4.1 Final publishable summary report

Executive summary.

TNF receptor associated periodic syndrome (TRAPS) is a rare dominantly inherited disorder (OMIM 142680), caused by mutations in the p55 TNF Receptor (or TNFR1), encoded by the TNF Super Family Receptor 1A gene (*TNFRSF1A*). It is a disabling condition characterised by recurrent attacks of fever and pain. Renal amyloidosis is the most serious complication in untreated patients and makes TRAPS a potentially fatal disease. The disease usually starts in infancy, although a wide range of ages at onset has been reported. Corticosteroids, and more recently anti-TNF therapeutics have not been consistently effective. Since TRAPS is a recently identified condition (identification of the gene in 1999), it is largely under diagnosed and little data was available on its pathophysiology, disease course, epidemiology, and best practice for patient diagnosis and care before the start of the EUROTRAPS project. This multidisciplinary collaborative project, by combining ideas, skills, resources and data from 9 centres (6 countries), aimed to gain insights into the natural course and pathophysiology of TRAPS, and develop early diagnostic and innovative therapeutic models applicable to hereditary paediatric recurrent fevers.

We have achieved most of our goals and even more. We have delineated the distribution of TRAPS patients in Europe according to age, gender, their complete clinical pattern and their quality of life through the constitution of a formal network in collaboration with EUROFEVER. We have elaborated a diagnostic score for TRAPS in childhood, implemented a quality control for the genetic diagnosis of TRAPS and all recurrent fevers through the European Molecular genetics Quality network, established agreed treatment protocols and defined a scoring risk for amyloidosis. We have also improved our knowledge of the genetics and pathophysiology of TRAPS. A large number of new mutations have been identified, as well as possible new pathways involved in the disease and in TRAPS-like patients. We demonstrated that mutated TNFR1 aggregates in the cytoplasm, monocytes from TRAPS patients do not oversecrete IL-1β compared to healthy controls, there is an increased basal IL-6 levels in mutant cell lines compared to wild type cells, total ROS production is higher in TRAPS-associated TNFR1 mutants compared to transfected WT cells, there is impaired autophagy in mutant TNFR1 containing cells. In terms of development of innovative therapeutic strategy, we designed a vehicle able to specifically and efficiently trigger RNAi-mediated gene silencing within the inflammatory monocyte subset in vivo and to impact on an ongoing systemic inflammation in an experimental mouse model. Finally we have conducted dissemination actions including communications (>40) at congresses, during teaching and continuing medical education, information on websites, and 24 already accepted publications in four European languages in journals including 19 with peer reviews. A significant number of foregrounds have been generated: we have developed a dedicated website (available at http://fmf.igh.cnrs.fr/ISSAID/EUROTRAPS/), and 3 prototype kits for the genetic diagnosis and prognostic factors for hereditary recurrent fevers.

Our work will have important socio-economic impact and wide societal implications on wealth and quality of life of our patients. Chronic pain and potentially devastating complications caused by TRAPS (e.g. amyloidosis), due to repeated bouts of inflammation has a significant effect on the patients. The development of concerted approaches to diagnostic strategies, consensual therapeutic and preventive schemes, will undoubtedly result in a lowering of the negative economic impact on society. The development of all EUROTRAPS activities has generated a number of employment positions for young researchers, engineers and technical assistants. We have continuously overseen the gender ratio among the participants of this consortium at all levels.

EUROTRAPS *objectives*

Summary of the state of the art that lead to the development of the project

TNF receptor associated periodic syndrome (TRAPS) is a rare dominantly inherited disorder (OMIM 142680), caused by mutations in the p55 TNF Receptor (or TNFR1), encoded by the TNF Super Family Receptor 1A gene (*TNFRSF1A*). The disease mainly affects people of Northern-European ancestry, but has been described in almost all ethnic groups, including those living in the USA and Mediterranean countries. The disease usually starts in infancy, although a wide range of ages at onset has been reported. TRAPS is a disabling condition characterised by recurrent attacks of fever and pain, often leading to developmental delay, and absenteeism from work. Renal amyloidosis is the most serious complication in untreated patients and makes TRAPS a potentially fatal disease. Corticosteroids, and more recently anti-TNF therapeutics have not been consistently effective.

Rational of the project

Since TRAPS is a recently identified condition, it is largely under diagnosed, thereby jeopardising the chance of patients ever receiving optimal treatment. Therefore, very little was known about the pathophysiology of TRAPS before the start of the EUROTRAPS project. As a result of discussions during specialised conferences dealing with these issues, and comprehensive reviewing of the literature we have decided to create a EUROTRAPS consortium in order to optimise research on this disease in Europe, and to develop further clinical experience in treating this condition, as well as tools and models that may be applicable to all hereditary recurrent fevers. Indeed, we believed that it was realistic to hope that advances in our understanding of the mechanisms underlying this disease would lead to earlier and more accurate diagnosis, and provide better molecular targets for the development of improved therapeutic intervention. The discovery of the TRAPS gene in 1999 has prompted a number of independent laboratories to develop research protocols to address these issues. However, conflicting results have been reported, which were probably due to the number of patients studied being too small, with contrasting study designs in different centres, and also the possibility that the experiments were conducted using non-physiological models.

Detailed objectives of EUROTRAPS

Gain insight into the natural course of TRAPS

- Create a European network and registry of paediatric and adult TRAPS patients
- Understand the prevalence, clinical presentation and natural course of TRAPS
- Evaluate disease features specific to the paediatric group of TRAPS patients
- Evaluate the overall quality of life of TRAPS patients and their families

Acquire knowledge for future development of diagnosis, therapeutics and prevention

- **Diagnosis**
  - Improve early diagnosis of TRAPS in childhood
  - Establish a quality control for molecular diagnosis of TRAPS
  - Develop a prototype kit for easy screening of TRAPS mutations
  - Identify *TNFRSF1A* mutation negative patients and search for novel genes

- **Therapeutics**
  - Collect data on the effects of immunomodulators and cytokine modifying drugs
  - Identify possible outcome measures for future therapeutic trials
  - Identify genetic susceptibility factors which modulate clinical response to treatment
  - To develop a prototype kit assay for the identified genetic treatment response factors

- **Prevention**
  - Define clinical and genetic features predisposing to complications of TRAPS
  - Develop tools for predicting occurrence of these complications that can be translated into the clinical setting
  - Identify at-risk patients and assign them to preventive and therapeutic approaches
  - To develop a prototype kit assay for the identified genetic factors related to development of amyloidosis

Pathophysiology of TRAPS

- Investigate TNFR1 expression and trafficking associated with *TNFRSF1A* mutations
- Assess intracellular mechanisms related to impaired apoptosis in TRAPS
- Delineate the pattern of IL1-β secretion in leucocytes from TRAPS patients

Development of innovative therapeutics and humanized animal models

- Design a simple and relevant humanized mouse model of TRAPS
- Determine the therapeutic efficiency of the RNAi-based therapy *in vivo*
- Evaluate and validate alternative drugs interfering with IL-1ß secretion in experimental models

Results EUROTRAPS ***aimed to achieve***

Improvement of TRAPS patient diagnosis, care and quality of life

Overview of TRAPS patients in Europe:

To assess the actual prevalence of this disease, together with the distribution of cases according to age, gender, origin and way of life.

Improvement of knowledge on the clinical features of the patients:

To provide a patient registry as a unique worldwide accessible tool to define a complete clinical pattern and chronology of disease symptoms.

Improvement in our knowledge of the genetics and pathophysiology of TRAPS

TRAPS and TRAPS-like mutations:

- Update of the registry of TRAPS mutations
- Expansion of the number of genes associated with the TRAPS phenotype
- Improvement of the sensitivity and quality of the genetic diagnosis

Clarification of the role of normal and mutated *TNFRSF1A* variants

To resolve the apparent contradictions between independent studies into the pathophysiologic mechanisms of TRAPS by the use of the same system for all *TNFRSF1A* mutations and cells coming directly from the patients as well as cells transfected with the corresponding mutated protein.

Improvement of present and future treatment

Evaluation of conventional anti-cytokine therapies

To provide review of the current trials or anecdotal experiences. Indeed, none have been reported prior to the EUROTRAPS project. This aim is important as some TRAPS patients are refractory to TNF blockade, depending on the mutation present. Moreover, there are deleterious side effects and additional co-morbid conditions associated with a systemic blockade of IL-1B or TNF, including uncontrolled fevers and increased susceptibility to infections (such as tuberculosis).

Definition of new markers of disease severity and response to treatment (outcome measures)

We anticipated that monitoring of a selected range of acute phase reactants in well characterised TRAPS patients may highlight a set of easily investigable indicators of disease course. The patient registry was designed to include a set of variables (days of fever, intensity of clinical manifestations on a visual analogue scale, dose of steroids…) that can be used as outcome measures. We also aimed to complement this by the search for, and validation of, a genetic signature associated with susceptibility to amyloidosis, and treatment resistance to help patient care.

Development of innovative therapies

To evaluate the therapeutic potential of drugs interfering with proteins involved in different steps of IL-1ß secretion as well as RNAi-based technologies using mice models. New therapeutic approaches that induce a prolonged remission with limited side effects by targeting the mediators of inflammation were needed.

Application of the EUROTRAPS model to hereditary recurrent fever (HRF) diseases as a whole

We reasoned that registries, data, biological resources, *in vitro* and *in vivo* models would serve for all other hereditary recurrent fevers, notably, improvement of HRF diagnosis and better understanding of HRF pathophysiology.

Description of the main S & T results/foregrounds.

WP2: Characterization of the natural course of TRAPS patients

Creation of a European clinical registry for TRAPS patients

One of the main limitations in the development of a single registry for each specific disease is the impossibility to compare the clinical manifestations occurring in different diseases for the final elaboration of diagnostic or classification criteria and/or for the identification of evidence-based parameters for the indication to the molecular analysis for each disease. For this reason EUROTRAPS decided to make a consortium with another parallel initiative working in the field of autoinflammatory diseases: EUROFEVER (www.printo.it/eurofever). In its original version, EUROFEVER was intended to set up a network of international registries for all those diseases lacking a web-based method for data collection ("Orphan Diseases"). Since TRAPS (EUROTRAPS), Hyper IgD (HyperIgD registry www.hids.net) and FMF (metaFMF) were already covered by international registries; these latter diseases were not originally included in the EUROFEVER Project. During the last few years an intense collaborative work has raised among various members of both EUROFEVER and EUROTRAPS projects that belong to the same scientific community involved in the study of Autoinflammatory diseases. For this reason it was chosen to elaborate a single registry for all Autoinflammatory diseases. In such a new approach EUROTRAPS provided the specific knowledge related to TRAPS.

The inherited Autoinflammatory diseases (AID) included were: FMF, CAPS, TNF-receptor associated periodic syndrome (TRAPS), Mevalonate kinase deficiency (MKD), Blau syndrome, Pyogenic Sterile Arthritis, Pyoderma Gangrenosum and Acne (PAPA) syndrome, DIRA and FCAS2. Other multifactorial autoinflammatory conditions, such as chronic recurrent multifocal osteomyelitis (CRMO), Behcet's disease, periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA) and undefined periodic fevers were included. As a first step experts in AID were asked to identify the variables they considered of interest for each disease. A draft data collection form was then sent to all the experts for their review. Five main categories were considered: i) baseline information, ii) clinical manifestations, iii) laboratory examinations, iv) imaging and other diagnostic procedures, and v) response to treatments. Further revisions of the forms were subsequently evaluated by the experts with a final approval of the definitive version during a Consensus Meeting with nominal group technique in March 2009. Data were collected through a secured web-based case report forms (CRFs) on an https platform located in the member area of the PRINTO website (www.printo.it).

