PROGRESS REPORT

RESEARCH PROGRAM: "LA DIAGNOSI PRECOCE DELLA SCLERODERMIA**"**

RESEARCH UNIT: Department of Biomedical Sciences and Human Oncology, University of Bari.

TITLE: CORRELATION OF HLA-G EXPRESSION WITH PRE-CLINICAL MANIFESTATIONS OF EARLY SYSTEMIC SCLEROSIS.

PRINCIPAL INVESTIGATOR:

Name: Federico
Last name: Perosa
Place of birth: Bari, Italy
Date of birth: July 2, 1958
Nationality: Italian
Work address: Department of Biomedical Sciences and Human Oncology (DIMO), Rheumatologic and Autoimmune Diseases Unit, University of Bari Medical School, Piazza G. Cesare 11, 70124 Bari, Italy
Phone: +39.80.547.88.91- Fax: +39-80-547.88.20.
E-mail: federico.perosa@uniba.it.

LIST OF PARTECIPANTS

Federico Perosa
MD, PhD; Full professor in Rheumatology

Marcella Prete
MD, PhD;Researcher

Elvira Favoino
BS, PhD

Liria Digiglio
BS, PhD

Isabella Eleonora Favia
BS; PhD student

1 ABSTRACT OF RESEARCH PROGRAM

Systemic sclerosis (SSc) is an orphan, complex, multisystem connective tissue disorder characterized by three main pathologic processes, namely vascular abnormalities, collagen deposition and activation of the immune system. The disease causes fibrosis of the skin and internal organs, with a marked impairment of their functions. Its clinical expression and severity are highly variable, ranging from mild to severely disabling clinical forms. In both cases, the Raynaud's phenomenon (RP) occurs in about 95 percent of SSc patients, and it may precede by several years the onset of disease, though not all individuals affected by RP develop SSc. The long-term goal of this project is to identify biomarkers predictive of both evolution of RP to SSc and aggressiveness of SSc itself. The specific hypothesis behind the proposed research is that the serum levels and/or gene polymorphism of HLA-G, a molecule with immunomodulatory properties, is able to provide predictive information in pre-clinical phase of the disease. This hypothesis is based on the following observations: i) the soluble form of HLA-G is involved in the mechanisms of vascular remodeling; ii) low plasma levels of sHLA-G are associated with increased activity of autoimmune disease; iii) HLA-G expression in the skin of SSc patients is associated with a better prognosis. Based on these observations, the specific aims of this study are to analyze:

i) the association of genetic polymorphisms of HLA-G with RP and SSc-related RP (RP-SSc).
ii) the association between HLA-G-specific alleles and its plasma levels with other prognostic markers, in patients with RP and with RP-SSc .
iii) any correlation between gene and plasma HLA-G expression profiles with patient's exposure to environmental factors (i.e., exposure to benzene and particulate) and with clinical manifestations of SSc.

KEYWORDS: Systemic sclerosis, Raynaud's phenomenon, HLA-G, sHLA-G, typing, genetic polymorphisms.

2 INTRODUCTION

In this first part of the research project funded by GILS, we have evaluated the genetic polymorphism of HLA G, namely the presence (Ins) or absence (Del) of a 14bp polymorphism in exon 8 at the 3'UTR of the HLA-G gene, which regulates the transcription and stability of HLA-G mRNA. Genomic DNA was collected from 15 patients with SSc, 11 with RP and 8 healthy blood donors (HBD). In addition, we measured serum expression of HLA-G in 20 patients with SSc, 10 patients with RP and 10 HBD. Then, we have also analyzed the distribution and expression of the transcription factor forkhead BOX E3 (FOXE3) , which has never been implicated in the pathogenesis of SSc and which is the target of some anti-centromeric-associated protein A (CENP-A) antibodies (Ab). Patients with Ab to FOXE-3 appear to have a more favorable prognosis as compared to those which are anti- FOXE3 negative (Perosa *et al.*, Arthritis Research and Therapy, 2004).

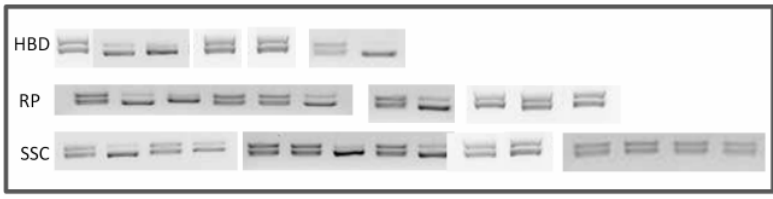
3 RESULTS RELATED TO AIM 1 AND 2:

3.1.1 Genomic analysis

Genomic DNA, reverse-transcribed with appropriate primers, were found to be either heterozygous (Del/Ins; Del^+/Del^-) or homozygous (Del^+/Del^+) for the deletion at exon 8. Specifically Del^+/Del^- was found in 11 SSc, 7 RP and 5 HBD, while Del^+/Del^+ was found in 4 SSc, 4 RP and 3 HBD. None of the patients from the three groups bore the homozygous Ins/Ins (Del^-/Del^-) genotype.

