HELLENIC REPUBLIC
National and Kapodistrian University of Athens
EST. 1837

"ALEXANDER FLEMING"
Biomedical Sciences Research Center

International M.Sc. in "Molecular Biomedicine - Disease Mechanisms, Molecular and Cellular therapies, and Bioinnovation"

Medical School, University of Athens – BSRC "Alexander Fleming"

"*BAFF*, *APRIL* and *BAFF-R* in the pathogenesis of sarcoidosis"

Maria Martinou

Supervisor: Maria Gazouli, Professor of Biology, Medical School, University of Athens

Athens, 2022

ACKNOWLEDGEMENTS

This dissertation was conducted in the context of the Master in Science (M.Sc.) program “Molecular Biomedicine – Disease Mechanisms, Molecular and Cellular therapies and Bioinnovation”, jointly organized by the Medical School of the National and Kapodistrian University of Athens and the Biomedical Sciences Research Center “Alexander Fleming”. The experiments took place in the Laboratory of Biology, Medical School of Athens, National and Kapodistrian University of Athens.

First of all I would like to thank Prof. Maria Gazouli for accepting me a bit more than a year ago for a rotation at her lab, and then again for this project. The time I spent as part of her team will always be invaluable to me. I would also like to thank Prof. Maria Roubelakis for the time I spent in her own lab as well, and for being part of my dissertation’s three-member committee, and Prof. Karamanolis, who also gladly accepted evaluating this work.

I am truly thankful to all current and former members of the lab for working with me, and especially to Hector Katifelis, Nikos Andreou, Matina Grammatikaki, Maria Nikou, as well Lydia Ntari and Antrianna Psaraki, for making every day better by working with them.

Finally, I would like to thank my family and friends, who supported me in many ways during the last two years of this Master.

Περιεχόμενα

ABSTRACT

Sarcoidosis is a rare, inflammatory disease, with no know etiology up to date. It is believed to be caused by a combination of genetic and environmental factors, none of which however, have been yet completely elucidated. Although, it has been considered a disease mediated by cellular immunity, lately evidence shows that humoral immunity may also have a role in its development. Thus, the purpose of this study is to determine whether single nucleotide polymorphisms (SNPs) of 3 humoral immunity genes, *BAFF*, *BAFF-R* and *APRIL*, are associated with sarcoidosis.

One hundred and seventy-three blood samples from sarcoidosis patients as well as one hundred and sixty-four blood samples from a control group were collected. The samples were genotyped for the following polymorphisms: *BAFF* rs2893321, rs1041569 and rs9514828, *BAFF-R* rs61756766, and *APRIL* rs11552708.

Genotypic frequency comparison showed no significant association of the *BAFF* SNPs with sarcoidosis; a higher frequency of the T allele of rs1041569 and rs9514828 however, was indicated in sarcoidosis patients. In the case of *BAFF-R* rs61756766 the CT genotype and T allele had a marginally significant association with the disease. The genotypic and allelic frequencies of *APRIL* rs11552708 were not calculated, due to insufficient data. Patients were also separated in groups of cardiac and no cardiac involvement, but no associations could be deduced. Lastly, when haplotype analysis of the *BAFF* polymorphisms was performed, 3 haplotypes (ATT, GTA, and GTT) were found to be over-represented in the group of patients with cardiac involvement.

In conclusion, a possible relationship between *BAFF* SNPs, rs1041569 and rs9514828, and *BAFF-R* SNP rs61756766 with sarcoidosis susceptibility is suggested by the results, and these SNPs could serve as potential biomarkers for the disease .

ΠΕΡΙΛΗΨΗ

Η σαρκοείδωση είναι μια σπάνια, φλεγμονώδης νόσος, χωρίς γνωστή αιτιολογία μέχρι σήμερα. Πιστεύεται ότι προκαλείται από έναν συνδυασμό γενετικών και περιβαλλοντικών παραγόντων, κανένας από τους οποίους ωστόσο δεν έχει ακόμη πλήρως διευκρινιστεί. Αν και θεωρείται ως μια ασθένεια που διαμεσολαβείται από την κυτταρική ανοσία, πρόσφατα στοιχεία δείχνουν ότι η χυμική ανοσία μπορεί επίσης να παίζει ρόλο στην ανάπτυξή της. Έτσι, ο σκοπός αυτής της μελέτης είναι να προσδιορίσει εάν οι πολυμορφισμοί ενός νουκλεοτιδίου (SNPs) 3 γονιδίων χυμικής ανοσίας, των *BAFF, BAFF-R* και *APRIL*, σχετίζονται με τη σαρκοείδωση.

Συλλέχθηκαν εκατόν εβδομήντα τρία δείγματα αίματος από ασθενείς με σαρκοείδωση καθώς και εκατόν εξήντα τέσσερα δείγματα αίματος από μια ομάδα ελέγχου. Τα δείγματα γονοτυποποιήθηκαν για τους ακόλουθους πολυμορφισμούς: *BAFF* rs2893321, rs1041569 και rs9514828, *BAFF-R* rs61756766 και *APRIL* rs11552708.

Η σύγκριση γονοτυπικών συχνοτήτων δεν έδειξε κάποια στατιστικά σημαντική συσχέτιση των *BAFF* SNPs με τη σαρκοείδωση. Ωστόσο, υποδεικνύεται υψηλότερη συχνότητα του αλληλόμορφου T των rs1041569 και rs9514828 σε ασθενείς με σαρκοείδωση. Στην περίπτωση του *BAFF-R* rs61756766 ο γονότυπος CT και το αλληλόμορφο T είχαν οριακά σημαντική συσχέτιση με τη νόσο. Οι γονοτυπικές και αλληλικές συχνότητες του *APRIL* rs11552708 δεν υπολογίστηκαν, λόγω ανεπαρκών δεδομένων. Οι ασθενείς χωρίστηκαν επίσης σε ομάδες με καρδιακή και χωρίς καρδιακή εμπλοκή, αλλά δεν υπήρξε κάποια συσχέτιση. Τέλος, όταν πραγματοποιήθηκε ανάλυση απλότυπου των πολυμορφισμών *BAFF*, βρέθηκαν 3 απλότυποι (ATT, GTA και GTT) να υπερεκπροσωπούνται στην ομάδα ασθενών με καρδιακή εμπλοκή.

Συμπερασματικά, υποδηλώνεται από τα αποτελέσματα μια πιθανή σχέση μεταξύ των *BAFF* SNP, rs1041569 και rs9514828 και του *BAFF-R* SNP rs61756766 με την ευπάθεια στη σαρκοείδωση και αυτοί οι πολυμορφισμοί θα μπορούσαν να χρησιμεύσουν ως πιθανοί βιοδείκτες για τη νόσο.

1. INTRODUCTION

1.1. Epidemiology

Sarcoidosis is a multisystemic, inflammatory disease with unknown etiology, characterized by the formation of non-caseating granulomas [1]. Its prevalence varies depending on the geographic location (Figure 1), while it is the highest in Nordic countries and African Americans[2]. The annual incidence rate in Caucasians of the United States is 10.9 in 100,000 and in African Americans 35.5 in 100,000[3]. As of 2009 in Greece, the disease's prevalence was 7/100,000, making sarcoidosis one of the most frequent interstitial lung disease in the country[4]. Apart from ethnicity, age and sex are additional factors that influence the disease's incidence, since females develop sarcoidosis more frequently. The age where both sexes, however, are more likely to be affected is between 25 and 45[5]. Females not only have a higher chance of developing sarcoidosis, but they also exhibit different symptoms compared to men, and tend to experience the musculoskeletal manifestation of the disease[6].

