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Selected molecular events in the pathogenesis of sarcoidosis — recent advances

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Abstract

Sarcoidosis is an orphan inflammatory disorder that can virtually affect any organ or system in the body, although the lungs and lymph nodes are most frequently involved. Sarcoidosis is believed to derive from an interaction between environmental and genetic agents. Many studies emphasize a strong association between certain human leukocyte antigen (HLA) alleles and sarcoidosis susceptibility. Several new insights have allowed the further evaluation of other candidate genes with a potential function in the immunopathogenesis of sarcoidosis.

This review summarizes recent advances in the identification of novel molecular markers that may play a role in different stages of disease, such as the acute phase of inflammation, granuloma formation and fibrosis. Furthermore, this article elucidates the role of both TGF- β /Smad and (HIF)-1a-VEGF-ING-4 signaling pathways in the development of sarcoidosis. The potential epigenetic regulation of the processes occurring in sarcoidosis by miRNA is also discussed.

Key words: sarcoidosis, molecular markers, pathogenesis

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Introduction

Sarcoidosis is a chronic, multisystem, inflammatory disease of unknown etiology. Patients are commonly young or in middle adulthood (20–39 years) and can be either male or female. However a second peak above 50 year of age has been documented in female patients [1].

The most common universal clinical signs of sarcoidosis include pathological intrapulmonary lesions, such as those of the lung/thoracic lymph nodes (> 90%) [2], and extrapulmonary lesions of the skin (20–30%) [3], liver (impaired liver function tests in up to 80%) and/or spleen (5–10%) [4], eyes (20–50%) [5], cardiovascular system (2–27%) [6], central nervous system 10–25% [7] have been recognized. It is of note, that extrapulmonary sarcoidosis is recognized on the clinical basis much less frequently.

The current diagnosis of sarcoidosis is based on its clinical and radiological picture, with the presence of non-caseating granuloma in tissue biopsy. The diagnosis needs to be documented by endobronchial ultrasonography or esophageal ultrasonography transbronchial needle aspiration (EBUS/EUS-TBNA), bronchial mucosal biopsy, transbronchial peripheral lung biopsy (TBLB), mediastinoscopy, or extrathoracic biopsy (skin, peripheral lymph nodes, mucosa, other organs). Only in patients with typical clinico-radiological picture (bilateral hilar lymph nodes enlargement, Löfgren syndrome) biopsy is not obligatory. The typical bronchoalveolar lavage (BAL) results (increased percentage of lymphocytes with CD4/ CD8 > 3.5) substantially increase the probability of the diagnosis [1].

There is no reliable and validated "gold standard", which could be useful for a proper

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DOI: 10.5603/PiAP.2015.0076 Received: 14.05.2015 Copyright © 2015 PTChP ISSN 0867–7077 diagnosis of sarcoidosis. Several basic elements have been already determined to support the diagnosis of this granulomatous disease, the most important including (1) clinical parameters and radiological findings, (2) tissue biopsy specimen that exposes noncaseating granulomas, (3) the absence of established granuloma genic agents and (4) exclusion of similar diseases [1].

Both respiratory function tests and postero-anterior chest radiographs are commonly used to estimate the severity and course of pulmonary sarcoidosis. Pulmonary sarcoidosis is commonly staged according to the 0-IV staging system [1]. In the estimation of sarcoidosis activity, such biochemical and cellular parameters as serum angiotensin converting enzyme (SACE) [8], C-reactive protein (CRP), calcium metabolism index (such as serum calcium and 24 h urinary calcium loss) [9] as well as biomarkers such as lysozyme, soluble IL-2 receptor (sIL-2R) [10], chitotriosidase (CHIT1) [11], Krebs von den lungen-6 (KL-6) [12], neopterin [10], human cartilage glycoprotein 39 (YKL-40) [13], and 8-isoprostane (8-IP) [14] have been used. Although their prognostic value has been considered in many studies, their clinical value has never been validated by larger studies [10, 11, 14].

Interestingly, few studies on sarcoidosis epidemiology have been published worldwide. According to ACCESS (A Case Control Etiologic Study of Sarcoidosis) the morbidity is estimated at around 16.5/100 000 in men and 19/100 000 in women but with significant differences in frequency among races [15]. Sarcoidosis is recognized as occurring most frequently in African Americans and Scandinavians, and much more rarely in Southern European populations, such as those in France and Spain [16]. Recent data from years 2001–2005 presented in European Lung White Book estimate the mean prevalence in Europe at 4.68/100 000/year [17].

Remarkably, the background of sarcoidosis is not fully understood, but many hypotheses based on microbacterial [18], viral [19], autoimmune [20] and genetic-environmental theories [21, 22]. Of these, the most widely accepted appear to be the genetic-environmental theory.

Sarcoidosis is known to be connected with many different environmental agents. Some studies suggest that exposure to common environmental inorganic or organic agent/s can trigger its development [9].