There were no statistical association between Del^+/Del^- or Del^+/Del^+ with the SSc, RP or the status of HBD (Pearson x^2 $p>0.05$) (representative results in the figure below).

To increase the strength of the analysis, a higher number of patients with SSc with different clinical subset and patients with RP will be tested.

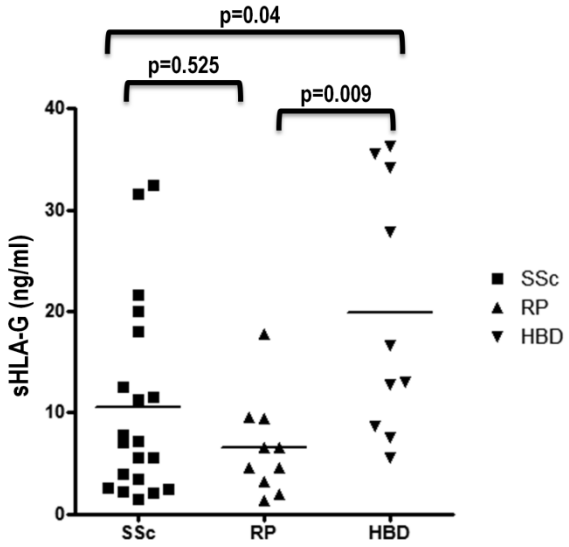


	Del+/Del-	Del+/Del+	Del-/Del-
HBD	5	3	0
Ray	7	4	0
SSc	11	4	0

Legend. Genomic DNA was extracted from the heparin blood using a QIAamp DNA Mini Kit (Qiagen, Crawley, UK). Exon 8 at the 3' UTR of the *HLA-G* gene of each sample was amplified by polymerase chain reaction (PCR) using the primers GE14HLAG-5'-GTGATGGGCTGTTTAAAGTGTCACC-3' e RHG4-5'-GGAAGGAATGCAGTTCAGCATGA-3'. Then, PCR products were analyzed by gel electrophoresis in 4%, agarose gel and stained with ethidium bromide. The PCR product sizes were 224 or 210 bp, according to the presence or absence of 14-bp insert at exon 8.
HBD: healthy blood donor; **RP**: raynaud phenomenon; **SSc** systemic sclerosis.