The Eurofever project surveyed involved all centers linked to the Pediatric Rheumatology International network organization (PRINTO), a non-profit organization devoted collaborative studies, that now encompass more than 400 centers in 60 countries worldwide12 asking about their experience of AID. The results of the survey are available on the Eurofever website (www.printo.it/eurofever). Further dissemination was performed among adult centers with interests in the diagnosis and management of AID, the International society of autoinflammatory diseases (ISSAID), the European league against Rheumatism (EULAR), the European society of immune-deficiencies (ESID), through personal contacts, lectures and scientific communications and links with the Eurofever website.

As a result, an international network on Autoinflammatory diseases was created. Up to September 2011, 1880 patients (M:F=916:964) from 66 centers in 30 countries have been entered in the registry. Participating countries, in alphabetic order, are: Albania, Argentina, Australia, Belgium, Croatia, Czech Republic, Denmark, France, Georgia, Germany, Greece, Hungary, Israel, Italy, Latvia,

Lithuania, Netherlands, Oman, Poland, Romania, Russia, Saudi Arabia, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Most of the enrolled patients (1388; 74%) were resident in western Europe, 294 patients (16%) in the eastern and southern Mediterranean (Turkey, Israel, north Africa), 106 (6%) in eastern and central Europe, 54 in Asia, 27 in South America and 11 in Australia. At the time of the enrollment, 1424 patients (76%) were children and 456 (24%) were adults.

In total, 1049 patients were enrolled with a diagnosis of an inherited AID; 703 had genetic results completely consistent with the diagnosis; 197 had a heterozygous mutation in a classically autosomal recessive disease; 93 had no mutations detected and 56 patients had no genetic testing. Most of these latter patients were not resident in Western Europe and the lack of testing was for technical or financial reasons. 831 patents with other Autoinflammatory syndromes have been also enrolled

Study on the characteristic of the disease at its onset and on its natural course

The Eurofever/EUROTRAPS consortium has collected data on 199 mutation positive patients from 11countries constituting the most extensive clinical series to date. The patients were largely contributed by European centres which may have contributed to the dominance of Caucasian ethnicity.

Complete data on clinical features on 124 patients (adults and children) has been evaluated to date. TRAPS usually presents in childhood with a median age at presentation of less than 5 yrs but 9% of patients present well into adult life. 60% of patients report a family history of disease and most reported kindreds are small and mutations are family specific. Median attack duration was 10 days (range: less than 1 day to continuous disease) and the median number of attacks a year was 6 (range: less than 1 to continuous), the median number of days with TRAPS symptoms per annum was 60 (range: 8 – 360).

There were no recognized attack precipitants in 34%, in contrast 31.5% of patients were sure that some of their attacked were induced. These triggers were most often: stress, the menstrual cycle, fatigue, infection and exercise. Mutations affecting cysteine residues in extracellular domains are over represented but the most widespread disease causing mutation is T50M. Symptomatic patients with the well recognized polymorphism of R92Q and P46L make up 38% of the patients and appear to have a later onset of disease symptoms with lower penetrance.

Study on the natural course, outcome and quality of life of TRAPS patients

Impact of disease on the quality of life of pediatric TRAPS patients

The Health related quality of life (HRQOL), the national language version of the parental administered 50-item version of the Child Health Questionnaire (CHQ also called CHQ-PF 50) was used to assess the quality of life of pediatric TRAPS patients with structural mutations and patients with R92Q substitution. The CHQ is a generic self-administered instrument designed to capture the physical, emotional and social components of health status of children aged 5–18 years. It comprises 15 health concepts (range 0–100): global health (GGH), physical functioning (PF), role/social limitations–emotional/behavioural (REB), role/social limitations–physical (RP), bodily pain/discomfort (PB), behaviour (BE), general behaviour (GBE), mental health (MH), self-esteem (SE), general health

perception (GH), change in health (CH), parent impact–emotional (PE), parent impact–time (PT), family activities (FA) and family cohesion (FC). In addition, there are two summary measures based on the US normative standard named the physical summary score (PhSPHs) and the psychosocial summary score (PsSPass); the summary measures are standardised to have a mean of 50 and SD of 10. An international sample of 3315 healthy children (52.2% female), with a mean (SD) age of 11.2 (3.8) years, constituted the healthy control group. 11 paediatric TRAPS and the 20 R92Q patients were available at the moment of molecular analysis. A worse quality of life was significantly observed for most of these concepts, especially in patients with structural mutations.

Impact of the disease on quality of life in TRAPS adult patients

The Medical Outcome Short Form (36) Health Survey (SF-36®) was used to assess quality of life in 8 adults with *TNFRSF1A* mutations who were managed with intermittent corticosteroids alone as their disease was relatively mild with infrequent TRAPS attacks and 15 patients who disease was sufficiently severe that treatment with biologics was planned (but not yet started). The results demonstrate that patients with mild TRAPS disease have a well preserved quality of life across all assessed domains. Patients with more severe and frequent TRAPS activity show very different results with poor scores in all domains before effective maintenance treatment was started.

WP3 Acquisition of knowledge for future development of diagnosis

A diagnostic score to increase rates of early diagnosis of TRAPS in childhood

Our main goal was to identify evidence-based criteria for the identification of patient at high risk to carry mutations for genes involved in inherited periodic fevers, including TRAPS.
In a preliminary experience performed in the Italian population of children with periodic fever we identified a set of clinical criteria for the differentiation of TRAPS from other periodic fever (Gattorno et al. Arthritis Rheum. 2008;58(6):1823-32.). The score is made by 6 independent variables (age at onset, abdominal pain, aphthosis, chest pain, diarrhoea and positive family history) (see *www.printo.it/periodicfever*). Its aim is to timely select those patients at higher risk to carry mutations of known genes.
In this project, the Diagnostic Score was performed in a population of children with periodic fever fulfilling the criteria for the PFAPA (Periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis) syndrome. PFAPA is the more frequent cause of recurrent fever in children and is characterized by the absence of a known genetic etiology and high rate of self-resolution. However, at the moment of its presentation it enters into the differential diagnosis with the monogenic periodic fevers, including TRAPS. We analyzed complete clinical and genetic information from 393 children with periodic fever. 82 were positive at the genetic test (FMF, TRAPS or MKD), 75 displayed an incomplete genetic test (low penetrance mutations or heterozygosis for autosomal recessive diseases), 236 were negative for *MVK*, *TNFRSF1A* and *MEFV* mutations. The "Gaslini" Diagnostic score was able to correctly identify 91% of the genetically positive PFAPA-like patients with a global accuracy of 66%. Therefore, t**he "Gaslini" diagnostic score represents a useful tool to identify patients at high risk to carry mutations for genes involved in inherited periodic fevers, including TRAPS.** These results have been published M. Gattorno et al, Paediatrics. 2009;124(4):e721-8.

Improvement of molecular testing of *TNFRSF1A* in European countries and beyond

External quality assessment for the molecular diagnosis of TRAPS and recurrent fevers

We have developed an external quality assessment (EQA) scheme for the molecular diagnosis of TRAPS. After an in house pilot study, we used the Organization for Economic Co-Operation and Development guidelines for quality assurance in molecular genetic testing to evaluate the reporting practices. **B1a** as the coordinator appointed two assessors, took contact with the EMQN executive administrator, updated the list of participating labs, provided all necessary information for this new scheme, and prepared samples and forms for the assessment meeting. Samples and mock clinical cases for FMF, TRAPS, MKD and CAPS were distributed for genetic testing via EMQN to the genotyping labs. Scores on genotyping and clerical accuracy comments were assigned according to pre-publicized criteria. The interpretation was scored for the first time in the year 2010.

In the pilot study, mutation nomenclature was wrong or incomplete in ~ 70%. The mutation/genotype error rate decrease from 30% to 4% over years. The combined performance on the basis of the correct identification of all genotypes by a given laboratory in all the 3 years was 40%. After affiliation to the EMQN network, the overall genotyping error rate improved over the two years from 2.2% to 1%. The mean 2010 scheme interpretation score was 1.94 of a maximum 2.00. A common interpretation oversight was alerting on the risk of amyloidosis in a TRAPS mock case that had a mutation predicted to disrupt a protein disulfide bond. **Overall, the performance of genetic testing of hereditary recurrent fevers has dramatically improved.** The results were disseminated individually and at various meetings. A second paper is considered.

Delineation of guidelines for the genetic diagnosis of hereditary recurrent fevers

We then delineated a framework for best laboratory practice and reporting for the genetic diagnosis of HRFs. A draft was written by **B1a** and used as a basis document for discussion. This draft was disseminated by EMQN and amendments were made during a Best Practice workshop meeting held in Bruges (Belgium) on September 18th and 19th, 2011. The following items were discussed: Indications for HRF testing, diagnostic strategy, interpretation of the mutations, reporting, recipient and content, clerical information. **An agreed practical set of best practice guidelines has been developed for molecular genetic testing of HRFs** that should be published by the end of 2011.

Development of novel tools for molecular testing of TRAPS and recurrent fevers

Complete screening of TRAPS (B8)

We aimed to set out a kit for easy and complete screening of *TNFRSF1A* mutations. The first technology that we chose was a newly developed electrophoresis approach (Enhanced Mismatch Mutation Analysis - EMMA). We screened about 140 samples that we received from the EUROTRAPS members. Although we assessed that the sensitivity and the robustness were excellent since we easily differentiated all variants, we chose to abandon the method because of difficulties that arose during the development stage and because it was necessary to use equipment dedicated only for EMMA.

We therefore decided to use **DNA Sequencing** approach, which is the most commonly-used approach for both mutation scanning and mutation testing, and is widely regarded as the gold standard. We have developed the ProntoSEQ™ TRAPS prototype kit, that was designed and developed to allow

screening and detection of both known and novel point mutations (substitutions and small indels), in all ten exons as well as part of introns in the *TNFRSF1A* gene. **The kit is now ready for manufacturing**!

Screening of HRFs

Hereditary recurrent fevers (HRF) are a group of inherited disorders of the innate immune system characterized by apparently unprovoked inflammation recurring at variable intervals. Diagnostic assesment of HRF includes clinical data, evaluation of acute phase reactants, response to specific drugs and presence of specific mutations. For the latter, the involvement of several genes implies a major challenge and requires diagnostic methods with the potential of screening rapidly for causative mutations in the genes for Familial Mediterranean Fever (FMF), Hyper-IgD Syndrome (HIDS), Tumor Necrosis Factor Receptor-associated Periodic Syndrome (TRAPS) and Cryopyrin-associated Periodic Syndromes (CAPS).

Sequencing (B8)

We consulted the EUROTRAPS consortium regarding the genes and relevant exons that need to be screened. The Differential HRF prototype kit was designed and developed to cover most common point mutations in the four main HRF genes by sequencing procedure,
Although not yet a commercial product, **we now offer, as a service, the complete screening of the relevant exons in the 4 HRF genes using sequencing technique**.

Strips (B9)

Aiming at a simple but powerful first-line screening tool for HRF mutations, we have developed and validated a reverse-hybridization assay (HRF StripAssay) for the rapid and simultaneous detection of 22 most common HRF mutations: H20N, H20P, I268T, V377I (HIDS); R260W, D303N, L305P, T348M, L353P, Y570C (CAPS); C30R, C33Y, D42Del, T50M, C70R, C73W, R92Q (TRAPS); M680I(G/A), M680I(G/C), M694I(G/A), V726A (FMF).

Reliable genotyping of recombinant mutant clones and a selection of reference DNA samples was achieved by means of teststrips presenting parallel arrays of allele-specific oligonucleotides. We demonstrated that the prototype HRF StripAssay is capable of detecting all 22 mutions, as well as identifying homozygotes by the absence of the corresponding wild-type signal. The entire procedure from blood sampling to final result required less than 6 h, and hybridization/detection can be performed manually or automated (up to 48 samples per run).

