Università Cattolica del Sacro Cuore
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Complesso Integrato Columbus

Clinica di Reumatologia
Scuola di Specializzazione in Reumatologia
SC Dottorato in Proteomica Clinica
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Sede del Progetto:
Università Cattolica del Sacro Cuore –
Istituto di Reumatologia con responsabile Prof GF. Ferraccioli
in collaborazione con Istituto di Microbiologia Professor Sanguinetti.

Coordinatore del progetto: Dottoressa Silvia Laura Bosello

Title: Role of microbiota in the pathogenesis of scleroderma: correlations with nutritional status and with gastroenteric involvement.

Report

Gastrointestinal involvement (GI) is frequent in systemic sclerosis (SSc) and it is the presenting feature of the disease in 10% of patients [1,2]. Whist severe gastrointestinal involvement affects only 8% of SSc patients, the associated mortality is very high and only 15% of these patients is alive 9 years after diagnosis. In particular, malabsorption is a very poor prognostic factor with a 50% mortality rate at 8.5 years. [3,4]. Gastrointestinal manifestations of SSc impact severely not only on prognosis but also on quality of life and are a cause of depression in SSc patients [3]. Unfortunately, gastrointestinal involvement is often noticed only when severe complications have already occurred and treatment options are limited. The pathophysiology of GI involvement in SSc is not clear and only few studies have investigated the pathology of the intestinal system. A four steps pathogenetic process, similar to that occurring in the skin, has been proposed. The first step is an early vascular lesion, which manifests as mild changes in intestinal permeability, transport and absorption, and is followed by neural dysfunction and then smooth muscle atrophy, while the last steps is muscle fibrosis [6].

The most frequent GI organ involved is the oesophagus, followed by the anorectum and the small bowel. The altered peristaltic activity with multiple secondary problems lead to oesophageal reflux, early satiety, nausea, vomiting, pseudo-obstruction, small intestine bacterial overgrowth, malabsorption and, ultimately, malnutrition.

Assessment of nutritional status is generally difficult and no tools are yet validated to measure gastrointestinal involvement in SSc [7]. It has been proposed the use of gastrointestinal questionnaire used by the Canadian Scleroderma Research Group (structured 14 questions on GI symptoms), the UCLA scleroderma clinical trial consortium GIT 2.0 questonnarie (34 items and 7 multitier scales focused on reflux, distension/bloating, diarrhea, fecal spoilage, constipation, emotional well being and social functioning), and the MUST (malnutrition universal screening tool) that combines BMI and weight loss measures [8]. Using this latest tool, a Canadian study demonstrates an important risk of malnutrition in SSc, with more than 28% of patients at medium- or high-risk [9].

The pathogenesis of systemic sclerosis is not well understood but it is thought that environmental factors determine the onset of the disease in genetically predisposed subjects and the major human organs that are exposed to environment are the skin and the gut. The human gut is an ecosystem consisting of a great number of commensal bacteria living in symbiosis with the host, number which far exceed the number of cells in one's human body. Several data confirm that gut microbiota is engaged in a dynamic interaction with the intestinal innate and adaptive immune system, affecting different aspects of its development and function [10]. The intestinal microflora has a protective, metabolic, trophic and immunological function and is able to establish a "cross-talk" with the immune component of mucosal immunity, comprising cellular (B cells with sIgA, mucosal T cells, epithelial cells) and soluble elements (i.e. interleukines) [11]. A shift in

composition of the gut microbiota may cause either a pathological or beneficial outcome mediated by the regulation of particular T cell subtypes influenced by the gut microbiota.

The gut commensal bacteria may dictate a pro- or anti-inflammatory environment that can have a substantial impact on the adaptive immune response away from the gut, suggesting that gut microenvironment, sex and genetic factors may be able to predict susceptibility to develop autoimmunity [10]. With the recent advances in technology, it is now possible to sequence the commensal bacteria present in the human gut and experimental data support the hypothesis that alteration of the gut microbiota is supposed to be involved in the development of different disease, such as atopic illness, inflammatory bowel disease (IBD) [13, 14], rheumatoid arthritis (RA) [15,16] and systemic lupus erythematosus [17].

Accumulating evidence suggests that IBD is the consequence of an inappropriate inflammatory response to intestinal bugs in a genetically susceptible host [13,14]. It is known that the microbiota of these patients is different from the one of healthy controls: comparison between 161 faecal samples from patients affected by Crohn's disease versus 121 healthy samples showed depletion of the class Clostridia and the genus Bifidobacterium and an increase of the genus Bacteroides; in addition, the composition of the microbiota was different between the two groups depending on the activity status of the intestinal illness. For this reason, fecal microbiota is may be a possible marker of disease activity [18]. The analysis of fecal microbiota of RA patients conducted by PCR of 16S RNA sequencing of microbes present in the stool showed a different pattern of bacteria respect with patients with fibromyalgia [15]. The studies on microbiota in human and in autoimmune disease mouse models suggest that the intestine may be a critical organ in triggering disease through an altered immune homeostasis and that a leaky gut with proinflammatory conditions may be an event that begins before the onset of clinical phenotype of a disease.

