PRECISESADS

Molecular Reclassification to Find Clinically Useful Biomarkers for Systemic Autoimmune Diseases

IMI Periodic Report

"Molecular reclassification to find clinically useful biomarkers for systemic autoimmune diseases"

PRECISESADS

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1. Executive summary

1.3 Summary of progress versus plan since last period

WP1 is the central Project Management and Coordination component of the Consortium and has achieved all the proposed tasks, milestones and deliverables according to plan. The Project Office has been established and all WPs report into this as well as the Steering Committee. The Project Handbook which defines consortium day to day operating procedures has been written and distributed to all members. This outlines how WPs will communicate as well as progress and financial reporting. An internet site for the project has been launched and is updated on a regular basis listing the different components of the Consortium as well as highlighting events, publications, etc. The project internal Intranet site can be accessed from here and is a forum for WP Leaders to upload documents and minutes of meetings, deliverables, etc. The 2 project advisory boards, scientific and ethical, have been formed and convened to attend the first General Assembly to be held in M12. We have also established links with other IMI projects, notably the Taxonomy partner in neurodegeneration, AETIONOMY, and signed the memorandum of understanding with them ahead of schedule.

WP2 is in charge of the logistics for the collection of samples from the recruited individuals and of the storage of the samples. It is also responsible for the preparation of the derivatives from blood and urine and their transportation to the various laboratories that will be performing the laboratory analyses. WP2 has accomplished all of the deliverables set forth for the first year and: a) has prepared the ethical protocols for the cross-sectional and the inception cohorts; b) has performed a pilot study to determine the viability of the sample collection and to define a preliminary SOP that was tested and modified according to the achieved experience; c) has developed the SOPs for the sample collection and designed the kits necessary for sample collection in coordination with all WPs, to establish the needs with regards to types of samples, quantities, processing requirements and storage requirements. This has been done for the cross-sectional and the inception cohorts. By the end of the first period, almost all ethical approvals were obtained, kits had begun to be distributed and sample collection had started.

WP3 has fulfilled its expected achievements during this first year. There are no delayed or partially completed Milestones and Deliverables. WP3 was in charge of defining the items for the eCRF of the PRECISEASAD study, both for the cross-sectional and for the inception cohorts. One of the main challenges for WP3 when building the eCRF was to limit the collection of disease details so as to avoid potential biases in the cluster analyses. In this process, WP3 took into account the input from WP8 with respect to two main principles: a) The number of items for the cross-sectional cohort should not exceed 40-50 in total; b) The items should be limited as much as possible to fields yielding a “yes/no” answer. Additionally, the items for the eCRF had to be measurable in every systemic

autoimmune disease of PRECISESADS to avoid the occurrence of missing data and be measurable on the same scale for every disease. According to these principles a first subset of 130 potential items for the cross-sectional study was constructed; this subset was sent to a clinical working group of 5 people (L. Beretta, R. Cervera, R. Hoffmann, L. Laigle, J. Hervouet) and by means of a Delphi exercise was then reduced to 27 core items. This reduced subset was then further discussed during several teleconferences by all the recruiting clinical centres and the final CRF for the cross sectional study that included 52 items plus details about co morbidities and demographics was eventually generated. The proposed items were inserted into the Inform system by Servier and internally tested. The final version of the eCRF was presented and tested by the clinical centres during the training at Servier headquarters in September 2014.

The CRF for the inception cohort was devised after internal discussion with the clinical recruiting centres and starting from the items used in the cross-sectional study. Further items to capture changes and/or activity of clinical conditions were added taking into account broadly-used and validated questionnaires, examples include but are not limited to: the Raynaud's condition score, the sicca symptoms score, the active/chronic lupus cutaneous changes, the activity of central/peripheral nervous system and others. No disease-specific questionnaires or composite scores were used as they are not "transversal" to the different systemic autoimmune diseases and not functional to the general aim of PRECISESADS. The items for the inception cohort were inserted into the Inform system by Servier and internally tested.

WP4 has progressed across the year having advanced on their tasks. With respect to three main areas, the following update can be given.