The prototype HRF StripAssay proved to be a fast, easy-to-perform and reliable screening method for most common HRF mutations. Additional mutations of interest can be rapidly integrated, which keeps it a flexible tool within the complex field of HRFs. The simple protocol allows its use also in basically equipped laboratories.

Identification of additional pathways and genes likely to be involved in patients affected by TRAPS-like disorders

Identification of a novel exon 2-skipped TNFR1 transcript: regulation by *TNFRSF1A* rs1800692 and role in patients with TNFR-associated periodic syndrome (TRAPS) phenotype by B1a

Our lab recently identified a splicing isoform of the *TNFRSF1A* gene that we named TNFR1-d2. Because a defect in gene expression regulation may play a role in TRAPS physiopathology, we decided to carry on this expression analysis within the EUROTRAPS project. Quantitative studies of

TNFR1-d2 expression revealed that the level of TNFR1-d2 expression seems to be higher in TRAPS patients. We also showed that its expression was associated with the genotype of the 473-33C>T polymorphism in intron 4 of the *TNFRSF1A* gene.

The rs1800692 (c.473-33C>T) T/T genotype is rare in the TRAPS group

The rs1800692 genotypic distribution was comparable in our controls and in two Caucasian populations (CAUC1 and PGA-European-Panel) available from the NCBI SNPdb database (National Center for Biotechnology Information). Moreover controls and the TRAPS-like group showed similar genotype distributions and were both in Hardy-Weinberg equilibrium. In contrast, the T/T-genotype frequency dropped dramatically in TRAPS patients. Indeed, we had no T/T homozygote patient in the RQ study-group ($p=0.02$), and only one in 112 available genetically confirmed TRAPS patients (51 patients from Montpellier and 57 from the EUROTRAPS consortium) ($p<10-4$).

Using allele-specific PCR or RFLP strategies, we observed that the T allele was always located in cis with the wild type allele, apart from 3 patients, carrying a cysteine mutation. This result, together with the fact that one T/T TRAPS patient was identified suggests that the combination of two T alleles plus one TRAPS mutation is not lethal.

To check rs1800692 (c.473-33C>T) as a possible modifier, we compared C/C homozygote patients to those carrying at least one T allele for the major clinical signs but observed no significant difference in either TRAPS or TRAPS-like patients (data not shown). When we consider the genotype at rs1800692 in TRAPS-like patients, we found a significantly result between the fever and the T/T genotype ($p=0.02$), suggesting that the T/T genotype could be protective for fever

Effect of rs1800692 (c.473-33C>T in intron 4) on *TNFRSF1A* exon 2 skipping

To assess the relative impact on exon 2 skipping of these three polymorphisms, we performed an *in vitro* alternative splicing assay using minigene constructs. In SW480 cells, the presence of the C or T allele of c.473-33C>T did not modify the level of exon 2 splicing whatever the promoter inserted in the transfected construct. In HEK293 cells however, the T allele together with the viral promoter resulted in a significant decrease of exon 2 skipping as compared to C-containing construct, whereas the exon 2 spliced transcript was over-expressed when the T allele was together with the *TNFRSF1A* promoter and exon 1. These results demonstrate that the polymorphism c.473-33C>T in intron 4 (rs1800692) can modulate *TNFRSF1A* exon 2 splicing at least in HEK293 cells and that this modulation seems dependent on the sequence (promoter and exon) in front of *TNFRSF1A* exon 2. We conclude that these two *TNFRSF1A* regions may have a functional and combined effect on exon 2 skipping in a cell-specific manner.

The *TNFRSF1A* intron 4 sequence acts on transcriptional level

Our in vitro alternative splicing assay revealed that the difference in expression level of exon 2-spliced products with T allele of c.473-33C>T seems to be dependent on the *TNFRSF1A* promoter in HEK293. These results suggest that the regulation of exon 2 skipping may also implicate another mechanism such as regulation of the gene transcription. Indeed, the pre-mRNA splicing process is complex and it is well documented that splice site selection can also be influenced by regulation of transcription as transcription and splicing processes are coupled.

To address whether the *TNFRSF1A* sequence intron 4 had an effect on gene transcriptional activity, we inserted it into a pGL3 luciferase reporter vector containing a constitutively active SV40 promoter . The relative luciferase activity of these constructs was increased around 2 fold as compared to that of the pGL3-promoter vector in both cell lines. The two alleles at c.473-33C>T (rs1800692) resulted in similar luciferase activity. This increased transcriptional activity was abolished after removal of the region extending from c.473-72 to c.473-33 in intron 4, indicating that this sequence

probably interacts with one or more transcriptional factor(s). When compared to the SV40 promoter, the *TNFRSF1A* promoter appeared more efficient in HEK293 cells (2.8 to 2.6 fold for the G and T allele at rs4149570 respectively) than in SW480 (1.3 and 1.2 fold), with no significant influence of rs4149570. Our results suggest that intron 4 contains transcriptional regulatory element(s) that are c.473-33C>T - and cell-independent, and that the *TNFRSF1A* promoter activity is independent of rs4149570 but cell-dependent.

Identification of novel genes by B5b

Candidate gene approach

In order to identify other genes involved in TRAPS-like patients, we carried out a mutation search on a number of candidate genes based on their possible involvement in cell processes believed to play a role in TRAPS pathogenesis. Candidate genes screened are: (i) ARTS-1 (aminopeptidase regulator of TNFR1 Shedding) is a type II integral membrane protein that binds full-length TNFR1 and regulates both the constitutive release of TNFR1 exosome-like vesicles and the proteolytic cleavage of soluble TNFR1 ectodomains; (ii) Nucleobindin 2 (NUCB2), a calcium-dependent ARTS-1 binding partner; (iii) RBMX (RNA-binding motif gene, X chromosome) associates with ARTS-1; (iv) TNFAIP3 (tumor necrosis factor, alpha-induced protein 3), also known as A20, is involved in the regulation of TNFR1 signalling by ubiquitination; (v) CARP-2 is a protein with ubiquitin ligase activity, recruited to early TNFR1 complex inside the endocytic vesicles; (vi) TTP, also called ZFP36, is a zinc finger-containing protein that destabilizes mRNA by binding to an AU-rich element. It has been shown that mice deficient in TTP develop a severe inflammatory syndrome mainly because of overproduction of tumor necrosis factor alpha. Finally, (vii) the Caspase 1 gene (CASP1), proteolytically cleaving the precursor of interleukin-1, has also been analyzed. A total of six patients were selected among a large set of autoinflammatory syndromic patients, on the basis of

i) presence of TRAPS-like clinical manifestations and/or positive diagnostic score indicative for a TRAPS phenotype

ii) exclusion of causative mutations of the coding sequence of the *TNFRSF1A* gene and

iii) evidences of a defect, assessed in patients' monocytes, of either shedding and/or TNF-induced apoptosis. Though the patients were not homogeneous regarding shedding of the TNFR1 receptor and TNF-induced apoptosis, they were regarded as belonging to a same TRAPS-like patients set, and thus underwent, without further distinction, to the molecular analysis of candidate genes possibly involved in the pathogenesis of their disease phenotype.

As a result, no gene could be identified. This work is published

***Borghini S**, Fiore M, Di Duca M, Caroli F, Finetti M, Santamaria G, Ferlito F, Bua F, Picco P, **Obici L**, Martini A, **Gattorno M**, **Ceccherini I**. Candidate Genes in Patients with Autoinflammatory Syndrome Resembling Tumor Necrosis Factor Receptor-associated Periodic Syndrome Without Mutations in the TNFRSF1A Gene.*J Rheumatol. 2011 Apr 1.

Expression microarray approach

To look for novel genes, we have undertaken a strategy making use of hybridization to Affymetrix expression arrays of transcription products from different individuals. In particular, we have profiled expression of LPS-treated and -untreated monocytes obtained from: 1) healthy controls (C), 2) TRAPS patients, before starting anakinra treatment (TRAPS-pre), 3) TRAPS patients, after starting anakinra treatment (TRAPS-post) and 4) TRAPS-like patients. A total of 38 samples have been subjected to this

analysis and, though data are already available and statistical elaboration nearly complete, at present validation has just started on a small subset of potentially relevant genes.

Several gene families and canonical pathways have been found to be enriched. The top 15 pathways containing genes differentially expressed between TRAPS and control monocytes were deduced after running our data in the KEGG (http://www.genome.jp/kegg/) and DAVID EASE (http://david.abcc.ncifcrf.gov/) softwares.

This has confirmed the involvement of the genes belonging to these gene families and canonical pathways in the pathogenesis of TRAPS and their response to pathogens. A first conclusion we can draw from this first observation is that, already without any treatment, TRAPS monocytes show in their basal level expression of genes reflecting enrichment for pathways involved in innate immunity, inflammation, stress response and apoptosis, a picture which precisely recapitulates our present knowledge about TRAPS pathogenesis (involvement of ER trafficking, defective apoptosis and/or shedding, induction of oxidative stress etc.). These pathways result to be enriched at a different extent of genes which modulate their expression after LPS-treatment in TRAPS compared to Controls, an observation consistent again with the known response to pathogens in TRAPS patients. Involvement of miRNAs, protein intracellular trafficking, cytokines and cytokine receptors interactions and other pathways has also been confirmed to play a role in TRAPS compared to Controls.

WP4: Acquisition of knowledge for future development of therapeutic approaches

Collection of data on the effects of various treatments (immuno-modulators and cytokine modifying drugs)

As TRAPS is both a very rare and relatively newly recognised disease there are few centres with substantial treatment experience. There are no drugs specifically licensed for use in TRAPS and no prospective trials to guide treatment choices. All the therapies used carry risks of significant side effects and the newer anti cytokine agents are high cost therapeutics which difficult to fund. A literature search of all reported treatments in patients with TRAPS found 50 publications describing response to treatment, results were analysed and incorporated into the data resource which was utilised to build the consensus treatment guidelines. Data on treatments used and their effect was sought from the EUROTRAPS and EUROFEVERS web-based registries. Physicians were asked to define responses as a complete, partial, none or worse based on combination of patient symptoms, clinical findings and laboratory parameters. Data on 159 patients with clearly pathogenic variants/variants of debated clinical significance was available from 14 countries. The major treatments used are : symptomatic treatment, corticosteroids, colchicine, anakinra, etanercept.

This is the largest survey of treatment of TRAPS to date. Patients were collected from a number of centres in Europe and beyond but with a marked predominance of patients from Western Europe. This may be reflected in the high use of biologic agents which are not necessarily widely available. The most significant findings are that intermittent corticosteroids are effective in treating acute TRAPS attacks in more than 40% of patients initially but almost 80% of patients with structural mutations and 36% of those with the R92Q variant are eventually treated with anti cytokine agents.

In patients with structural mutations anti IL-1 treatment with anakinra is completely effective in 89% of cases and continued long-term in 92% of cases. Its use is associated with a 90% reduction in the requirement for corticosteroids to treat acute attacks. Etanercept is significantly less effective and is

discontinued in almost 75% of cases. In patients with the R92Q polymorphism the treatment efficacy was considerably less striking and not significantly different between the 2 agents.

Consensus treatment recommendations for patients with TRAPS associated with *TNFRSF1A* mutations and for patients with the common R92Q variant have been formulated based on a literature review and data from the combined registries which comprises the largest treatment series to date. Recommendations were generated for: the general aims of treatment; treatment of TRAPS associated with pathogenic variants and with the R92Q variant and for 2 specific groups: children and patients with TRAPS complicated by AA amyloidosis.