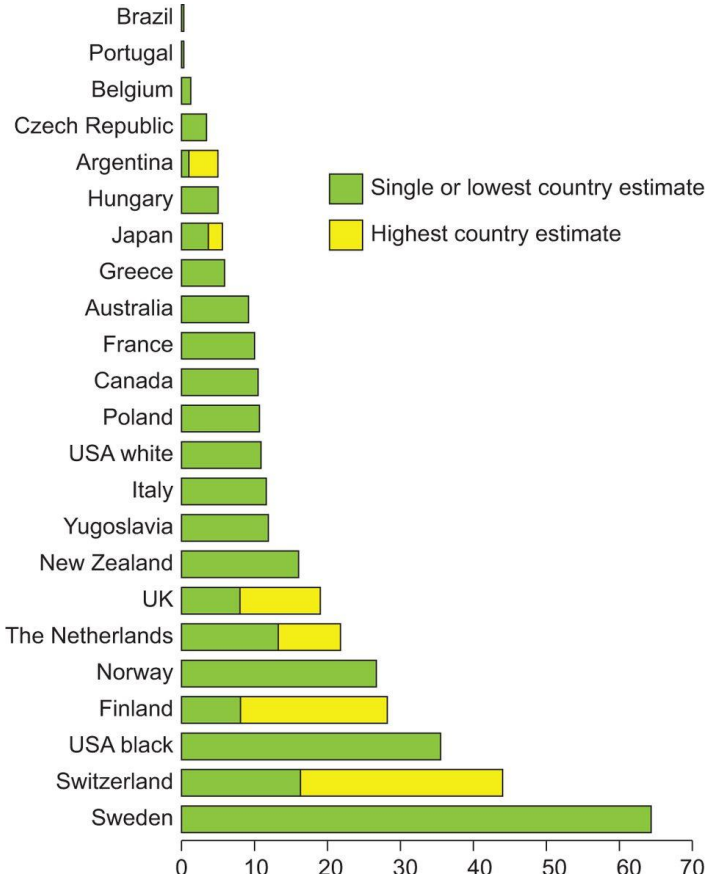


Figure 1: Prevalence of pulmonary sarcoidosis around the world. ***Source: Denning, D. W., Pleuvry, A. & Cole, D. C. Global burden of chronic pulmonary aspergillosis complicating sarcoidosis. Eur. Respir. J. 41, 621–626 (2013)*** [85]

1.2 Mortality

Sarcoidosis mortality is calculated to be 9-14 cases per 1000 persons, while 5-year survival is 93-95%. The graph on the right (Figure 2) presents the survival probability after follow-up, and its decrease is evident in patients that are in need of treatment [7]. The comorbidities that can accompany the disease are haematological cancers skin cancers, upper digestive tract cancer, kidney, liver and colorectal cancer[8].

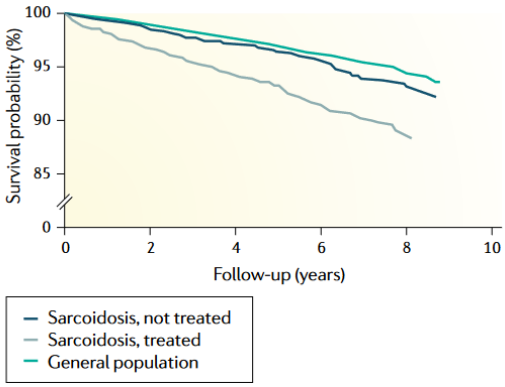


Figure 2: Percentage of survival probability (%) after follow-up. ***Source: Grunewald, J. et al. Sarcoidosis. Nat. Rev. Dis. Prim. 5, (2019).***

1.3 Granulomas

The histological characteristic of sarcoidosis is the formation of non-caseating granulomas (Figure 3). These granulomas consist of a core and a crust. The core segment is composed of macrophages, epithelioid cells, and multinucleated giant cells, while the crust segment is usually full of T-cells, and a few B-cells [9]. When granuloma formation is still at an early stage, the macrophages are transformed to epithelioid cells, and aggregate into a cluster to form immature granuloma. Then, multinucleated giant cells are formed by the fusion of macrophages and monocytes/dendritic cells, that will later fill the core section of the granuloma, as mentioned[10].

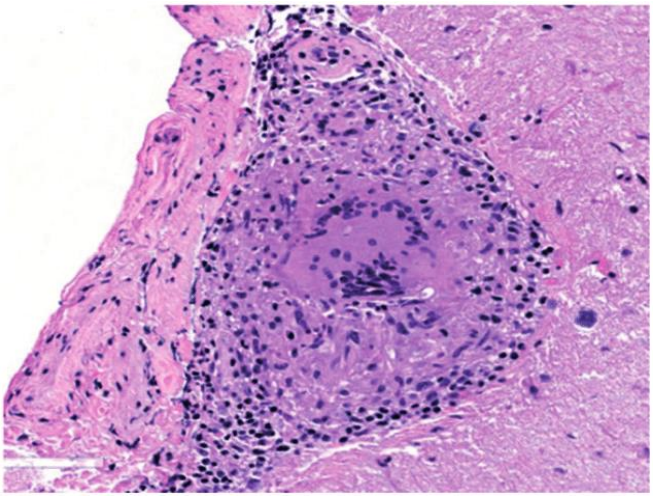


Figure 3: Sarcoid granuloma in the subarachnoid space of the cerebellum (H&E stain, original magnification Â200). ***Source: Tana, C. et al. Challenges in the diagnosis and treatment of neurosarcoidosis. Annals of Medicine 47, 576–591 (2015).*** [11]

1.4 Clinical Representation

1.4.1 Pulmonary Sarcoidosis

Although sarcoidosis can affect multiple organs most patients have intrathoracic involvement, making pulmonary sarcoidosis the most common disease manifestation, and highlighting the significance of chest radiographic imaging during diagnosis[12]. Typical symptoms are dyspnea, coughing and chest tightness, most patients with stage I disease however, are asymptomatic. Stage I patients have a good overall prognosis, as less that 5% of them will develop chronic respiratory impairment in a 10-year period, while in more advanced stages there is a 5-fold increase of chronic respiratory impairment risk[13]. Even though it may be considered outdated compared to computed tomography (CT), chest X-rays are used as a tool of high prognostic value, classifying the disease into 5 stages (Figure 4).

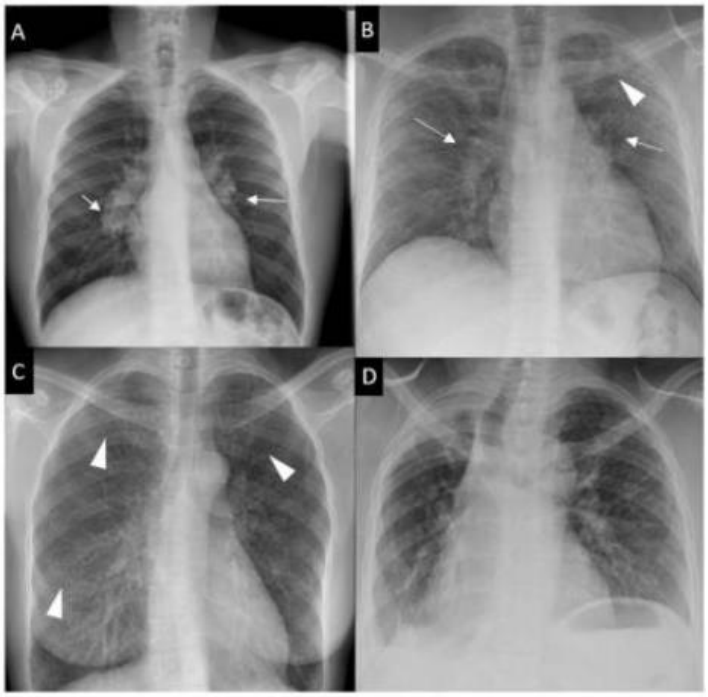


Figure 4: Five stages of sarcoidosis can be recognized using Scadding's classification on Chest x-rays(CXR). Stage 0, which is not shown, represents a normal X-ray, Stage 1 features bilateral hilar lymphadenopathy (A); Stage 2 feautures bilateral hilar lymphadenopathy and pulmonary infiltrates in upper lobes (B); Stage 3 features pulmonary infiltrates without bilateral hilar lymphadenopathy (C); and Stage 4 is pulmonary fibrosis (D). *Source: Sève, P. et al. Sarcoidosis: A clinical overview from symptoms to diagnosis. Cells 10, (2021).*[16]

