TOLL-LIKE RECEPTOR 7 GENE
POLYMORPHISMS IN SARCOIDOSIS

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Dove tutte le mie ricerche trovano una risposta.
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SUMMARY

Sarcoidosis is a multi-factorial systemic disease with increased activity of the cellular immune components which is responsible of the formation of non-caseating granulomas in involved organs. The aetiology of sarcoidosis is currently unknown. Recent views on the aetiology indicate interactions between inherited susceptibility and environmental or lifestyle factor. Concerning genes that may influence susceptibility to sarcoidosis, Toll-like receptors (TLRs) may represent plausible candidates. TLRs is a family of transmembrane proteins expressed in most immune cells and are main components of innate immunity and they play a crucial role in the recognition of components derived from a wide range of pathogens. Furthermore, imbalanced host immune response via TLR-dependent signaling pathways may be responsible of inflammatory and autoimmune diseases. In particular, TLR7 (which is located on chromosome X) has been implicated in several autoimmune disorders. We analyzed TLR7 gene polymorphisms in order to establish if this gene could have a role in sarcoidosis development. The TLR7 polymorphisms we analyzed were SNP rs179008 (A/T), relative to start codon ATG on exon 3; SNP rs3853839 (G/C) relative to the TRL7 3’untranslated region; SNP rs5935436 (C/T) relative to the TRL7 promoter. The analysis was performed by TaqMan allelic discrimination using the Assay-by-Design SNP Genotyping Assays. Polymerase Chain Reaction (PCR) was conducted according to the manufacturer’s protocols on StepOnePlus™ Real-Time PCR System. 149 sarcoidosis patients and 151 sex- and age-matched healthy controls were included in the study. Among the 149 sarcoidosis patients, 85 were women and 64 men. All the participants in this study were Caucasian.
The data were analyzed separately in males and females. In females, frequency of the TLR7 rs179008/Gln11Leu polymorphism differed between patients and controls. The incidence of the TT/AT genotypes of the polymorphism was significantly lower in sarcoidosis patients compared to control subjects, which was mainly ascribed to an increased AT genotype frequency and decreased AA genotype frequency (P=0.01). We could observe in control subjects a significant preponderance of the T allele of the TLR7 rs179008/Gln11Leu polymorphism compared to sarcoidosis female patients (P=0.008). In males, no significant differences between patients and controls emerged in allele frequencies of the TLR7 rs179008/Gln11Leu polymorphism (P>0.05). SNP rs3853839 (G/C) relative to the TRL7 3’untranslated region and SNP rs5935436 (C/T) relative to the TRL7 promoter did not show any statistically significant association compared to healthy controls (P>0.05).

Our data is consistent with a protective role of TLR7 rs179008/Gln11Leu polymorphism in female patients affected by sarcoidosis. Studies of TLR7 signaling in the development of autoimmunity have revealed, anyway, complex roles for this pathway. Therefore it is plausible that together with the TLR7 rs179008/Gln11Leu polymorphism other genetic alterations of the TLR7 signaling pathway should be present to determine a cumulative protective effect against sarcoidosis.
SARCOIDOSIS

Sarcoidosis is a chronic multisystem granulomatous disease of unknown aetiology reported for the first time in 1899. Ethnicity and specific HLA haplotypes are in some cases evidently linked to many phenotypic manifestations of the disease. Pulmonary involvement is the most common manifestation requiring treatment, but specific treatment indications, measures of response and choice of medications are still to be well-defined due particularly to the presence of different disease patterns of sarcoidosis.

Epidemiology
Sarcoidosis affects males and females of all ages and ethnic groups but appears more common in young adults. It is rarely present in children and affects most severely African and Asian patients. The real prevalence of the disease is difficult to be estimated but it is valued that less than 3/100,000 person years are affected by sarcoidosis with high incidence in Scandinavia and in Afro Caribbean people. There is a peak presentation at around age 30 years in both men and women, although in some populations a second peak at around 60 years is observed for women. Certain disease presentations are associated with ethnicity and HLA assessment like erythema nodosum, which is more common in young women of Northern European ancestry carrying the haplotype DRB1*0301. Extrapulmonary disease seems to be more frequent in patients of African and Polynesian origin, while patients of Asian, particularly Indian, descent may have more severe pulmonary disease. Many studies have been performed to link environmental exposure with
the development of sarcoidosis, particularly in the face of early reports of geographical and seasonal clustering\textsuperscript{8-10}. A large multicenter case control study (named ACCESS) found no clear association with any environmental or occupational agent although it underlined higher odds ratios for occupations involving exposure to organic material\textsuperscript{11}. An infective aetiology has been proposed because of the characteristic immune response with discarding reports of the presence of mycobacterial antigens, cell wall deficient bacteria and propionibacteria in tissues from people with sarcoidosis\textsuperscript{12-15}. To date, no consensus has emerged supporting a specific microbial agent as a causative factor in the disease\textsuperscript{16,17}. The ACCESS investigators have confirmed a familiar susceptibility, even if a familial association is insufficient to explain the prevalence of sarcoidosis and it is considered that both genetic susceptibility and environmental trigger are necessary for the development of the disease\textsuperscript{18-22}.

**Immunopathology**

Sarcoidosis is characterised by the accumulation of CD4 T helper cells in affected tissues with release of IL-2, IFN-\(\gamma\) and TNF-\(\alpha\)\textsuperscript{23,24}. It has been shown that two other key cytokines, IL-12 and IL-18, may conduct what appears to be a dysregulated immune response to an environmental factor. More recently another subtype of T cells, T regulatory cells, have been identified and these cells may play a role in the formation of granulomas, which are the characteristic histologic feature of sarcoidosis\textsuperscript{25-27}. The presence of a restricted T cell receptor repertoire has been confirmed by many studies, supporting the hypothesis that exposure to a particular environmental trigger promotes the inflammatory response\textsuperscript{28}. It still not well-defined the role of infectious agents in sarcoidosis development, especially mycobacterial antigens (particularly catalase-peroxidase)\textsuperscript{29}. More data are required to identify precisely any
particular infectious agent or other specific environmental trigger for the immune response that is characteristic of this disease. Another important immunological phenomenon consists that patients with sarcoidosis demonstrate peripheral anergy and this feature explains why they fail to recognise key antigens such as PPD (the Mantoux test). Their susceptibility to opportunistic infections such as those caused by Cryptococcus sp is thought to be the clinical manifestation of such anergy.\(^{30}\)

Primary cutaneous sarcoidosis may be considered a very interesting immunopathogenetic model of sarcoidosis. In this context, the proposed immunological pathway (Figure 1) may also represent a potential mechanism for explaining systemic involvement following primary cutaneous sarcoidosis. An exogenous antigen penetrated through the skin is captured and processed by immature Langerhans cells. The subsequent systemic involvement of sarcoidosis could be determined by the spread through lymphatic vessels of antigen-bearing mature dendritic cells. This concept is supported by the clinical observation that specific cutaneous lesions in the course of sarcoidosis are generally observed before the onset of systemic disease.\(^{31}\)

**Clinical features**

The clinical features of sarcoidosis are heterogeneous and, although the lung is involved in over 90% of patients, multisystem involvement is predominant in the disease.\(^{32,33}\) Many patients are asymptomatic and the diagnosis is posed in consequence of incidental chest radiograph, but there are also classical clinical presentations such as Lofgren’s syndrome (the triad of erythema nodosum, hilar lymphadenopathy and lower limb periarticular arthritis) and Heerfordt’s syndrome (acute parotitis and 7th nerve paralysis). These presentations are linked to ethnicity, and in the
Figure 1. The basic immunopathological mechanisms that can be expected to be operative in primary cutaneous sarcoidosis (Bordignon et al\textsuperscript{31}).
case of Lofgren’s syndrome, gender (with females predominantly affected) and season\textsuperscript{8,34}. Non-specific systemic symptoms such as fever, anorexia, weight loss and fatigue are present in approximately a third of patients with sarcoidosis\textsuperscript{32}. The fever is usually low grade and lasts less than 6 weeks but sarcoidosis should be considered in the differential diagnosis of a fever of unknown origin. Fatigue is a very disabling and often under-recognised symptom; its exact incidence is unknown but varies from 30\% to 70\% depending on age, gender, ethnicity and organ involvement\textsuperscript{35,36}. It is strongly related to all aspects of quality of life independently of other symptoms, organ involvement and demographics\textsuperscript{37}. An acute phase response associated with fatigue in sarcoidosis has been reported but the exact mechanism of fatigue has not been established\textsuperscript{38}.

