function in inflammation mediated by pathogens and recent data

suggested that it plays a role in human infection [203]. Their role in Blau syndrome could be deeply investigate.
Our data for IL12 showed that the cytokine concentration significantly decrease in patients. This down-regulation of IL12 level could explain or be linked to the low level of IFNγ observed in BS patients. Since the interferon is known to be involved in granulomas formation and BS presents granulomatous features, IFNγ would be expected to be higher in Blau syndrome patients than in controls. Further investigations will be necessary to clarify this result.

2 Evaluation of possible biomarkers in autoinflammatory diseases

Despite major advances in genetics and pathophysiology in autoinflammatory diseases, not all patients show the expected clinical features or respond favourably to drugs, leading to a high morbidity in these diseases.
In this context, the use of biomarkers can help improving the identification of autoinflammatory diseases and developing personalized or therapeutic strategies. Biomarkers are defined by the European Health Commission as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". Their strength lies in providing an objective measure to assess disease severity, progression, risk, and survival. Identification of such pattern is a sizeable problem, but using an integrative approach to analyse a great number of possible biomarkers would represent an enormous advance in the diagnosis and/or profiling of disease. To enable this, microarray-based systems will be the high-throughput assays for measuring cytokines or other serum proteins and signalling intermediates possibly associated to autoinflammatory diseases.

2.1 Signalling intermediates

Reverse phase protein microarray (RPPA) is a relatively new, sensitive and high throughput functional proteomic technology that offers many potential advantages over other current methods used to detect known protein markers such as Western blotting assay. The high sensitivity and miniaturized format of lysate microarray technology, makes it suitable for of large clinical trials where limited quantities of thousands of samples may need to be interrogated for the expression level of a defined set of protein markers. In this thesis work , RPPA was the eligible technique to study inflammatory signalling pathways in PBMC of different autoinflammatory diseases patients. Advantages of RPPA are the ability to provide a descriptive picture of the ongoing state of the signalling networks from different cellular samples, and also provide information about the post translational modifications such as phosphorylations that can not be provided

using gene study [227]. The success of RPPA experiments depends mainly on the presence of specific primary antibodies. Antibodies were selected for studying NF-κB, PI3K/Akt, MAPK, JAK/STAT and inflammasome signalling pathways in the different autoinflammatory diseases.
Since a lot of data have been obtained for each disease, we focused on description of the proteins that better characterize each pathway in Behçet disease, Adult Onset Still disease, TRAPS, FMF and Blau syndrome. The other autoinflammatory syndrome patients (Schnitzel, MKD and NALP12 disease) were not statistically analyzed, due to the low number of patients enrolled for each disease.

Behçet disease
NF-κB is an important transcription factor that is necessary for the transcription of many genes involved in inflammatory and immune responses. Analysis of microarray data on NF-κB pathway for Behçet disease showed higher level of phospho NF-κB (active form of NF-κB) and other upstream component of the pathway in Behçet patients compared to healthy controls. We also noted that A20 was upregulated in patients than in controls and this can reflect the effect of the higher level of NF-κB in patients. A20 is in fact an ubiquitin-editing enzyme expressed as a result of activation of NF-κB, even if it can also has a negative feedback on NF-κB activation.
The role of PI3K/Akt in regulation of inflammatory process is well established, as several studies have demonstrated the role of PI3K/Akt in several inflammatory diseases such as rheumatoid arthritis, asthma, chronic obstructive pulmonarydisease, psoriasis, multiple sclerosis, and atherosclerosis. The results obtained from this study indicated higher levels of active Akt, phosphorylated at its key sites (Threonine 308 and Serine 473) and PI3K p85 in patients than in controls, indicating activation of this pathway. PI3K/Akt activation in turn activates the NF-κB pathway. So, the results obtained are consistent with the ones described previously.
MAPK is an important inflammatory signalling pathway that regulates gene expression, cell survival, cell proliferation, and apoptosis. The high level of phospho ERK1/2 seen in patients PBMC in comparison to controls reflects the activation of this MAPK pathway in our cases of Behçet disease. Phospho HSP27 level was higher in patients than in controls. In literature, it is reported that the level Hsp27 phosphorylation correlated also with ERK1/2 activation [228], confirming our results.
The data obtained from STAT3/JAK2/c-Src pathway showed a higher level only of phospho STAT1 in patients than in control. No literature to date was found that correlate this finding with Behçet disease. However, it is known that STAT1 is a target gene of ERK1/2, central protein of the MAPK pathway previously described active in Behçet. Further analysis should be performed in order to understand better this datum.