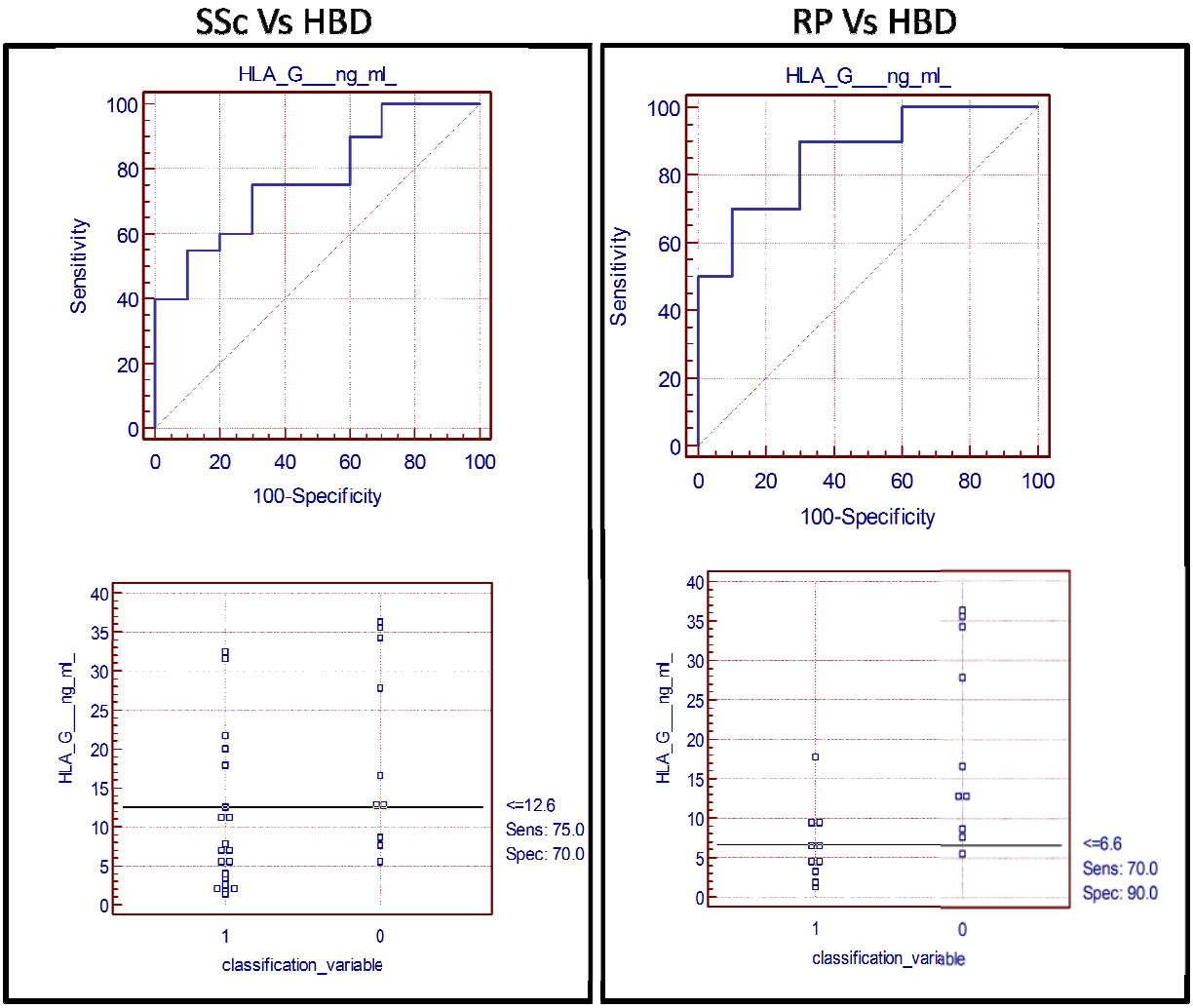
3.1.2 Serum expression of HLA-G

Different mean values of soluble HLA-G (sHLA-G) were obtained among the groups. Specifically the mean (ng/ml±SD) value of sHLA-G in HBD (19.8±12.3) was higher than in SSc (10.51±9.5) and RP group (6.51±4.8). There was an overall statistical difference among groups (one-way analysis of variance [Anova] p=0.009). The Tukey's HSD post test indicated that sHLA-G was significantly higher in HBD than in SSc group (one-way anova p=0.04) and in RP group (p=0.009), while no statistical difference was observed between the last group and SSc (p=0.525).



Then, we used ROC analysis to define cut-off concentration of the sHLA-G that could discriminate SSc from HBD and RP group (see the Figure below). The HLA-G cut-off

concentration that best discriminated SSc and HBD was ≤12.5 ng/ml [sensitivity, 75%; specificity, 70%; area under the curve (AUC), 0.77]. If instead of SSc group, we used RP group, we obtained a similar sensitivity (70%), but higher specificity (90%) at a lower cut off value of sHLA-G (≤6.6 ng/ml).



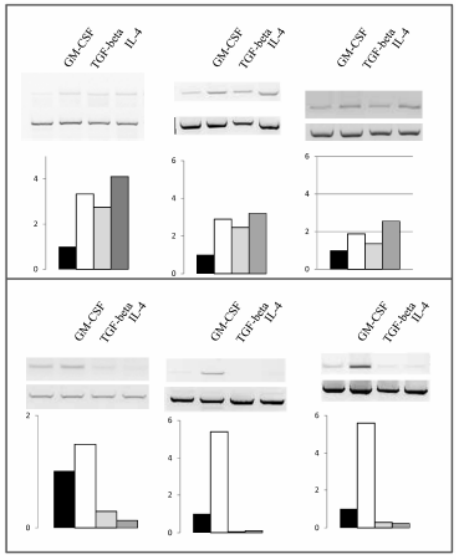
Overall, the data suggest that levels of HLA-G better discriminate HBD from RP condition and that when SSc occurs, other factors related to heterogeneity of disease state might influence HLA-G levels. These finding will prompt us to extend the analysis of levels of HLA-G to a higher number of RP and SSc patients and to analyze their relationship with disease parameters (including disease duration) and other markers which correlate to disease state, namely the levels of anti-FOXE-3 Ab (Perosa *et al*, Arthritis Research and Therapy, 2013). In this context, we have already collected clinical data, including disease duration, from a total of 148 patients with SSc. It will be of interest also to analyze the relationship between HLA-G levels, disease duration and/or activity and expression of FOXE-3.