The micobacterial theory of sarcoidosis, based on the tuberculosis pathogenesis involved in its development, is widely understood. No studies have so far confirmed the presence of viable *Mycobacteria* in pathologically altered tissue specimens obtained from patients with clinically confirmed sarcoidosis [23]. However, studies based on molecular biology confirmed the presence of mycobacterial products such as superantigens (Sags) and membrane fragments or bacterial DNA in patients with sarcoidosis [24]. Therefore M. tuberculosis complex (MTC), M. smegmatis, *M. avium-intracellulare* have been proposed as possible etiological links with sarcoidosis [25]. Despite, there are many studies focused on M. tuberculosis as an etiological factor in sarcoidosis, but many results are ambiguous. So far, in the literature there is a lack of direct evidence that M. tuberculosis is the causative agent of sarcoidosis, but it is known that Mycobacterium tuberculosis heat shock proteins (Mtb-hsp) may be involved in the immune response both in patients with sarcoidosis (SA) and tuberculosis (TB). Some important differences in these responses were shown, most importantly in Mtb-hsp-stimulated T-cell cultures IL-4 levels were lower and those of IL-10 were higher in SA vs. TB. $CD8^+\gamma^+IL-4^+$ T-cell were detected significantly less often in Mtb-hsp-stimulated cultures in SA, whereas $CD4^+\gamma^+TCR$ cells were more frequent in stimulated cultures in TB. Similarly, a different profile of secreted cytokines (IL-4, IL-6, IL-10, $TNF\alpha$, INF- γ) in the three treatment groups were found, showing higher levels of $\text{TNF}\alpha$, IL-6 in stimulated SA cells [26].

Propionibacteria are also worthy of attention as etiological factors of sarcoidosis. Studies conducted by Abe et al. confirmed the presence of *P. acnes* in 78% of biopsied lymph nodes of Japanese patients with sarcoidosis compared to 21% of control tissue [27]. Similar results were also obtained by Eishi et al. based on studies using polymerase chain reaction (PCR) methods. They found much higher total numbers of genomes of *P. acnes* or *P. granulosum* than those of *M. tuberculosis* [28]. Moreover, high titers of antibodies against lymphotropic DNA viruses: *Epstein-Barr virus* (EBV) [29] *human herpesvirus* (HHV) [30] and rubella have been found in patients with diagnosed sarcoidosis [31].

Oswald-Richter et al. report that peripheral anergy and an exaggerated, pulmonary CD4+ Th1 response is involved in sarcoidosis pathogenesis [32]. They demonstrate CD4+ anergic responses to polyclonal TCR (T-Cell Receptor) stimulation both peripherally and within the lungs of sarcoid patients [32]. The discovery of T-regulatory (Treg) cells subset (CD4+CD25+FOXP3+ and

CD4+CD39+) and later Th17 cells has changed the traditional concept of Th1/Th2 bipolarized adaptive immune response. In general, Treg cells are responsible for maintaining immune homeostasis and preventing autoimmunity, whereas Th17 cells are a source of proinflammatory IL-17 cytokine, and play crucial role in host immunity towards extracellular bacterial and fungal pathogens [33]. Both cell subsets were shown to be involved in the pathogenesis of sarcoidosis: Treg cells may play a crucial role in abating the inflammatory process, whereas Th17 cells, by promoting a proinflammatory milieu (IL-17, IL-6, TNF α , GM-CSF) may be responsible for sustained inflammatory reaction. Huang et al. have shown the increased Th17/Treg ratio in peripheral blood and BAL fluid in active sarcoidosis, which was normalized with treatment [34]. The same results were reported by Tondell et al. [35], who revealed that IFN- γ^+ Th17 cells may represent a pathogenic subset of Th17 cells, which are stimulated to IFN- γ production by Th1 cells. An increased activity of Treg cells in peripheral blood, which may be overwhelmed by Th17-related response in lungs and other involved organs may be responsible for peripheral anergy [36, 37].

Autoimmune theory is based on the observation that macrophages can present antigens and release cytokines (IL-2, IL-4, IL-6, IL-10, IL-12, TNF- α , INF- γ , TGF- β) [38] involved in polarization of CD4⁺ T-cells to Th1 in the blood of patients with sarcoidosis. In sarcoidosis patients, oligoclonal expansion of CD4⁺ causes secretion of Th1 cytokines [39]. It is commonly recognized that immunopathological factors are very important in the development of sarcoidosis, but that genetic factors also contribute to this disease [40].

An interesting and universal hypothesis on the pathogenesis of sarcoidosis was proposed by Dubaniewicz [20]. The theory is based on the Matzinger consept, according to which the antigen presenting cells (APCs) responding to endogenous cell products (so called "danger signals") released from host cells under stress caused by different infectious and non-infectious agents, may induce the immune response [41]. These external agents referred to as pathogen-associated molecular patterns (PAMPs) induce the release of damage-associated molecular patterns (DAMPs) from injured cells. According to "Danger theory" Mycobacterial hsp may act as PAMPs, whereas human hsp may act as DAMPs, and linking to pattern recognition receptors (PRR) on APCs both may induce the immune response leading to accumulation of Th1 lymphocytes and formation of granuloma. It is of note, that there is significant homology between many bacterial and human HSPs, therefore autoimmunity may play a role.

