APPEL D'OFFRES 2015
SCIENTIFIC PART
Application for research grant

Name of the applicant	MIYARA Makoto
Project's title	The search for a serologic diagnostic test in relapsing polychondritis
Grant requested	20 000 Euros
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French Citizen coming back from abroad	NO

1- Introduction and aim of the project

Relapsing polychondritis (RP) is a severe, episodic, and progressive inflammatory condition involving cartilaginous structures, predominantly those of the ears, nose, and laryngotracheobronchial tree [1]. Other affected structures may include the eyes, cardiovascular system, peripheral joints, skin, middle and inner ear, and central nervous system. The diagnosis of RP mainly relies on criteria established by Michet et al. [2]. These criteria require the presence of a proven inflammation in 2 of 3 of the auricular, nasal, or laryngotracheal cartilages, or the proven inflammation in 1 of 3 of the auricular, nasal, or laryngotracheal cartilages plus 2 other signs including ocular inflammation, vestibular dysfunction, seronegative inflammatory arthritis, and hearing loss.

Relapsing polychondritis is reported to be one of the less frequent rheumatic diseases. Because RP is a very rare condition, only a few medical teams have the opportunity to investigate the pathogenesis of this disease, which remains largely unknown. While the international incidence of RP is still unknown, the annual incidence in Rochester, Minnesota, was noted to be 3.5 cases per million population [3]. In France, it is estimated that approximatively 300-400 patients could be affected by this rare condition. Many of these patients are followed in our tertiary care center (Internal medicine department 2, Pitié-Salpêtrière hospital, Paris, France) which is specialized in the care of RP patients. Many of these patients have also regrouped in an association named "Association Francophone contre la Polychondrite Chronique Atrophiante" (AFPCA), which aim is to promote translational research on this very rare disease and where we play an active role.

RP is well known to be a severe condition. This flaring-remitting disease can cause painful cartilaginous inflammation and deformity, arthritis, epiglottitis, scleritis, vision loss, blindness, deafness, vertigo, acute and chronic respiratory failure, arteritis, aortic dissection and valvular heart disease. Tracheolaryngeal symptoms are present at the time of diagnosis in a quarter of RP patients, with about half of them presenting such symptoms at some time in the course of the disease [2]. A collaborative international score for assessing disease activity in RP has been recently developed [4]. It has been shown that life expectancy in RP patients is significantly decreased as compared with age- and sex-matched healthy individuals. The 5-year survival rate associated with RP has been reported to be as low as 66%, and a 10-year survival rate of 55% has been

reported [3]. The most frequent causes of death associated with RP include acute respiratory failure due to laryngotracheobronchial chondritis and infections secondary to the immunosuppressive treatments. **Actually, there is no accurate test for the diagnosis or the prognosis of RP.**

The exact causes of RP are still unknown. However, a few lines of evidence have suggested that RP may be an immune-mediated disease as there is a well-documented overlap of RP with other rheumatic and autoimmune diseases [2, 5]. Moreover, both "humoral" and "cellular" immune responses directed against cartilage components have been demonstrated in RP patients.

No controlled trials of therapy for RP have ever been conducted or published. The systemic corticosteroid therapy is the mainstay of treatment. Prednisone (20-60 mg/day) is administered in the acute phase and is tapered to 5-25 mg/day for maintenance. Intravenous bolus of high dose methylprednisolone are sometimes used for the treatment of severe flare. Long term treatment includes azathioprine, methotrexate, cyclophosphamide, cyclosporin A and therapeutic monoclonal antibodies. However, none of these treatments has ever been formally validated and the optimal therapeutic strategy is yet unknown.

The objectives of this study are:

Primary objective : To determine autoantibodies and/or combinations of autoantibodies diagnostic for relapsing polychondritis.

Secondary objective : To determine whether some autoantibodies and/or combinations of autoantibodies are correlated to specific phenotype of relapsing polychondritis, especially those related to severe phenotypes (e.g. laryngotracheobronchial involvement) or related diseases such as myelodysplastic syndrome