Identification of outcome measures for future therapeutic trials

A consensus conference held in London in Feb 2010 agreed that that disease activity in TRAPS should be assessed by symptoms combined with biochemical measures of inflammation, specifically CRP and if available SAA and assessment of quality of life using the Medical Outcome Short Form (36) Health Survey (SF-36®) for adults aged over 18 years and the Child Health Questionnaire – Parent Form (CHQ-PF50©) for children. There was a significant improvement across all domains after introduction of biologic treatment, anakinra (IL-1Ra).

Clearly this does not exclude even better long term outcomes with effective maintenance treatment of TRAPS but the current data from adults who have not had effective maintenance treatment in childhood or adolescence suggests that it may be difficult in short term or small scale studies to see a significant improvement in these with the current generation of highly effective treatment.

The most deleterious outcome in TRAPS is the development of AA amyloidosis. This was seen in 22 patients and was the sole cause of mortality detected in the EUROTRAPS registry to date and appears to be the single most important complication of the disease.

Identification of genetic susceptibility factors which modulate clinical responses to treatment

Recent evidences that micro(mi)RNAs are detectable in body fluids and represent a novel class of biomarkers in several cancers and autoimmune disorders pushed EUROTRAPS consortium to investigate this potential for patients with TRAPS syndrome. Partner 2 thus initiated a novel task in WP4 aiming at including the miRNAs in the identification of a genetic signature associated with response to treatments. First, partner 2 optimized the parameters for the isolation, detection and quantification of miRNAs from total blood and serum samples. Using RT-qPCR, partner 2 then compared the expression levels of several miRNAs in the blood and serum of TRAPS patients with those in other forms of rheumatic diseases (RA, USpA, AS, OA and PsA) and identified 3 miRNAs differentially expressed in TRAPS patients. Results showed higher expression levels of miR-16 and miR-197 in TRAPS patients compared with other diseases, while those of miR-125b were lower. Importantly, the work of the EUROTRAPS consortium on optimizing the extraction and quantification of miRNAs in serum and blood suggests that future tracks for research of miRNA-based TRAPS signatures shall focus on identifying miRNA patterns in the whole blood as opposed to the serum.

A prototype kit assay for the identified genetic treatment response factors

As no genetic marker associated with response to treatment could be identified the resources of this team have been redeployed to investigate the role of a new transcript likely to be involved in TRAPS physiopathology.

WP5: Acquisition of knowledge for future development of preventive approaches

Identification of patients who develop AA amyloidosis or who have a strong family history of AA amyloidosis complicating TRAPS.

During the 42 months of the project, a periodic clinical follow-up has been performed, as a standard of care for this disease, for TRAPS patients previously diagnosed at UCL and OSM. Up to 70 patients were specifically monitored for AA amyloidosis development at UCL by means SAP scan and/or renal and liver function blood tests and 24-hour proteinuria. Overall, 20 patients were followed at Pavia Amyloid Centre. Periodic clinical assessments included renal and liver function tests, 24-hour proteinuria and an abdominal fat aspirate for the search of amyloid deposits. During the project, 6 patients were identified that developed AA amyloidosis. It should be noted that the increasing availability of effective anti-IL-1 treatments has likely changed the natural history of this disease, probably preventing or reducing the occurrence of this long-term complication in our cohort in the past three years. Additionally, 2 new patients were diagnosed with TRAPS and AA, bringing up to 24 the total number of patients with AA recruited into the study. The final number of AA patients available within the project allowed us to perform statistical analysis.

Wide genome search for factors modulating the risk of development of AA amyloidosis in TRAPS

This is the first task within this work-package focusing on the identification of possible genetic modifiers associated with an increased risk of developing of AA amyloidosis. We searched for copy number variations (CNVs), which include large deletions and duplications in the genome, possibly associated with AA amyloidosis. This study was performed by array-Comparative Genomic hybridization (Array-CGH) performed on an Agilent 4x180K platform, with a resolution of about 40 kb. 59 patients with AA amyloidosis (15 with TRAPS, 4 with FMF, 2 with HIDS, 23 with chronic inflammatory arthritis and 15 with a still undefined underlying disease) were studied. Several common CNVs, according to Database of Genomic Variants (http://projects.tcag.ca/variation/) were found in all samples. The frequency of these CNVs in patients with AA amyloidosis did not differ from the frequency reported in controls. Some less common CNVs were also identified, each in 2 or 3 patients. The frequency of these variations in the studied population was not significant.

In one patient with AA amyloidosis secondary to a still unknown underlying disease we found by CGH array a large (4.6 Mb) deletion on the long arm of chromosome 15. The analysis of the functional role of the proteins encoded by the genes located in this region highlighted the potential significance of *SELS* gene, coding for a 189 amino acid protein expressed in many tissues and containing a selenocysteine residue at its active site. In humans, SELS has been shown to regulate cytokine production. The possible relationship between SELS deletion and AA amyloidosis prompted us to search for other types of mutations (missense mutations, small deletions) in *SELS* in other 14

patients with AA amyloidosis of unknown origin, by means of direct sequencing. However, no variants of clinical significance have been identified in these patients. Additionally, we studied three *SELS* SNPs polymorphisms previously associated with increased cytokine and CRP levels in other inflammatory conditions as potential modifiers of AA risk in TRAPS, as mentioned in Task 3.

A candidate gene approach for the identification of modifiers affecting the occurrence of AA amyloidosis in TRAPS

The second approach to the search for possible genetic modifiers of AA amyloidosis risk in TRAPS patients was based on the analysis of single nucleotide polymorphisms in a set of selected genes.

The following SNPs were selected according to either previous evidence in similar diseases or because of their potential role as modulators of inflammation: *SAA1* alleles, *MBL2* alleles, -13 T>C polymorphism in *SAA1* promoter, macrophage migration inhibitory factor gene (*MIF*) polymorphism in the 5' region (-173*G/C), TNFα promoter polymorphisms -308 G/A, -1031T>C, -863C>A, -857C>T, c. 473-33C>T in *TNFRSF1A* intron 4 (rs1800692) and three SNPs in *SELS* gene, namely -105G>A (rs28665122), 3705G>A (rs4965814), 5227C>T (rs4965373).

Determination of the genotype for *SAA1* and mannose-binding lectin 2 (*MBL2)* alleles and for single-nucleotide polymorphisms (SNPs) was performed by either direct sequencing or RFLP analysis. The Fisher exact and χ^2 tests were used to compare categorical variables, as appropriate. **Allele and genotype frequencies for all SNPs were in Hardy-Weinberg equilibrium.**

A significant association between genotype and AA risk was observed only for SAA1. The genotype distribution and allele frequencies among TRAPS patients with and without AA amyloidosis showed that the frequency of the *SAA1.1* allele was significantly higher in patients with AA amyloidosis compared to patients without AA ($p < 0.001$) and homozigosity for *SAA1.1* was significantly associated with development of AA (HR 3.7, 95% confidence interval 1.64-8.39, $p = 0.002$). For all the other SNPs analyzed no significant association with AA risk was found.

Development of a scoring system for evaluation of the risk of AA amyloidosis in TRAPS

We evaluated the relative contribution of clinical and genetic factors to the occurrence of AA amyloidosis in TRAPS, with the aim of identifying patients at higher risk and to assign them to preventive and tailored therapeutic approaches. For this study, data were obtained from the EUROTRAPS website and the EUROFEVER registry. Written informed consent was obtained from patients. Data were entered in an anonymous fashion. Inclusion criteria were: age ≥ 18 years at last follow-up, identification of a *TNFRSF1A* mutation, availability of both "baseline" and "clinical data" in EUROFEVER. For patients included in both EUROFEVER and EUROTRAPS, results of genetic analyses were also available.

We considered the following clinical variables as potential predictors of amyloid-free survival: sex, family history for AA, type of mutation (clearly pathogenic variants vs. low-penetrance variants, namely R92Q and P46L), disease course (recurrent vs. continuous), age at TRAPS onset, age at TRAPS diagnosis, frequency of attacks, duration of attacks and clinical manifestations at attacks, namely fever >38°C, arthralgia, myalgia, conjunctivitis, fasciitis, abdominal pain, aseptic peritonitis, skin rash. As a marker of inflammation, CRP level > 20 mg/L in between attacks was also analyzed. Additionally, treatments with steroids and biological agents were considered. Genetic variables included genotype for *SAA1.1* allele, that was previously proved to be significantly associated with AA, as detailed in task 3, and the presence of any additional mutation in *MEFV* and *NLRP3*. All

variables were dichotomized except for age at TRAPS diagnosis and age at TRAPS onset. Cox model was fitted to compute hazard ratios and 95% confidence interval for the onset of amyloidosis for a series of potential predictors. Multivariate analysis was performed including predictors showing a $p < 0.05$. Survival curves were plotted according to Kaplan-Meier and differences in survival tested for statistical significance. Statistica 8 and MedCalc 11 were used for computation.

By matching data from EUROTRAPS and EUROFEVER, 104 patients were included that satisfied all inclusion criteria. Of these, 21 had AA amyloidosis. SAA genotype was available in 89 patients. 80 patients were from both EUROTRAPS and EUROFEVER, 14 from EUROTRAPS only and 10 from EUROFEVER only. 77 patients (20 with AA) had a clearly pathogenic variant. 27 had either P46L or R92Q and only one of these had AA amyloidosis.

At univariate analysis, family history for amyloidosis, *SAA1.1* homozygosity, disease course, age at TRAPS onset and the type of mutation were significantly associated with AA amyloidosis. These variables were included in the multivariate analysis. In spite of its significance at univariate analysis, CRP level > 20 mg/L was not included in the multivariate analysis for the limited number of observations available.

In the final model, homozygosity for *SAA1.1* and age at TRAPS onset independently predicted development of renal amyloidosis. *SAA1.1/1.1* genotype was the variable with the strongest influence on AA development, with a 5.3 fold increased risk. On the contrary, increasing age at TRAPS onset was associated with a reduced risk of AA amyloidosis.

Survival according to *SAA1* genotype was estimated by Kaplan-Meier analysis. Median amyloid free survival was 47 years vs. not reached (p=0.01). By the age of 53 years 75% of patients with SAA 1.1/1.1 are projected to develop AA amyloidosis.

Development of a diagnostic test prototype for the genetic factors modulating the risk of development of AA amyloidosis

We developed and validated a teststrip-based reverse-hybridization assay (Amyloidosis StripAssay) for the rapid and simultaneous detection of genetic factors modulating the risk of developing AA amyloidosis**.** Genes and polymorphisms/mutations covered by the prototype StripAssay were: 1. c.473-33C>T in intron 4 of *TNFRSF1A* gene*; 2.* c.2080A>G (M694V) of *MEFV* gene, which is a mutation found in patients suffering from Familial Mediterranen Fever (FMF); 3. c.209C>T and c.224T>C in *SAA1* gene, which define three different alleles, namely SAA1.1, SAA1.2 and SAA1.3; 4. c.154C>T, c.161G>A and c.170G>A of *MBL2* gene, defining four different alleles indicated as B, C, D and O respectively.

DNA samples from individuals previously typed positively for one of these genetic markers were obtained from EUROTRAPS partners 1, 3, 4, 5 and 6. They were used to generate recombinant plasmid clones for each of the two alleles at all loci of interest (TOPO TA Cloning Kit; Invitrogen). We applied these plasmid clones as homozygous reference samples to evaluate and optimize reverse-hybridization probes. Using the NCBI Reference Sequence databank (*TNFRSF1A*: NM_001065.2, *MEFV*: NG_007871.1, SAA1: NG_021330.1, MBL2: NG_008196.1), a series of candidate 15-25mer oligonucleotides, encoding all alleles of interest, were defined and chemically synthesized. After multiple rounds of hybridization, oligonucleotides which differentiated best between the two alleles at each polymorphic site, were used to prepare a prototype probe array including a 5´-biotinylated control

oligonucleotide to allow performance control of the detection system. This membrane-bound array was finally sliced into 3-mm, ready-to-use teststrips.