3.1.3 Expression of FOXE-3 in monocytes from patients with SSc and correlation with their serological profile

Besides analyzing HLA-G, which is constitutively expressed on monocyte, we have also explore on these cells the expression of FOXE-3 in patients with SSc. This transcription factors has been extensively studied in the process of epithelial-mesenchymal transition (EMT) of lens epithelial cells (LEC). Its expression progressively decreases with the migration of LEC from the anterior to the equatorial region. FOXE-3 expression cessation marks initiation of fiber differentiation, suggesting that the loss of FOXE3 expression favors a pro-fibrotic phenotype. It is noteworthy that EMT has been regarded in SSc as one of the possible mechanism favoring tissue accumulation of monocyte-derived fibrocytes or myofibroblasts, hence fibrosis.

Given the possible implication of FOXE-3 in the pathogenesis of SSc , its possible prognostic significance and its involvement in EMT, we have investigated the FOXE3 mRNA expression in unstimulated and TGF-β- or IL-4-stimulated monocytes from SSc patients and HBD, to established whether i) FOXE3 is constitutively expressed in human monocytes; ii) FOXE3 expression can be modulated *in vitro* by cytokines involved in SSc profibrotic process; iii) there is any association between FOXE3 expression and a particular SSc serological profile.

The results of these analysis, which have been summarized in the attached abstract presented to EULAR 2014 - Paris, demonstrated 1) the FOXE3 mRNA expression in monocytes from HBD and SSc patients, and 2) a differential expression, following TGF-β and IL-4 stimulation, which correlate with the patient's serological profile. Specifically, we found that TGF-β and IL-4 markedly enhanced FOXE-3 mRNA expression of CD14+ from anti-ScL70^{-} SSc patients (example in the upper panel of the figure below), while the expression was down-regulated in CD14^{+} cells from anti-Scl-70^{+} SSc patients (example in the lower panel of the figure below). In both panel the stimulation with GM-CSF was used as positive control.



The data suggest that the down-regulation of FOXE3 induced by TGF-β and IL-4 may direct monocytes toward a more profibrotic phenotype in anti-Scl70^{+} as compared to anti-Scl70^{-} patients.

In the second part of the project, we plan to evaluate whether a correlation exist between levels of HLA G and the differential regulation of FOXE3 mRNA expression following TGF-β and IL-4 stimulation.

4 RESULTS RELATED TO AIM 3

Increased amount of evidence indicates that environment play a role in the pathogenesis of RP and SSc. Among risk factors, benzene, vinyl-chloride monomer, crystalline silica, white spirit, solvents (chlorinated and ketones), all have been reported to be associated to an increase number of RP events or in the evolution of RP to SSc. Finally, mice submitted to subcutaneous injection of oxidative substances develop SSc-like disease and SSc-specific auto-Ab, their specificity being dependent on the type of oxidative substance injected.

To get insight the role of environment factors in RP onset or its evolution to SSc, we set up an epidemiological self-administered questionnaire for about 500 patients affected by primary RP and SSc-RP and to an equal number of healthy individuals. This would be one of the largest Italian patients data banks available. Currently, seven Rheumatology (or Internal Medicine) centers have joined the project.

The questionnaire analyzes several characteristics of RP patients (e.g. clinical features, influence on the quality of life, disease activity), family history of disease (e.g. for autoimmune diseases or RP), socioeconomic features, aspects of social life (e.g. social status, place of residence), medical history (i.e., thyroid disorders, arthritis, dermatitis, carpal tunnel syndrome), voluptuary habits (drugs, smoke), habits of daily living (pro-oxidative or chlorinated compounds (i.e., nail polish), sport practiced (e.g. tennis, golf, cycling), exposure to implants (prosthesis, silicon implants, intrauterine device and contact lenses), work history and possible exposure to pollutants (type of work and exposure to pollutants and chemicals). When possible, sera and lymphocytes are also being collected, for evaluation of genomic and sHLA-G.

Data are being included in a Microsoft Office Excel sheet. When completed, they will be elaborated through the SPSS statistical analysis software. Association between variables and the disease will be evaluated with Fisher exact test corrected for alpha error; variables found to be statistically associated to the clinical and laboratory features of disease will be analyze by linear regression to establish their interdependences.

PUBLICATIONS REPORTING GILS FUNDING.

- Prete, M., Fatone M. C., Favoino E., and Perosa F.. Raynaud's phenomenon: from molecular pathogenesis to therapy. Autoimmun. Rev. 13:655,2014. IF:7.97.
- Favoino E, Favia IE, Digiglio L, Perosa F. (Abstract) Expression of the transcription factor forkhead box E3 (FOXE3) in peripheral blood mononuclear cells of patients with systemic sclerosis. Clinical and experimental Rheumatology 2014; 32 (Suppl81), s63
- Favoino E, Favia IE, Valentini G., Perosa F. (Abstract) Expression of the transcription factor forkhead box E3 (FOXE3) in monocytes from patients with systemic sclerosis and correlation with their serological profile. Ann. Rheum. Dis. 2014;73(Suppl2) (Abstract).