2- Situation of the research topic in the literature and results by the investigator in this area

a. Pathogenesis of relapsing polychondritis

i. Pathology of relapsing polychondritis

Auricular chondritis is one of the hallmarks of RP, as it is seen in 90–95% of patients [2, 5]. Physical examination typically reveals swelling, erythema, and tenderness of the cartilaginous part of the ear. Early RP lesions seem to be characterized by a pleomorphic infiltrate with various proportions of lymphocytes, macrophages, activated antigen-presenting cells, neutrophils, and plasma cells in the perichondrium, while the cartilage is mostly normal at this initial stage. Infiltrating T lymphocytes are mainly CD4+ T cells. Direct immunofluorescence examination show inconstant deposits of immunoglobulins (Ig) and C3 at the junction between the perichondrium and the cartilage [6]. As the disease progresses, the cartilage is invaded by inflammatory cells. At that stage, proteolytic enzymes such as Matrix metalloproteinase (MMP)-3, -8, -9, elastase and cathepsins K and L are highly expressed [6] and deposits of Ig and C3 can be found in the matrix of the cartilage which is being progressively destroyed and loses its basophilia as the glycosaminoglycans are degraded [6]. The elastic and collagen fibers are disorganized and fragmented and the chondrocytes eventually disappear. The cartilage matrix is severely destroyed and/or replaced by a fibrous connective tissue. Macroscopically, the shape of the cartilage may be severely impaired with the development of cauliflower ears, saddle nose deformity, laryngotracheobronchial stenosis or even fatal airway collapse [7].

ii. Etiology of relapsing polychondritis

RP is an autoimmune-mediated disease. The typical cartilaginous involvement of RP suggests a role for cartilage antigens. Historical studies have shown evidence of humoral and/or cellular responses directed toward cartilaginous components [6]. Several reports suggest that chemical or mechanical insults to the cartilage may trigger the onset of RP in susceptible subjects presumably through the release of cartilage antigens [6]. Susceptibility to RP has been reported to be significantly associated with HLA-DR4 [8]. The primary target of

autoimmune response in this disease is yet unknown. However, data from human studies and murine models strongly support a role of both Collagen Type II (CII) and matrilin-1 as potential candidates (see chapter 2.b.).
Immunization against cartilage antigens parallels a cascade of events including inflammatory cell recruitment mediated by different cytokines. RP is likely a Th1-mediated disease as serum levels of interferon (IFN)-γ, interleukin (IL)-12, and IL-2 parallel changes in disease activity, while the levels of Th2 cytokines do not [9]. Serum levels of sTREM-1, interferon-γ, chemokine (C–C motif) ligand 4, vascular endothelial growth factor, and matrix metalloproteinases-3 are significantly higher in RP patients than in healthy donors [10]. Using a multiplexed approach, Stabler et al. [11] measured 17 cytokines serum levels in 22 patients with active RP and an equal number of age- and sex-matched healthy controls and RA patients. While RA patients showed a general increase in all cytokines measured, patients with active RP only showed significantly higher levels of MCP-1, MIP-1β, and IL-8 than controls. MCP-1, MIP-1β, and IL-8 are pro-inflammatory chemokines involved in the modulation and recruitment of monocytes and neutrophils. Serum level of macrophage migration inhibitory factor (MIF), another cytokine involved in the modulation of macrophage function, is also elevated in RP [12]. Altogether, these data suggest that a cytokine and chemokine network is orchestrating the recruitment of the infiltrating cells in cartilaginous tissues. Cytokine modulation using TNFα blockers, anakinra, tocilizumab, and abatacept has recently been shown effective in some cases of RP but further data are needed [13].

b. Known target auto-antigens in relapsing polychondritis

There are three types of cartilage tissue (hyaline, elastic and fibrocartilage), which are characterized by different composition of the intercellular matrix. The cartilage intercellular matrix consists of various proportions of collagens and elastic fibers interlaced in a ground substance composed of hyaluronan-, chondroitin- and keratin-sulfate, as well as matrilin-1, among many other molecules playing a key role in cartilage homeostasia.

i. Collagens

Type II collagen (CII) represents 95% of the total collagen content of the cartilage. Type VI, type IX (CIX), type X and type XI collagens (CXI) represent only 5% of the collagen content [14]. The ability to induce an inflammatory polyarthritis in various animal species following intradermal injection of homologous or heterologous CII has been known for years [15, 16] and is mediated by both cellular and humoral collagen type-specific immunities. Certain rat strains immunized with native CII develop ear lesions characterized by intense chondritis, positive immunofluorescence with deposits of IgG and C3, and circulating IgG reactive with native CII [17, 18].
Foidart et al. reported the presence of anti-CII antibodies in the serum of five (33%) of 15 RP patients [19]. These antibodies were detected at the onset of RP and their titers appeared to correlate with the severity of the disease. It is, however, important to underline that anti-CII antibodies are not specific to RP as they are also found in RA patients [20]. Buckner et al. Demonstrated a T-cell response directed against CII in a patient with RP [21]. Oral administration of CII as a tolerogenic treatment has been shown effective in a case report [22]. The role of minor collagens is more speculative, but strong immune responses against CIX and CXI have been demonstrated in a RP patient, who also had immune responses toward CII [23].