1.- **Shared genetic component in systemic autoimmune diseases (SAD): rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and systemic sclerosis (SSc).**

In order to decipher the shared genetic component of SAD, we designed a strategy based on the meta-analysis of genome wide association studies (GWAS) data of several SADs. As a proof of concept GWAS data of systemic lupus erythematosus (SLE) and scleroderma (SSc) were analysed in the first instance and novel common genes for both diseases, SLE and SSc, were identified (Martin JE et al., Hum Mol Genet, 2013). Recently, we have analysed extensive GWAS data of RA and SLE identifying nine putative new risk loci for both diseases that currently are being replicated in independent cohorts including around 10.000 RA patients and 5.000 SLE patients. Similarly, a meta GWAS of RA and SSc is in progress where five common genes found in the discovery phase were included in the replication phase that is ongoing at present. Final data of both meta GWAS, RA vs SLE, and RA vs SSc, will be delivered in three months.

2.- **Shared signatures between systemic autoimmune disease (SAD): rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Sjögren's syndrome (SjS).**

The shared biological mechanisms among SAD are not yet fully characterised. The objective of this study was to perform a meta-analysis using publicly available gene expression data about RA, SLE and S, to identify shared gene expression signatures and overlapping biological processes. The gene expression meta-analysis revealed a SAD signature composed of 371 differentially expressed genes in

patients and healthy controls, 187 of which were under expressed and 184 over expressed. Functional analysis showed that over expressed genes were involved mainly in immune and inflammatory responses, mitotic cell cycles, cytokine-mediated signaling pathways, apoptotic processes, type I interferon–mediated signaling pathways and responses to viruses. Under expressed genes were involved primarily in inhibition of protein synthesis. In conclusion, we define a common gene expression signature for SLE, RA and SjS. The analysis of this signature revealed relevant biological processes that may play important roles in the shared development of these pathologies. (Toro-Dominguez D et al., Arthritis Research Therapy, 2014, Dec 3;16(6):489)

3.- Trans-ethnic genome-wide analysis reveals MHC variability in rheumatoid arthritis genetic susceptibility

We have conducted a meta-analysis of Amerindian-enriched Latin American and Spanish rheumatoid arthritis cohorts with the aim of finding novel shared genetic risk loci and loci unique to each cohort. A total of 5,533 subjects were genotyped with the immunochip custom array and included in the final data set. This analysis showed that four amino acids in the 11, 13, 70 and 86 positions of the HLA-DRβ1 explain all association in our combined analysis. Interestingly, we also observed differential associations each cohort separately. In the Spanish cohort, only the amino acids in positions 13 and 86 of HLA-DRβ1 are necessary to explain all associations in the MHC region, while for Hispanic population amino acids in positions 13 and 70 are necessary. Outside of the HLA region, the most interesting signals were observed on the 2p25.3, 3q25.33 and 7p12.2loci where the genes MYTL1, IL12A and IKZF1 are located. In summary, we have performed a comprehensive analysis of association between the HLA region and RA in an European and Amerindian populations, allowing the detection of differences as compared to the model previously described for northern European populations for RA susceptibility (Teruel M et al,, Arthritis Rheum, 2015 –submitted-).

WP5 all expected deliverables (D5.1 to D5.3) have been reached without any deviations from the objectives of the project.

Our first milestone was the design and the approval of compatible antibody panels to be available for the different cytometers across the consortium. Consequently, to reach this milestone, three challenges were identified: 1-Fluorescence compatibility between Beckman-Coulter and Becton-Dickinson flow cytometers resolved by the use of 8-colour flow-cytometers with compatible optical benches; 2-Panel optimisation (compatibilities between dyes and markers for each panel). Nine panels have been designed; 3-Minimise variation between every sites resolved by the use of the Duraclone® tubes. This product consists in a one test dried antibodies « premix » tube. The benefits are the production of big batch to avoid inter lot variability, a long time stability, a fluorochrome intensity preserved, ready to use tubes (one per panel) and the avoid of pipetting errors.