**Pulmonary disease**

Respiratory symptoms such as dyspnoea, dry cough, wheezing and chest pain are present in 30\%-50\% of patients\textsuperscript{39}. There is poor correlation between symptoms, especially dyspnoea, and objective measurements of lung involvement and many patients with significant pulmonary disease are asymptomatic\textsuperscript{40}. Notably, dyspnoea often is more severe referring to radiologic findings and might reflect unsuspected cardiac disease or pulmonary hypertension. Despite significant radiological abnormality, lots of patients with sarcoidosis have normal auscultatory findings. Crackles are rare and wheezing may reflect proximal bronchostenoses.

**Radiology**

A radiographic staging system for sarcoidosis was developed many decades ago by Scadding\textsuperscript{41}: Stage 0, normal; Stage 1, bilateral hilar lymphadenopathy (BHL); Stage 2, BHL and parenchymal infiltrates; Stage 3, parenchymal infiltrates; Stage 4, fibrosis. Typically, the infiltrates are
bilateral and most common in the upper lobes. However, this system has restricted use because of the considerable overlap of the clinical features related with each stage. High resolution chest CT (HRCT) is superior to a Chest X-Ray (CXR) in particularizing disease pattern and extent and is more sensitive, often revealing parenchymal disease or adenopathy not visible on plain radiograph. The characteristic parenchymal abnormalities are nodules, typically 1-5 mm in diameter, with a perilymphatic distribution seen along the bronchovascular bundles, interlobular septa, major fissures and subpleurally. These small nodules may coalesce to form macro nodules measuring 1-4 cm. Other patterns of disease commonly observed are reticular opacities, ground glass opacification and consolidation. Bronchial abnormalities such as bronchial wall thickening can be observed in up to 65% of patients. Pleural effusion, pleural thickening and pneumothorax are uncommon. Early findings of fibrosis include posterior displacement of the main and upper lobe bronchi, with irregular reticular opacities. Septal thickening develops with progression. Honeycombing and massive conglomerate fibrosis may be seen. With exclusion of the fibrotic changes, there is evidence that the other patterns of disease can be reversible. Some studies trying to correlate functional markers and symptoms with CT manifestations have produced controversial results. In selected centres, the presence of classical sarcoidosis changes on HRCT combined with typical clinical features is often enough to make a diagnosis of sarcoidosis without the need for histological support.

Extrapulmonary disease
Sarcoidosis may affect any organ. Extrapulmonary manifestations are often clinically relevant, and in many cases indicates a more chronic course of the disease. The ACCESS study defined criteria for definite, probable and possible organ involvement that can be used as a clinical tool.
Skin

Cutaneous involvement in sarcoidosis occurs in about one-quarter of the patients and is generally observed at the onset of the disease process although it may occur coincident with or after systemic involvement\textsuperscript{46,47}. Therefore, cutaneous lesions can be an initial presentation and are probably an important factor in the investigation of the etiology of sarcoidosis\textsuperscript{31}. Skin lesions may be classified in specific, when histology shows typical noncseating granulomatous inflammation, or non specific, in presence of reactive process without granulomas.

The frequency of specific skin involvement ranges from 9% to 37\%\textsuperscript{48}. All specific cutaneous lesions exhibit noncaseating granulomas on biopsy. Histological findings in specific sarcoid lesions show aggregates of epithelioid histiocytes with occasional Langhans giant cells and few or no other inflammatory cells, the so-called naked or sarcoidal noncaseating granulomas. Frequently, there are inclusion bodies in giant cells\textsuperscript{49}. The centre of granulomas is typically surrounded by CD4+ lymphocytes, rare CD8+ lymphocytes and mature macrophages. Despite the same histologic appearance, clinical manifestations of primary cutaneous sarcoidosis may be variable.

The most common types of specific skin manifestations are maculopapular lesions. They commonly appear on the face with a purple or red-brown appearance but may also be observed on lips, neck, upper trunk, extremities, and rarely mouth; these lesions show typical apple-jelly colour when examined by diascopy\textsuperscript{48}. Plaques are larger, red-brown, infiltrated lesions that are present on face, scalp, shoulders, arms, and buttocks (Figure 2). The lesions may be single or multiple and are associated with chronic course of disease. When plaques are multiple, distribution of the lesions tends to be symmetric. They may be associated with large telangiectatic vessels or may exhibit thick scaling\textsuperscript{50}. Specific cutaneous lesions of sarcoidosis may take form of mobil and indolent subcutaneous nodules that appear generally late in the course of the disease. The
patients may present single or multiple nodules with a diameter between 0.5 and 2 cm without clinical alteration of the epidermal compartment. These nodules may be associated with sarcoidal involvement of lung, spleen, and liver. Scar sarcoidosis is characterised by the development of red-purple infiltrated lesions at the site of previous scar; this phenomenon, of unknown etiology, may precede the onset of lung involvement or be simultaneously with systemic sarcoidosis. Lupus pernio is characterized by an indolent, infiltrated red-brown or purple shiny plaque on nose, lips, cheeks, and ears, more frequent in African American women. Lupus pernio can be followed or be associated with chronic fibrotic disease, notably chronic fibrotic sarcoidosis of upper respiratory tract, lung fibrosis, chronic uveitis, and bone cystis.

**Eye**

Sarcoidosis can affect any part of the eye and eye involvement may occur in up to 89% of patients. Anterior uveitis is the most common lesion often presenting with symptoms of blurred vision, red eye, painful eye or photophobia. It may however be asymptomatic and all patients should be evaluated for ocular involvement, with slit lamp examination performed if patients are symptomatic.

**Cardiac**

Clinical evidence of cardiac involvement may be present in 5% of patients, but autopsy incidence is higher. The clinical features are variable and include congestive heart failure, supraventricular and ventricular arrhythmias, conduction defects and sudden death. The diagnosis of cardiac involvement is not easy as, although endomyocardial biopsy is the gold standard with close to 100% specificity, the sensitivity is poor with
Figure 2. Cutaneous sarcoidosis involving hands and forearms of a middle-aged man. The lesions are reddish plaques, firm, indurated, with fine scaling and non-itching.

Figure 3. Model of Lupus pernio conserved St. Louis Hospital in Paris.
sampling error due to heterogeneous distribution of granulomas\(^{57}\). The diagnosis is often posed when a typical clinical presentation is associated with an abnormal 24-hr Holter monitor, echocardiogram or cardiac MRI. All patients diagnosed with sarcoidosis should have a 12-lead electrocardiogram at least, but if there is clinical suspicion (for example progressive dyspnoea not otherwise explained) then further investigations should be performed. Cardiac PET may offer greater sensitivity than MR or radionucleotide studies\(^{58}\).

**Neurological**
Clinically involvement of the nervous system occurs in less than 10% of patients\(^{59}\). Cranial nerve involvement, particularly facial paralyses, and hypothalamic and pituitary lesions are the commonest abnormalities and usually respond to treatment. Mass lesions, peripheral neuropathy and neuromuscular involvement tend to occur later in the story of the disease and respond less favourably to conventional treatment\(^{60}\). CT and MRI are used to support the diagnosis and the majority of patients will have a CSF lymphocytosis and elevated proteins. Where possible, however, histological confirmation of the diagnosis should be given.