ii. Matrilin-1

Matrilin-1 is a 148-kd cartilage-specific protein found in the intercellular matrix consisting of 3 identical disulfide-bonded subunits assembled into a trimer via a coiled-coil domain in the C-terminal end [24]. Matrilin-1 is highly expressed in tracheal, nasal, auricular and xiphosternal cartilages, and thus might represent a potential target autoantigen in RP patients [25]. Of note, it is not expressed in normal adult articular cartilage. Immunization with matrilin-1 induced both tracheal and nasal chondritis in rats, while the joints were not affected [26]. Very interestingly, 17% of the affected animals developed an antibody response to CII, suggesting

that this response is most likely elicited by CII released as a result of the destruction of the target cartilage. The likelihood of developing the disease appeared to be governed at least in part by MHC genes, and was dependent upon alpha beta T cells [27] as well as upon anti-matrilin-1 antibodies [28]. Monoclonal anti-matrilin-1 antibodies injected into neonatal mice bound specifically to the cartilage of the respiratory tract, and adult B-cell-deficient mice injected with the same antibodies developed erosive respiratory chondritis [28], providing a direct link between anti-matrilin-1 antibodies and onset of lesions of the tracheal cartilage in this murine model. Additional data from murine models suggest that at least in some cases, matrilin-1 rather than CII may be the putative auto-antigen in polychondritis [29].
In RP patients, Saxne and Heinegard reported [25, 30] that increased serum level of matrilin-1 could be found in patients in the active phase of the disease, suggesting that the release of matrilin-1 resulted from the destruction of the involved cartilage. Buckner et al. described [31] a RP patient who had both humoral and cellular immune responses directed toward matrilin-1. Hanson et al. showed [32] that 13 (13.4%) of 97 RP patients had increased titers of matrilin-1 antibodies and that these antibodies correlated with the presence of respiratory symptoms in 69% of the cases. In this study, serum antibodies from the RP patients bound tracheolaryngeal and nasal cartilage of neonatal mice in vivo.

iii. Other possible target auto-antigens

- Cartilage oligomeric matrix protein (COMP) is a 100-kd matrix protein found only in cartilage [33]. In RP patients, serum concentrations of COMP were found below normal when the disease was active, and became normal when the patients had recovered, suggesting that the release of COMP might reflect repair and de novo cartilage matrix synthesis. Anti-COMP antibodies were detected in 7 (7.2%) of 97 RP patients [32]. In this study, all the patients with anti-COMP antibodies had anti-matrilin-1 antibodies. Rats immunized with matrilin-1 showed increased titers of antibody to COMP [26]. Altogether these data suggest that immunization against COMP results in cartilage destruction by a matrilin-1 mediated process.
- Autoantigen in the cochlea and the vestibular organ (anti-labyrinthine antibodies) and in the corneal epithelium [34-36].

iv. Conclusion

Altogether, these data suggest that immunization against various cartilage targets can be elicited in RP patients. This phenomenon might be the primary events leading to the auto-inflammatory reaction. It also likely reflects indirectly the undergoing cartilage destruction process. Whatever the role they play in the pathogenesis of the RP, these autoantibodies helps to better understand the pathogenesis of the disease and can be used as diagnosis and prognosis tools. Actually, the known autoantigens/autoantibodies in RP are detected only in a minority of the RP patients as they are detected in only 33.0%, 13.4% and 7.2% of the patients for anti-CII antibodies, anti-Matrilin-1 and anti- COMP autoantibodies, respectively [19, 32]. **Using new multiplex technology we make the assumption that new autoantigens/autoantibodies can be found associated with RP and that their use, perhaps in combination in arrays, will help for the diagnosis and the prognosis of this disease.**