The level of MyD88 was upregulated in patients than in controls. The upregulation of MyD88 may be related to activation of a TLR pathway. Future microarray studies should deeply analyze this pathway in Behçet disease. None of the components of inflammasome pathway analyzed were detected at a significant higher level in our cohort of Behçet disease patients.

Adult Onset Still's disease (AOSD)

NF-κB is considered to be the master regulator of both innate and adaptive immunity. Analysis of microarray data showed increased phospho NF-κB (active form) level in patients than in controls. High level of other upstream component of the pathway, in particular phospho IKBα, was seen in AOSD patients compared to healthy controls. The results also showed that increased IKB Kinase (IKK) levels were associated with more active IKBα (phosphorylated form). A20 was upregulated in patients than in controls and this can reflect the effect of the higher level of NF-κB in patients, as previously explain for Behçet patients. The net results of investigating NF-κB signalling pathway demonstrated upregulation of this pathway in AOSD patients compared to controls. No data are available in literature to confirm our findings.

PI3K/Akt pathway has a role in several complex inflammatory diseases. The results obtained from this study indicated higher levels of active Akt, phosphorylated at Threonine 308 residue, in patients than in controls. Upstream (PI3K p85 and PI3K p100a) and downstream (phospho Bad, phospho GSK-3b and phospho c-Raf) activators of Akt were also present at higher level in patients than in controls, indicating activation of this pathway. Since PI3K/Akt activation in turn activates the NF-κB pathway through phosphorylation of IKBα, the results are consistent with the ones described previously. The observation of high level in patients of phospho PTEN, known to inhibit PI3K and Akt signalling cascade, have to be deeply investigated. We could speculate that Akt pathway, due to the high level of most of the compounds, may be activated by alternative way rather than via PI3K.

The upstream regulators of MAPK pathways such as TRAF2 and phospho MKK3/MKK6 were studied and found at higher level in patients. TRAF2 signals through MAPK induction, primarily through activation of JNK [229]. Level of phospho SAPK/JNK are higher in patients than in controls, leading to activation of this MAPK pathway. Higher level of MKK7, observed in AOSD patients, was also related to SAPK/JNK pathway activation. Phospho ATF2 level were higher in patients compared to controls. ATF2 is normally activated in response to signals that converge on JNK [230].

High level of phospho ERK1/2 was seen in AOSD patients PBMC in comparison to controls, underlining the activation of even this MAPK pathway. Higher level of phospho MEK1/2, observed in patients, was upstream related to ERK1/2 MAPK pathway.

Concerning JAK/STAT/c-Src pathway in AOSD, we observed a higher level of phosho STAT3 and phospho c-Src in patients rather than in controls. The role of STAT3 as a mediator of inflammation is well documented in the literature, since its activation is promoted by pro-inflammatory agents as IL6 and TNFα, and there is a correlation between STAT3 pathway and NF-κB pathway. STAT3 is known to be a target of the non-receptor tyrosine kinase c-Src [231], so we can hypothesize that STAT3 may be activated by phosphorylation of c-Src in our patients. The high level of SOCS3 observed in patients deals with the activation of STAT3, since SOCS3 is a target protein of STAT3 signalling.
Our results showed also higher level of MyD88 in AOSD patients than in controls. This finding may be related to the activation of a TLR pathway MyD88-dependent. In literature, TLR7 MyD88-dependent signaling pathway is described to be involved in the pathogenesis of adult-onset Still's disease [232]. To confirm, future analysis could focus on this other pathway components.
The inflammasome pathway related studies presented higher level of caspase1, cleaved caspase1, BCL2 and phospho BCL2 in patients compared to controls. The activation of caspase1 is the main downstream event in this pathway after inflammasome formation and it is responsible for increased IL1β production; furthermore, a higher concentration of IL1β was observed in our patients. BCL2 activation is reported in literature to inhibit NALP1 inflammasome through inhibition of caspase1 activation in a concentration-dependent manner [233]. Further experiments should be performed in order to evaluate better NALP1 and maybe also NALP3 inflammasome related pathway, in order to understand the molecular cause of caspase1 activation in our patients.