1.4.2 Cardiac sarcoidosis

Another organ typically affected by sarcoidosis is the heart, a condition that causes conduction abnormalities and arrhythmias, congestive heart failure, and potentially sudden death [14]. Clinically, cardiac sarcoidosis is rare to be diagnosed, with only 5% of sarcoidosis patients being diagnosed with this subtype. In autopsies however, it is present in up to 25% specimens, a much higher percentage [15]. So it seems that the actual prevalence of cardiac sarcoidosis is highly underestimated, since some patients may be asymptomatic, especially at early stages, have non specific symptoms or subclinical disease [16]. The characteristic granulomas that form during disease progression usually affect the left ventricle of the heart, and histologically three stages can be defined : edema, granulomatous inflammation, and finally fibrosis that will lead to post-inflammatory scarring [17].

1.4.3 Other manifestations

A very common manifestation of the disease involves the skin, and is present in about 30% of patients. Skin lesions can be divided into two categories; sarcoidosis-specific and non specific[18]. Erythema nodosum (Figure 5) is part of the second category, as a non-specific lesion, and when presented with bilateral hilar lymphadenopathy, and a series of other possible symptoms like fever, arthritis, and anterior uveitis, a diagnosis of Löfgren's syndrome can be made. This syndrome is an acute form of sarcoidosis, and usually is an indicator of good prognosis[19].

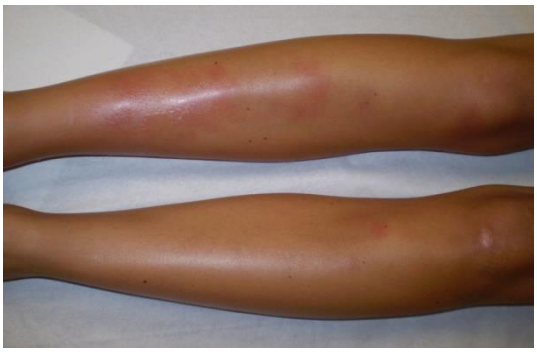


Figure 5: Erythema nodosum in a woman with Löfgren's syndrome. ***Source: Sève, P. et al. Sarcoidosis: A clinical overview from symptoms to diagnosis. Cells 10, (2021).16***

Ocular involvement is another frequent manifestation, with uveitis being the most common disease. Its prevalence varies from 12 to 50% of patients, who usually experience symptoms of blurred vision, eye pain and floaters. In fact, in a study of Caucasian patients, eye symptoms were the symptoms that led to diagnosis of systemic sarcoidosis, indicating the significance of ophthalmologic examinations in this context[20].

Other affected organs include the lymph nodes, endocrine and exocrine glands, joints, kidney, heart, nervous system, spleen and liver [21]. In the table below (Table 1) there is a summary of the most affected organs along with the respective symptoms and prevalence of involvement.

Table 1: Common manifestations of sarcoidosis; affected organs, examples of symptoms and prevalence of organ involvement. ***Source: Grunewald, J. et al. Sarcoidosis. Nat. Rev. Dis. Prim. 5, (2019).***

Affected organ	Examples of related symptoms	Prevalence of organ involvement (%)
Lung	Cough, dyspnea, wheezing and stridor	89-99
Skin	Lupus pernio, papules, nodules, plaques and infiltrated scars and tattoos	16-32
Eyes	painful and/or red eye and vision loss	5-23
Liver	Abdominal pain and elevated liver functions	12-20
Lymph nodes	Peripheral lymphadenopathy	13-15
Spleen	Abdominal pain	5-10
Nervous system	Facial palsy, fatigue, gait disturbance, headache, hearing loss, numbness or paraesthesia, seizure, trigeminal neuralgia, vertigo, visual loss, weakness and/or paresis	3-9
Heart	Conductance disturbances, arrhythmias, dyspnea, fatigue and syncope	2-5

1.5 Risk factors

Genetics

Genetic predisposition seems to contribute to disease development, an observation that can be based on the differences in susceptibility to sarcoidosis among ethnic groups, familial clustering , and a higher risk of developing sarcoidosis in monozygotic twins [22].

Several genes and their variants have been detected through Genome-wide association studies (GWAS) and investigated for their influence on this pathologic phenotype. BTNL2 is one of those genes, whose protein acts as a co-stimulatory molecule to T-cell activation. The BTNL2 SNP, rs2076530, has been characterized as a disease variant, and when the risk-associated allele is transcribed the protein has a truncated form, due to a premature stop in the spliced mRNA[23]. The mutation has been confirmed to affect susceptibility to sarcoidosis, and is especially linked to the likelihood of developing granulomatous diseases in Caucasian populations[24].

Sarcoidosis is a polygenic disease, so a more extended genetic profile would be appropriate in order to understand the true risk for its development. The ACCESS project (A Case Control Etiologic Study of Sarcoidosis) involved the enrollment of 474 cases of both black and white populations, and proved that two HLA class II alleles, HLA-DRB1 (*1101) and HLA-DPB1 (*0101), can be considered risk factors for sarcoidosis [25]. Various HLA gene alleles have been associated with the involvement of different organs , therefore they are mostly useful as a biomarker of phenotypic classification and predictor of organ manifestation [22].

Other genes and their polymorphisms that have been studied for their association with sarcoidosis are *NOTCH4* (neurogenic locus notch homolog 4), *TAP2* (transporter 2, ATP binding cassette subfamily B member), *TNFα* (tumor necrosis factor α), *LTA* (lymphotoxin α), *HSPA1L* (heat shock 70 kDa protein 1L) [26], though many more appear to contribute to a sarcoidosis patient's genetic predisposition.

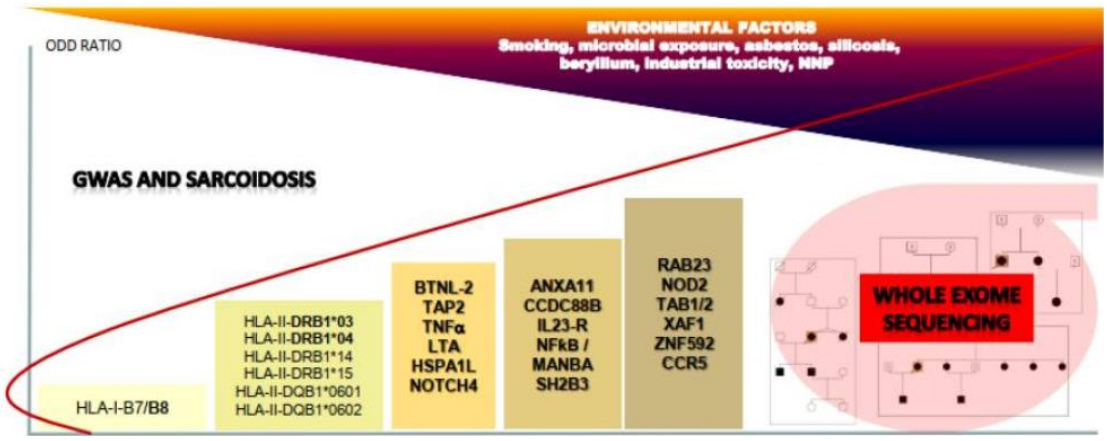


Figure 6: Genes identified by genome wide association (GWAS) studies in sarcoidosis. The graph represents the accumulative risk of acquiring the particular variants. ***Adapted from : Calender, A., Weichhart, T., Valeyre, D. & Pacheco, Y. Current insights in genetics of sarcoidosis: Functional and clinical impacts. Journal of Clinical Medicine 9, 1–21 (2020)***

Environmental factors

Along with genetic risk factors, environmental and lifestyle factors can act as triggers in a combinatorial way. For instance, exposure to insecticides due to occupational obligations is strongly related to sarcoidosis, whereas exposure to them in industrial settings does not have the same effect. Agricultural employment is generally considered to be a significant factor, perhaps because of the exposure to various chemicals, while it is also hypothesized that moldy environments and microbial bioaerosols can have the same effect [27].