**Other**
Peripheral lymphadenopathy, sino-nasal disease and dactyliitis/joint disease are all common manifestations of sarcoidosis, but there are no specific clinical features. A lot of patients with extrapulmonary disease may have involvement of various organ systems and not all could display parenchymal lung disease. Histologically, hepatic involvement is a normal feature although most patients are asymptomatic, with elevation of ALP and GGT suggesting the clue to diagnosis\(^{61}\). Hypercalcaemia occurs in 2%-10% of patients, with hypercalciuria more common. Disordered calcium metabolism correlates to dysregulated production of 1,25
Dihydroxy vitamin D, which in turn can cause renal stones, nephrocalcinosis and renal failure. However, most renal calculi in sarcoid patients are oxalate, and Vitamin D restriction may in turn result in secondary hyper-parathyroidism and increased oxalate absorption. Other studies of vitamin D metabolism and bone turnover in sarcoidosis are necessary.

**Diagnosis**

The diagnosis of sarcoidosis requires a compatible clinical and radiologic findings, usually accompanied by histologic evidence of non-caseating granuloma, in the absence of positive mycobacterial and fungal cultures. No specific diagnostic tests are available and sarcoidosis is therefore a diagnosis of exclusion. In addition to establishing the diagnosis, it is important to determine the extent and severity of organ involvement, to assess disease activity and to determine whether treatment is indicated.

**Biopsy**

The choice of biopsy site should be guided according to which involved organ is most accessible and most suitable to minimally invasive sampling. Careful inspection should be made for any skin involvement, including granulomatous reactions on the site of an old scar. In patients with respiratory symptoms or radiographic evidence of pulmonary involvement (including BHL), transbronchial lung biopsy is the recommended procedure with a yield of 44%-90%, dependent on the number of samples taken, site sampled and area of parenchymal disease on CXR and CT scan.

**Supporting investigations**

Serum angiotensin converting enzyme (ACE) is secreted by epithelioid cells of sarcoid granulomas and a variable percentage of patients with
clinically active sarcoidosis displays elevated levels. ACE gene polymorphisms influence the concentrations of ACE in individual patients and several conditions can cause false positives (other granulomatous diseases, diabetes mellitus, hyperthyroidism)\textsuperscript{65}. Even if a normal level does not exclude sarcoidosis, patients with an elevated level of serum ACE can be followed using this enzyme as a marker of disease activity\textsuperscript{66}. Bronchoalveolar lavage (BAL) is a safe and minimally invasive technique, which (although not diagnostic) can support other findings. Characteristically, a normal or only mildly elevated total cell count with a lymphocyte predominance (>15\%) is seen. A CD4/CD8 ratio in the lymphocyte subpopulation of greater than 3.5 has been reported to show a high specificity (94\%) for sarcoidosis but low sensitivity (52\%)\textsuperscript{67}.

THERAPY
Corticosteroids have always been the core of treatment for sarcoidosis. Oral corticosteroids are suggested for patients with progressive pulmonary disease or important extrapulmonary disease, with risk of end organ failure\textsuperscript{59,68}. Treatment with oral corticosteroids is generally warranted in patients with clear evidence of falling lung function, particularly if associated with low baseline lung function or symptoms. In general, steroid treatment is not based on radiologic features alone as there may be some patients with relative preservation of lung function but troublesome symptoms such as cough. Equally, in extrapulmonary disease, treatment is generally started in discussion with experts in the organ system involved, for example, hepatologist, cardiologist, and is aimed at reducing physiologic dysfunction and alleviating symptoms. A starting dose of prednisolone 0.5 mg/kg/day is regarded as maximum, with a tapering dose to that required to control symptoms and/or lung function with total treatment period of 6-24 months. Bone protective therapy should be considered for all patients.
There are many alternatives to corticosteroids. Immunosuppressive agents such as azathioprine and methotrexate are most commonly used. Chloroquine and hydroxychloroquine have been used in skin, neurologic and hypercalcemia manifestations of sarcoidosis. Other immunosuppressants such as cyclophosphamide, cyclosporine and chlorambucil have been reported to be of benefit in small case series or single case reports and cannot be recommended over methotrexate. Pentoxifylline, a xanthine, has been studied in a case series with reported benefit and appears safe and well tolerated. Thalidomide is hypothetically toxic and needs rigorous regulatory control but has been reported to improve skin symptoms in a small case series. The development of monoclonal antibody therapies targeting TNF-α has yielded considerable data regarding potential benefit for this class of drugs. Particularly, infliximab, a chimeric antibody blocking TNF, has been reported to be of benefit in numerous case reports, particularly in extrapulmonary disease. Further trials are necessary particularly in extrapulmonary disease and in patients with declining pulmonary status.

**Prognosis**

Sarcoidosis has an elevated rate of spontaneous remission but many patients will necessitate treatment at some point in the disease. Mortality is rare even if historical series reported up to 10% in patients attending hospital clinics. Females and patients of Afro-Caribbean ancestry appear to have more severe disease. Pulmonary hypertension carries greater mortality as does cardiac, neurologic and hepatic disease. For patients with end-stage pulmonary disease and respiratory failure, lung transplantation may be appropriate.
Innate immunity and acquired immunity are the two components developed by the immune system for host defense against invading microbial pathogens. Both components of immunity are able to identify invading pathogens as non-self antigens, stimulating immune responses to eliminate them. To date, both parts have been described independently, and the principal research interest in the immunology field has been restricted to acquired immunity where B and T lymphocytes use antigen receptors such as immunoglobulins and T cell receptors to recognize non-self molecules.

At the end of the 20th century, it has been shown that Toll was an necessary receptor for host defence against fungal infection in Drosophila, which only has innate immunity and after few months a mammalian homolog of the Toll receptor (now called Toll-Like Receptor - TLR) was shown to induce expression of genes involved in inflammatory responses. Moreover, a point mutation in the TLR4 gene has been identified in a mouse strain that is unresponsive to lipopolysaccharides (LPS). These studies have just started the great interest towards innate immunity which in recent years has been shown to possess a skillful system that senses invasion of microbial pathogens by TLRs. From microbial pathogens, several evidences have demonstrated that TLRs may recognize also self components, and that an appropriate manipulation of these components of the innate immune system might eventually provide the means to treat a broad range of chronic inflammatory conditions sustained by an immune system impairment.
Identification of the TLR family

TLR4 was the first mammalian TLR characterized and subsequently numerous proteins that are structurally correlated to TLR4 were identified and named Toll-like receptors. Mammalian TLRs comprise a family composed by 14 members. TLR1–9 are conserved between the human and mouse whereas the expression of TLR10 and TLR11 is presumably functional in the human, but it still to be clarified. The role of TLR12-14 is still to be determined. The cytoplasmic portion of TLRs is very similar to that of the IL-1 receptor family, and is termed a Toll/IL-1 receptor (TIR) domain. Despite this correspondence, the extracellular domains of both types of receptors are not structurally related. The IL-1 receptors have an immunoglobulin-like domain, whereas TLRs possess leucine-rich repeats (LRRs) in the extracellular domain. Regarding functionality, a critical role of TLR4 in the identification of the microbial component LPS was firstly characterized. Subsequently, it has been stated that individual TLRs play primary roles in recognizing specific microbial components of bacteria, fungi, protozoa and viruses (Figure 4).

Among TLRs, the most relevant studies on functions and activities have been focused on TLR1-10.