TNF-receptor associated periodic fever (TRAPS)
Activation of NF-κB in TRAPS is a controversial issue as some investigators demonstrated reduction of the NF-κB signalling pathway upon expression of TRAPS-associated mutations, while others showed up-regulation [234, 235]. Shedding light on the activation status of NF-κB in TRAPS is an important goal in understanding the pathogenesis of the disease. Analysis of microarray data showed increased level of NF-κB and phospho NF-κB (active form) in patients compared to controls. Higher level of upstream component of the pathway, in particular phospho IKBα and IKK complex, was seen in patients rather than in controls. Increased IKB Kinase (IKK) levels was associated with more active IKBα (phosphorylated form).
PI3K/Akt pathway has a main role in several inflammatory diseases. The results obtained from this study indicated higher levels of active Akt, phosphorylated at both the key residues (T308 and S473) in patients compared to controls. Studying the upper regulators of Akt (PI3K p85 and p100), the results demonstrated up regulation of both proteins in patients in comparison to controls, again indicating activation of the PI3K/Akt pathway. Higher level of the phosphorylated downstream activator GSK-3β in patients fits with the activation of Akt exposed

above. GSK-3β is important in improvement of NF-κB transcriptional activity and prevention of apoptosis. Since PI3K/Akt activation in turn activates the NF-κB pathway through phosphorylation of IKBα, the reported results are consistent with the ones described previously for NF-κB pathway.

Two main MAPK pathways (ERK1/2 and SAPK/JNK) were up regulated in our cohort of TRAPS patients in comparisons to the controls. These results are in agreement with a study by Stjernberg-Salmela and colleagues who demonstrated low level of phospho P38 MAPK in leukocytes of patients carrying different TRAPS associated mutations [236]. Among the upstream stimulators of MAPK, TRAF2 was observed upregulated in patients. TRAF2 is known to activate NF-κB by interaction with IKK complex [237]. So, overexpression of TRAF2, as in our findings, may correlate with higher levels of NF-κB detected under the same conditions. The observed higher levels of MKK7 and phospho MEK1/2 in patients correlate with the activation of the two MAPK pathway, since their role as upstream activators. We also observed higher level of phospho ATF2 in patients, a target protein of the SAPK/JNK pathway. To warrant better understanding of this complex pathway, further studies on a bigger cohort of TRAPS patients have to be performed.

The expression of phosphorylated Jak2 was higher in patients than in controls. Phosphorylated Jak2 is reported to activate phosphorylation of several downstream inflammatory signaling pathways and components, such as PI3K p85, NF-κB, MAPK and STATs [238]. Our results seem to present a similar activation trend, related to the observed activation of inflammatory pathways.

Activated Jak2 also supports STAT3 phosphorylation. So, the high level of phospho Jak2 in patients was associated with the observed high level of STAT3 and the related high level of SOCS3, target of STAT3. c-Src, an important activator of PI3K/Akt and NF-κB pathway mediated by TNF receptor 1 [239], was found upregulated in patients rather than in controls.

Our results showed also higher level of MyD88 in TRAPS patients than in controls. This finding may be related to the activation of a TLR pathway MyD88-dependent. Further investigation have to be performed, since TLR signalling can initiate production of ROS, which are known to be elevated in TRAPS patients [240].

The inflammasome related pathway analysis resulted on higher level of cleaved caspase1 and BCL-XL in patients compared to controls. The reason of the presence of activated form of caspase1 and its inhibitor in patients should be further evaluated.