Infectious agents have long been suspected to be associated with sarcoidosis. A meta-analysis of Esteves et al. indicates an etiological link between *Propionibacterium acnes* and sarcoidosis, as well as an association with mycobacteria infection. Interestingly, mycobacteria are present in about a quarter of patients with skin lesions[28]. Moreover, studies have pointed to *Borrelia burgdorferi* and Human herpesvirus 8 (HHV-8) as risk factors, but the combination of results in the meta-analysis tells otherwise [28].

1.6 Immunopathology

As it was previously mentioned, the hallmark of sarcoidosis histologically is the formation of non-caseating granulomas. It is speculated that the interaction of and antigen-presenting cell with an antigen is what triggers the disease, leading to the activation and aberrant response of T and B cells. The constant migration of lymphocytes to the site of inflammation, along with the macrophage differentiation caused by the antigen's persistence result in the formation of granulomas[10].

The lymphocyte population at the inflammation loci consist mainly of CD4-positive (CD4+) T helper cells that differentiate into T_{H1} and $T_{H17.1}$ effector cells, as well as T_{H17} cells. From the first type of cells IFNγ is produced, and the second type produces IL-17[29]. Other pro-inflammatory cytokines, like tumour necrosis factor (TNF), IL-12, IL-6, IL-18, as well as regulatory cytokines like transforming growth factor-β (TGF-β) and IL-10 are upregulated[7]. The excessive immune responsethat is triggered enables macrophage accumulation, and the expansion of innate T_{reg} cells[30]. The multiplication of T_{regs} could explain the immunological paradox of sarcoidosis, which is that even though the intense immune response causes major inflammation in the affected organs, simultaneously there is a state of anergy[31]. Moreover, the accumulating macrophages differentiate into the anti-inflammatory M2 type, producing the profibrotic chemokine CCL18, and thus prolonging the process of fibrosis[32].

Although sarcoidosis is considered to be a mainly T-cell mediated disease, evidence shows that the role of humoral immunity is not negligible. In patients with severe chronic sarcoidosis, B-cell populations are characterized by signaling defects and have a distribution similar to that of primary Sjögren's Syndrome (pSS) patients. It is also suspected that the lack of memory B-cells in sarcoidosis and the consequent insufficient production of suppressive cytokines could support the formation of granulomas, and that the ineffective B-cell responses could be responsible for the persistence of antigens that speculated to be the trigger of the condition[33]. Another finding that serves as proof of humoral immunity's involvement in sarcoidosis is the aggregation of a large number of B-cells and plasmatic hypergammaglobulinemia in sarcoid pulmonary lesions[34], and the good response of sarcoidosis patients to

monoclonal antibodies (anti-CD20) antibodies, which deplete B-cell populations[35]

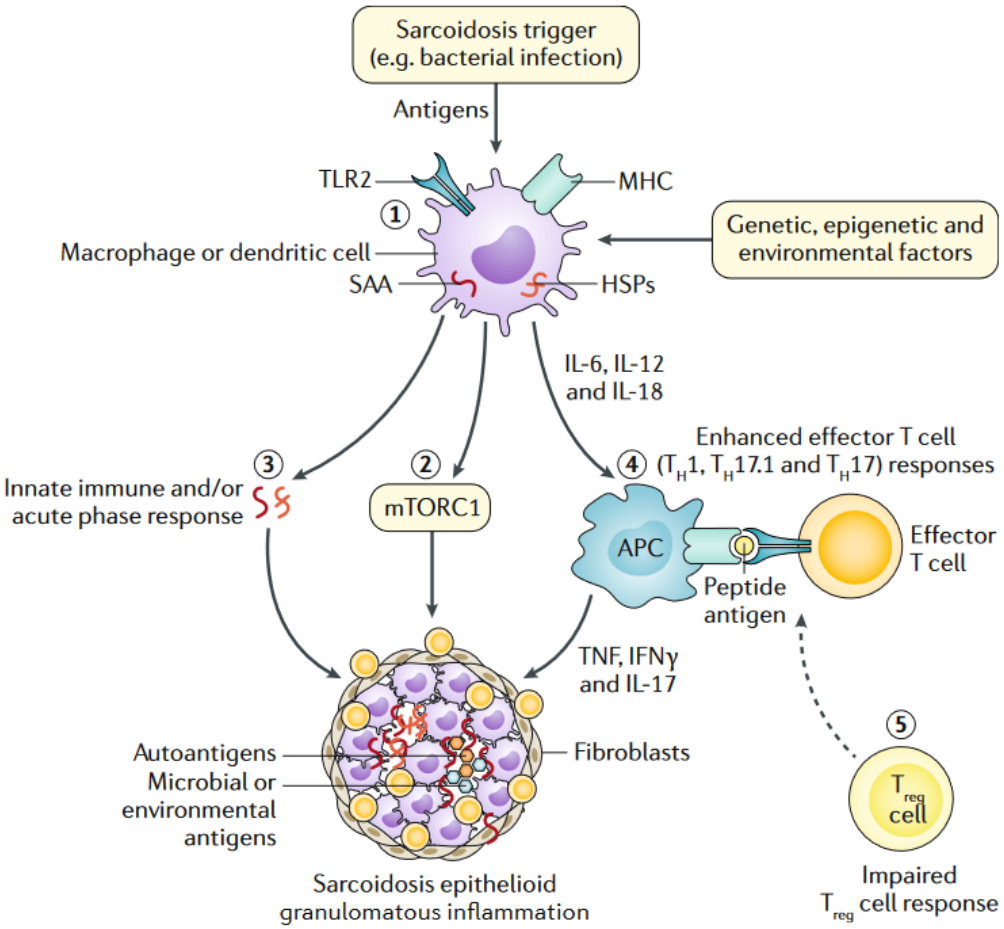


Figure 7: An environmental agent, such as a bacterial infection, is thought to be what triggers sarcoidosis. The interaction of genetic, epigenetic and enivonmental factors lead to the activation of macrophages and dendritic cells and their translocation to the inflammation site. The cytokines released from these cells result in the upregulation of the mTORC1 pathway, which will then lead to the differentiation of macrophages to epithelioid cells. Moreover, serum amyloid A (SAA) and heat shock proteins (HSPs) are upregulated. Aggregation of SAA in granulomas promotes enhanced effector T cell responses which when IL-12, IL-18, IL-6 and transforming growth factor-β (TGFβ) are present, the polarization of T helper 1 (TH1), TH17 and TH17.1 responses in affected site is promoted. Lastly, an impaired regulatory T (Treg) cell response results in chronic sarcoidosis.

Source: Grunewald, J. et al. Sarcoidosis. Nat. Rev. Dis. Prim. 5, (2019).