Our second milestone was the optimisation and standardisation of immunostaining protocols, the establishment and daily monitoring standard instrument settings for a common bright signal placed at the same level in different flow cytometer instruments (calibration of MFI with beads). To reach this milestone, two methods of calibration have been designed: 1- The use of 8 peak beads. These rainbow calibration particles are a mixture of negative beads and seven bead populations, each

containing a known amount of a broadly fluorescent dye (calibrated in equivalents of a given fluorochrome). The MFI of the dimmest peak as well as the brightest peak was determined at Brest, validated in Granada and all centres have to mirror their PMT according to these values defined as references; 2- The use of capture beads. Due to intrinsic differences (mirrors, filters, electronic…) between flow cytometers, the intermediate fluorescence intensities cannot be matched exactly (e.g. KO-LOG). Consequently, we have optimised the calibration procedure with the Versa Comp antibody capture beads. This procedure helped us to adjust more precisely the PMT voltage for each channel.

Our third milestone was the establishment of a protocol for purification of specific cell subsets (T cells, B cells, monocytes and neutrophils). This has been established using magnetic beads commercially available according to the manufacturer's instructions and optimized by FPS and UBO. The rationale of using this method is that it will avoid cell activation during the procedure. The procedure is described in SOP#5.

WP6 has progressed according to the DoW plan. The objective of the work group is to profile metabolites, biological mediators, and auto-antibodies of relevance to systemic auto-immune disease.

A sub-contractor has been identified by UNIMI for cytokine analysis and a pilot study completed comprising analysis of a representative sub-population of analytes in patient serum. Execution of the contract is expected by end March 2015 (delayed by one month due to administrative procedures).

The task 6.2 is related to the determination of routine autoantibody (Ab) profiles in a European Reference Center (Brest University Medical School) using serum of 288 selected SADs individual and healthy controls, and the inception cohort. Seven panels will be used, which have been verified, including autoantibodies and complement.

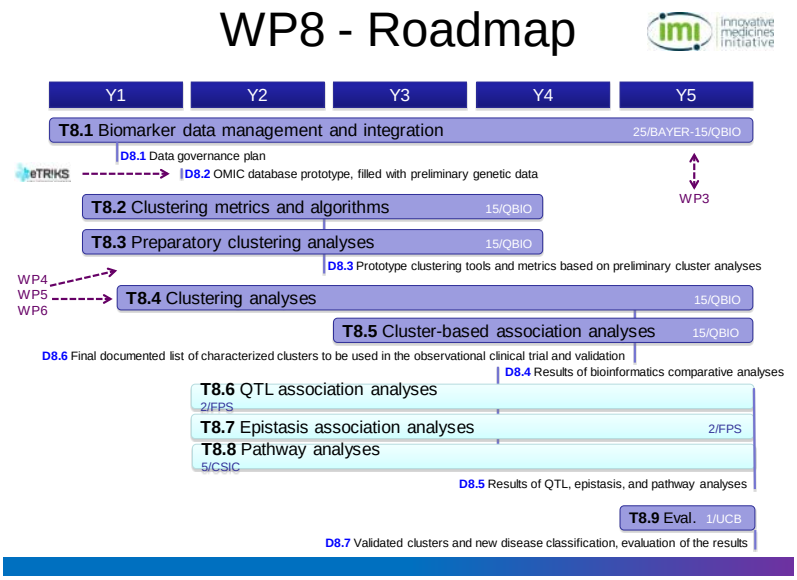
Task 6.3 involves analysis of metabolites in plasma and urine by HPLC-ESI-QTOF-MS which will be carried out in three main steps: sample preparation, HPLC-ESI-QTOF-MS analysis and data pre-processing and analysis. The methods used in every step have been optimised by using healthy control plasma and urine samples. Regarding plasma samples preparation, the composition of the precipitation agent as well as plasma/solvent ratio and precipitation time has been optimized to enhance the protein precipitation and minimize the sample preparation time and analyte losses. Regarding urine samples, since they do not contain any component incompatible with the analytical technique but their salts concentration is high, the sample preparation has been limited to a dilution with milli-Q water and the dilution factor has been also optimized. Once established the sample treatment procedures, the analytical methodology for the analysis of those samples by HPLC-ESI-QTOF-MS has been developed. In this way, the mobile phase composition, elution gradient, flow rate, volume of injection and column temperature have been optimized. Furthermore, the effect of operating parameters of QTOF on metabolites detection such as drying gas flow rate, drying gas temperature, nebulizer pressure, capillary voltage and fragmentor have been evaluated in-depth. The continuous infusion of reference ions to correct each mass spectrum has been also tested. The metabolic fingerprinting data conversion into a standard and uniform format as a pretreatment

previous to statistical analysis, have been assessed. This pretreatment have included mass calibration, noise and background reduction, alignment of chromatograms and automated peak finding.
The complementary NMR analysis will be carried out in a similar fashion: sample preparation, 1H NMR spectra acquisition and data pre-processing and analysis.