Genetic factors in sarcoidosis

Many previous studies have confirmed that genetic factors have a role in determining the risk, as well as clinical course, of sarcoidosis [1]. The pivotal genetic predisposition to sarcoidosis was confirmed by observation of familial clustering in monozygotic twins, which are more often predisposed to disease than dizygotic twins. However, this genetic effect is multiple, small or moderate [42].

This theory is also supported by diversity of frequencies in the incidence of sarcoidosis in different populations. African Americans are known to be more frequently affected by sarcoidosis than Caucasians [1].

The risk of sarcoidosis is generally accepted as being associated with both HLA class I and class II human leukocyte antigens. Some alleles, *HLA-DRB1*11*, *HLA-DRB1*12*, *HLA-DRB1*14*, *HLA-DRB1*15* [43, 44], are known to be strongly associated with the risk of sarcoidosis, while others may play a protective role: e.g. *HLA-DRB1*01*, *HLA-DRB1*04* [45]. Additionally, HLA genes such as *HLA-DRB1*03* can be related to acute onset disease (Löfgren's syndrome) [46], whereas others such as *HLA-DQB1*0201* and *HLA-DQB1*0602* are associated with the chronic course of disease [47].

It was documented that some other MHC loci may also increase the risk of sarcoidosis. Interestingly, Morais et al. suggest that in Caucasians, *BTNL2*, genetically independently inherited factor of the HLA class II genes, may act as a T-cell co-stimulatory molecule [48] and their SNP variants displays in different populations, both negative and protective role in the development of sarcoidosis [48–50]. Dubaniewicz et al. reported [51], that DQB1*02, DQA1*0501 in Löfgren syndrome, and DRB1*15 in stage II sarcoidosis than in tuberculosis, what could explain — according to the authors — different disease patterns in patients subjected to the same etiologic factor [52].

Non-HLA genes have been also explored in terms of disease susceptibility and prognosis. For instance, Typiak et al. [53] found increased frequency of F allele of FCGR3A gene encoding receptor for Fc fragment of immunoglobulin G FcgRIIIa (F allele connected with increased receptor affinity) in stage I sarcoidosis, but increased frequency of V allele (connected with decreased receptor affinity) in stage II disease. The authors conclude, that V158F polymorphism of FCGR3A gene may be responsible for the clearance efficiency of immune complexes (like those containing Mtb-hsp epitope), what may be important in terms of disease duration and chronicity. These observations also support the concept of autoimmune pathogenesis of sarcoidosis.

The authors of the present paper found strong association between MMP-9 T-1702-A gene polymorphism, with higher frequency of T allele and TT homozygotic genotype and sarcoidosis morbidity, however no associations with clinical parameters and prognosis were found [54].

Although genetic factors are recognized as important etiological elements of the disease, their interactions with environmental or immunological factors have not been fully explained. It has been generally accepted that sarcoidosis occurs in genetically predisposed patients with an altered immune response to unknown environmental factors [55].

The most probable interaction model between important genetic and environmental factors that influence the incidence of sarcoidosis is shown in Figure 1.

Risk regions and susceptibility genes

Progress in molecular technologies such as high density genotyping chips, SNP analysis, and genetic mapping have resulted in the discovery of several new susceptibility genes, risk *loci* or allele variants which may be relevant in the diagnosis and treatment of sarcoidosis. The whole genome association assay (GWAS) technique has had a huge influence on current knowledge concerning the pathogenesis of many complex human diseases, including sarcoidosis [22, 44]. The technique has identified genes located on 6 chromosome (*locus* 6p12.1) which are impor-



Figure 1. The most likely model of the interactions between the most important factors influencing the global incidence of sarcoidosis

tant for risk and course of sarcoidosis: *C6orf65* (*BEND6*), *BAG2*, *KIAA1586*, *ZNF415* and *RAB23*. *RAB23* was confirmed as a particularly important vital diagnostic genetic marker which may be helpful in the diagnosis of sarcoidosis. Significant differences in the relative expression of *RAB23* between sarcoidosis patients and healthy controls have been observed [56].

Moreover, *RAB23* was found to interfere with Sonic hedgehog (Shh)Treg signaling pathway molecules, contributing to the overactivation of CD4⁺ T-cells and stimulation of epithelial repair in patients with sarcoidosis [56]. Other important new risk *loci* for sarcoidosis are 11q13.1 (candidate risk genes: *KCNK4*, *PRDX5*, *PCLB3*, with *CCDC88B* being the most promising) [57] and 12q13.3, with the most important genomic region appearing to be rs1050045 in the 39-untranslated region (39 UTR) of *OS9* (*Osteosarcoma amplified 9*) [58].

During the effector phase of granuloma formation in sarcoidosis, inflammatory cells are recruited to the granuloma by specific chemokines (IP-10, CCR2, RANTES, MCP-1) [59]. It has been reported that polymorphisms in the CCR2 gene are important factors in determining the risk of disease in some populations. It was documented that SNP G/A in position 64 (rs1799864) of the *CCR2* gene is protective against sarcoidosis in a Japanese population [60].