1.7 BAFF

B-Lymphocyte-activating factor (*BAFF*) is a member of the tumor necrosis factor (TNF) cytokine family [36], and is also known as BLyS, THANK, TALL-1, TNFSF13B, and ZTNF4. It is a type II transmembrane protein expressed by monocytes, macrophages, dendritic cells, bone marrow stroma cells, and T cells[37] as a membrane-bound ligand, as a soluble trimer, or as a soluble 60mer following cleavage by furins[38]. Its expression is increased by the presence of type I IFNs, and by Toll-like receptor 3 (TLR3), TLR4 and TLR9 stimulation[39].

The two forms of BAFF, soluble and membrane-bound, have a variety of effects and functions on different cell types. The main biological activity of the molecule is to promote B-cell survival and development, which has been validated with experiments as simple as culturing human peripheral blood B cells with and without soluble BAFF[36], and more complex, such as the production of BAFF-Tg mice, that exhibited B-cell hyperplasia and an Systematic Lupus Erythematosus (SLE)-like autoimmune phenotype[40].

The human *BAFF* gene locus is on chromosome 13 (13q33.3), has 6 exons, 5 introns and a molecular weight of 39kb. Variants of *BAFF* include *ΨBAFF* , a non functional transcript[41], *ΔBAFF*, an alternative splice isoform lacking exon 3 that can suppress *BAFF* function by competitive co-association [41], and *Δ4BAFF*, a splice variant that lacks of exon 4 and can regulate the expression of many innate immune system genes[42].

Patients with several inflammatory diseases, such as Crohns's Disease (CD)[43], SLE[44] and Multiple Sclerosis (MS)[45] have elevated BAFF levels in the blood. In the case of sarcoidosis, *BAFF* levels are increase in both serum and bronchial alveolar lavage fluid increased and positively correlated with the disease's severity[46,47] and activity [48]. For these reasons, it is believed that *BAFF* could potentially be significant in the pathogenesis of sarcoidosis, and is ought to be investigated genetically as well.

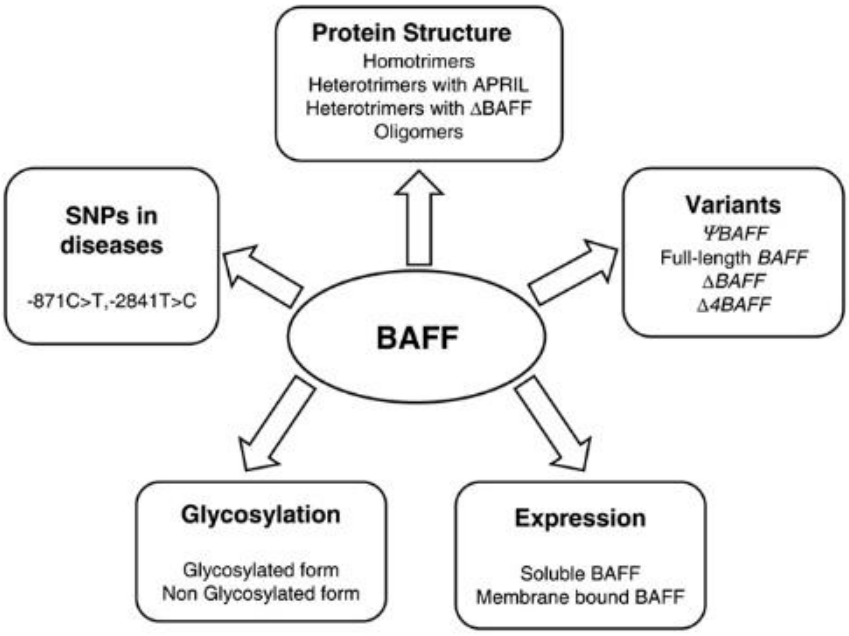


Figure 8: The various forms of *BAFF*; variants, protein structure, expression, glycosylation and SNPs.

Source: Lahiri, A. et al. The complexity of the BAFF TNF-family members: Implications for autoimmunity. J. Autoimmun. 39, 189–198 (2012).

1.8 APRIL

APRIL, also known as TNFSF13 and CD256, is another member of the TNF cytokine family, and is expressed by lymphoid cells and certain tumor cells[49]. It shares about 50% of structural similarity with *BAFF*, in addition to high genomic organization similarity, without however necessarily sharing biological functions[50]. APRIL has been implicated in functions such as plasma cell survival, T-cell independent and dependent IgA switch and T-cell costimulation[51]

APRIL levels in the blood have been found to be significantly higher in patients with SLE[52], pSS[53], and MS[54], though its influence in autoimmunity remains under investigation.

1.9 BAFF-R

BAFF can interact with three receptors belonging to the tumor necrosis factor receptors (TNFR) family: *BAFF-R* (also known as *BR3*, *CD268* and *TNFRSF17*), TACI (CD267, TNFRSF13B), and BCMA (CD269, TNFRSF13C). Of these receptors, *BAFF-R* is specific for *BAFF*, whereas TACI and BCMA can bind with APRIL as well[49,55].

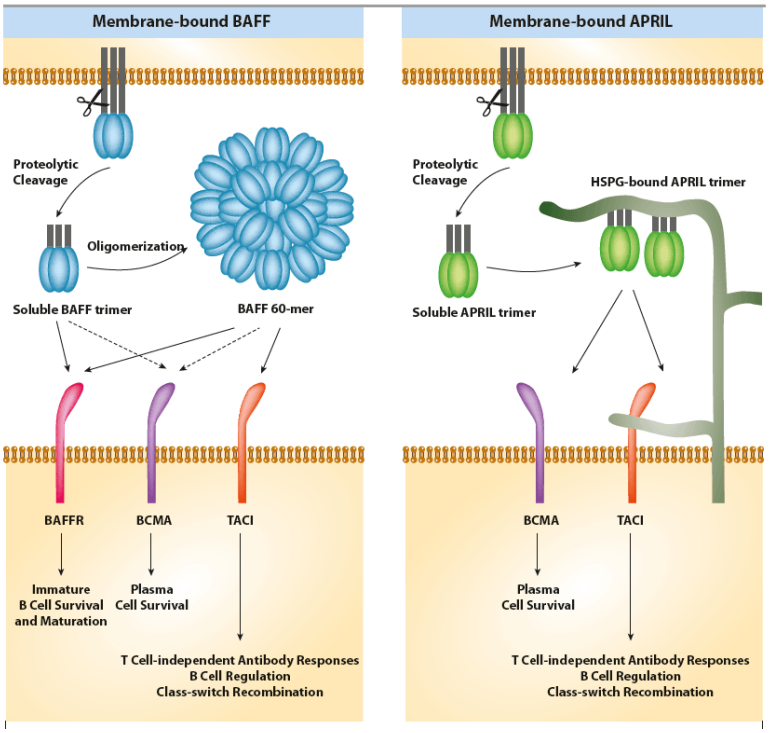


Figure 9: Membrane bound and soluble BAFF and APRIL and their respective receptors.

Adapted from: https://www.biomol.com/resources/biomol-blog/autoimmune-disease-biomarkers-APRIL-BAFF

BAFF-R expression begins when the immature B-cells develop to transitional B-cells[56], and as a protein it is expressed on the surface of all human peripheral B cell subsets with the exception of plasma cells and centroblasts located in the dark zone of germinal centers[37].

The binding of *BAFF* to *BAFF-R* activates the non-canonical NF-κB2-dependent pathway, which is much slower compared to the activation of the canonical NF-κB1 pathway[57]. When the *BAFF* ligand is present and binds to *BAFF-R*, an aggregation of *BAFF* receptors is initiated, and as a result TRAF3 is recruited to the intracellular part of the receptors. Thus, the NIK-TRAF2/3-cIAP1/2 is exposed, and TRAF3 can be degraded by proteasomes. NIK, that is now free, accumulates and phosphorylates IKK1, which in turn phosphorylates NF-κB2 p100. NF-κB2 is ubiquitinated by βTrCP, it binds to the proteasome and cleaves the p100 precursor into the p52 form. This then, binds to relB and moves to the nucleus where gene regulation is happening[37]. At the same time, *BAFF* can induce activity of the PI3K pathway[58].