We validated the specificity of the Amyloidosis StripAssay by analyzing a series of amplification products obtained from plasmid clones carrying defined alleles of the selected polymorphic sites. Our results demonstrated that the StripAssay is capable of specifically detecting the presence of any of the 14 alleles of interest, as well as correctly identifying homozygous states by the absence of a signal for the corresponding other allele. For a negative PCR product obtained on water instead of DNA, only the biotinylated control probe, which is expected to produce color irrespective of the presence of hybridizing DNA fragments, stained positively.

In conclusion, the Amyloidosis StripAssay will be a helpful tool for the rapid and reliable diagnosis of modifying risk factors in periodic fever syndromes and related disorders. The test follows a simple protocol and requires only cheap and commonly available instrumentation, such as thermocycler, waterbath and shaker. For higher throughput, the test may also be run essentially automated on existing equipment (e.g. TECAN ProfiBlot T48). Very small amounts of DNA (approx. 50 ng) are sufficient for comprehensive genetic analysis, and the current type of teststrip can easily be extended to include additional markers once they are identified.

As soon as more extensive validation by external laboratories has been concluded, and CE/IVD registration has been filed, the Amyloidosis StripAssay shall become available as a commercial test through ViennaLab Diagnostics and its international network of distributors.

WP6 : Functional consequences of TRAPS mutations in physiological in vitro models

TNFR1 expression and trafficking associated with *TNFRSF1A* mutations by B3

A panel of full-length wild type TNFR1 (WT) and TRAPS-associated mutants expressed in pcDNA6 and pGT vectors was generated to study the functional consequences of TRAPS mutations in physiological in vitro models. The pcDNA6 vector was used to over-express the receptors in HEK 293T cells and the pGT vector designed so that the level of the receptor expression is controlled by doxycycline using a tet-on system. **Constructs were generated for the following *TNFRSF1A* mutations by team 3: (Exon 2) H22Y, C29Y, C33Y; (Exon 3) T37I, P46L, T50M, T50K (Exon 4) C88R, plus an intron 4 splice mutation (c.472+1G>A) (C158delinsYERSSPEAKPSPHPRG).** All of these were expressed in pcDNA6 with and without C-terminal fusion protein tags. **In addition constructs for C43Y, C55Y, a 27 base pair deletion,** c.612del27 (DEL27), **and R92Q were generated in the pcDNA3 vector by team 5.** The WT and mutant TNFR1 expressed in pcDNA and pGT vectors were used to transfect HEK 293T cells to study receptor expression and downstream signalling, along with their associated biochemical and cellular consequences such as apoptosis, autophagy, IL-1β release and NF-κB activation. We first established transient cell lines and later stable cell lines (clones) expressing wild type (WT) and a number of mutant TNFR1 mutant. In the stable cell lines the expression is longterm.

Stably transfected cell lines expressing the WT and mutant receptors were studied for receptor expression and localisation. We also performed NF-κB subunit activation and reactive oxygen species (ROS) studies. NF-κB activation prevents apoptosis and increased ROS levels have been shown to

mediate the increased proinflammatory cytokine secretion that is often seen in TRAPS patients (Bulua 2011).

Accumulation of mutant TNFR1 in the cytoplasm; **Immunofluorescence revealed accumulation of TNFR1 in the cytoplasm of T50M, C88R and intron 4 splice mutants compared to the WT, T50K and P46L cell lines**. All mutant cell lines tested showed higher levels of overall ROS and mitochondrial ROS production than the WT cells. **In addition team 5 showed aggregates in the cytoplasm of C43R, T50M, C55Y, and c.612del27 mutant cells**.

Intracellular mechanisms related to impaired apoptosis in TRAPS by B5

A) Monocytes from TRAPS patients display the same defect of TNF-induced apoptosis already observed in circulating neutrophils

Consistent with our previous study, we showed that not only circulating neutrophils but also monocytes from TRAPS patients display impaired activation of Complex II leading to defective induction of apoptosis after TNF stimulation.

B) Impaired internalisation of TNFR1 in cells from TRAPS patients and in transfected cells after TNF stimulation

The binding of TNF ligand to TNFR1 on the cell surface activates the pro-inflammatory intracellular pathways leading to NF-κB transcription factor activation (Complex I). Conversely, activation of the pro-apoptotic cascade (Complex II) is secondary to the compartmentalization and internalization of the TNFR1-TNF complex. Since a defect of the metalloprotease-dependent shedding of membrane TNFRI after a nonspecific stimulus, such as Phorbol Myristyl Acetate (PMA), has been described in TRAPS patients, we investigated TNFR1 internalization after direct stimulation with TNF. The main limitation of the *in vivo* approach using primary cells from TRAPS patients is the impossibility of clearly dissecting the actual functional impact of mutated proteins from that of WT proteins expressed on the cell surface. For this reason we analyzed, in the same experimental setting, the pattern of expression and internalization of TNFRI in HEK 293T cells transiently transfected with the same mutant TNFRI as we had detected and studied in our TRAPS patients.

After 1 hour of incubation monocytes from healthy individuals with TNF induced a drastic down-regulation of surface expression of TNFR1. This effect was not observed in monocytes from TRAPS patients carrying mutations such as T50M, C29Y, C55Y and the 27aa interstitial deletion, whose membrane TNFR1 expression was not affected by TNF stimulation. Monocytes from patients carrying the R92Q mutation displayed a pattern of TNF-induced TNFR1 down-modulation at the plasma membrane similar to that observed in healthy individuals. No soluble TNFR1 was detected in monocyte cell culture supernatants of TRAPS patients and healthy controls. A significantly lower down-modulation of membrane expression of TNFR1 was observed in TRAPS patients.

We transfected four TNFR1 constructs, three of which carry cysteine mutations (the already reported C43R, C55Y mutations and a novel C29Y mutant) and the fourth characterized by a 27aa deletion of exon 6, already observed in a TRAPS patient. WT and R92Q mutant TNFR1, along with a mutant TNFR1 carrying an amino acid substitution (Y207A), known to be associated with impaired internalization of TNFR1, were also analyzed. As previously described, surface TNFR1 was expressed on GFP-positive cells transfected with the WT and R92Q mutations, whereas mutations of cysteine residues were associated with defective membrane TNFR1 expression, as also observed by others. Conversely, the 27aa deletion of exon 6 and the Y207A mutant construct showed normal surface expression of TNFR1 on transfected 293T cells. After TNF stimulation, HEK 293T cells transfected

with wild-type and R92Q mutated TNFR1 showed a down-modulation of the receptor as already observed in monocytes from healthy controls and R92Q mutated TRAPS patients. Conversely, 293T cells transfected with the 27aa deletion displayed the same defect of internalization observed in 293T cells transfected with the Y207A mutant form of TNFR1. Of course, lack of TNFR1 expression on the surface of HEK 293T cells transfected with cysteine mutants did not allow us to analyze their pattern of internalization after TNF stimulation.

Thus, transfection experiments confirmed the alteration in the intracellular trafficking of mutations affecting the extracellular cysteine residues. On the other hand, non-cysteine mutations of the *TNFRSF1A* gene showed normal expression of the mutated receptor at the cell membrane. Interestingly, these latter mutations cause an internalization defect, similar to that observed in monocytes from the TRAPS patients carrying the same mutation.

These data support the possible role of defective internalization of TNFR1 receptor as a possible cause for the resistance to TNF-induced apoptosis in TRAPS patients.

Pattern of secretion of IL-1β in monocytes and neutrophils isolated from TRAPS patients by B5

Monocytes from 5 chronic infantile neurological, cutaneous and articular (CINCA) syndrome and 4 TRAPS patients selected for treatment with anakinra were activated with 1μg/ml of LPS for 3 hours, both at baseline and after 7 days from the beginning of the treatment. For comparison, monocytes from 24 healthy donors were also studied. Intracellular pro-IL-1β and secreted IL-1β were analysed by Western blotting (WB) and ELISA before and after a short exposure (15 min) to exogenous ATP that accelerates IL-1β secretion. Unstimulated monocytes from healthy donors express very low if any pro-IL-1β (not shown). Conversely, CINCA and TRAPS patients exhibited variable levels of intracellular pro-IL-1βin the absence of stimulation. In healthy subjects LPS-induced IL-1β secretion was variable but consistently ≤5ng/ml and it was markedly increased by exposure to exogenous ATP (up to 20ng/ml). Monocytes from CINCA patients secreted abnormally elevated amounts of IL-1β after LPS stimulation (up to 40ng/ml) that were not further increased by ATP. Conversely, monocytes from TRAPS patients did not secrete more IL-1β than healthy controls in response to LPS, but similarly to CINCA patients presented a low response to ATP.

Thus, at least in this experimental setting, monocytes from TRAPS patients do not show a clear oversecretion of IL-1β compared to healthy controls.

For this reason, we decided to merge the resources available for the present WP6 with WP7 to focus our experimental approach on the impact of the defective intracellular trafficking and elevation of ROS on the process of cellular autophagy in TNFR1 mutated cells. The possible consequences on IL-1β secretion and the possibility of modulation with geldanamycin have also been investigated.

Effects of TRAPS-associated *TNFRSF1A* mutations on autophagy, apoptosis and NF-κB is prevented by geldanamycin treatment

The molecular pathogenesis of TRAPS is not yet completely understood and defects in TNFR intracellular trafficking and receptor shedding have already been reported.

During the final year of the project, we have focused on the intracellular trafficking defect to explain the possible effects of TNFR1 mutations on TRAPS pathogenesis. We have been able to demonstrate,

for the first time, the presence of a defective autophagy mechanism in TRAPS, a defect which can be related to a lack of control in the IL-1β production and secretion pathways in TNFR1 mutated cells.

Starting from the observations that TNFR mutations cause an incorrect localization of the mutant protein, with retention in the endoplasmic reticulum (ER) and accumulation of the mutant proteins due to impaired protein elimination, found both *in vitro* cell models and in leukocytes obtained from TRAPS patients, and following recent observations suggesting a link between inflammation and autophagy, we have investigated whether defective autophagy may play a role in TRAPS pathogenesis. In particular, by using 293T cells transiently expressing WT or mutant forms of TNFR we have demonstrated that:

TNFR1 mutations lead to intracellular inclusions

Fluorescent microscopic analysis of transiently transfected 293T cells revealed that, while the WT protein could be observed both within cells and on cellular membranes, mutant proteins, were detected almost exclusively in intracellular inclusions. These observations were confirmed also in blood cells derived from a healthy individual and a TRAPS patient. Retention of mutant TNFR proteins inside the transfected cells was also assessed by cytofluorimetric analysis

As mechanisms underlying TNFR intracellular accumulation seem to rely on defective mutant protein elimination rather than excessive protein production, we have investigated whether the TNFR protein levels might be regulated by the ubiquitin proteasome system (UPS) and autophagy. Unexpectedly, proteasome inhibition by MG132 caused a marked reduction of both WT and mutant TNFR proteins rather than their accumulation, while, on the contrary, the use of the autophagy inhibitor, 3-MA, induced a marked accumulation of all TNFR proteins, thus suggesting that autophagy is the main mechanism whereby cells proceed to remove mutant misfolded TNFR proteins in TRAPS patients. Consistently, in the presence of proteasome inhibition, the elimination of the TNFR protein occurred together with an increase of the LC3B-II protein, the active pro-autophagic form of the LC3B protein.