TLR2 and TLR1-TLR6-TLR10 cluster

A wide range of microbial components are recognized by TLR2. These include lipoproteins/ lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, lycosylphosphatidylinositol anchors from Trypanosoma cruzi, a phenol-soluble modulin from Staphylococcus epidermis, zymosan from fungi and glycolipids from Treponema maltophilum. Moreover, TLR2 may also recognize LPS preparations from non-enterobacteria such as Leptospira interrogans, Porphyromonas gingivalis and Helicobacter
These LPS are structurally different from the typical LPS of Gram-negative bacteria recognized by TLR4; these differences are focused in the number of acyl chains in the lipid A component, which presumably confers differential recognition\textsuperscript{101}. More studies will be required to elucidate if some LPS are recognized by TLR2, but not TLR4.

There are two mechanisms that may elucidate why TLR2 recognizes various microbial components. In the first model, TLR2 forms heterophilic dimers with other TLRs such as TLR1, TLR6 and TLR10 (structurally similar to TLR2). Any production of inflammatory cytokines in consequence to mycoplasma-derived diacyl lipopeptides could be noted in macrophages derived from TLR6-deficient mice. However, these cells displayed an usual production of inflammatory cytokines in response to triacyl lipopeptides derived from Gram-negative bacteria\textsuperscript{102}. From the opposite side, macrophages from TLR1-deficient mice demonstrated a normal response to mycoplasma-derived diacyl lipopeptides, but an impaired response to triacyl lipopeptides\textsuperscript{103}. Consequently, TLR1, TLR6 and TLR10 are functionally associated with TLR2 and they are able to discriminate between diacyl or triacyl lipopeptides. The second model involves identification of fungal-derived molecules by TLR2\textsuperscript{104}. This feature explain why TLR2 functionally cooperated with different types of receptors such as dectin-1, a lectin family receptor for the fungal cell wall component b-glucan. Therefore, TLR2 is able to identificate various microbial components through functional collaboration with several proteins that are either structurally related or unrelated.

TLR2 gene is localized in chromosome 4q32; TLR1, TLR6 and TLR10 gene is localized in chromosome 4p14.
Figure 4. Toll-Like Receptors ligands.
**TLR3**

Mammalian TLR3 recognizes dsRNA (produced by most viruses during their replication), and enhance the activation of NF-kappaB and the production of type I interferons (IFNs). TLR3-deficient (TLR3/-) mice demonstrate low responses to polyinosine-polycytidylic acid (poly(I:C)), resistance to the lethal effect of poly(I:C) when sensitized with d-galactosamine (d-GalN), and reduced production of inflammatory cytokines. MyD88 is an connector protein that is common to all the TLRs discovered. When triggered by poly(I:C), TLR3 induces a MyD88-dependent signaling pathway cytokine production. In addiction, poly(I:C) may promote activation of NF-kappaB and mitogen-activated protein (MAP) kinases independently of MyD88, with consequent maturation of dendritic cells\textsuperscript{105}. TLR3 gene is localized in chromosome 4q35.

**TLR4**

TLR4 is an essential receptor for LPS recognition\textsuperscript{94,106}. Moreover, TLR4 is involves in the identification of taxol, a diterpene purified from the bark of the western yew (\textit{Taxus brevifolia})\textsuperscript{107,108}. Furthermore, it has been demonstrated that TLR4 recognizes endogenous ligands, such as heat shock proteins (HSP60 and HSP70), the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen. Nevertheless, all of these endogenous ligands need very high concentrations to trigger TLR4. Moreover, it has been shown that contamination of LPS in the HSP70 preparation confers the capacity to activate TLR4\textsuperscript{109}. LPS is a very powerful immunostimulator, and therefore TLR4 can be stimulated by a very small quantity of LPS, contaminating these endogenous ligand solutions. TLR4 gene is localized in chromosome 9q33.1.
TLR5
TLR5 has been reported to identify an evolutionarily conserved domain of flagellin through close physical interaction between TLR5 and flagellin\textsuperscript{110}. Expression of TLR5 has been acknowledged on the basolateral (but not the apical side) of intestinal epithelial cells\textsuperscript{111} and in the intestinal endothelial cells of the subepithelial compartment\textsuperscript{112}. In addition, flagellin activates lung epithelial cells to induce inflammatory cytokine production\textsuperscript{113}. These findings denote the important role of TLR5 in microbial detection at the mucosal surface. The presence of a stop codon polymorphism in the ligand-binding domain of TLR5 has been related with susceptibility to pneumonia caused by the flagellated bacterium Legionella pneumophila\textsuperscript{113}. TLR5 gene is localized in chromosome 1q41-q42.

TLR7, TLR8 and TLR9
TLR7 and TLR8 are proteins with a highly conserved structure and they share in some cases the recognition of the same ligands. TLR7 and human TLR8 were projected to recognize a nucleic acid-like structure of the virus. It has been reported that they recognize guanosine or uridine-rich single-stranded RNA (ssRNA) from viruses like human immunodeficiency virus, vesicular stomatitis virus and influenza virus\textsuperscript{114-116}. ssRNA is abundantly present in the host, but normally the host-derived ssRNA is not recognize by TLR7 or TLR8. This feature is consequent to the fact that TLR7 and TLR8 are expressed in the endosome, and host-derived ssRNA is usually excluded by the endosome. Analysis of TLR9-deficient mice revealed that TLR9 is a receptor for CpG DNA\textsuperscript{117}. Bacterial DNA contains unmethylated CpG motifs, which confer its immunostimulatory activity. In vertebrates, the occurrence of CpGmotifs is strictly reduced and the cysteine residues of CpG motifs are mostly
methylated, leading to abolition of the immunostimulatory activity. There are at least two types of CpG DNA, termed A/D-type CpG DNA and B/K-type CpG DNA. B/K-type CpG DNA is conventional and it was firstly identified; it is a potent stimulator of inflammatory cytokines production, such as IL-12 and TNF-α. A/D-type CpG DNA is structurally different from conventional CpG DNA and has a greater ability to induce IFN-α production from plasmacytoid dendritic cells (pDC), but less ability to induce IL-12\textsuperscript{118,119}. TLR9 has been shown to be essential for the recognition of both types of CpG DNA\textsuperscript{120}. Since TLR9 identification of A/D-type CpG DNA leads to production of an anti-viral cytokine such as IFN-α in pDC, this feature indicates that TLR9 is involved in viral recognition.

The ability of TLR7 and TLR9 to recognize RNA and DNA makes these genes very important in autoimmune disease development, as the recognition by these receptors of self-RNA and self-DNA could lead to autoimmunity.

TLR7 gene is localized in chromosome Xp22.2; TLR8 gene is localized in chromosome Xp22.2; TLR9 gene is localized in chromosome 3p21.3.

Subcellular localization of TLRs

TLRs have a different distribution within the cell. It has been demonstrate by positive staining of the cell surface with specific antibodies that TLR1, TLR2 and TLR4 are expressed on the cell surface. In contrast, TLR3, TLR7, TLR8 and TLR9 have been detected in intracellular compartments such as endosomes\textsuperscript{121,122,124}. TLR3-, TLR7- or TLR9-mediated identification of their ligands has been shown to need endosomal maturation\textsuperscript{114-116,123,125,126}. CpG DNA (TLR9 ligand) is first unspecifically taken into endosomes, where TLR9 is engaged from the endoplasmic reticulum upon non-specific uptake of CpG DNA\textsuperscript{124,125,127}. Thus, it can be speculated that in the presence of bacterial infection, macrophages and dendritic cells endocytose bacteria by phagocytosis. CpGDNA is then
presented after degradation of bacteria in phagosomes/lysosomes or endosomes/lysosomes, where TLR9 is recruited or expressed. Regarding viral infection, viruses invade cells by receptor-mediated endocytosis, and the viral components are exposed to the cytoplasm by fusion of the viral membrane and the endosomal membrane. Rarely, degradation of the viral particles occurs in the endosomal compartment, with the exposure of TLR ligands such as dsRNA, ssRNA and CpG DNA. Also TLR2 (expressed on the cell surface) is engaged into the phagosomal compartment of macrophages after exposure to zymosan. Thus, TLR recognition of microbial molecules may be mainly activated in the phagosomal/lysosomal or endosomal/lysosomal compartments.