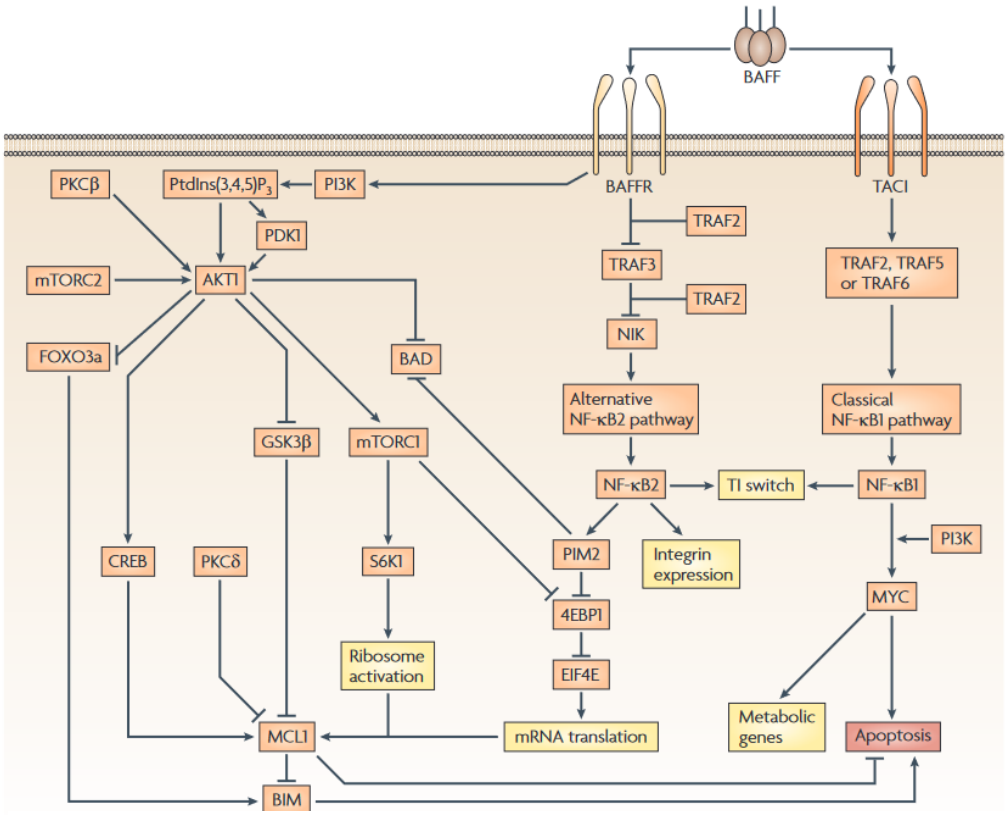


Figure 10: Signaling cascades through *BAFF* receptors.

Source: MacKay, F. & Schneider, P. Cracking the BAFF code. Nat. Rev. Immunol. 9, 491–502 (2009).

1.10 BAFF SNPs

In addition to the examination of serum *BAFF* levels, which have been correlated with active forms of diseases, several genetic variants of the gene have also been investigated for their association with various diseases. In the next sections, three SNPs of *BAFF* are presented, which were chosen to be studied in the context of sarcoidosis, with the criteria being their previous association with autoimmune and autoimmune-like diseases, and the absence of evidence that they have been studied before for sarcoidosis in a Caucasian population.

1.10.1 rs2893321

rs2893321 is a SNP located at intron 3 of the *BAFF* gene. Lin et al. have associated this polymorphism with the development Grave's disease (GD) and autoimmune thyroid diseases, especially in the female gender , when the SNP was studied in an ethnic Chinese population[59]. In another study focusing on GD, the AA genotype of rs2893321 in women was linked to higher *BAFF* mRNA and protein levels, a finding which shows that this SNP may have a role in *BAFF* mRNA and protein production[60]. Myasthenia Gravis is another autoimmune disease where rs2893321 is implicated, again in a gender-specific manner[61]. Lastly, it could be a predictor of Graft versus host disease (GvHD) subtypes and of the recipient's response to rituximab[62].

1.10.2 rs1041569

The heterozygosity of the *BAFF* promoter SNP rs1041569 has been speculated to have a protective role in chronic lymphocytic leukemia (CLL)[63], while a possible association of the SNP with lupus related atherosclerotic risk has also been found[44]. Moreover, susceptibility to Crohn's Disease (CD) seems to be strongly influenced by rs1041569[43].

1.10.3 rs9514828

Another promoter SNP, rs9514828, has been associated with a higher risk for familial lymphoproliferative disorders [64], while it could also serve as a predictor for the survival of patients with T-cell lymphomas[65]. The response of rheumatoid arthritis patients to rituximab also seems to be determined by this SNP, either when examining the mutation alone or in combination with alleles of other polymorphisms[66],[67]. Furthermore, in patients with SLE, rs9514828 seems to increase *BAFF* gene expression, which makes it a potential follow-up marker of SLE, since elevated *BAFF* levels imply that the disease is active[68].

1.11 BAFF-R SNPs

Next follows a short description of the *BAFF-R* SNP that was selected, using the same criteria as the *BAFF* SNPs. Since *BAFF-R* is involved in the signaling cascades of several pathways, it was deemed necessary to examine the influence of this gene's variants in sarcoidosis susceptibility.

rs61756766

This *BAFF-R* SNP, rs61756766, was first identified in tumor and germline tissue of non-Hodgkin lymphoma patients and appears to cause increased NF-κB activation when the mutated genotype is present[69]. Higher mRNA and protein expression of NF-κB was also detected in patients with Sjogren's syndrome (SS), and the mutation was more frequently found in those with SS and MALT lymphoma[70]. The variant has also been proposed to be a risk factor for CLL[63], and more recently, the heterozygous genotype has been found to appear with increased frequency in patients suffering from severe COVID-19[71].

1.12 APRIL SNPs

Although serum *APRIL* levels do not correlate to those of *BAFF*, they have been found to be elevated in numerous disorders, and the fact that this protein is part of many immune functions that could underlie pathogenesis of a disease, led to the investigation of the following SNP.

rs11552708

Koyama et al. discovered a polymorphism, rs1152708, located at the extracellular domain of *APRIL*, which was significantly associated with SLE in a Japanese population. The mutation results in a Gly to Arg substitution that would alter the structure and function of *APRIL*, either by inserting a new furin cleavage site, by changing the binding affinity to receptors or by modifying the reverse signal transmitted through the TNF family ligands in their membrane form[72]. This SNP has been studied for its association with SLE in Chinese populations[73], where no association was found. SLE in African-American, Hispanic and Japanese populations however, seems to be linked with rs1152708[74]. Therefore, we chose to study this polymorphism of *APRIL* in the context of sarcoidosis, given the common basis of autoimmunity that underlies both sarcoidosis and SLE, and the potential involvement of *APRIL* in the immunopathogenesis of the disease.

2. AIM OF THE THESIS

Considering that the genetic basis of sarcoidosis is greatly undiscovered, and that the aforementioned SNPs have been previously linked with autoimmune and inflammatory disorders, the aim of this thesis is to investigate the potential association of *BAFF* SNPs rs2893321, rs1041569, rs9514828, *BAFF-R* SNP rs61756766 and *APRIL* SNP rs1152708 with susceptibility to sarcoidosis in Greek cohort of patients. A secondary aim is to uncover putative biomarkers for the detection of cardiac sarcoidosis, given the high percentage of asymptomatic patients. This will be the first time that the particular polymorphisms will be studied for their relationship with sarcoidosis in a European population, making this work potentially a useful addition to the genetic knowledge of this complex disease.