Mutant TNFR proteins do inhibit autophagy

- Electron microscopy (EM) investigation confirmed an autophagy defect in cells expressing the mutant TNFR with respect to cells expressing the WT protein
- co-localization of the autophagic protein beclin-1 with mutant TNFR aggregates as well as detection of beclin-1 in the insoluble fraction of cells expressing the mutant TNFR protein, confirmed the association between autophagy impairment and the presence of TNFR mutant proteins expression of LAMP-1, a gene positively regulated by autophagy induction, was significantly reduced in cells expressing mutant compared to WT proteins
- The nuclear income of TFEB, a transcription factor which up-regulates lysosomal genes following autophagy induction, is impaired in 293T cells transfected with the mutant C55Y construct and treated with the autophagy inducer trehalose.

Geldanamycin can restore correct TNFR localisation

HSP90 inhibition by the antibiotic geldanamycin (GA) and the consequent overexpression of heat shock proteins has been reported to induce protein refolding in cellular models of several diseases. We have investigated whether GA could restore the correct localization of mutant TNFR proteins by cytofluorimetric analysis of TNFR expression on cells' surface showing that 1) GA induced formation of a diffused distribution of the C55Y protein with respect to the pattern of aggregation shown by untreated cells and 2) large aggregates were significantly reduced in the presence of the antibiotic, in both transfected cells and monocytes derived from TRAPS patients.

Geldanamycin affects TNFR-mediated NF-κB activation, thus rescuing apoptosis

Expression constructs encoding the WT and mutant TNFR proteins were co-transfected with a plasmid containing several NF-κB regulatory sequences cloned upstream of a Luciferase reporter gene. Luciferase activity detected 48 hour later showed that TNFR WT was strongly able to induce NF-κB activity with respect to the empty vector while all mutant constructs were shown to trigger a higher level of NF-κB with respect to the WT protein. Consistent with our expectations, 293T cells transfected with the same constructs and added with GA for 24 hours showed a dramatic reduction of NF-κB activation compared to untreated cells. Moreover, GA was able to induce apoptosis and addition of TNF, together with GA, were shown to enhance such effect, thus suggesting that NF-κB inhibition could rescue the responsiveness to apoptotic driving forces.

Geldanamycin decreases IL-1β production by LPS-treated monocytes

Recent studies have demonstrated a novel role of autophagy in the regulation of inflammatory immune response. Autophagy controls the production of IL-1β through two mechanisms: 1) targeting pro-IL1β for lysosomal degradation and 2) regulating activation of NLRP3 inflammasome (Harris et al. JBC 286:9587-97, 2011). However, how autophagy regulates cytokine secretion is poorly understood. In TNFR1-associated periodic syndrome (TRAPS) defective trafficking of TNFR1 mutations promote production of pro-inflammatory cytokines. In preliminary experiments we found that monocytes produce IL-1β after 6-24h of LPS stimulation; remarkably, addition of GA inhibits IL-1β secretion. Defective autophagy may thus lead to inflammatory IL-1β production in TRAPS patients, taken together our data suggest a possible role for GA in induction of autophagy and to preventing inflammation. These latter data relaunch the possible pivotal role of IL-1β in the pathogenesis of TRAPS, as shown by preliminary reports on the dramatic clinical response to anti-IL-1 treatment in TRAPS patients. The pattern of secretion of IL-1β in TRAPS patients will be therefore further analyzed using different experimental settings in respect with those already described above.

Summary - Advances in the pathophysiology of TRAPS

Among the main findings from the transfected HEK293T cells expressing WT TNFR1 and the cell lines expressing various TRAPS-associated TNFR1 mutants include

1) **mutated TNFR1 aggregates** in the cytoplasm of C43R, T50M, C55Y, C88R, c.612del27 and intron 4 splice mutant cell lines compared to the WT, R92Q, T50K and P46L

2) **monocytes from TRAPS patients do not oversecrete IL-1**β compared to healthy controls.

3) **increased basal IL-6 levels** in mutant cell lines compared to WT cells.

4) **total ROS production** was found to be higher in TRAPS-associated TNFR1 mutants compared to transfected WT cells. All mutant cell lines tested showed higher levels of mitochondrial ROS production than the WT cells. This work corroborates the report by Bulua *et al.* showing that mitochondrial ROS promote production of proinflammatory cytokines and are elevated in TRAPS patients.

5) **impaired autophagy is present in mutant TNFR1** containing cells, thus possibly accounting for TRAPS molecular pathogenesis.

6) the above described defective autophagy mechanism in TRAPS may be related **to a lack of control of IL-β production and secretion pathways** in mutated cells.

All these observations can be reconciled in a complex series of effects which were further validated through the use of Geldanamycin treatments. TNFR1 mutations lead to intracellular inclusions, with increased ROS production and inhibition of autophagy. These defective intracellular mechanisms cause NF-κB activation and secretion of pro-inflammatory cytokines. These studies have increased our understanding of the pathophysiology of TRAPS but have not yet impacted on the development of new therapies.

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WP7: Development of innovative therapeutics and humanized animal models

Objectives : Inconsistent and partial efficiency of current biotherapies used in TRAPS, associated with problems of tolerance and potential serious infectious side effects, as well as refractory patients, support the need for the development of alternative therapeutic approaches that induce a prolonged remission with limited side effects by targeting the mediators of inflammation. WP7 thus aimed at evaluating the therapeutic potential of drugs interfering at the protein or mRNA levels with genes involved in different steps of the TNF-α and IL-1β secretion, using relevant mouse models. Major steps were the following :

Development of a liposome-based vehicle targeting human monocytes (D7.1)

Monocytes are a heterogeneous cell population with subset-specific functions and phenotypes. The inflammatory monocyte subset, as a first line of defense of the innate immunity, controls the immune response to pathogens and forms a prominent constituent of inflammatory infiltrates in the tissues targeted by autoimmune and autoinflammatory disorders. In patients with TRAPS, there is a specific involvement of inflammatory blood monocytes and macrophages of the inflamed tissues in the disease pathogenesis. They are a major source of proinflammatory mediators including TNF and IL-1, and their numbers are increased both systemically and in the inflammatory tissues.

Recent publications suggest that the inflammatory monocyte subset may represent a valuable cellular target for innovative immunotherapeutic strategies against immune-mediated inflammatory disorders. Delivery systems able to discriminate between the various monocyte subsets are however still missing and it remains crucial to demonstrate in vivo the cell-specific targeting, as well as efficient immunomodulation using a relevant animal model.

Using a previously developed cationic liposome formulation DMAPAP (previously named RPR209120/DOPE) partner 2 showed that **the siRNA-containing lipoplexes were preferentially taken up by the mouse inflammatory Ly6C^{high} monocyte subset upon intravenous injection.** This

systemic strategy was not only able to target Ly6Chigh monocytes from the blood but also from the spleen, liver, inflamed joints and draining lymph nodes of mice with collagen-induced arthritis.

Importantly, in view of potential biomedical applications, partner 2 replaced the plasmid DNA cargo of the formulation by various non-toxic, biodegradable and FDA-approved anionic polymers and selected the sodium alginate as optimal on the basis of in vitro studies performed on the human monocytic THP1 cell line. Using total blood leukocytes, partner 2 demonstrated ex vivo that **the novel DMAPAP lipoplex formulation preferentially targets the human $CD14^+/CD16^-$ inflammatory monocytes and efficiently mediated the RNAi-based gene silencing** of master proinflammatory cytokines within this specific subset.

Validation of siRNA sequences specific for the TNF and IL-1 gene silencing in TRAPS monocytes (D7.2)

Mutations of the *TNFRSF1A* gene have been shown to underlie hereditary TRAPS diseases, characterized by increased TNF-α and IL-1β expression in peripheral blood leukocytes compared with normal controls, responsible for the manifestations of the disease. Since both TNF-α and IL-1β have a fundamental role in the pathogenesis of inflammation associated with TRAPS, and mRNA stability is a critical check-point for bioactive protein synthesis, partner 2 designed and **validated siRNA sequences that efficiently silence (i) human IL-1 and TNF-α in human primary monocytes in vitro and (ii) mouse IL-1 and TNF-α in inflammatory monocytes in vivo**.

Development of a humanised model of activated TRAPS leukocytes in SCID mice (D7.3)

For 2 years partner 2 together with partner 3 have tried to set up conditions for reconstituting the myeloid compartment of the human immune system in SCID/Bg mice with normal and genetically-modified monocytes. Several protocols previously published have been tried, but none of them led to significant amount of human monocytes in the mouse circulation within the few days following injections. Considering the huge numbers of mice already used in this work (the planed numbers have been already reached) and the large volumes of human blood requested, that would represent ethical issues for TRAPS patients, the consortium decided to stop this work at the 2nd EUROTRAPS annual meeting in Amsterdam (2010, September).

As the D7.3 was not delivered because of technical reasons, the milestone M7.2 “design of a humanized SCID mice model of TRAPS monocytes” could not be reached either and impacted on D7.4. In agreement with the consortium, partner 2 modified D7.4 such as the proof-of-concept for therapeutic intervention using RNAi-based lipoplexes will not been carried out in a humanized mouse model of TRAPS, but in a simple existing mouse model of systemic and joint inflammation, i.e. the mouse collagen-induced arthritis.

Evaluation of the therapeutic potential of RNAi-based lipoplexes for TRAPS syndromes (D7.4)

In order to optimize the repression of master proinflammatory cytokines within the inflammatory monocyte subset, partner 2 tested the possibility to target only one gene to impact on several

deleterious cytokines at the same time. Pre-B-cell colony enhancing factor (PBEF), also named Visfatin or Nampt, is not only an essential enzyme in the NAD biosynthetic pathway but also exerts numbers of additional effects, including a key role in the persistence of inflammation. Indeed, its extracellular form acts as a proinflammatory cytokine through induction of the production of inflammatory cytokines including IL-1, TNF-α and IL-6. PBEF also functions as an inhibitor of cell death by apoptosis in response to a variety of inflammatory stimuli including IL-1 and TNF-α. Finally, PBEF is highly expressed in patients with a variety of inflammatory disorders and its elevated expression in body fluids correlates with disease activity. Since most of the cytokines deregulated in TRAPS'monocytes are under the regulation of PBEF, partner 2 therefore hypothesized that targeting PBEF/Visfatin in the inflammatory monocyte subset might be a promising alternative therapeutic strategy to interfere with chronic inflammation in TRAPS. Partner 2 thus evaluated the feasibility of triggering RNAi-mediated gene silencing of PBEF in Ly-6Chigh monocytes in vivo in the context of established inflammation.

Partner 2 demonstrated for the first time that the of the cationic liposome DMAPAP formulation enable targeted delivery of siRNAs agaisnt PBEF to the inflammatory mouse Ly6C^{high} monocyte subset upon intravenous injection. Moreover, such strategy triggers efficient and specific silencing of PBEF within the inflammatory Ly6C^{high} monocytes and results in the reduction of down-stream proinflammatory cytokines TNF and IL-6, and successfully impaires disease progression in collagen-induced arthritis by decreasing the frequency of pathogenic Th17 cells. These results bring the **proof-of-concept that inflammatory monocytes are key target cells for efficient immuno-intervention in inflammatory disorders and shows that targeting a relevant gene within this specific cellular subset by RNAi provides a therapeutic benefit in vivo**. These data also opened promising perspectives for the development of innovative strategies for the treatment of TRAPS.