Recently, some attempts have been made to identify the relationship between VEGF gene polymorphisms and susceptibility to sarcoidosis. A case-control study of Japanese patients [61] did not confirm any correlation between polymorphism VEGF (C/G in position 627) and predisposition to sarcoidosis. Another study on the genetic variation of TGF- β isoforms emphasizes the linking of several SNP in TGF- β 1: C/T in position 509 (rs 1800469), T/C in position 29 (rs 1800470), G/C in position 74 (rs 1800471) [62] and in TGF- β 2: A/G in position 59941 (rs 1891467) [63].

An additional important candidate on sarcoidosis susceptibility gene is *CC10*, encoded Clara cell 1-kD-protein, which may inhibit such inflammatory mediators as *INF-* γ , *TNF-* α or (*IL*)-1 β [10]. It has been proven that SNP A/G in position 38 in *CC10* gene (rs 3741240) may be related to progressive sarcoidosis [64].

Expression markers associated with clinical course

It has been claimed that many diverse potential genes may be involved in different clinical stages of sarcoidosis: e.g. acute phase inflammation (*TNF-* α , *INF-* γ , *IL-2*) [65], granulomatous inflammation and granuloma formation (*IL-1*, *IL-6*, *IL-8*, *IL-15*, *TNF-* α , *INF-* γ , *GM-CSF*, *RANTES*, *IP-10*) [39, 66] or fibrosis (*IL-4*, *IL-5*, *IL-9*, *IL-10*, *IL-13*, *TGF-* β) [66, 67]. These genes are identified as a considerable in various signaling pathways involved in lung cell physiology.

Acute phase of inflammation

IL-1, IL-6, IL-11, TNF- α , and other chemokines, G-CSF and GM-CSF play a pivotal role in the acute inflammation of sarcoidosis [67]. In this phase of the disease, some cytokine genes, such as *IL-1*, *IL-2* [68], *IL-6* [68, 69], and *TNF-α* [68, 70], have demonstrated altered mRNA expression levels. Interestingly, the expression of *TNF-* α was decreased in acute phase of inflammation [71], whereas higher levels of *IL-2*, IFN- γ and *IL-12* mRNA expression was noted in patients with active sarcoidosis compared to individuals with the non-active form of the disease [69]. Additionally, in the non-active form of sarcoidosis, mRNA expression of IL-4, IL-5 and IL-10 was decreased [69]. Lower levels of *IL-17A* mRNA expression was found in the CD4⁺ T-cells of patients with Lofgren's syndrome, compared with those of healthy patients [72].

Granuloma formation

A prolonged state of inflammation in the presence of chronic antigenic stimulation leads to the formation of granulomas [1]. In sarcoidosis, the formation of granulomas requires an interplay between APCs, antigen and T-cells [73]. Ziegenhagen et al. observed the spontaneous release of greater amounts of TNF- α in patients with active sarcoidosis than those with the inactive form of disease [74]. Similarly, elevated levels of *TNF-\alpha* gene expression was observed in the BAL fluid of sarcoidosis patients compared with control subjects [70].

So far, vascular endothelial growth factor (VEGF) has been considered an important cytokine involved in the pathological processes of sarcoidosis [75, 76]. *VEGF* has been identified as a relative factor playing an important role in the recruitment and activation of monocytes towards granuloma formation [77]. Only one report has noted a change in the transcription pattern and protein production of the *VEGF* gene in patients diagnosed with sarcoidosis in comparison to control subjects. Koyama et al. report the presence of significantly lower *VEGF* levels in BALF specimens obtained from sarcoidosis patients compared to healthy controls [78]. Such low *VEGF* levels may contribute to the reduction of angiogenesis and induction of apoptosis in lung cells during the development of sarcoidosis. In contrast, a study by Sekiya et al. [76] revealed a high increase of VEGF level in the serum of patients with extrapulmonary sarcoidosis. In addition, Tolnay et al. [77] reported the overexpression of *VEGF* receptors in activated macrophages, in multinuclear giant cells and in epithelioid cells of sarcoid granulomas. In the light of these findings, it has been suggested that VEGF can be considered a prognostic indicator of the severity of a disorder [61].

Fibrosis

Local shifts in the Th1/Th2 regulatory networks tilting the balance of Th2 lymphocytes and increasing the amount of anti-inflammatory cytokines such as IL-4, IL-9, IL-10, IL-13 and TGF- β can encourage progression to fibrosis [39]. As a result, fibrogenesis via expansion of the mesenchymal cell population with increased deposition of matrix compoments has been observed [79]. Increased levels of *IL-10* [69], *IL-13* [70] mRNA have also been observed in the BALF of patients with sarcoidosis who have recently developed growing systemic symptoms.

As a multifunctional cytokine, TGF- β plays an important role in the initial steps of chronic inflammation and fibrogenesis. Moreover $TGF-\beta$ is a key cytokine involved in inflammatory processes: while it acts as a pro-inflammatory cytokine, stimulating the T cells and macrophages, in the early stages of inflammation, it demonstrates anti-inflammatory and immunosuppressive properties and stimulates the process of fibrosis in the later stages [80]. Increased immunoexpression of *TGF-\beta* has been found in alveolar macrophages, lung epithelial cells and myofibroblasts. Among the mammalian *TGF-\beta* isoforms (*TGF-\beta1-\beta3*), *TGF-\beta1* leads to increased deposition of extracellular matrix (ECM) and is most strongly involved in the pathology of fibrosis involved in sarcoidosis [81]. TGF- β 2 has been suggested to have a wound healing effect in lung inflammation [82]. Although the role of the third isoform (*TGF-\beta3*) is not fully understood, it is possible that it acts as an anti-fibrotic factor [83].