3. MATERIALS AND METHODS

3.1 Sampling

For the purposes of this study peripheral blood samples from 173 patients with sarcoidosis were acquired. The subjects were recruited from the Outpatient Department of Respiratory Medicine, "Attikon" University Hospital, Athens, Greece. They were of Greek origin, and were diagnosed based on clinical and radiological criteria, having first excluded that granulomatosis resulted from a different possible cause[75]. In order to evaluate the presence of cardiac involvement in these patients the modified criteria of the Japanese Ministry of Health and Welfare were used[76].

Similarly, 164 peripheral blood samples were collected as healthy controls from of healthy volunteers recruited to attend a health survey at Aeginition Hospital, National and Kapodistrian University of Athens, Athens, Greece. All subjects signed an informed consent regarding their participation in the study and the study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the participating centers.

The clinicopathological characteristics of both groups, sarcoidosis patients and healthy controls are listed at the following table (Table 2).

Table 2: Clinicopathological characteristics of sarcoidosis patients and healthy controls.

	Sarcoidosis patients (n=173)	**Healthy Controls (n=164)**
Male/Female	72/101	68/96
Age at diagnosis (years), mean ±SD	51.5±13.99	
Smokers	55	42
Löfgren's Syndrome	5	
Chest radiographic stage		
0	5	
I	55	
II	69	
III	28	
IV	14	

3.2 Genotyping

3.2.1 DNA extraction

Firstly, genomic DNA from peripheral blood samples was extracted using the Nucleospin Blood Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). A volume of 200 μl per sample was lysed by adding 25 μl of Proteinase K and 200 μl B3 buffer to the eppendorf tube. The mix was vortexed and incubated at 70 °C for 15 minutes. Then, 210 μl of ethanol (100%) was added to each sample, the tubes were vortexed and the samples were loaded into NucleoSpin® Blood Columns, which were placed in Collection Tubes. After centrifugation at 11,000 x g for 1 minute all collection tubes containing the flow-through were discarded, and the columns were placed into new collection tubes. The next steps involved two washes of the silica membrane, first by the addition of 500 μl of BW buffer to the column, centrifugation at 11,000 x g for 1 minute and discarding the collection tube, and secondly by the addition of 600 μl of B5 buffer, and again centrifugation at 11,000 x g for 1 minute. The flow-through was discarded, the column was placed back to the collection tube and centrifuged at 11,000 x g for 1 minute, so as to dry the silica membrane. Finally, the column was placed into an 1.5 mL eppendorf tube, 100 μl of elution buffer that was preheated at 70 °C was added directly onto the membrane and with a last 1 minute centrifugation at 11,000 x g the DNA was eluted. The quality and concentration of the eluted DNA was evaluated using a spectrophotometer and checking the A_{260} / A_{280} ratio. All DNA samples were stored at -20 °C for later use.

3.2.2. Polymerase Chain Reaction-Restriction fragment length polymorphism (PCR-RFLP)

For the determination of the samples' genotype the method of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was selected. The SNPs for which the samples were genotyped with this method were the three SNPs of the *BAFF* gene (rs2893321, rs1041569 and rs9514828), as well as the *BAFF-R* polymorphism (rs61756766).

Each PCR reaction was set up in a total volume of 50 μl using 5 μl DNA, 5 μl Taq Buffer A (Kapa Biosystems, USA), 1 μl dNTPs (200μM), 0.25 μl primers (0.5μM), 0.25 Taq Polymerase (Kapa Biosystems ,USA) and 38.25 μl H20. All reactions were run under the following conditions: initial denaturation at 95°C for 5 minutes, then 35 cycles of denaturation at 95°C for 35 seconds, annealing for rs2893321 at 55 °C (rs2893321) for 35 seconds (rs2893321) or at 58°C for 45 seconds (rs1041569 and rs9514828), extension at 72°C for 35 seconds, and a final extension at 72°C for 5 minutes. The primers (Eurofins Genomics AT GmbH, Vienna, Austria) that were used for all reactions are derived from former studies[59,77,78], and are listed in Table 3.

Table 3: Primer sequences and Tm of each primer set used for genotyping rs2893321, rs1041569, rs9514828 and rs61756766.

SNP	Primer Sequence (5'-3')	Tm(°C)
rs2893321	F: TTTTTCGTTGGACTTGGTCA R: CAACCCAAATCCAGAATCCT	55
rs1041569	F:ATTCCCTGTCTTCAGAATTTTCTCT R:CCTATAACTCCCACAATAAGGTGAC	58
rs9514828	F: TTGTACACCGACCTGTTAGGC R: TGGAAGTAAGTCCACTGGGAAT	58
rs61756766	F: CCTCCAGAGGAGTCTTCTAG R: TCCAAGCCCCTGGCTGGG	57

After the reactions were done and the desired DNA segments were amplified, the PCR products were digested with restriction enzymes at 37°C overnight. The restriction enzymes used were chosen, so as to have their restriction site on the same site of the studied polymorphism and produce a different number of smaller segments, depending on the alleles that are present. In order to visualize the digestion products electrophoresis in 3% agarose gels stained with Gel Red (Biotium, USA) was performed. The restriction enzymes for each SNP and the products of enzyme digestion are listed in the following table (Table 4).

Table 4: Base changes, restriction enzymes and digested products of each *BAFF* and *BAFF-R* polymorphism.

SNP	Base Change	Restriction Enzyme	Digested Products(bp)
rs2893321	A/G	AseI	AA: 121,62 AG: 183,121,62 GG: 183
rs1041569	A/T	DpnII	AA: 207,167,94 AT: 261,207,167,94 TT: 261,207
rs9514828	C/T	AciI	CC: 262,131 TC: 392,262,131 TT: 392
rs61756766	C/T	EaeI	CC: 178,70,62 CT: 310,178,70,62 TT: 310

3.2.3 Allele-Specific PCR

For the *APRIL* SNP, rs11552708, the literature search yielded no primer sequences, therefore new ones had to be designed. The polymorphism's locus does not contain any known enzyme's restriction site, and that is why Allele-Specific PCR was chosen as a method to study rs11552708.

The primers (Eurofins Genomics AT GmbH, Vienna, Austria) that were used had the following sequences:

R: 5'-CCTTTAACAGTTTCCTTTCCT-3'

F1: 5'-AGGGGCTCCTGTCAT-3'

F2'-5'-AGGGGCTCCTGTCAC-3'

They were designed, so that the common reverse primer would hybridize with the 5'- end of the DNA segment chosen to be amplified, and that the two forward primers would differ only at their last nucleotide of their sequence, which also happens to be the polymorphic site. Therefore, for each sample two PCR reactions were made, one for each allele, containing the common reverse primer and one of the two forward primers depending on which allele was studied.

All PCR reactions were set up in a total volume of 50 μl using 5 μl DNA, 5 μl Taq Buffer A (Kapa Biosystems, USA), 1 μl dNTPs (200μM), 0.25 μl primers (0.5μM), 0.25 Taq Polymerase (Kapa Biosystems, USA) and 38.25 μl H20. The reactions were run under the following conditions: initial denaturation at 95°C for 5 minutes, then 35 cycles of denaturation at 95°C for 35 seconds, annealing at 67 °C for 35 seconds, extension at 72°C for 35 seconds, and a final extension at 72°C for 5 minutes. The PCR products were visualized by electrophoresis in 3% agarose gels stained with Gel Red (Biotium, USA).

Since many unspecific bands appeared to have been amplified in reactions for both alleles, A and G, gradient PCR was performed in a temperature range from 62°C to 67°C. The best results were acquired at 67°C, without, however, eliminating all unspecific bands. In another attempt to correct the issue Taq Buffer B (Kapa Biosystems, USA) was used, instead of Taq Buffer A. Some reactions were also set up with the addition of 5% and 10% DMSO. None of these attempts unfortunately increased the band specificity sufficiently enough, therefore no data could be collected for rs11552708 that would produce a reproducible conclusion.