Familial Mediterranean Fever (FMF)

Among all NF-κB pathway components studied in FMF patients, only IKKβ presented a higher level compared to controls. This could indicate that in our cohort of patients NF-κB pathway was not activated, unlike the literature data that displayed activation of NF-κB by N-terminal pyrin [241]. One possible

explanation of the different result in our cohort fits with the wide heterogeneityof FMF patients in analysis, also related to the mutations associated with the disease.
Concerning the PI3K/Akt pathway, only the upstream activator PI3K p85 was shown with higher level in patients than in controls. This could mean that this pathway may be truncated at the beginning of the signalling cascade. This preliminary finding has to be confirmed.
Our data on MAPK pathway showed higher level of phospho ERK1/2 and phospho SAPK/JNK in patients PBMC in comparison to controls. Finding a high level of phospho HSP27, known to be linked to the activation of ERK1/2, confirms the activation of ERK1/2 MAPK in our cohort. Related to the phosphorylation of SAPK/JNK, we could further study this MAPK signalling cascade in FMF evaluating other upstream and downstream regulators of this kinases.
JAK/STAT/c-Src pathway analysis resulted with higher level of phosho c-Src in patients rather than in controls. To date, no study in literature presents an evaluation of this pathway in FMF. Future analysis should involved this pathway in a wider group of FMF patients.
Our results concerning the inflammasome-related pathway showed a higher level of ASC, NALP1 and BCL-XL in patients than in controls. Upregulation of ASC at transcriptional level was also observed in a big cohort of FMF patients carrying different mutations [242]. Another study focused on the role of inflammasome in FMF using animal models. By generating pyrin-mB30·2knock-in mice in a background deficient in inflammasome components, Kastner and colleagues proved that the disease was dependent on caspase1, ASC and IL1 signalling but was independent of NLRP3, NLRC4 and AIM2 [243]. Our findings about ASC activation and also IL1β high concentration (presented below) may provide some evidences for the ASC-dependent NLRP3-independent inflammasome causing FMF, as reported in Kastner work. Further investigating in our cohort of patients may be helpful to elucidate inflammasome and FMF relation. The higher level of both NALP1 and its inhibitor BCL-XL in patients need to be better inquired, since it was the first time they were evaluated in FMF patients.

Blau syndrome
In addition to NF-κB pathway, we have also evaluated other signalling pathway in Blau syndrome patients.
The results obtained from PI3K/Akt pathway study indicated higher levels of Akt phosphorylated at serine 473 in patients than in controls. This finding is consistent with the activation of the pathway. Moreover, an upstream regulator of Akt activation, PI3Kp85 were found with higher level in patients. Downstream Akt activators, phospho BAD and phospho GSK-3b, were higher in Blau patients than in controls. BAD is also a pro-apoptotic signalling molecule but when it is phosphorylated it loses this function. In literature a recent study on *wild-type* NOD2 presented that the PI3K/Akt pathway negatively regulates Nod2-mediated

NF-κB pathway [244]. Our data on patients carrying mutation p.E383K showed instead a positive correlation between these two pathways. Future analysis could deeply investigate this aspect.
P38 MAPK pathway seems to be activated in Blau syndrome, due to the presence of higher level of phospho P38 in patients than in controls. The level of phospho HSP27 as a down regulator of phospho P38 MAPK was also observed higher in patients than in controls. HSP27 is known to be related to NF-KB activation, enhancing the proteolysis of phosphorylated IKBα [245].
Analyzing the JAK/STAT/c-Src pathway, the only protein at higher level in patients was phospho STAT1. The biological activity of STAT1 correlated with pro-inflammatory and antiproliferative responses. In literature, we did not find to date any studies correlating the disease or NOD2 protein to this protein activated. Further investigation may be helpful to explain our result. Furthermore, none of the components of inflammasome pathway analyzed were detected at a significant higher level in our cohort of Blau syndrome patients.