The focus of task 6.5 is to determine the role of antibodies to small lipid-related epitopes, in the autoimmune diseases included in the PRECISESADS. Validated methods have been developed and published using ELISA platforms for most subclasses and isotypes.

WP7 has the aim of extracting of RNA, proteins and DNA from tissue samples (kidney biopsies in the case of systemic lupus erythematosus, skin biopsies in systemic sclerosis patients) in order to perform high-throughput transcriptomic, proteomic and methylomic analyses on major target organs in autoimmune diseases. The source of the samples is the biobank at UCL (Brussels), and the major challenge during the first months of this project was to set up procedures in order to extract enough material of enough quality from very small residual tissue fragments, initially obtained and stored for diagnostic purposes. Several protocols were tested, and led to the development of a procedure enabling concurrent RNA and protein extraction from the same biopsy samples for further analyses.

WP8: The Data Governance Plan was approved and delivered on schedule. The 'omic server is up-and-running. Test data transfers were performed with preliminary genetic data. A Standard Operating Procedure was approved for flow cytometry raw data transfer. Clustering methods (Principal Component Analysis, Density-based clustering, Similarity Network fusions) and feature reduction methods, based on available genetic data, were explored (work in progress). Public available gene expression data sets were analysed to identify common genes in SLE, RA, and SjS (work published, see dissemination activities).



WP9 has managed to achieve all the objectives planned for this period: A key activity in PRECISESADS is the dissemination of the experience gained in the project to maximise its impact and guarantee a wider deployment and transferability of results. PRECISESADS aims at becoming a globally recognised project in the field of autoimmune diseases, genomics, rheumatology, internal medicine, genetics, biomarker technology, pharmacogenetics, and disease classification. To achieve this, we have, in our first year, prepared a Communication Plan defining the activities that will be undertaken throughout the project to reach the audiences more efficiently with our messages, and provide the tools for doing so.

We have also worked towards developing the PRECISESADS website and Intranet at: www.PRECISESADS.eu (also in www.PRECISESADS.com and www.PRECISESADS.org). The web contains general information such as: a description of the project, objectives, structure, consortium partners, agenda, information for patients, etc.

It was designed to be user friendly and responsive to the needs of the partners, stakeholders and outsiders and to allow for the best possible dissemination of PRECISESADS results. Apart from the web itself, attention was also paid to the dissemination through social media by the preparation of Twitter and Facebook accounts and a YouTube channel linked to the project website:

- Twitter: https://twitter.com/PRECISESADS
- Facebook : https://www.facebook.com/people/PRECISESADS-Imi/100008204445009
- YouTube PRECISESADS: https://www.youtube.com/channel/UC7uy7fENL_QTnInGlzj5R4g

In order to improve the researcher skills for the accomplishment of the project objectives, namely the clinical studies recruitment, WP9 organised with WP3 a clinical training in Paris, held in September 2014.

Goad is to develop interactions with other EU consortia (for example, IMI project AETIONOMY) and patient Associations (Lupus Europe). The contacts with Lupus Europe were achieved through the Spanish FELUPUS. Ms Blanca Rubio, member of the Spanish association and Vice President of Lupus Europe has become a contact for the project and joined the Ethics Advisory Board. Lupus Europe includes also patient associations for other systemic autoimmune diseases so provides for close contact with them.

1.4 Significant achievements

WP1: The consortium after 2 meetings was able to sign the MoU with AETIONOMY ahead of schedule. The Coordinators from both consortia also had a publication accepted of the Taxonomy Project in Nature R & D (see dissemination activities).