Design of alternative IL-1 blocking drugs (D7.5)

In WP6, partner 5a demonstrated an impaired autophagy in in TRAPS mutated cell lines and hypothesized that this could be linked to the dysregulation of IL-1 production and secretion. Taking advantage of the **Geldanamycine** (GA), an antibiotic that was described to exert a broad action, including protein refolding and induction of apoptosis, partner 5a investigated the anti-IL-1 potential of GA. Indeed, upon LPS stimulation, the use of GA **was able to down-modulate the IL-1 secretion by monocytes isolated from both healthy donors and TRAPS patients**. Although further study on the pattern of secretion of the GA-treated cells is still required, these data suggest that GA might represent an alternative strategy for TRAPS. Notably, the 17-AAG (Allyl Amino Geldanamycin) is a less toxic analogue of the geldanamycin that has completed phase I and is currently entering phase II clinical trials for cancer applications.

Description of the potential impact and the main dissemination activities and the exploitation of results.

Socio-economic impact and wider societal implications D1.8

The EUROTRAPS consortium has reached the performance foreseen in the initial project as summarised below:

Impact on wealth

Thanks to the EUROTRAPS project, we have established a comprehensive pan-European figures outlining TRAPS distribution in Europe and in the world, and impact on wealth. This project provided a clear picture concerning this matter. The development of concerted and manufactured approaches to diagnostic strategies (WP3), consensual therapeutic (WP4) and preventive (WP5) schemes, will undoubtedly result in a lowering of the negative economic impact on society. Indeed, chronic pain and potentially devastating complications caused by TRAPS (e.g. amyloidosis), due to repeated bouts of inflammation and by auto-inflammatory disorders in general has a significant effect on the patients. Severe financial burdens can be incurred on many levels (costs of healthcare services and medication, job absenteeism and disruption in the workplace, loss of income, non-productivity in the economy and in the home, impact on family, friends and employers, worker compensation costs and welfare payments…). For example, we could demonstrate that with anakinra, the number of days with TRAPS symptoms fell from a mean of 40.4 to 0 .57 and the number of days of lost work fell to a mean of 0.5 from 10 days per annum.

Impact on quality-of-life

- We have created a specific registry which helps define a complete clinical pattern and chronology of the symptoms (WP2). This registry, with contributions from all clinicians of the European network assembled within the framework of this project, served to develop a paediatric score and assess patient quality of life, treatment effects, and complication occurrence. The efficiency and side effects of cytokine modifying drugs have been reviewed in adults and children (WP4), and serum, genetic and epidemiologic indicators and risk factors for disease severity and response to treatment (WP5) have been evaluated as foreseen. We believe that EUROTRAPS, with its clinically oriented approach, will contribute to slowing down and eventually neutralising the disease, as well as improving the physical, emotional and economical wellbeing of the patient and his family.

- We have developed discriminative score and criteria for TRAPS diagnosis and differential diagnosis with other hereditary recurrent fevers (WP2). Many patients have experienced prolonged delay in reaching a diagnosis (up to 20 years) due to lack of recognition of this condition. Absence or delay in correct diagnosis is a source of anxiety, with increased costs and enhanced risk of amyloidosis. Recurrent attacks of fever with acute pain lead to school and professional absenteeism. Our study demonstrated a significant decrease in diagnosis announcement.

- Through numerous actions (see D1.7), we have promoted the dissemination of advice and information, and supported the teaching of good practice in clinical and diagnostic

assessment. Since most of participants are members of scientific committees of patient associations, we are now able to encourage health structures to recognise the existence and importance of TRAPS and to cover care costs incurred by this disease.

Impact on employment

As foreseen, a significant impact on employment has resulted, in the later stages of the project, from the transfer/hiring of young investigators from academic to the corporate laboratories.

- The development of all EUROTRAPS activities has generated a large number of employment positions for young researchers, engineers and technical assistants. Coupled with the cross-national project culture this will underpin the next generation of broadly informed and well-trained European researchers in this important area.
- Secondly, the hiring of new staff by the SMEs, who are project partners, as well as by the wider industrial sector resulting from exploitation of the biological information generated by EUROTRAPS has strengthened the European economy in the area of drug discovery and biotechnology.

Main dissemination activities and exploitations of results D1.7

Dissemination activities

They are described in details in the public area of our website
http://fmf.igh.cnrs.fr/ISSAID/EUROTRAPS/public.php

They include communications (>40) at congresses, during teaching and continuing medical education, information on websites, and 25 already accepted publications in four European languages in journals including 20 with peer reviews. We of course intend to submit more publications within the next two years related to this project.

Actions of communication

• Presentations at meetings:

- **July 5th, 2011:** Abstract of the The second national FMF, autoinflammatory diseases and amyloidosis meeting, , Tel Hashomer (Israel): Quality assurance in genetic testing of hereditary periodic fevers (HRFs): Genotyping and beyond. Y. Shinar, I. Aksentijevich, J. Arostegui, I. Touitou.
- **May 25-28, 2011:** Presentation at EULAR 2011, London (United Kingdom). The Eurofever registry for Autoinflammatory disease: results of the first 15 months of enrolment. By partner 5.
- **May 15-18, 2011:** The 13th TNF International Conference (TNF 2011), Hyogo (Japan): Tumour Necrosis Factor Associated Periodic Syndrome (TRAPS) is associated with unprovoked sustained high levels of proinflammatory cytokines in ex vivo and in vitro models. Azad M Aziz, Laura J Dickie, Dennis McGonagle, Philip Robinson and Michael F McDermott. By partner 3.
- **2010:** Chapter in the book: Clinical Genomics: Practical Applications in Adult Patient Care, McGraw Hill Publishers and the American College of Physicians editors: Familial autoinflammatory diseases. By partner 1.
- **December 4-5, 2010:** Presentation at the Golden Helix Symposium, Genetic Analysis in Translational Medicine, Athens (Greece): Combined mutation and rearrangement screening by

quantitative PCR high-resolution melting: Is it relevant for hereditary recurrent fever genes? By partner 1.

- **December 1-3, 2010:** Poster presentation at the 5th European workshop on immune-mediated inflammatory diseases, Sitges (Spain). Myeloid cell subsets dynamic during progression of mouse collagen-induced arthritis. Presumey J., Jorgensen C., Courties G., Apparailly F. By partner 2.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): Quantitative PCR High-Resolution Melting (qPCR-HRM) for Hereditary Recurrent Fever Genes: Is It Relevant to Screen Both Punctual Mutations and Large Rearrangements? By partner 1.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): Infevers and ISSAID: Gateway and tools for clinicians and scientists working on FMF and other Autoinflammatory Diseases. By partner 1.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): A novel TNFR1 spliced transcript translated by an internal initiation codon induces NF-kB pathways : Implication in TNF receptor associated periodic syndrome (TRAPS)? By partner 1.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): The *TNFRSF1A* polymorphism c.473-33C>T (rs1800692) in intron 4, and its influence on gene expression. By partner 1.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): The Eurofever/EUROTRAPS registry: results of the first 6 months of enrolment. By all EUROTRAPS partner.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): A mutation in more than one Inherited Periodic Fever Syndrome gene is associated with greater prevalence of AA amyloidosis. Jethwa H, Rowczenio DM, Russell T, Trojer H, Loeffler J, Hawkins PN, Lachmann HJ. By partner 4.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): AA Amyloidosis Complicating the Inherited Periodic Fever Syndromes. Lane T, Rowczenio DM, Russell T, Trojer H, Gillmore JD, Wechalekar A, Hawkins PN, Lachmann HJ. By partner 4.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): Experience with anakinra in 120 patients with systemic autoinflammatory diseases. Melo Gomes S, Loeffler J, Rowczenio D, Trojer H, Russell T, Rannigan L, Lane T, Woo P, Brogan P, Hawkins PN, Lachmann HJ. By partner 4.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): Anakinra for the treatment of TNF Receptor Associated Periodic Syndrome. Melo Gomes S, Loeffler J, Rowczenio D, Trojer H, Russell T, Drewe E, McDermott E, Rannigan L, Lane T, Woo P, Brogan P, Hawkins PN, Lachmann HJ. By partner 4.
- **September 2-6, 2010:** Presentation at the 6th International conference on Autoinflammatory disease, Amsterdam (The Netherlands): Characterisation of the genes associated with hereditary periodic fevers syndromes in patients with AA amyloidosis of undetermined aetiology. Rowczenio DM, Russell TL, Trojer H, Bybee A, Hawkins PN, Lachmann HJ. By partner 4.
- **August 27, 2010**: MicroRNAs in RA: Novel track for biomarkers and innovative drug discovery, 1st UCL symposium of translational research in rheumatology Bruxelles (Belgium). By partner 2.
- **June 22, 2010:** MicroRNAs as novel blood-based prognostic and diagnostic biomarkers for treatment outcome, Sanofi meeting « focus on miRNA », Montpellier (France). By partner 2.
- **June 4-5, 2010:** MicroRNAs, novel serum-based biomarkers in rheumatoid arthritis, Annual meeting South rheumatology MTM, Montpellier (France). By partner 2.
- **June 13-15, 2010:** Poster presentations at the French society on gene and cell therapy (Paris): A liposome-based vehicle for preferential targeting of the mononuclear phagocyte system in

arthritic mice. G Courties, J Presumey, V Escriou, D Scherman, C Jorgensen and F Apparailly. By partner 2.

- **March 19-20, 2010:** New Breakthroughs in Systemic IL-1β–Driven Autoinflammatory Diseases', Berlin (Germany): Auto-inflammatory diseases Update on Genetics: advances in diagnostics. By partner 1.
- **March 4-6, 2010:** Poster presentation at the EWRR (European Workshop of Rheumatology Research), Bamberg (Germany); A liposome-based vehicle for preferential targeting of the mononuclear phagocyte system in arthritic mice. G Courties, J Presumey, V Escriou, D Scherman, C Jorgensen and F Apparailly. By partner 2.
- **January 14, 2010:** Oral presentation at the French evaluation committee AERES, Montpellier (France).
- **November 27, 2009:** Seminar UK primary immunodeficiency network annual meeting given by partner 4.
- **November 20, 2009:** Memorial Lecture – 20Th Graham Watson Study Day (UK paediatric meeting) given by partner 4.
- **November 18 2009:** Lecture Wessex Regional Rheumatology meeting given by partner 4.
- **October 20, 2009:** To analyze the actual impact of R92Q mutation on *TNFRSF1A* gene in children with periodic fever in comparison with TRAPS patients with structural mutations and periodic fever of unknown origin (PFAPA). Philadelpia (USA). By partner 5.
- **October, 2009:** Building a registry for the Autoinflammatory diseases in childhood (The Eurofevers project). These results has been presented by partner 5 at the EULAR Meeting, Copenhagen, June 2009
  and 75TH ACR meeting, Philadelpia, October 2009. Ann Rheum Dis 2009;68(Suppl3):508. By partner 5.
- **September 11, 2009:** Partner 6 organized a meeting with doctors and patients of the Italian Association for Periodic Fevers, Giardini Naxos (Italy).
- **September 4-5, 2009:** Séminaire maladies autoinflammatoires pratiques pour les francophones, Montpellier (France) : "Diagnostic génétique, pour qui, pourquoi, comment? By partner 1.
- **July 24, 2009:** Lecture UK Summer Allergy School given by partner 4.
- **July 9, 2009:** Plenary Lecture Third Kidney and Collagen Disease Conference given by partner 4.
- **June 29, 2009:** The first year meeting of the EUROTRAPS project was held in Montpellier.
- **June 10-13, 2009:** Annual European Congress of Rheumatology EULAR 2009, Copenhagen (Denmark); The clinical spectrum of 20 children with the R92Q (TRAPS) mutation. By parter 1.
- **May 23-26, 2009:** European Human Genetics Conference 2009, Vienna (Austria); High throughput, complete genotyping of the *TNFRSF1A* gene; Shira Lezer, Aline Yakir, Nir Navot.
- **May 15, 2009:** A meeting of the SOFREMIP was held in Paris (France). The program included a session by the French Reference Center for Auto-Inflammatory diseases (CeRéMAI). The EUROTRAPS project, and its connection with the Eurofevers project was presented by the coordinator (Pr I Touitou).
- **April 26-29, 2009:** 12th International TNF Conference, San Lorenzo de El Escorial, Madrid (Spain); Functional consequences of mutations in *TNFRSF1A*, comparison of in vitro cellular events to findings from tissue samples isolated from patients with tumour necrosis factor receptor-associated periodic syndrome (TRAPS); Azad M. Aziz, Laura J. Dickie, Paul Emery, Sinisa Savic, Michael F. McDermott.
- **March 31, 2009:** Presentation at the Eurofever meeting , Camoglie (Italy): Definitions and policy for genetic analysis. By partner 1 and 5.
- **January 16, 2009:** National meeting for pediatric rheumatologists to coordinate registration of patients with periodic fever syndromes in Germany, in Essen.
- **November 24-25, 2008:** The 2nd RIMO meeting, Montpellier (France); Presentation of the EUROTRAPS project; Pr I Touitou.