Looking at the heatmaps for the different signalling pathways, it is clear that all patient groups have raised the levels of signalling molecules compared to the control group. Moreover, the heatmaps (Fig. 45, 50, 56, 62 and 68) suggest that the patterns of the signalling compounds may relate also to individual patients. For example, in the TRAPS cohort, patient 39 has raised levels of signalling molecules across virtually all pathways, whereas patient 22 has relatively low levels across all pahways. Similar comparisons can be made in the other disease cohorts. In Behçet group, patient 7 presented a higher level of signalling molecules across all pathways, whereas both patients 27 and 28 showed relatively low levels across all pathways. In AOSD patient 13 the expression level of all signalling molecules evaluated raised up; for patients 19, 20 and 29, it was quite the contrary. In the FMF cohort, patient 6 has raised levels of all signalling molecules across the studied pathways, whereas patients 21 and 31 presented low levels.
A possible explanation of this patient-related pattern for signalling pathways may lie in the peculiar characteristics of each subject, such as treatment type, disease activity or possible mutations.
Concerning the TRAPS group, higher levels of all signalling molecules observed in patient 39 could be associated with the rare mutation p.D12E carried by this subject and found in few other cases in literature. This mutation is reported to be linked with complex symptoms [246], that may be related to the pathway activation observed in this work. We need more information about disease activity to confirm this hypotesis for patient 39. Lower levels of all signalling pathways compounds were observed in patient 22, similar to ones showed by healthy controls. This subject presents some polymorphisms in *TNFRSF1A* gene and no therapy. In this case, it would be useful to know the symptom presented by this patients, to infer if the clinical diagnosis was correct at the light of these results.

Regarding the Behçet group, there is not enough information about the patient 7 to discriminate which one could be associated to the increased levels of all the pathways components. Then, we also observed low levels in patients 27 and 28, whose only peculiarity compared to other Behçet patients is belonging to the same family. Concerning the Still's group, the higher levels in all pathways observed in patient 13 could be related to the absence of treatment at the time. Further information about the disease characteristic needs to be collected, in order to understand whether the absence of therapy correlates with higher disease activity. In patients 19, 20 and 29, the observed lower levels of molecules in all pathways may be related to the treatment. These patients are female subjects treated with kineret (anti IL1ra), proven to be effective in a number of autoinflammatory diseases. Also in this case, additional information about disease activity could confirm the results. For the FMF group, there is not enough information about the patient 6 to discriminate which one could be associated to the increased levels of all the pathways components. The absence of treatment could maybe be related to these results, but activity disease information should be obtained to justify the hypothesis. Patients 21 and 31, instead, presented lower levels of compounds across all pathways. Since there are no clearly peculiar characteristics among the features collected for these patients, further information has to be gathered in order to understand if there are any clinical characteristics that may be linked to the results.
It is clear that more information about disease activity, age of onset and other clinical features needs to be collected in future for all autoinflammatory patients involved in this studies, to understand better to what extent patients characteristics might be related with differences in signalling pathway activation.

2.2 Cytokine profile

Antibody microarray is a high throughput and sensitive technique, used in this work to study the cytokines and chemokines mainly presented in sera samples obtained from different autoinflammatory disease affected patients. Amplification was the main task for using this technique with serum samples. Due to the low abundance of some cytokines, an amplification method that balances signal to background had to be provided. Even though Genisphere Ultra Amp are 3DNA dendrimers customized with a variety of labels to increase the sensitivity, the simple Tyramide amplification provides a higher sensitivity with a lower background. A possible explanation deals with a longest application time and an increased number of steps the Genisphere technique required. This maybe increases the possibility of aspecific binding, leading to a higher background compared to Tyramide amplification.
There are also high correlations between antibody microarray and ELISA, indicating that this microarray technique can potentially be used as a diagnostic

tool for quantification of specific antigen in clinical samples, especially if the sample amounts are limiting [70].

Cytokines represent a group of important signalling molecules whose primary function dwells in immunomodulation of both innate and acquired immunity. The finely tuned network of cytokine signalling has been a subject of many investigations studies on autoinflammatory diseases. In particular, the pro-inflammatory cytokines (IL1β, IL6, TNFα, IFNγ) are targeted for therapeutic intervention because of their role in promoting or sustaining harmful inflammation. Spreading the range of investigated cytokines to also IL8, IL12, IL17, IL22 and IL23, we would analyse the cytokinic profile of the different autoinflammatory diseases studied in this thesis.