**Involvement of TLRs in immune disorders**

Several lines of evidence indicate that TLRs are implicated in inflammatory and immune disorders. It is reasonable to assume that the association between infection and autoimmunity is often caused by TLR-mediated induction of proinflammatory cytokine and chemokine expression and upregulation of co-stimulatory molecule expression by APCs. Various experimental studies have demonstrated the ability of microbial TLR ligands to activate disease development in experimental models of arthritis, multiple sclerosis (experimental allergic encephalomyelitis, in mice), myocarditis, diabetes and atherosclerosis. Whether endogenous TLR ligands contribute markedly to the onset or maintenance of these diseases is not well-known. TLR2 and/or TLR4 have been reported to be stimulated by these molecules: breakdown products of the extracellular matrix, such as hyaluronate and heparan sulphate; molecules that have been released from cellular damage or death, such as high-mobility group box 1 protein (HMGB1), fibronectin and heatshock proteins; fibrinogen; and modified low-density lipoprotein. Many of these molecules may reach very high levels in the joints of patients affected by rheumatoid
arthritis and at other sites of inflammation. It is still to be clarified the exact mechanism by which TLR2 and TLR4 recognize such various molecular structures, and the extent of redundancy between TLR2 and TLR4 in the recognition of these molecules where cell injury or inflammation are present\textsuperscript{137}.

The innate immune response to viruses and other intracellular pathogens depends critically on plasmacytoid dendritic cells (pDC), which are highly specialized for producing large amounts of type I IFN, required to control viral replication and promote the development of a Th1 response\textsuperscript{138}. Probably, in order to avoid any non-necessary activation by extracellular pathogens, pDC do not express any of the cell-surface TLRs but they just express the intracellular TLR7, specific for RNA, and TLR9, specific for unmethylated DNA. Thus, pDC secretion of large amounts of type I IFN can be triggered by either DNA or RNA viruses\textsuperscript{138}. Unfortunately for the host, it is possible that self RNA and DNA may activate TLR7 and TLR9 if the RNA penetrates in the endosomal compartment of the pDC (or B cells, in which both of these TLRs are also present). A rich potential source of self-RNA and -DNA is the remains of host cells that have died via necrosis or apoptosis. Normally such apoptotic debris appears to be cleared rapidly by macrophages, which in humans do not express TLR7 or TLR9. Anyway, if the apoptotic clearance is not quickly completed, or if there are autoantibodies responsive to the antigens presented on the apoptotic cells, then these complexes can be wrongly guided to pDC and B cells, where they can trigger TLR7 and/or TLR9 (Figure 5). The result of this autoactivation is the production of type I IFN by pDC, the B cell receptor is able to activate the B cell, it is possible that B cell differentiates into a plasma cell\textsuperscript{139}. In SLE-affected patients, both mechanisms are involved in the stimulation of pDC secretion of type I IFN: firstly, apoptotic cells are numerically elevated, and secondly, autoantibodies against RNA- and DNA-associated antigens are present in lupus sera and they are supplemented with apoptotic blebs, such as small nuclear
ribonucleoprotein (snRNP) and chromatin\textsuperscript{140}. The subsequent RNA-containing immune complexes trigger pDC through TLR7, and DNA-containing immune complexes activate pDC through TLR9 to secrete IFN-\(\alpha\). With this mechanism, SLE patients show an increased serum concentrations of IFN-\(\alpha\), which has association with disease activity and probably contributes to disease pathogenesis\textsuperscript{140}. Taken together, these data suggest that various phases of immune responses, even in the lack of infection, are probably involved in TLR-mediated pathways.
Figure 5. TLR7 is activated by self-nucleic acids and stimulates IFN secretion, which promotes autoimmunity.
AIM OF THE STUDY

Sarcoidosis is a multi-factorial systemic disease with increased activity of the cellular immune components. Recent views on the aetiology indicate interactions between inherited susceptibility and environmental or lifestyle factor. Concerning genes that may influence susceptibility to sarcoidosis, TLRs represent plausible candidates since imbalanced host immune response via TLR-dependent signaling pathways may be responsible of inflammatory and autoimmune diseases. In particular, TLR 7 (which is located on chromosome X) has been implicated in several autoimmune disorders.

The aim of our study was to analyse TLR 7 gene polymorphisms in order to establish if patients affected by sarcoidosis and bearing one or more TRL7 gene polymorphisms could be more or less affected by the disease.
MATERIALS AND METHODS

Selection of patients

One hundred-forty-nine (149) sarcoidosis patients and one hundred-fifty-one (151) sex and age matched healthy controls were included in the study. Written informed consent was obtained from all subjects and the study was performed under a protocol approved by the Ethical Committee. The validation of a sarcoidosis diagnosis was made on the basis of the International Consensus Statement on Sarcoidosis. The criteria comprehended: 1. History (occupational and environmental exposure, symptoms); 2. Physical examination; 3. Posteroanterior chest radiography; 4. Pulmonary function tests: spirometry and diffusion capacity DLCO; 5. Peripheral blood counts: white blood cells, red blood cells, platelets; 6. Serum chemistries: calcium, liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase), creatinine, blood urea nitrogen; 7. Urine analysis; 8. Electrocardiogram; 9. Routine ophthalmologic examination; 10. Tuberculin skin test. All subjects were interviewed about the course of their disease and agreed to release relevant medical records for research review. Among the 149 sarcoidosis patients, 85 were women and 64 men. Among healthy controls, 86 were women and 65 men. All the participants in this study were Caucasian.

DNA extraction

The DNA extraction was performed from both peripheral blood and saliva. In the first case, the high molecular weight genomic DNA was extracted using a chromatographic approach. In this regard, we used the "spin
Figure 6. “Spin columns” method for DNA extraction.

Figure 7. Kit for DNA extraction from saliva.
columns” method (Figure 6), that involves the use of columns of resin that binds DNA. The whole blood was incubated with a lysis solution and loaded onto a column. The DNA extracted from nucleated cells binds to the membrane of silica in the presence of chaotropic salts that create a bridge between the membrane and the cationic DNA. The remaining lysate was cleared by centrifugation. After a series of washes the DNA was eluted with an appropriate buffer at low salt concentration. With this method we obtained good quality DNA with molecular weights between 20 and 50 kb as demonstrated after agarose gel electrophoresis. DNA collection from healthy controls was obtained by sputum. The process involves first mixing the saliva sample, collected either by spitting or cotton swab, with the provided Preservation Solution (Norgen Biotek) (Figure 7). The resulting preserved saliva is stable for an extended period of time even at room temperature, and is resistant to further bacterial contamination. When DNA isolation is required, binding solution is then added to the samples and the solution is loaded onto a spin-column. Norgen’s resin binds DNA in a manner that depends on ionic concentrations. Thus only the DNA will bind to the column, while most of the RNA and proteins will be removed in the flowthrough. The bound DNA is then washed twice with the provided wash buffer in order to remove any remaining impurities, and the purified total DNA is eluted with the elution buffer.

**TRL7 analysis**

The TLR7 gene is located on chromosome Xp22.2. The TLR7 polymorphisms we analyzed were SNP rs179008 (A/T), relative to start codon ATG on exon 3; SNP rs3853839 (G/C) relative to the TRL7 3’untranslated region; SNP rs5935436 (C/T) relative to the TRL7 promoter.
Figure 8. StepOne™ Real-Time PCR System (Applied Biosystems).

Figure 9. Scheme of 5’ Nuclease assay process.
These polymorphisms have been implicated in the onset of several autoimmune disorders\textsuperscript{141-146}.