c. Preliminary results

In a previous study performed in our laboratory, using Invitrogen Protoarray v5.0 protein chip, we have differentially analyzed serum samples from 8 RP and 4 healthy controls. Thirteen of more than 9000 proteins were significantly more targeted in patients than in controls. Unfortunately, the subsequent ELISA assays performed with individual autoantigens could not confirm the specificity of those autoantigens. The peptidic nature of autoantigens immobilized on these chips may account for the absence of confirmation in subsequent specific analyses. **The HuProt arrays recently developed by the company Cambridge Protein Arrays** enables the analysis of more than 20 000 autoantibodies. In addition to a significantly higher number of autoantigens, the HuProt arrays are spotted with total proteins produced by saccharomyces cerviciae instead of

short length peptides in the Invitrogen system, enabling the detection of autoantibodies recognizing conformational epitopes.
We first verified the diagnostic validity of the HuProt array by analyzing sera from patients with well-defined clinical and biological diagnosis of autoimmune disease such as primary biliary cirrhosis, systemic lupus erythematosus and anti-Jo1 associated inflammatory myositis. Antimitochondria antibodies (dihydrolipoamide S-acetyltransferase which is the component E2 of pyruvate dehydrogenase complex) was found in the sample of the patient with primary biliary cirrhosis, while anti-ribosomal protein P2 was found in the sample of the patient with SLE which was positive for anti-RiboP with conventional routine assays and anti-histidyl-tRNA synthetase (HARS) antibodies was found in the patient with anti-Jo1 associated autoantibodies (See figure 1).

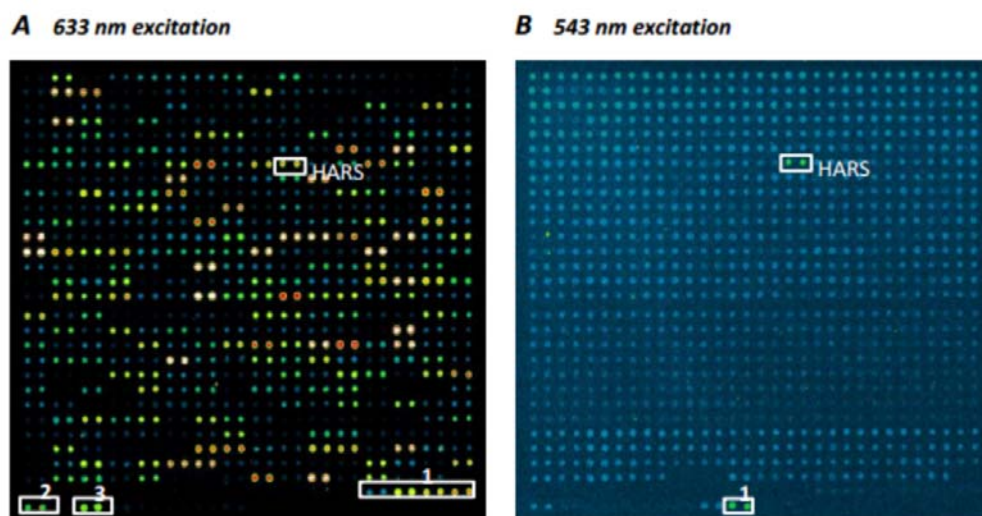

Figure 1. Detection of the anti-histidyl-tRNA synthetase (HARS/Jo1) by the HuProt array. A) Fluorescence detection of anti-GST antibodies by excitation of GST at 633 nm enabling the quality control of the array. Controls are GST alone spotted with increasing concentrations (box1), anti-biotin antibodies (box2) and biotinylated BSA (box 3). HARS position is also indicated with the confirmation of the presence of the protein on the slide detected by anti-GST antibodies. **B)** HARS detection by anti-human IgG at 543 nm excitation. Internal positive control (Box1) is a fluorescent protein not coupled with GST.

We therefore performed the Huprot array analysis of 10 sera obtained from RP patients and compared the results with 6 controls devoid of autoimmune diseases. While we found that no shared autoantibody could be detected in all patients, 10 autoantibodies recognizing CAST, CTDP1, MAGI1, OPTN, PPP1R12B, RBPJ, SHISA3, SLC16A4, TXNDC2, WT were detected in at least two different patients. Of note, among the 13 autoantibodies screened in the previous study using the Invitrogen Protoarray v5.0 protein chip, the autoantibodies recognizing PELI1 and CCDC11 were also found once in two different patients with the Huprot array. **Our preliminary results indicate that not a single autoantibody but a combination of several autoantibodies may be diagnostic for RP.**

The objectives of the project are therefore to consolidate our preliminary results by performing the HuProt array analysis on the sera of 20 patients and to confirm by ELISA, in a large cohort of patients with RP, the autoantibodies that we will have detected with the chip. We will compare the results with those obtained with

the sera of patients with other connective tissue diseases and rheumatoid arthritis in order to determine whether some combinations can be either sensitive or specific for the biological diagnosis of RP.