Behçet disease

It is doubtless that cytokines play some crucial roles in the initiation and perpetuation of this disease. We showed a statistically higher level of IL8, IL17 and IL22, along with a lower level of IL12. The upregulation of IL17 was already documented in literature [247], as well as the relation between IL8 and Behçet disease . IL8 was reported at higher level in active stage of BD in a number of studies thus far; Gür-Toy G *et al*. reported that the association of IL8 level with disease activity was better than that of acute phase ESR and CRP [186]. The extremely high level of IL22 may points to a possible role of this lymphokine in the pathogenesis of this disease, as suggested also by Cai and colleagues [248]. All the other evaluated cytokines have not statistically different levels from healthy controls. These results move away from literature, that described elevated levels of IL1β, IL6, IL12, TNFα [179]. Even IFNγ was highly expressed and considered an important mediator involved in the development of Behçet [249]. A possible explanation of these different results should be found in the small cohort of patients in analysis. The patients presented clinical and genetic differences and the most of them were under treatment at the time of the analysis. The therapy may have affected the cytokines' profile in those patients.

Adult Onset Still disease (AOSD)

The antibody microarray data showed higher level of all the cytokines and markers studied in patients compared to healthy controls. This is the first time to our knowledge that IL17, IL12, IL22 and IL23 are evaluated in AOSD patients. The elevated levels point to a possible role in the pathogenesis of this complex disease. In literature, elevated levels of proinflammatory cytokines such as IL1β, IL6, IL17, IL8, IL18, and TNFα were previously described in untreated AOSD patients, often in association with disease activity and/or distinct clinical phenotypes and serological features [36, 250]. Since half of our patients underwent to IL1β blocking treatment, our finding of elevated levels of proinflammatory cytokines (IL1β in primis) appear strange. To understand the data, we need further information, concerning how long patients were on therapy

and if the dosage is sufficient to give amelioration in their conditions. In fact, since disease severity varies significantly among affected individuals and, even, within the same individual, patients require continuous and aggressive immunomodulatory treatment.

TNF-receptor associated periodic fever (TRAPS)

The antibody microarray data were a mean of all data obtained from all TRAPS patients carrying different mutations. Interesting would be the analysis at cytokines levels for each mutation, if the cohort of patients had been more consistent. To a better analysis of the disease, future studies with a bigger cohort of patients will be performed.

The data showed higher level of IL8, IL17, IL22, IL23, IFNγ and TNFα in patients compared to healthy controls. IL1β level was not increased, due to the fact that most of the patients are currently under IL1β blocking treatment (canakinumab or anakinra). IL8 and TNFα increased levels were also presented in literature for a mutation implied in TRAPS [251].

The extremely high level of IL22 may points to a possible role of this cytokine in the systemic inflammation in the joints, skin and muscles, as suggested also by Nakamura and colleagues [252]. In literature, the role of IFNγ, IL17 and IL23 has not been investigated yet. Their upregulation may be correlated with the disease.

Familial Mediterranean Fever (FMF)

The antibody microarray data showed higher levels of IL1β, IL6, IL8, IL17, IL22 and IL23 in patients compared to healthy controls. Most of the patients were under colchicine treatment. Haznedaroglu *et al* reported that IL17 levels were significantly higher in regularly treated FMF patients compared with healthy controls, as shown by our data [253]. Levels of IFNγ were higher in patients than in controls, as presented in literature by Köklü and colleagues. Moreover, they reported that colchicine did not affect circulating IFNγ levels [254]. Concerning IL6 and IL23, a study by Pamuk *et al* did not show any increase in the level of these cytokines in treated patients compared to controls, unlike our data [255]. The incredibly high level of IL22 may play a significant role in the pathogenesis of FMF, but there is no data in literature to confirm this finding. Then, it would be interesting to evaluate the cytokines secretion pre- and post- febrile attack, to assess any changes in the cytokinic profile.