Higher serum levels of TGF- β 1 in patients with pulmonary sarcoidosis may be associated with the progression of the disease, as well as with an increased risk of lung fibrosis [84].

Signaling pathways important in sarcoidosis

$TGF-\beta/Smad$ signaling pathway

The TGF- β /Smad signaling pathway is involved in many key cellular processes in both the adult organism and developing embryo, including those concerned with cell growth, differentiation, apoptosis and cellular homeostasis. In lung tissue, the TGF- β /Smad signaling pathway participates especially in airway remodeling, peribronchial fibrosis and wound healing [85]. This pathway is a distinctive signal transduction pathway which can transmit signals directly from cell surface receptors by interacting with DNA binding molecules and with transcriptional coactivators and corepressors. Abnormalities in $TGF-\beta$ expression are very common in fibrotic diseases, including sarcoidosis, characterized by excessive production, admission, and reduction of extracellular matrix (ECM) [84, 86].

As a result of TGF- β receptors (T β RI and T β RII) activation *via* phosphorylation following binding to the TGF- β ligand, Smad 2/3 proteins complex, the pathway may be sequentially activated. During Smad 2 and 3 activation, Smad complex formation with Smad 4 is initiated. The Smad2/Smad3/Smad4 complex migrates to the cellular nucleus and interacts with the SBE (Smad binding element) as well as transcription factor binding element (TFBE). The interaction between Smad proteins complex and transcription factors may control the expression of certain genes, including genes encoding the extracellular matrix (see Figure 2).

Interestingly, TGF- β also induces Smad 7 through a Smad 3- and Smad 4-dependent mechanism. It was suggested that TGF- β can suppress Smad 3/4 action *via* the overexpression of Smad 7 [87]. Smad 7 can act as an inhibitor by suppressing the phosphorylation of Smad 2 and Smad 3 *via* ubiquitination by Smurf2 ubiquitin-protein ligase. This process contributes to the degradation of the TGF- β R1 and R2 receptor complex, inhibiting TGF- β signaling as a consequence [88]. It is also suggested that Smad 7 gene expression influences lung airway remodeling and lung injuries, leading to fibrosis through regulating the magnitude of TGF- β signaling [89].

There is considerable evidence that Smad 2 and Smad 3 play different biological roles in $-\beta$ /Smad signaling in both epithelial cells and fibroblasts [90]. Particularly in fibroblasts, Smad 3 is known to be required for gene expression induced by TGF- β , as it has been documented in



Figure 2. TGF- β /Smad signaling pathway in the remodeling of ECM in fibrosing diseases, including sarcoidosis. Abbreviations: TGF- β — transforming growth factor- β , TGF- β I — transforming growth factor- β receptor 1, TGF- β II — transforming growth factor- β receptor 1, Smad 2 — mothers against decapentaplegic homolog 2 protein, Smad 3 — mothers against decapentaplegic homolog 3 protein, Smad 4 — mothers against decapentaplegic homolog 4 protein, Smad 7 — mothers against decapentaplegic homolog 7 protein, SBE — Smad binding element, TFBE — transcription factor binding element

gene expression profiles of adult *Smad3_/_* mice models [91].

It has been also documented that TGF- β / Smad2/3 plays a crucial role in inflammation processes. It acts as inhibitor of such inflammatory cytokines as IL-2, IL-4 and INF- γ in T-cells by binding many important regulatory proteins to Smads, such as the antiproliferative factor Tob [92] or the T-bet [93] molecule: a critical transcription factor for INF- γ production and Th1 differentiation of CD4⁺ T-cells.

There is evidence that the TGF- β /Smad signaling pathway plays a crucial role in the progression from acute inflammation to chronic fibrosis. Bonniaud et al. suggested that the process of inflammation may be sufficient to induce fibrosis when it proceeds through the TGF- β /Smad 3 pathway [94]. Recently up-regulation of *TGF*- β_{T} and *SMAD3* genes in sarcoidosis patients presenting signs known to be related to worse prognosis (insidious disease onset, parenchymal involvement, lung function impairment) confirmed that elements of this pathway may be critical for the development of unfavorable outcome in sarcoidosis [95].

Current findings show that the activity of sarcoidosis can be marked by the process of ECM remodeling, related to the balance between immune cells and tissue remodeling [96]. It is commonly suggested that ECM is a Smad 3-dependent process [97]. Several studies have documented that TGF- β promotes fibrosis both by synthesis and the inhibition of ECM degradation through downregulating expression of matrix-degrading enzymes and increasing expression of MMP inhibitors, mainly *via* Smad 3 [98]. These processes are shown in Figure 2.

To summarize, the TGF- β /Smad signaling pathway may be an important molecular mechanism in the controlling contortion of inflammatory processes and fibrogenesis during the development of pulmonary sarcoidosis.