WP2 has achieved in conjunction with WP3 and WP8 the elaboration of the ethical protocols. It has also achieved the organisation of the sample collection for all the cohorts and individuals, and performed a pilot test for optimisation of the procedures. WP2 has prepared the SOPs for the collection of samples from the clinical groups closely coordinating with WP4, WP5 and WP6 to know the type and amount of sample required. Based on the former, WP2 has prepared the kits and started their sending for the collection of the samples. WP2 has also established the infrastructure for the storage of the samples at the Granada Andalusian biobank, the negotiation with vendors for materials and shipment of samples and a competitive bidding for the transportation of the samples.

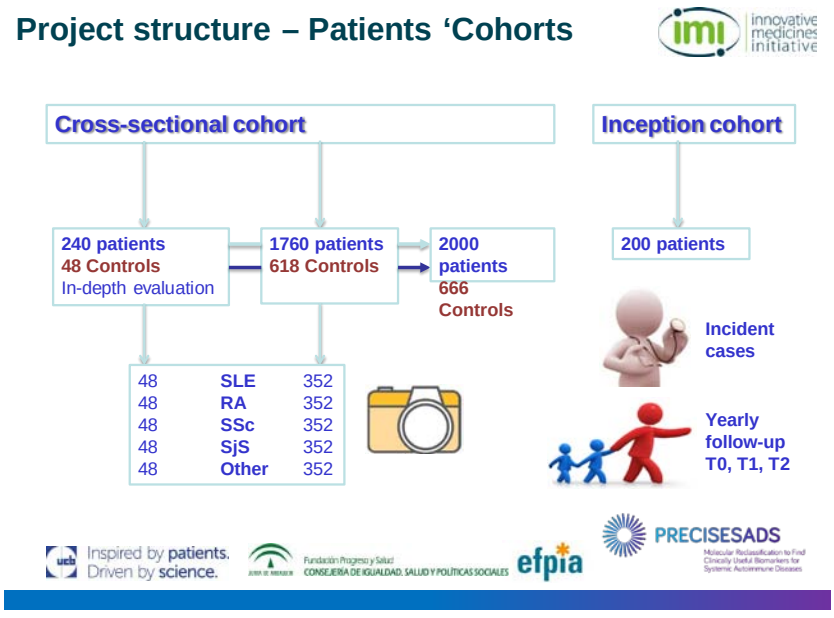
WP3 team Milestones and Deliverables were achieved thanks to the strong communication between WPs and partners.

The team worked closely and WP3 successfully produced a list of CRF items for both studies in line with the two study objectives and manageable by the recruiting sites. Then the eCRF database was developed according to each study structure.

A training of investigator sites staff was held on September 26th 2014 at Servier site in France (Suresnes). Presentations covered protocol, biobank procedures, GCP and eCRF functioning.

The clinical data bases of both the cross-sectional and the prospective inception cohort studies are now operational and data collection has begun.

Recruitment started in December 2014 in the Cross-Sectional study and the sites confirmed that the eCRF is an easy to use web-based application. Sites are waiting for approvals to start the inception cohort.



The **WP4** participants have defined a common gene expression signature for SLE, RA and SjS. The analysis of this signature revealed relevant biological processes that may play important roles in the shared development of these pathologies (Toro-Dominguez D et al., Arthritis Research Therapy, 2014).

Recently, we have analysed large GWAS data of RA and SLE identifying nine putative new risk loci for both diseases that currently are being replicated in independent cohorts including around 10.000 RA patients and 5.000 SLE patients. Similarly, a meta-GWAS of RA and SSc is in progress where five common genes found in the discovery phase.

In **WP5** five standard operating procedures (SOP) have been established and have been uploaded on the PRECISESADS intranet website (http://www.PRECISESADS.eu/workpackage/workpackage-5/).