SNP genotyping of these TLR7 polymorphisms was performed by TaqMan allelic discrimination using the Assay-by-Design SNP Genotyping Assays (Applied Biosystems, Foster City, CA) (Figure 8). Both alleles were scored in one well by using Primers and TaqMan minor groove binder probes labeled with VIC and FAM dye. PCR was conducted according to the manufacturer’s protocols on StepOne™ Real-Time PCR System (Applied Biosystems) (Figure 9).

\textit{Statistical analysis}

Differences in genotype frequencies among the two groups were tested by chi-square test for independent data. A statistically relevant value was considered if $p \leq 0.05$. 
RESULTS

The data of the three TRL7 polymorphisms (SNP rs179008 (A/T), relative to start codon ATG on exon 3; SNP rs3853839 (G/C) relative to 3’untranslated region; SNP rs5935436 (C/T) relative to TRL7 promoter) were analysed separately in males and females. Only the TLR7 rs179008/Gln11Leu polymorphism differed between female patients and controls. To this regard, the incidence of the TT/AT genotypes of the TLR7 rs179008/Gln11Leu polymorphism was significantly lower in sarcoidosis patients compared to control subjects, which was mainly ascribed to an increased AT genotype frequency and decreased AA genotype frequency (P=0.01) (Table I, Figure 10). Comparing just TT genotype of the TLR7 rs179008/Gln11Leu polymorphism, we could not observed a statistically significant association compared to control subjects (Table II, Figure 11). Distribution of TLR7 rs179008/Gln11Leu polymorphism was then analysed in the male patients of our study population; in this case, the polymorphism is studied in hemizigosis because the TLR gene is expressed in chromosome X; a statistically significant association was not achieve (Table III, Figure 12). Subsequently, we evaluated the TLR7 rs179008/Gln11Leu polymorphism in the female patients of our study population where we noted that the incidence of the TT/AT genotypes was significantly lower in sarcoidosis patients compared to control subjects (Table IV, Figure 13). Also in this case, there was an increased AT genotype frequency and decreased AA genotype frequency (P=0.0001) (Table V, Figure 14).

We could observe in control subjects a significant preponderance of the T allele of the TLR7 rs179008/Gln11Leu polymorphism compared to sarcoidosis female patients (P=0.008) (Table VIII, Figure 17) In males and in the general population, no significant differences between patients and
controls emerged in allele frequencies of the TLR7 rs179008/Gln11Leu polymorphism (P > 0.05) (Tables VI and VII, Figure 15 and 16).

The other two polymorphisms SNP rs3853839 (G/C) relative to the 3’untraslated region and SNP rs5935436 (C/T) relative to the TRL7 promoter analysed in our study did not show any statistically significant association (P>0.05) with sarcoidosis.
Table I. TLR7 rs179008/Gln11Leu polymorphism: distribution of TT/AT and AA genotype in our study population.

<table>
<thead>
<tr>
<th></th>
<th>Sarcoidosis</th>
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<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>TT/AT</td>
<td>31</td>
<td>(20,8)</td>
<td>50</td>
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<tr>
<td>AA</td>
<td>118</td>
<td>(79,2)</td>
<td>101</td>
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<tr>
<td>Total</td>
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<td>151</td>
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</table>

TT/AT vs AA: OR=0,53; CI95% 0,30-0,92; \(p=0,01\)

Figure 10. Histogram representation of % data of TT/AT and AA reported in Table I.
Table II. TLR7 rs179008/Gln11Leu polymorphism: distribution of TT and AA genotype in our study population.

<table>
<thead>
<tr>
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<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
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<tr>
<td>TT</td>
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<td>(13,2)</td>
<td>14</td>
<td>(12,2)</td>
<td>32</td>
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<tr>
<td>AA</td>
<td>118</td>
<td>(86,8)</td>
<td>101</td>
<td>(87,8)</td>
<td>219</td>
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<td>Total</td>
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<td>115</td>
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</table>

TT vs AA: OR=0,90; CI95% 0,43-1,91; p=0,80

Figure 11. Histogram representation of % data of TT and AA reported in Table II.
Table III. TLR7 rs179008/Gln11Leu polymorphism: distribution of T and A genotype in the male patients of our study population.

<table>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>T</td>
<td>14</td>
<td>(21.9)</td>
<td>12</td>
</tr>
<tr>
<td>A</td>
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<td>53</td>
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<td>Total</td>
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<td>65</td>
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T vs A: OR=0,80; CI95% 0,34-1,91; p=0,23

Figure 12. Histogram representation of % data of T and A reported in Table III.
Table IV. TLR7 rs179008/Gln11Leu polymorphism: distribution of TT/AT and AA genotype in the female patients of our study population.

<table>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>TT/AT</td>
<td>17</td>
<td>(20,0)</td>
<td>38</td>
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<tr>
<td>AA</td>
<td>68</td>
<td>(80,0)</td>
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<tr>
<td>Total</td>
<td>85</td>
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**TT/AT vs AA: OR=0,32; CI95% 0,15- 0,66 p=0,0007**

Figure 13. Histogram representation of % data of TT/AT and AA reported in Table IV.
Table V. TLR7 rs179008/Gln11Leu polymorphism: distribution of TT, AA and AT genotype in the female patients of our study population.

<table>
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<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>TT</td>
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<td>(4,7)</td>
<td>2</td>
<td>(2,3)</td>
<td>6</td>
<td>(3,5)</td>
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<tr>
<td>AA</td>
<td>68</td>
<td>(80,0)</td>
<td>48</td>
<td>(55,8)</td>
<td>116</td>
<td>(67,8)</td>
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<tr>
<td>AT</td>
<td>13</td>
<td>(15,3)</td>
<td>36</td>
<td>(41,9)</td>
<td>49</td>
<td>(28,7)</td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
<td></td>
<td>86</td>
<td></td>
<td>171</td>
<td></td>
</tr>
</tbody>
</table>

TT vs AA: OR=0,70; CI95% 0,12-4,02 p=0,69
TT vs AT: OR=0,18; CI95% 0,02-1,10 p=0,04
AT vs AA: OR=0,25; CI95% 0,12-0,53 p=0,0001

Figure 14. Histogram representation of % data of TT, AA and AT reported in Table V.
Table VI. TLR7 rs179008/Gln11Leu polymorphism: distribution of T and A alleles in our study population.

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<tbody>
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<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>T</td>
<td>49</td>
<td>(16,4)</td>
<td>64</td>
</tr>
<tr>
<td>A</td>
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<td>238</td>
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<tr>
<td>Total</td>
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<td>302</td>
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</table>

T vs A: OR=0,73; CI95% 0,47-1,13; p=0,13

Figure 15. Histogram representation of % data of T and A alleles reported in Table VI.
Table VII. TLR7 rs179008/Gln11Leu polymorphism: distribution of T and A alleles in the male patients of our study population.

<table>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>T</td>
<td>28</td>
<td>(21.9)</td>
<td>24</td>
</tr>
<tr>
<td>A</td>
<td>100</td>
<td>(78.1)</td>
<td>106</td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
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<td>130</td>
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</table>

T vs A: OR=0.80; CI95% 0.43-1.48; p=0.49

Figure 16. Histogram representation of % data of T and A alleles reported in Table VII.
Table VIII. TLR7 rs179008/Gln11Leu polymorphism: distribution of T and A alleles in the female patients of our study population.

<table>
<thead>
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<th>Total</th>
</tr>
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<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>T</td>
<td>21</td>
<td>(12,4)</td>
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<td>A</td>
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<td>(87,9)</td>
<td>132</td>
</tr>
<tr>
<td>Total</td>
<td>170</td>
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</table>

T vs A: OR=0.46; CI95% 0.26-0.82; p=0.008

Figure 17. Histogram representation of % data of T and A alleles reported in Table VIII.
Sarcoidosis is a cell–mediated immunological disorder characterized by granuloma development and production of cytokines by inflammatory cells. This may implicate that the innate branch of the immune response is not implicated in this autoimmune condition. On the other hand, a large body of evidence suggests that in certain autoimmune diseases, the deregulation is not exclusively in the adaptive branch of the immune response but in the innate one as well. A deregulated innate response by boosting antigen presentation and suppressing regulatory T cells activity can results in an overacting adaptive response against self antigens. A key role in this phenomenon is played by dendritic cells.