(HIF)-1A-VEGF-ING-4 axis

Although the exact mechanism of *VEGF* overor under-expression in sarcoidosis has not yet been well established, a basic explanation of its transcriptional regulation has been proposed [99].

HIF-1 α is a subunit of hypoxia-inducible factor 1 (HIF-1), which is a heterodimer based on a helix-loop-helix structure. The function and stability of *HIF-1A* might be immediately regulated by oxygen availability [100]. Under normoxic conditions, HIF-1A undergoes degradation via the ubiquitin-proteasome pathway. It has been proven that the von Hippel-Lindau tumor suppressor gene product (pVHL) plays a pivotal role in this process [101]. In turn, decreased O_2 levels result in *HIF-1a* accumulation within the cell due to the impaired action of pVHL [101]. HIF-1A is recognized as the most critical regulator of *VEGF* transcription [100]. Moreover, VEGF was identified as a pleiotropic factor in sarcoidosis. It has been documented as an important regulator of monocyte recruitment towards granuloma formation. The expression of VEGF within sarcoid granuloma was found to be raised [77]. These observations support the concept of "immunoangiostasis" explaining sarcoid granuloma formation [77].

HIF-1A expression is modulated not only by changes in cellular oxygen concentration, but also by the activity of distinct factors including insulin growth factor (IGF), tumor necrosis factor (*TNF-a*), as well as inhibitor of growth 4 (*ING-4*) expression. [99].

ING-4 is a nuclear factor: a tumor suppressor gene that interacts with nuclear factor-kappa B (NF- κ B) and represses its transcriptional activity. *ING-4* is known to belong to a family of proteins containing a highly conserved C-terminal plant homeodomain (PHD)-like zinc-finger domain [102]. The action of *ING-4* is strongly implicated in several important cellular processes including angiogenesis, apoptosis, cell cycle control. proliferation and DNA repair [99, 102]. Under hypoxic conditions, *ING-4* directly interacts with HIF prolyl-hydroxylase 2 (HPH-2) leading to *HIF-1A* downregulation, which may represent a possible mechanism for the promotion of angiogenesis [102]. Tzouvelekis [99] reported an impairment of the *HIF-1A-VEGF* axis resulting in ING-4 overexpression. The combination of the downregulation of *HIF-1A* within sarcoid granulomas with VEGF and ING-4 up-regulation may be the cause of the angiostatic environment found within granulomas, as well as the macrophage chemotaxis within sarcoid lesions [99]. In contrary, up-regulation on transcriptional level of proangiogenic factors such as HIF-1A and VEGF genes in PB lymphocytes of sarcoidosis patients has been documented [103]. Moreover the negative correlation between expression level of mRNA *HIF-1A* and lung function test results (FEV₁/FVC) in sarcoidosis patients with parenchymal involvement has been documented [103]. This is an important observation due to the fact that observed up-regulation of *HIF-1A* may inhibit the epithelial cell repair and therefore may render the epithelial lung injury towards pulmonary fibrosis in sarcoidosis patients.

Wnt/β-catenin signaling pathway

It has been documented that this signaling pathway is necessary for lung branching and distal airway cell specification [104, 105]. The Wnt/β -catenin regulate many biological processes in epithelial cells such as: fate determination, motility and polarity. Recent reports show that deregulated Wnt/ β -catenin signaling has disastrous consequences in embryonic development [106]. It is recognized that Wnt/β -catenin signaling may play an important role in the lung inflammation and fibrotic processes and responses. Several of the target genes of the Wnt/ β -catenin pathway such as: COX-2 (cyclooxygenase-2) [107], MMPs (matrix metalloproteinases) [108], VEGF [109] as well as c-myc [110] have been shown to be increased in sarcoidosis patients. In aspect of sarcoidosis pathogenesis, there are many published evidences confirming Wnt/β -catenin signaling

involvement in the epithelial-mesenchymal transition (EMT), an pivotal process maintain mainly the fibrosis occurring in the progression of sarcoidosis [111].

Programmed death-1 pathway

PD-1 was mentioned for the first time in 1992 by Ishida and co-workers [112]. PD-1 is expressed in various types of cells such as T-cells. Tregs. exhausted T-cells, B-cells, activated monocytes, DCs, and natural killer (NK) cells [113]. It is known that inhibitory receptors, such as programmed death-1 (PD-1), negatively impact CD4⁺ Tcell function [114]. PD-1 signaling is mediated by two different phosphatases: src homology region 2 domain-containing phosphatase (SHP)-1 and -2. When PD-1 is activated, these phosphatases block the PI3K/Akt (phosphatidylinositol 3 kinase/Akt) signaling pathway, leading to reduced T-cell activation. Ligation of PD-L1 or PD-L2 on an antigen -presenting cell with PD-1 on the T-cell results in the inhibition of PI3K-AKT, leading to a decrease of cytokine production, especially IL-2, and inhibition of LAT-Zap70 leading to a reduction of T-cell proliferation, and ultimately, inhibition of C3G-Rap1signaling, which causes a reduction of T-cell adhesion [115, 116]. In some granulomatous diseases such as chronic beryllium disease (CBD), inhibition of the PD-1 signaling pathway restores T-cell function [117]. Braun et al. reported increased numbers of PD-1⁺ CD4⁺ T-cells in patients with sarcoidosis, especially in the BAL fluid, connected with spontaneous expression of such cytokines as IL-2 and IFN- γ , compared with systemic T-cells. According to the authors, PD-1 expression carries immunologic consequences in sarcoidosis pathogenesis [118].

miRNA — a new promising epigenetic regulator of sarcoidosis?