3- Research program

Using HuProt array, autoantibodies found in at least 2 RP patients and not found in healthy controls will be considered as potentially diagnostic tool for this disease. ELISA will be designed and performed to detect these autoantibodies in additional sera of RP patients with well-defined disease. Sera from patients with connective tissue diseases and with rheumatoid arthritis will be analyzed as well as samples from healthy donors. Clustering and ROC Curves analysis will be performed in order to determine whether some combinations of autoantibodies can be determined as sensitive and/or specific for the diagnosis of RP. We will also determine whether some particular antibodies or some combinations of autoantibodies are correlated with specific phenotype of RP, especially those related to severe phenotypes (laryngotracheobronchial inflammation, cochleovestibular involvement, ocular inflammation…) or associated conditions (e.g. myelodysplastic syndrome).

a. Description of patients and of samples

Sera of patients with well-defined RP diagnosis, stored at -20°C at the Immunochemistry laboratory of Pitié-Salpêtrière (Dr Lucile MUSSET), will be screened for the presence of autoantibodies using the HuProt array. For ELISA, sera from about 100 RP patients will be prospectively collected by the physician of the department of internal medicine 2 (Pr Zahir Amoura) and by Pr Jean-Charles Piette in his outpatient clinic. The study was already approved by the local ethics committee, and written informed consent was obtained from all participants.

b. Description by task

The work will be divided in 3 tasks

Task 1 : large screening of autoantibody repertoire in relapsing polychondritis using a 20 000 autoantigen array (HuProt, Cambridge protein array).

Objective : detection of a larger panel of candidate autoantigens in relapsing polychondritis patients;

Methods : 10 sera of patients with well-defined RP according to the criteria established by Michet et al. [2] and stored at the immunochemistry laboratory of Pitié-Salpêtrière hospital will be analyzed using the HuProt array developped by the Camgbridge protein array compagny. Obtained data data will be merged with those shown in preliminary results and compared with those obtained from the sera of healthy controls. The HuProt™ v2 Human Proteome Microarray contains more than 20,000 full-length human recombinant proteins. These full-length recombinant proteins are expressed as GST fusions in the yeast S. cerevisiae, purified, and printed on glass slides in duplicate, along with a control set of proteins (GST, BSA, Histones, IgG, etc). Sera of patients are incubated on the glass slide at a 1:2000 dilution. The secondary detection reagent used is a goat anti-human IgG (H+L) conjugated to a fluorophore with excitation at 543 nm (anti-human IgG-543). Negative and positive control assays are performed in parallel with the patients samples. For the positive control, the microarray exposed to the sample are co-incubated with 0.5 µg/ml goat polyclonal anti-GST biotinylated antibody followed by incubation with Streptavidin conjugated to a fluorophore with excitation at 633 nm (Streptavidin-633). This provides data on the relative amount of all the proteins on the array and allowed for normalisation of the results. In the negative control assay, one additional microarray is treated identically to the experimental arrays but IVIG (1:20000 dilution) are added to the slide instead of the auto-immune serum sample. On completion of the assay the arrays are scanned at 543nm (detection of interactions of from samples) and 633nm (detection of GST staining of all proteins) with a resolution of 10 µm and fluorescence data collected. Autoantibodies detected in at least two distinct patients and not present in the sera of not autoimmune patients will be kept for confirmatory specific tests;

Milestones: To determine the repertoire of autoantibodies that are present in at least two distinct patients and not present in the sera from healthy controls;
Partner contribution: Partner 2: patients recruitment; Partner 1: sample preparation & analysis;
Risk analysis: The risk of the occurrence of false-positivity due to non-specific binding of antibodies on the HuProt array is lowered by intrinsic controls (presence of numerous IgG spots with increasing concentrations, proteins spotted in duplication);
Planned schedule: Results should be obtained within 3 months.