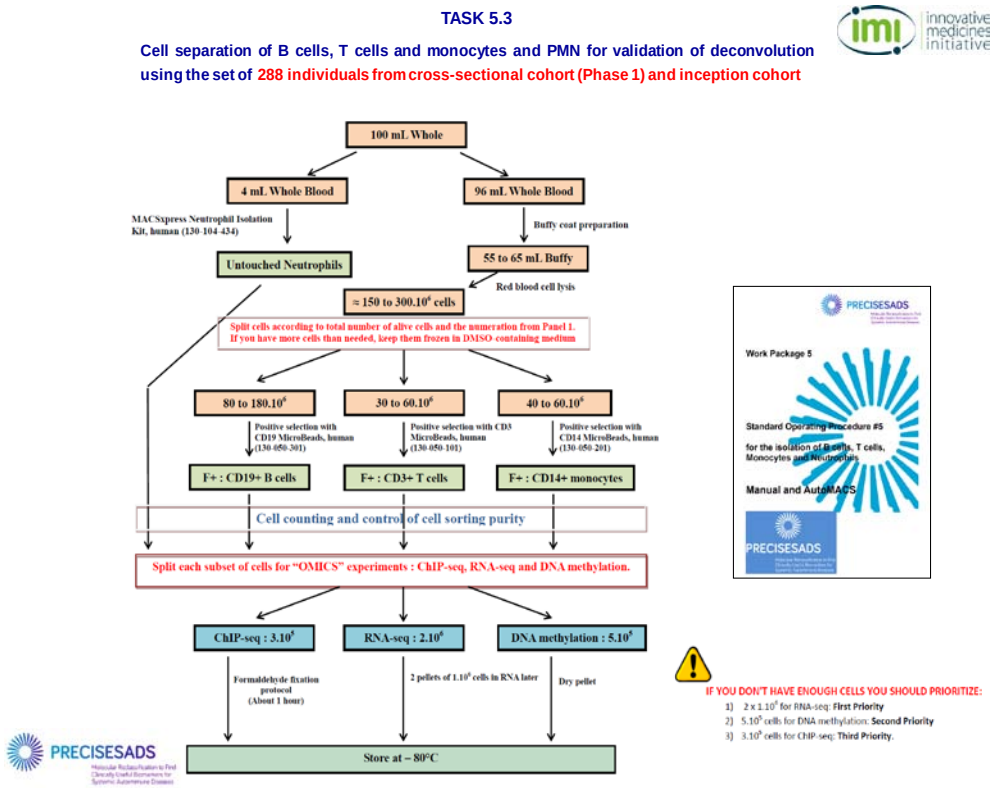
SOP#1 to provide instructions and procedures required for the generation of the proper settings of the different cytometers with 8 Peaks beads.

SOP#2 to provide instructions and procedures required for the creation of the different flow-cytometry sheets dedicated to the PRECISESADS project.

SOP#3 to provide instructions and procedures required for the generation of data during the acquisition of PRECISESADS cytometry panels in whole blood samples.

SOP#4 to provide instructions for the transfer of raw flow-cytometry files to QuartzBio.

SOP#5 to provide instructions for the isolation of B cells, T cells, Monocytes and Neutrophils.



The **WP6** participants have established platforms and have assays in place to fulfill the tasks outlined in the DoW. UNIMI participants have defined the best combination of mediators to be measured simultaneously. We have designed two mediator panels allowing the measurement of 88 mediators out of 100 considered in DoW. The method of measurement was validated in a pilot study (40 mediators in 10 frozen AI samples). Informal agreement has been reached with a Luminex Technology provider in accordance with allocated budget. All groups are prepared for sample processing immediately on receipt of clinical samples. Processing timelines will be determined by minimum batch specifications.

WP7 participants were successful in establishing a protocol enabling simultaneous high-quality RNA and protein extraction from small tissue fragments (residual corporal material) collected in SLE and SSc patients for diagnostic purposes. High-quality RNA could be extracted easily from the majority of the fragments tested. However, protein purification was more problematic. The extraction procedure was informed by the processing of similarly sized fragments from SLE-prone mice, which enabled the participants to estimate the expected protein yield (2,000 different proteins) in SLE kidneys. The protein extraction procedure was adapted until a similar yield was found in human samples. Half the kidney specimens were processed until now, and the remaining kidney and skin samples will be available within the next few months, according to plan.

WP8: Delivered the Data Governance Plan and Installation of the 'omic server.

WP9: One of our significant achievements was the establishment of an active and high-quality clinical research training together with WP3. The training took place in September 2014 and the training team was composed of academic and EFPIA members according to their specific roles within the project.

This full day training was used to explain the PRECISESADS eCRF tool to all investigators and their staff involved in the eCRF completion. During this day, the protocol inclusion/exclusion criteria and the visits procedures were reviewed as well as the Biobank sampling and shipment organization and related procedures.