The microbiota not only maintains a balanced mucosal immune system but also aids in digesting certain food and harvesting energy and increasing evidences indicate that the plays a significant role in maintaining a satisfactory nutritional state as well as in the development of obesity, obesity-associated inflammation and insulin resistance. Differences in composition and in genetic and metabolic activities of the gut microbiota seems to be able to stratify patients with different nutritional status, suggesting that gut "dysbiosis" contributes to the development of obesity and malnutrition and their complications. In obese individuals and in animal models, there is a deregulation of gut permeability and bacterial translocation to the host, that probably promotes the systemic inflammation, that is the hallmark of the obese state [19]. Finally, some evidences support the use of probiotics and prebiotics, with beneficial effect for the treatment of the diseases caused by the dysregulation of the immune system [12, 20-21], opening possible intervention in autoimmune diseases characterized by modification of microbiota.

To investigate the characteristics of gut microbiota in systemic sclerosis patients we enrolled in these 6 months 40 patients with Systemic sclerosis. The mean age is 40.0±14.0 years and the mean disease duration is 7±6 years, in particular 30% of the patients has an early disease. 40% presented a positivity for antitopoisomerase antibodies, and 30% have a positivity of anticentromere antibodies and 30% was only ANA positive. For all enrolled patients we collected serum sample, stool sample and we investigated the metabolic characteristics. The mean BMI is 24.0 ±5.0 kg/cm2, 46.1% of the patients were normal-weight, 42.2% were overweight, 10.4% were obese, while only 1.3% were underweight. Now we will try to increase the number of underweight patients as well as the obese group.

In our enrolled cohort the mean levels of cholesterol is 164.0 ± 41.0 mg/dl, the mean HDL levels is 50.0 ±16.0 mg/dl, the mean albumin levels is 4.0 ±1.3 g/dl, the mean haemoglobin levels is 12.0 g/dl ±2.0, the mean triglycerides levels is 89.0 ± 44.0 mg/dl. The mean levels of vitamin D is 27.0 ±

14.0 ng/ml, the mean levels of folates is 10.0 ±6.0 ng/ml and the mean levels of B12 vitamin is 457.0 ± 271.0 pg/ml.

All patients answered the Canadian Scleroderma Research Group and the GIT 2.0 questionnarie (34 items and 7 multitier scales focused on reflux, distension/bloating, diarrhea, fecal spoilage, constipation, emotional well being and social functioning). The mean UCLA GIT2.0 score is 0.4±0.3 and the mean CSR score is 2.9±1.8.

Fifty age, gender and weight matched healthy controls were also enrolled and the stool specimens were available.

All stool specimens were collected to characterize the composition of the intestinal microflora in the different subsets of SSc and in patients with different nutritional status to study if specific microbial species may be responsible of dysbiosis in SSc patients.

In the next 4 months the stool microbioma will be analyzed with the most recent and accurate molecular technologies available, to allow a complete analysis of the complex bacterial communities present in gut. The technology is based on pyrosequencing, a particular type of sequencing that identify the inorganic pyrophosphate molecules released in each nucleic acid neo-synthesis reaction. In this case the aim will not be to sequence the entire genome but only a particular gene, the 16S rDNA, which is present in all bacteria. As every bacterial specie present specific genomic regions, some with high homology and some with high variability, it is possible to distinguish one species from another due to the presence of these specific region. The analysis will be performed with the Roche 454 GS Junior instrument.

The PCR process consists in a multisequence approach characterized by the following step:

1. Extraction of total bacterial DNA from a defined number of samples (according to the material to be sequenced);

2. Specific amplification of all the 16S genes in the samples. Using degenerate primers engineered in order to have an "adapter" fragment and a "MID fragment" to distinguish all the sequences obtained from a single sample over another;
3. Combination of all amplified 16S genes into a single pool and processing according to the protocol with a whole series of purifications and techniques to prepare the DNA to be sequenced;
4. Analysis of the DNA sample by a sequencer, which returns a single file containing the data of all the sequences found in the sample. A dedicated software analyses extensively this data to align and find matches on the online database.

A successful run is able to return approximately 150,000 reads (sequences), each of which could potentially belong to a single bacterium. A comparative analysis of gut microbioma between SSc patients and healthy controls and in different subgroups of patients will then be performed.

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