MicroRNAs (miRNAs) are identified as small, endogenous non-coding RNAs (20–25 nucleotides) able to negatively regulate gene expression at the post-transcriptional level [119, 120]. Even though up to hundreds of human microRNAs are known, knowledge of their pathological role in human diseases including sarcoidosis is still limited.

Recent studies suggest that miRNAs play a significant role in immune responses via the indirect control of their own production, as well as their influence on the actions of other miRNAs [121]. However the role of particular miRNAs in inflammatory and fibrosis processes accompanying sarcoidosis remains unexplored [122].

A recently conducted study confirmed that miRNAs are included in the process of altering the immune response to bacterial components such as lipopolysacharide (LPS), which stimulate toll-like receptors (TLRs) [123]. Recent studies have shown that miRNAs are involved in the regulation of immune cells, including the development and differentiation of B- and T-cells (e.g. miR-155 [124], miR-181a [125] and cluster miR-17~92 [126]), as well as in the regulation of immune response (miR-155) [127], and the maintenance of the overall balance of the proand anti-inflammatory signals determining the progress or chronic state of inflammation (miR -146a) [128]. Moreover, many classes of miRNAs are recognized as pivotal in lung development, cell lung homeostasis (miR-155, miR-26a, miR-29, let-7f, miR-223, miR-146a) and act as regulatory factors in several pulmonary diseases (miR-155, miR-126) [129]. The interaction of miRNAs and cytokines contributes to the immune response mediated by T and B lymphocytes.

The most important miRNAs involved in pathological processes in the lung include miR-126, miR-7, miR-21, miR-29, miR-30, miR-17-92, let-7d, miR-155, miR-223. It has been documented that miR-126 is downregulated in cystic fibrosis airway epithelial cells [130]. Moreover, miR-155 and miR-21 have been recognized as being involved in allergic lung inflammation, with decreased expression being documented therein (see Figure 3) [129, 131].

Due to the fact that non-caseating granulomatous changes may be found in the lung in 90% of diagnosed sarcoidosis cases, many studies focus on miRNAs which are involved in the pathological processes occurring in the lungs. These studies mainly concern uncontrolled lung inflammation [128], pulmonary fibrosis and cystic fibrosis (see Figure 3) [132].

It should be stressed that expression of miR-NAs in the lung is very specific, highly conserved and dependent on physiological and pathological conditions. However, knowledge concerning this issue is still limited and typically based on animal models [133].

Pulmonary fibrosis is associated with extracellular matrix (ECM) remodeling and also regulated by miRNA. Degradation of ECM components leading to fibrosis is associated with excessive accumulation of collagens, fibronectins and elastin [134].

In the development of pulmonary fibrosis (both in IPF and in the course of sarcoidosis), some classes of miRNA can be downregulated



Figure 3. The deregulated classes of miRNA involved in the pathological processes occurring in the lungs. Abbreviations: miR — microRNA, decreased expression, ↑ increased expression

(let-7, miR-29, miR-30, miR-17-92 while others can be upregulated (miR-21, miR-155). Remarkably, miR-155 is involved in the regulation of many inflammatory processes. Its expression can be regulated by transcription factors NF-k β or AP-1 and cytokines such as TNF- α and IL-1 β . Upregulated expression of this miRNA leads to the reduction of keratinocyte growth factor (KGF) in normal pulmonary fibroblasts, leading to increased fibroblast migration *via* activation of caspase 3 [134]. Upregulated expression of miR-21 in pulmonary fibrosis is associated with miRNA-21 regulation of the *TGF-\beta* signaling pathway. Both factors, miR-21 and *TGF-\beta*, work in a feed-forward loop. *TGF-\beta* induces miR-21 expression and miR-21 in turn promotes $TGF-\beta$ -induced fibrogenic activation of pulmonary fibroblasts via the inhibition of Smad 7 and the reduction of Smad 2 [136, 137].

A study conducted by Xiao et al. found that miR-29 is the downstream target gene of Smad 3 and is negatively regulated by the TGF- β /Smad signaling pathway [138]. Moreover, miR-29 is also involved in EMT [139].

The miR-17~92 cluster is known to encode six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) [126, 140], which are decreased in patients with IPF. These six miR-NAs are also important in the development of lung epithelial cells with high expression during the embryonic phase which then declines into adulthood [126]. This is surprising, as so far no studies on sarcoidosis have been published concerning the modulation of this miRNA cluster. Our current finished studies (data not published) focus on many selected classes of miRNAs has documented that some classes of miRNAs (e.g. miR-192 and miR-221) are upregulated in sarcoidosis patients. Moreover, miR-16 and miR-20a were significantly increased in patients with lung volume restriction while miR-let7f increased in patients with bronchial obstruction. Our results suggest regulatory role of miRNAs in sarcoidosis and indicate that some of them may have a negative prognostic value.