Dendritic cells (DC) are a heterogeneous population of antigen-presenting cells (APC) that are uniquely capable of stimulating clonal expansion of naïve T cells. As the productive stimulation of naïve T cells by DC results in the initiation of primary immune responses and leads to the generation of memory, DC are often described as being professional APC or nature's adjuvant\textsuperscript{147-149}. The interaction of DC with a naïve T-cell not only leads to proliferation of the T cell but also can govern the type of T-cell response that develops, i.e. IFN-γ producing TH1 cells, IL-4 producing TH2 cells or IL-10 producing regulatory T-cells. Therefore, the critical role of DC in the initiation and development of an immune response suggests that they play a key role in the development of autoimmune inflammatory disorders by transporting autoantigen to the draining lymph node where DC then encounter and prime naïve T cells\textsuperscript{150}. Interferons are very important molecules for this feature.
DC can be derived from monocytes in vitro by culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. It is unknown whether this regimen reflects DC differentiation from blood precursors under physiological conditions. Induction of DC development from monocytes by IFN-α may occur in vivo during infection or inflammation and thus may represent a more physiological approach to DC differentiation in vitro. It has been show that incubation of GM-CSF-cultured monocytes with IFN-α does not induce DC differentiation: cells maintain their original phenotype and cytokine secretion pattern. Even after stimulation with pro-inflammatory or T-cell-derived activation signals, IFN-α-treated monocytes do not develop DC characteristics. Addition of IL-4 during stimulation of IFN-α-treated monocytes results in the rapid development of DC-like cells expressing co-stimulatory molecules, CD83 and chemokine receptor CCR7, indicating that some degree of developmental plasticity is preserved\textsuperscript{151}. However, DC pre-activated with IFN-α are less effective in inducing allogeneic or antigen-specific autologous T-cell proliferation, produce less IL-12 and express lower levels of CCR7 compared to DC generated by culture with GM-CSF and IL-4. Incubating GM-CSF-cultured monocytes simultaneously with IFN-α and IL-4 does not affect phenotypic maturation of DC, but reduces IL-12 production upon pro-inflammatory activation\textsuperscript{151}. Thus, the presence of IFN-α prior to or during differentiation of DC from monocyte precursors alters their response to maturation stimuli and may affect their capacity to stimulate T helper type 1 immune responses in vivo; this feature could lead to the activation of T cell towards self-antigens, initiating the way to autoimmunity.

Among the main components of immune system, one of the best understood pathways inducing innate immune activation specially with interferons production is signaling through the TLRs, which detect highly conserved components of pathogens that are not present in our own cells.
Various human TLRs have been identified and shown to detect pathogen-specific molecules such as lipopolysaccharides and lipopeptides. Although the TLRs appear to have evolved as a warning system to detect infections, in some cases they can be triggered accidentally by self molecules leading to autoimmunity. This is best established for the TLRs that detect nucleic acids, including TLR3 (activated by dsRNA), TLR7 and TLR8 (activated by ssRNA), and TLR9 (activated by unmethylated CpG motifs within ssDNA). To some degree, specificity in the detection of foreign nucleic acids by these TLRs may be provided by the localization of these TLRs in an endosomal compartment, from which self nucleic acids are normally excluded but through which pathogens commonly transit, during which time the TLR may be able to detect the pathogen. An additional level of specificity seems to be provided by the fact that vertebrate nucleic acids have several modifications that functionally reduce the probability of activating TLRs. Modified nucleosides that are commonly present in vertebrate rRNA and tRNA decrease the activation of TLR3, TLR7, and TLR8, and the methylation and suppression of CpG dinucleotides in vertebrate DNA avoids it from having the same immune stimulatory activity as bacterial DNA\textsuperscript{152,153}. Nevertheless, self RNA and DNA retain some ability to autoactivate TLR-driven immune responses. For example, some studies reveal that inappropriate activation of the TLR7, TLR8, and/or TLR9 pathways by chromatin- or RNA-containing immune complexes can lead to autoantibody production\textsuperscript{154-157}.

To this regard, TLR-7 might exhibit dangerous cross-reactivity with both self-and non-self constituents resulting in direct or indirect stimulation of autoreactive T and B lymphocytes. A strong evidence supporting a potential role for the increased expression of TLR7 in contributing to the development of autoimmunity is given by analysis of SLE models. Particularly, studies of the Y-chromosome-linked autoimmune accelerator (Yaa) mutation show that TLR7 promotes the development of lupus in
susceptible mouse strains. Careful genetic studies into the basis for Yaa have shown that it is a duplication and translocation of a portion of the X chromosome containing TLR7 onto the Y chromosome, resulting in a doubling of the level of TLR7 protein and mRNA expression and in increased responsiveness to TLR7 ligands. In many mice with genetic backgrounds that otherwise are phenotypically relatively normal, Yaa induces a lupus-like disease with the prominent production of autoantibodies against RNA-associated autoantigens. These results demonstrate that simply doubling the level of TLR7 expression can induce autoimmunity, presumably as a result of the endogenous stimulation of TLR7 by endogenous RNA-containing complexes. The fact that Yaa only induces systemic autoimmunity in certain susceptible mouse strains is consistent with a requirement for other background genes for autoimmunity development.

Even if almost all autoimmune diseases are more common in women than in men, SLE shows the greatest sex imbalance, with a prevalence in women that is nearly 10 times compared to men. To analysed this feature, it has been observed that peripheral blood mononuclear cells (PBMCs) from female subjects secrete significantly more IFN-α in response to the small-molecule TLR7/8 synthetic ligands than do PBMCs from males. The mechanism of this increased responsiveness is unclear, and it has still to be demonstrated its evidence with natural RNA TLR7 ligands. When taken together with the evidence that TLR7-mediated IFN-α secretion has a role in SLE pathogenesis, it seems likely that the increased responsiveness to TLR7 agonists in women may contribute to the higher prevalence of SLE.

As in SLE, TLR7 may have a role also in sarcoidosis linking innate immunity to adaptive immunity. An unidentified initiating factor triggers lymphocyte activation and proliferation in the first place. The triggering antigen in sarcoidosis leads to preferential induction of autoreactive Th-1
type CD4. A Th1 predominant profile of cytokine production has been observed in systemic sarcoidosis, where only low levels of Th2-associated mediators have been detected. In particular there is clinical evidence indicating a role for some cytokines associated with a Th1 immune response, including IFN-α and TNFα. It is plausible that activation of TLR7 leads to IFN-α production which in turn stimulates DCs generation determining enhancement of the activity of Th1 T lymphocytes that cause cell-mediated immunity. In our study we have shown that an altered TLR7 receptor may have a protective affected with regard to development of sarcoidosis. It is plausible that the TLR7 rs179008/Gln11Leu polymorphism is responsible of a functional alteration of the TLR7 molecule. Consequently, the innate mechanisms which are operative in the immunopathogenesis of sarcoidosis could be modulated by this genetic alteration.