- **October 10, 2008:** National meeting for pediatric rheumatologists to coordinate registration of patients with periodic fever syndromes in Germany, Würzburg on the annual meeting of the German Society of Pediatric Rheumatology (Gesellschaft für Kinder- und Jugendrheumatologie, GKJR)
- **September 10, 2008:** The Orpheme pole of competitivity, Lançon de Provence (France); Presentation of the EUROTRAPS project; Pr I Touitou.
- **September, 2008:**
  - Different pattern of synthesis and secretion of IL-1beta in patients with *CIAS-1* and *TNFRSF1A* mutations responding to IL-1 blockade.
  - Prevalence of monogenic autoinflammatory diseases among Pediatric Rheumatology centers: the Eurofever PReS/PRINTO survey. This work was presented by partner 5 at 15th Paediatric Rheumatology European Society (PreS) Congress, London September 2008. Pediatric Rheumatology 2008, 6(Suppl 1):P212.
- **June 5-6, 2008:** Workshop on Biotechnologies of Heath, Brussels; Presentation of the EUROTRAPS project; Pr I Touitou.
- **June 2, 2008:** A meeting with patients was held in Versailles (France) by the French Reference Center for Auto-Inflammatory diseases (CeRéMAI). The EUROTRAPS project, and its connection with the Eurofevers project was presented by the coordinator (Pr I Touitou).

• Project publications

- **Touitou I**, Dodé C, Jéru I, Cuisset L, Réseau national pour le diagnostic génétique des maladies auto-inflammatoires GenMAI.
  Genetic diagnosis of auto-inflammatory diseases: Indications and interpretation.
  j.monrhu.2011.09.002 (In press)
- **Savic S**, **Dickie LJ**, Battellino M, **McDermott MF**.
  Familial Mediterranean fever and related periodic fever syndromes/auto-inflammatory diseases
  Current opinion in rhumatology. (In Press)
- Quillinan N, Mannion G, Mohammad A, Coughlan R, **Dickie LJ**, **McDermott MF**, **McGonagle D**.
  Failure of sustained response to etanercept and refractoriness to anakinra in patients with T50M TNF-receptor-associated periodic syndrome.
  Ann Rheum Dis. 2011 Sep;70(9):1692-3. (PubMed)
- Piram M, Frenkel J, **Gattorno M**, Ozen S, **Lachmann HJ**, Goldbach-Mansky R, Hentgen V, Neven B, Stojanovic KS, Simon A, Kuemmerle-Deschner J, Hoffman H, Stojanov S, Duquesne A, Pillet P, Martini A, Pouchot J, Koné-Paut I; EUROFEVER and EUROTRAPS networks.
  A preliminary score for the assessment of disease activity in hereditary recurrent fevers: results from the AIDAI (Auto-Inflammatory Diseases Activity Index) Consensus Conference.
  Ann Rheum Dis. 2011 Feb;70(2):309-14. (PubMed)
- Wittmann M, Kingsbury SR, **McDermott MF**.
  Is caspase 1 central to activation of interleukin-1?
  Joint Bone Spine. 2011 Jul;78(4):327-30. (PubMed)
- Nedjai B, Hitman GA, Church LD, **Minden K**, Whiteford ML, McKee S, Stjernberg S, Pettersson T, Ranki A, Hawkins PN, Arkwright PD, **McDermott MF**, Turner MD.
  Differential cytokine secretion results from p65 and c-Rel NF-κB subunit signaling in peripheral blood mononuclear cells of TNF receptor-associated periodic syndrome patients.
  Cell Immunol. 2011;268(2):55-9. (PubMed)
- Shaw PJ, **McDermott MF**, Kanneganti TD.
  Inflammasomes and autoimmunity.
  Trends Mol Med. 2011 Feb;17(2):57-64. (PubMed)

- **Courties G**, **Presumey J**, Escriou V, Scherman C, **Jorgensen C** and **Apparailly F**.
  A liposome-based vehicle for preferential targeting of the mononuclear phagocyte system in arthritic mice.
  Annals Rheum Dis 2010 vol 69: A60 (Abstract)
- **Presumey J**, **Jorgensen C**, **Courties G**, and **Apparailly F**.
  Myeloid cell subsets dynamic during progression of mouse collagen-induced arthritis.
  Ann Rheum Dis 2011;70:A40-A41.
- Pelagatti MA, Meini A, Caorsi R, Cattalini M, Federici S, Zulian F, Calcagno G, Tommasini A, Bossi G, Sormani MP, Caroli F, Plebani A, **Ceccherini I**, Martini A, **Gattorno M**.
  Long-term clinical profile of children with the low-penetrance R92Q mutation of the TNFRSF1A gene.
  Arthritis Rheum. 2011 Apr;63(4):1141-50. (PubMed)
- Duroux-Richard I, **Jorgensen C**, **Apparailly F**.
  What do microRNAs mean for rheumatoid arthritis?
  Arthritis Rheum. 2011 Aug 26. (PubMed)
- **Borghini S**, Fiore M, Di Duca M, Caroli F, Finetti M, Santamaria G, Ferlito F, Bua F, Picco P, **Obici L**, Martini A, **Gattorno M**, **Ceccherini I**.
  Candidate Genes in Patients with Autoinflammatory Syndrome Resembling Tumor Necrosis Factor Receptor-associated Periodic Syndrome Without Mutations in the TNFRSF1A Gene.
  J Rheumatol. 2011 Apr 1. (PubMed)
- Pallares-Ruiz N, Philibert L, Dumont B, Fabre A, Cuisset L, Cointin E, **Rittore C**, **Soler S**, **Touitou I**. Combined mutation and rearrangement screening by quantitative PCR high-resolution melting: is it relevant for hereditary recurrent Fever genes?
  PLoS One. 2010 Nov 23;5(11):e14096. (PubMed)
- **Presumey J**, **Jorgensen C**, **Courties G**, and **Apparailly F**.
  Myeloid cell subsets dynamic during progression of mouse collagen-induced arthritis.
  J Transl Med. 2010; 8(Suppl 1): P54.
- **Touitou I** , **Grandemange S.**
  Advances in the genetics of auto-inflammatory diseases.
  Revue du Rhumatisme Monographies.Volume 77, Issue 4, August 2010, Pages 300-306
- Cook GP, Savic S, Wittmann M, **McDermott MF**.
  The NLRP3 inflammasome, a target for therapy in diverse disease states.
  Eur. J. Immunol., 2010. (PubMed)
- **Dickie LJ**, Savic S, **Aziz A**, Sprakes MS, **McDermott MF**.
  Periodic fever syndrome and autoinflammatory diseases.
  F1000 Reports 2:3, 2010. (PubMed)
- **Aziz A**, Wong CH, Emery P, **McDermott MF**.
  Comparison of auto-inflammation and autoimmunity using TRAPS and Rheumatoid Arthritis as prototypes of these conditions.
  Int J Clin Rheumatol 4: 681-695, 2009
- **Obici** & al.
  Susceptiblity to AA amyloidosis in rheumatic diseases: a critical overview.
  Arthritis Rheum., 2009. (PubMed)
- **Touitou** & al.
  An international external quality assessment for molecular diagnosis of hereditary recurrent fevers: a 3-year scheme demonstrates the need for improvement.
  Eur J Hum Genet., 2009. (PubMed)
- **Touitou** & al.
  Web resources for rare auto-inflammatory diseases: towards a common patient registry.
  Rheumatology, 2009. (PubMed)
- **McGonagle D**, **Aziz A**, **Dickie LJ**, **McDermott MF**.
  An integrated classification of pediatric inflammatory diseases, based on the concepts of autoinflammation and the immunological disease continuum. *Pediatric Research,* 2009. (PubMed)

- **Announce** of the **EUROTRAPS** project *(in French)* in the CHRU newsletter *Objectif Lettre n°217* of July, 2009, by J.Millot-Keurinck.
- **Announce** of the **EUROTRAPS** project *(in German)* in the newsletter of the German Society of Rheumatology (Deutsche Gesellschaft für Rheumatologie, DGRh), N° Q4, 10/2008; with a reminder linked to this article in N° Q2, 06/2009.
- **Announce** of the **EUROTRAPS** project *(in German)* in the newsletter of the German Society of Pediatric Rheumatology (GKJR), N° 7, 06/ 2008, and a reminder to this correspondent article in N° 8, 06/2009.

Websites

- Eurofever (including EUROTRAPS) patient registry: http://www.printo.it/eurofever/
- A diagnostic score for recurrent fevers: http://www.printo.it/periodicfever/
- **Announce** of the EUROTRAPS project *(in German)* in the newsletter on the Website of the Department of Pediatric Pneumology and Immunology, Charité, Berlin: http://www.charite-ppi.de ("Newschannel", "Archive").
- **Announce** of the EUROTRAPS project *(in English)* in the news and events session of the Website of the pronto diagnostics SME.

Exploitation of results

Kits and services for the genetic diagnostic and prognosis of TRAPS and other recurrent fevers

Several kits and services have been developed by the SMEs partners as foreseen and even more:

- Kit for sequencing of all TRAPS mutation by B8 see **D3.6**
- Service for sequencing of all mutations in hereditary recurrent fevers by B8
- Kit for the detection of the main HRF mutations by B9
- Kit for the detection of genetic prognosis factors in HRFs by B9 see **D5.3**

These tasks were delivered late at the end of the project mostly due to the scarcity of patients. Therefore discussions are still underway to define the finishing stages and distribution of these kits, participants who had a hand in the development of these kits, the distribution of intellectual property, and the probable establishment of an operating contract.

Development of guidelines for the future care of patients

Several guidelines have been issued after the meetings and consensus conferences held during this project and are or will be shortly disseminated through publications, conferences and websites:

- Consensus for the most efficient treatment of TRAPS with the less side effects
- Diagnostic score for the diagnosis of HRFs in children
- Guidelines for the best practice of genetic diagnosis of TRAPS and recurrent fevers
- List of the most relevant prognostic factors for TRAPS

They should represent gold standards for the future care of patients within the HRF medical community.

New collaborations and networks

Collaborations and optimization of resources between close projects conducted in Europe have been developed and will continue with time.

- Eurofever for the patient registry
- Infevers for the nomenclature and listing of HRF mutations
- AIDAI for the patient activity score
- ISSAID, the internal society of autoinflammatory diseases for the dissemination of guidelines and meeting information

Moreover, the success of this project has led some of the participants to continue their collaboration through their response to further European proposals.

PUBLIC WEBSITE ADDRESS AND RELEVANT CONTACT DETAILS.

Public website address: http://fmf.igh.cnrs.fr/ISSAID/EUROTRAPS
Contact details: Isabelle.Touitou@chu-montpellier.fr