Which miRNAs targeting signaling pathway may be important in sarcoidosis?

Smad 7, the signaling protein in the TGF- β / Smad signaling pathway thought to be important in fibrosis, is known to be a direct target of miR-21. Overexpression of this microRNA in primary pulmonary fibroblasts inhibits Smad 7 and as a consequence, regulates the TGF- β /Smad pathway. A molecule miR-21 is known to belong to a group of miRNAs (miR-21, miR-32, miR-136, miR-137, miR-192, miR-210, miR-211, miR-346) that participates in the regulation of TGF- β -dependent endothelial-mesenchymal transition (EMT) [141, 142]. During the process of fibrogenesis, various microRNAs such as miR-196b, miR-704, miR-717, miR-16, miR-195, miR-10a, miR-211, miR-34a, miR-367, miR-21 and let-7 are activated and target the TGF- β , Smad 3,6,7 and TGF- β Rs I and II genes from the TGF- β /Smad signaling pathway [143].



Figure 4. Important DE-miRNAs identified in lung sarcoidosis tissue, focusing on particular molecules participating in the TGF- β /Smad signaling pathway [110, modified]. Abbreviations: TGF- β — transforming growth factor- β , TGF- β I — transforming growth factor- β receptor 1, TGF- β II — transforming growth factor- β receptor 1, Smad 2 — mothers against decapentaplegic homolog 2, Smad 3 — mothers against decapentaplegic homolog 3, Smad 4 — mothers against decapentaplegic homolog 4, Smad 7 — mothers against decapentaplegic homolog 7, SP1 — transcription factor Sp1, E2F5 — transcription factor E2F5

Wnt/ β -catenin pathway can be targeted by so-called DEmiRNAs (differentially expressed microRNAs) detected in interstitial lung disease (ILD) [143, 144]. Ting Xie et al. propose that miRNAs exert an influence on the Wnt/ β -catenin signaling pathway: downregulated miR-16 and miR-195 influence Wnt7A, while *sFRP1/2* and *Wnt7B* (*Wingless-Type MMTV Integration Site Family, Member 7B*) are controlled via up-regulation of miR-351 [143].

However, only one original miRNA array study focusing on the significance of DEmiRNAs expression and their influence on TGF- β /Smad and Wnt/ β -catenin signaling pathways has been published, which may be important in pulmonary sarcoidosis [122]. The authors of this study confirm reduced expression of miR-20a and miR-302c in sarcoidosis patients and elevated expression of miR-92b and miR-206 in lymph node tissues in pulmonary sarcoidosis patients [122]. Many types of DE-miRNAs seem also to be involved in regulatory signaling pathway important for sarcoidosis [122]. These are summarized in Figure 4 and Figure 5.

Conclusions

Sarcoidosis has confirmed to have a complex environmental and molecular etiopathogenesis. In the previous decade, sarcoidosis development was recognized to have a genetic basis, including a number of susceptibility loci which were well recognized from both linkage and association studies. These are mainly associated with human leukocyte antigens, both HLA class I and class II. Many other candidate genes have since been confirmed by GWAS. Based on screening of the genome, chromosomal regions 6p12.1, 11.q13.1, 12q13.3 have been found to be associated with a significant risk of sarcoidosis.

Recently, an experimental study examined non-coding RNAs (ncRNA) including miRNAs which play an essential role in the posttranscriptional regulation of numerous human genes. Many class of miRNAs are recognized as important regulators of signaling pathways in lung cells.

There is evidence that lung cells have a very specific miRNA expression profile, which undergoes modifications during lung development. Current studies on animal models have



Figure 5. Important DE-miRNAs identified in the lung sarcoidosis tissue, focusing on particular molecules participating in the Wnt/ β -catenin signaling pathway [110, modified]. Abbreviations: APC — transcription factor APC, BTRC — beta-transducin repeat containing E3 ubiquitin protein ligase, CTNNB1 — catenin beta-1, DV1 — disheveled, FZD7 — frizzled class receptor 7, GSK1B — GSK-like kinase 1B, PPP2R2 — receptor for protein phosphatase 2, SIAH1 — Siah E3 ubiquitin protein ligase 1, WNT9A — wingless-type MMTV integration site family, member 9A

provided evidence that miRNAs participate in lung homeostasis and play a crucial role also in the control of pulmonary inflammation, TGF- β -dependent endothelial-mesenchymal transition (EMT) and fibrogenesis. Recent studies confirm that upregulated and/or downregulated expression of various miRNAs play a pivotal role in the pathogenesis of sarcoidosis — *via* their regulatory impact on signaling pathways (e.g. TGF- β /Smad and Wnt/ β -catenin). This extended knowledge of altered miRNA expression profiles in sarcoidosis may play a role in the development of new biological markers to predict disease development. Moreover, miRNAs may represent attractive novel diagnostic biomarkers and could potentially provide possibilities for therapeutic intervention.

Conflict of interest

The authors declare no conflict of interest.

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