Summarizing, in Behçet disease only IL8, IL17 and IL22 are significantly raised, whereas in AOSD all the measured cytokines are significantly heightened. Most cytokines are raised in TRAPS or FMF, although not all significantly. For example, IL12 is slightly increased in FMF, even if not at significant level; instead, it is clearly not raised in TRAPS. Thus, we could argue that the cytokines analyzed in this work could help to distinguish the autoinflammatory diseases in term of number of cytokines raised, as follow: Behçet<TRAPS<FMF<AOSD.

This hypothesis needs to be sustained and validated by further studies including bigger samples size and different autoinflammatory diseases, such as CAPS and MKD. So, if confirmed, the evaluation of the number of raised cytokines could be a new way to stratify autoinflammatory diseases patients, maybe in borderline cases.

Some considerations can be made observing the cytokine profile graphs across all the studied diseases (Fig. 73, 74, 75 and 76). The only cytokines significantly raised in all four autoinflammatory disorders (Behçet, TRAPS, FMF and AOSD) are IL8, IL17 and IL22. IL23 could be added at this list, even if it is not statistically significant in Behçet disease. What is particularly striking is the upregulation of IL17/22/23. This would clearly suggest an important role for Th17 or Th17-like cells in autoinflammatory diseases, since Th17 cells, which are directly involved in and mediate chronic inflammation, are characterized by the production IL17 and IL22 as well as the recruitment of neutrophils and other inflammatory cells. IL23, which promotes Th17 cell development as well as IL17 and IL22, plays essential roles in various inflammatory diseases [256]. Targeting Th17 cells and their related cytokines (IL17/22/23) may be an effective therapeutic approach for autoinflammatory diseases in future.

A linkage between Th17 cells and autoinflammatory diseases is discussed in literature on studies about cryopyrin-associated periodic syndromes, familial Mediterranean fever, Behçet and Adult onset Still's diseases. In gene-targeted mice expressing CAPS mutations, the inflammasome hyperactivation resulted from excess IL1β production potentiates Th17-dominant immune responses [257]. Ovadia and colleagues suggest the presence of a heightened Th17 response in FMF, related with frequent attacks and FMF genotype [258]. Upregulation of Th17 shows a correlation with Behçet [247] and Still's disease [225] progression. A better understanding of the involvement and possible pathogenic role of Th17 cells in our group of autoinflammatory diseases requires, however, further investigation and a bigger cohort of patients.

CONCLUSIONS

In conclusion, this is the first time that the mutation p.E383K in *CARD15/NOD2* associated with Blau syndrome has been functionally evaluated.

According to our *ex vivo* and *in vitro* studies on cytokines levels, it seems that there is not a primary mediation of IL1β and other pro-inflammatory cytokines in Blau syndrome patients carrying p.E383K, with the exception of IL17, IL22 and IL23. The role of these Th17-related cytokines has to be deeply investigated.

Concerning our *in vitro* and *ex vivo* data on NF-κB pathway, the reported contrasting results may indicate that this pathway activation should be regulated not only by NOD2 in carriers of p.E383K mutation. Further experiments need to be done in order to clarify these results, using different *in vitro* models.

From the microarray studies on a wide range of autoinflammatory diseases, we can firstly conclude that both techniques (reverse phase protein array and antibody microarray) are valuable in studying signalling pathways, cytokines and other inflammatory markers. Moreover, they are sensitive and accurate in quantification of different regulatory proteins.

When compared with healthy controls, the results led to the identification of raised levels of many of the tested signalling pathway components including NF-κB, PI3K/Akt, MAPK, Jak2/STAT3/c-Src and inflammasome, and also of Th17-related cytokines IL17/22/23. We acknowledge some limitations in these studies: (1) small samples size and (2) few information on disease activity and clinical parameters. However, this thesis work is the starting point for further biomarkers evaluation in autoinflammatory diseases, beginning from Th17 cells and their related cytokines. Analysis of the significant molecules from signalling pathways with biostatistical models will be required to generate specific diagnostic algorithms and to identify valuable biomarkers to be used as targets for specific therapeutic intervention.

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