However not only the T-cell mediated immunity, but also B-cell humoral immune responses are affected. Sarcoidosis frequently associates with hypergammaglobulinemia, autoantibody production, and circulating immune complexes. To this regard, TLR7 has been involved in autoantibody production and recognition of immune-complexes containing self-DNA and/or self-RNA. In mouse models of systemic lupus it has been shown activation of self-reactive B-cells through a TLR7-dependent mechanism. It is likely that TLR7 is activated by ligands derived from damaged cells which are responsible for autoreactive B-cell proliferation, inappropriate production of autoantibodies, and the subsequent development of autoimmune disease. Furthermore, it has been documented that lupus-prone mice deficient in TLR7 showed a reduction in the severity of the disease and that blockade of TLR7 prevented autoimmune kidney and lung injury. Overall, the presence of the TLR7 rs179008/Gln11Leu polymorphism in sarcoidosis may determine an alteration of TLR7 function hampering the signaling pathway involved in
the onset of both cellular and humoral autoimmunity. This is consistent with the view that in some circumstances genetic mutations affecting components of the immune system can prevent both organ-specific and systemic autoimmunity. Consequently, the genetic alteration observed in our study could be responsible of the protective effect against sarcoidosis by a mechanisms involving also the adaptive humoral response.

Alteration of the TLR7 function consequent to s179008/Gln11Leu polymorphism may have consequence on the complex TLR7 signaling pathway. Studies of TLR7 signaling in the development of autoimmunity have revealed complex roles for this pathway\textsuperscript{162}. The TLR7 signaling pathways have been extensively examined in pDCs in order to explain their ability to induce the production of type I interferon after viral infection. TLR7 and signaling pathways in pDCs are distinctive because they require MyD88 for the induction of type I interferon. In this context, IRF7, which is constitutively expressed by pDCs, binds MyD88 and creates a multiprotein signaling complex with IRAK4, TRAF6, TRAF3, IRAK1 and IKKα24. In this complex, IRF7 is phosphorylated by IRAK1 and/or IKKα, dissociates from the complex and translocates into the nucleus. In addition to necessitating phosphorylation, IRF7 activation probably requires TRAF6- and Ubc13-dependent ubiquitination. While IRAK1, IKKα and TRAF3 are specifically involved in the activation of IRF7, MyD88, IRAK4 and TRAF6 are important for the activation of both IRF7 and NF-κB\textsuperscript{162}. This complex network of cellular signaling has to be considered when dealing with TLR7 polymorphism when it is responsible of functional alteration of the protein. To this regard, the s179008/Gln11Leu polymorphism have been shown to affect the function of the TLR7 receptor and consequently its signaling pathway\textsuperscript{164}. 
Our study is the first investigation concerning TRL7 polymorphisms in sarcoidosis. Previous studies in sarcoidosis have analysed other TLRs polymorphisms with controversial results. In German patients, it has been found an association between TLR-4 polymorphism Asp299Gly (which lead to a change in the extracellular domain of the receptor and possible hyporesponsiveness to LPS) and a chronic course of sarcoidosis\(^{165}\). Anyway, in another study this polymorphism was genotyped in Dutch sarcoidosis patients and no differences were found in allelic distributions between patients and controls or within the different clinical subgroup of the sarcoidosis cohort. Importantly, no differences could be found between the Dutch and German sarcoidosis patients\(^{166}\).

In TLR2 gene, three single nucleotide polymorphisms and one dinucleotide repeat polymorphism were analysed by other authors divided into a study cohort and a validation cohort of sarcoidosis patients, and healthy controls. In the study cohort they found a significant increase in prevalence of the AA-genotype at promotor location -16934 in patients with chronic disease compared to patients with acute/self-remitting sarcoidosis). The validation cohort did not corroborate the results and consequently a possible role for TLR-2 genetics could be implicated in only a small percentage of sarcoidosis patients. Furthermore, a correlation statistically significant was found between the promotor polymorphism -16934 A/T and the number of GT repeats in intron 1. Considering functionality, after in vitro stimulation of PMBCs with different TLR-2 agonists, a correlation between induction of TNF-\(\alpha\), IL-12 and IL-6, and the number of GT repeats was noted. These data indicate that polymorphisms in TLR-2 could be relevant in a little group of sarcoidosis patients and that their functional consequences elucidate relatively some of the difference in cytokine pattern noted in different clinical phenotypes of this disease\(^{167}\).

TLR-9 single nucleotide polymorphisms were also analysed in sarcoidosis patients and divided into a study cohort and validation cohort and healthy
controls. No genetic differences were found between patients and controls. An interesting difference in TLR-9-induced IL-23 production among sarcoidosis patients and healthy controls was found and this observation could indicate that functional defects in the TLR-9 pathway of sarcoidosis patients play a role in disease predisposition or development\textsuperscript{168}. Another study focused on TLR10-TLR1-TLR6 gene cluster. A total of eight single nucleotide polymorphisms were genotyped in healthy controls and sarcoidosis patients (which comprehended also Löfgren's syndrome). Evaluating the total patient cohort with controls, it emerges that the allele frequencies of rs1109695, rs7658893 (TLR-10), and rs5743604 as well as rs5743594 (TLR-1) differed appreciably. The analysis of haplotype revealed that the most common haplotype found was significantly reduced in patients with chronic sarcoidosis. Furthermore, a significantly amplification of the less common haplotype was noted in patients with Löfgren's syndrome as well as sarcoidosis patients with self-remitting disease; this finding may indicate that this haplotype could act as a disease modifying haplotype. Lack of the common haplotype in the TLR10-TLR1-TLR6 gene cluster intensifies the risk of developing chronic disease in patients already affected by sarcoidosis\textsuperscript{169}. 
CONCLUSIONS

In our study we have demonstrated that the incidence of the TT/AT genotypes of the TLR7 rs179008/Gln11Leu polymorphism was significantly lower in female sarcoidosis patients compared to female control subjects.

However it is plausible that together with the TLR7 rs179008/Gln11Leu polymorphism other genetic alterations of the TLR7 signaling pathway would need to be present to determine a cumulative protective effect against sarcoidosis. This may explain the gender difference observed in our study and the fact that several patients bearing this polymorphism developed the disease.

Nevertheless, some limitations of the present study need to be addressed. First, we did not explore the effects of the SNP variant on the function of TLR7 protein, although previous investigations have shown a high impact of this polymorphism on protein function. The second limitation of our study was that X skewing might affect the interpretation of data in female. Finally, another limitation was the relative small sample size, that does not allow to speculate a role of this polymorphism referring to sarcoidosis clinical pattern. Thus, these results should be interpreted with a certain level of caution although they were corroborated by a high statistically significant p-value.
BIBLIOGRAPHY


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LIST OF ABBREVIATIONS

ACE: Angiotensin Converting Enzyme
APC: Antigen-Presenting Cells
BAL: Bronchoalveolar Lavage
BHL: Bilateral Hilar Lymphadenopathy
CCR: Chemokine Receptor
CXR: Chest X-Ray
DC: Dendritic Cell
pDC: plasmocytoid Dentric Cells
d-GalN: d-galactosamine
DNA: Desoxyriboonucleic Acid
dsDNA: double-strand Desoxyriboonucleic Acid
ssDNA: single-strand Desoxyriboonucleic Acid
HRCT: High Resolution Chest CT
HLA: Human Leukocyte Antigen
HMGB1: High-Mobility Group Box 1 protein
HSP: Heat Shock Proteins
GM-CSF: Granulocytomacrophage Colony-stimulating Factor.
IFN: Interferon
IL: Interleukin
LPS: Lipopolysaccharides
LRRs: Leucine-Rich Repeats
MAP: Mitogen-Activated Protein
MRI: Magnetic Resonance Imaging
PCR: Polymerase Chain Reaction
PET: Positron Emission Tomography
poly(I:C): Polynosine-Polycytidylic Acid
PBMCs: Peripheral Blood Mononuclear Cells
PPD: Purified Protein Derivative
RNA: Ribonucleic Acid
dsRNA: double-strand Ribonucleic Acid
ssRNA: single-strand Ribonucleic Acid
snRNP: Small Nuclear Ribonucleoprotein
SLE: Systemic Lupus Erythematosus
SNP: Single Nucleotide Polymorphism
TIR: Toll/IL-1 Receptor
TLR(s): Toll-Like Receptor(s)
TNF: Tumor Necrosis Factor
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