Task 2: Validation of the candidate autoantigen detected in task 1 using ELISAs
Objective: To confirm in a large cohort of RP patients the presence of the autoantibodies detected in task 1;
Methods: One ELISA for each presumed autoantigen determined in task 1 will be design by immobilizing purified native protein when possible or recombinant protein when they are not available at the native status. Alternatively the purified protein will be purchased from the company Cambridge Protein Arrays. Sera utilized in the preliminary results and in task 1 will be assessed as well as the sera of 60 more patients with defined RP according to the criteria established by Michet et al. [2]. Sera of patients with other connective tissue diseases (n=40) and with rheumatoid arthritis (n=40) will be used as control samples as well as sera from healthy donors (n=100). Threshold for positivity will be first determined as the 99th percentile of healthy donors.
Autoantibodies detected by ELISA in patients with RP but not in healthy donors or in patients with connective tissue diseases or RA will be considered as constituting the panel of specific autoantibody in RP. Sensitivity and Specificity will be calculated and a ROC curve will be built for each ELISA as well;
Milestone: determination of a panel of autoantibodies present in RP patients but not in patients with connective tissue diseases, RA and healthy donors;
Partner contribution: Partner 2 and 3: patients recruitment; Partner 1 and 3: sample preparation & analysis;
Risk analysis: The probability that numerous autoantibodies will be present in only a small proportion of the patients is high. Task 3 is meant to combine the different autoantigens obtained in task 2 to enhance the diagnostic power;
Planned schedule: completion of the task is expected within 6 months (collection of the sera: 9 months starting from the beginning of the project, design and control quality of ELISA: 3 months, starting at the end of task 1 analysis of sera and interpretation: 3 months).

Task 3: determination of the autoantibody combinations with a diagnosis value or associated with particular clinical features using clustering analysis
Objective: To determine combinations of autoantibodies associated with the diagnosis or specific clinical phenotype of RP. Actually, the known autoantigens/autoantibodies in RP are not useful for physician as they are detected only in a minority of the RP patients (e.g. anti-CII, anti-Matrilin-1 and anti- COMP autoantibodies are detected in only 33.0%, 13.4% and 7.2% of the RP, respectively). Combining the different auto-antigens that we will identify will improve the diagnosis and prognosis values of the serologic assays;
Methods: clustering analysis compiling the positivity of autoantibodies with the disease phenotype (e.g. laryngotracheobronchial inflammation, cochleovestibular involvement, ocular inflammation…), associated conditions (e.g. myelodysplastic syndrome), or the diagnosis of RP itself will be performed. Sensitivity and specificity will be calculated and a ROC curve will be built for each interesting combinations. The last step will be to design customized arrays with the ten or so autoantigens selected for having a diagnosis or prognosis value;
Milestone: determination of combinations of autoantibodies associated with a diagnosis value for RP
Partner contribution: Partner 1 and 2: statistical analysis;
Risk analysis: The sensitivity of the serologic assays will be impaired if several RP patients do not have autoantibodies at all in circulation;
Planned schedule: Completion of the task is expected within 3 months

c. Project management

- M Miyara will be in charge of scientific coordination together with the scientific committee composed of L Musset, A Mathian, K Dorgham, C Parizot, Z Amoura and G Gorochov. This group will discuss advances every month through lab meeting organized by the scientific coordinator.
- JC Piette, A Mathian and Z Amoura will be responsible for the patient recruitment and will supervise the clinical data collection.
- L Claër and M Miyara will perform the experimental work, the analysis and statistics.

d. Feasibility of the project

-The project is based on the use of techniques (Protein arrays and ELISA) that have solid internal and external controls to ensure the validity of obtained results;

-The serum samples for task 1 are already available while the samples for task 2 will be prospectively gathered. Sample gathering will not be an issue since the physicians in charge of the recruitment follow a large cohort of patients with RP, connective tissue diseases and RA. More than 150 patients are regularly seen at the outpatient clinic by Pr Jean-Charles Piette and the physicians of the internal medicine department

- Healthy control samples will be obtained from EFS (Etablissement Français du Sang);

-The "Association Francophone contre la Polychondrite Chronique Atrophiante" (AFPCA), which aim is to promote translational research on this very rare disease, will play an active role to boost this study;

e. Novelty

This project will determine the basis for the development of the first diagnostic assay in RP. In addition, the diagnostic tool is expected to segregate patients with risks to develop severe RP.

4. Expected consequences for translational research or disease treatment

The new data will help to develop a new diagnostic tool that will ensure the diagnosis of RP. This new test will exclude the diagnosis of RP in patients with symptoms resembling those occurring in RP. More importantly, patients with combinations of autoantibodies correlated with severe phenotype will benefit from closer follow up and early treatment.

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6. Expertise: mention the names of any referee you do not wish to examine this application due to known conflict of interest.

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