3.3 Statistical Analysis

The samples as presented in 3.1 were first divided into two categories; sarcoidosis patients and control group, and for further analysis they were divided again into sarcoidosis patients with and without cardiac involvement. All control group samples were tested for departure from Hardy-Weinberg equilibrium, and genotypic and haplotype frequencies for each SNP and gene accordingly were determined and compared using SNPStats[79]. P-Values of less than 0.05 were considered to be significant.

4. RESULTS

The determination of each sample's genotype by the methods described in the above sections, led to the accumulation of the data needed to extract the genotypic frequencies of the *BAFF* and *BAFF-R* polymorphisms (Table 5). All frequencies conformed to Hardy–Weinberg equilibrium (p>0.05).

Table 5: rs2893321, rs9514828, rs1041569 (*BAFF*) and rs61756766 (*BAFF-R*) polymorphism distribution in patients with sarcoidosis.

SNPs	**Sarcoidosis(%) n = 173**	**Controls(%) n = 164**	**P [OR; (95%CI)]**
rs2893321 (*BAFF*)			
AA (wt)	74 (42.77)	87 (53.05)	1
AG	75 (43.35)	59 (35.98)	0.10 [1.49; (0.94-2.37)]
GG	24 (13.87)	18 (10.97)	0.23 [1.57; (0.79-3.11)]
Alleles			
A	223 (64.45)	233 (71.04)	1
G	123 (35.55)	95 (28.96)	0.07 [1.35; (0.98-1.87)]
rs9514828 (*BAFF*)			
CC (wt)	24 (13.87)	32 (19.51)	1
TC	66 (38.15)	71 (43.29)	0.53 [1.24; (0.66-2.32)]
TT	83 (47.98)	61 (37.19)	0.08 [1.81; (0.97-3.38)]
Alleles			
C	114 (32.95)	135 (41.16)	1
T	232 (67.05)	193 (58.84)	0.03 [1.42; (1.04-1.95)]
rs1041569 (*BAFF*)			
AA (wt)	73 (42.20)	85 (51.83)	1
AT	70 (40.46)	61 (37.19)	0.24 [1.34; (0.84-2.13)]
TT	30 (17.34)	18 (10.97)	0.07 [1.94; (1-3.77)]
Alleles			
A	216 (62.43)	231 (70.43)	1
T	130 (37.57)	97 (29.57)	0.03 [1.43; (1.04-1.98)]
rs61756766 (*BAFF-R*)			
CC (wt)	118 (68.20)	128 (78.05)	1
CT	48 (27.75)	31 (18.90)	0.05 [1.68; (1-2.81)]
TT	7 (4.05)	5 (3.05)	0.56 [1.52; (0.47-4.92)]

Alleles			
C	284 (82.08)	287 (87.50)	1
T	62 (17.92)	41 (12.50)	0.05 [1.53; (0.99 – 2.34)]

4.1 Genotypic and allelic frequencies of *BAFF* SNPs

4.1.1 rs2893321

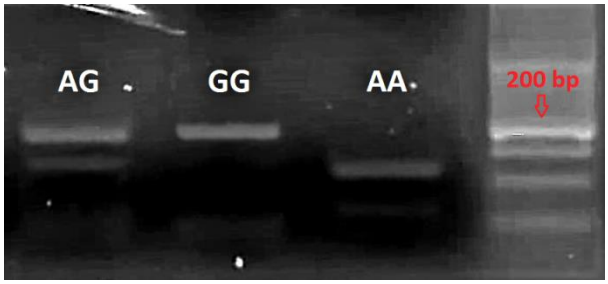
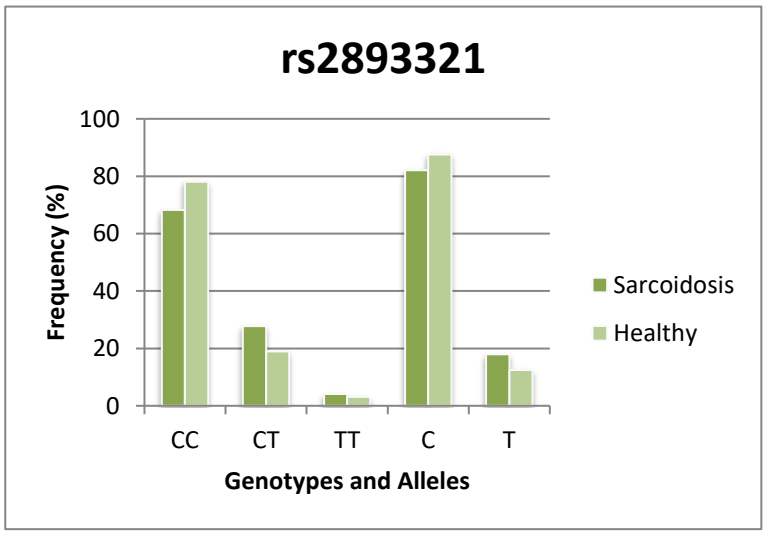


Figure 11 : Representative results of rs2893321 genotyping on a 3% agarose gel, stained with Gel Red.

Beginning with the *BAFF* SNPs, genotyping of rs2893321 resulted in the visualization of bands, a representative image of which can be seen on the left (Figure 11). The ladder that was used was a 50 bp step ladder (Nippon Genetics Europe, Düren, Germany), so that the smallest fragment of 62bp would be able to be discriminated after digestion.

According to the calculated frequencies for rs2893321, no significant difference between patients with sarcoidosis and controls was found. All genotypes of the polymorphism were detected in similar frequencies in both groups, and as for the allelic frequencies, the G allele was slightly more common in sarcoidosis patients, although with no statistical significance.



4.1.2 rs9514828

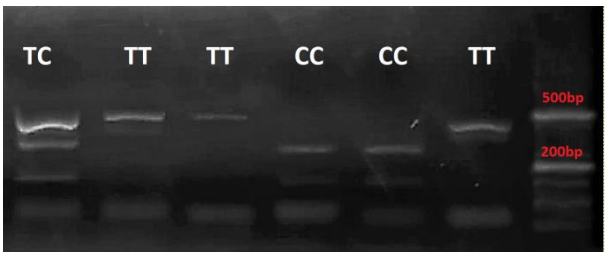
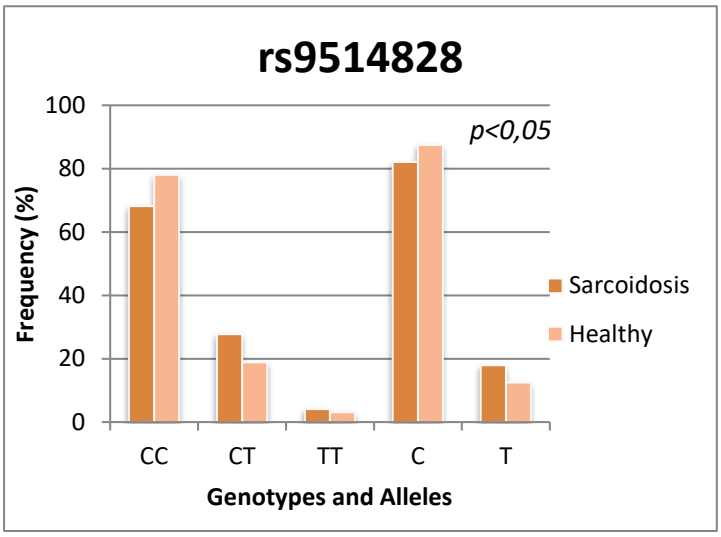


Figure 12: Representative results of rs9514828 genotyping on a 3% agarose gel, stained with Gel Red.

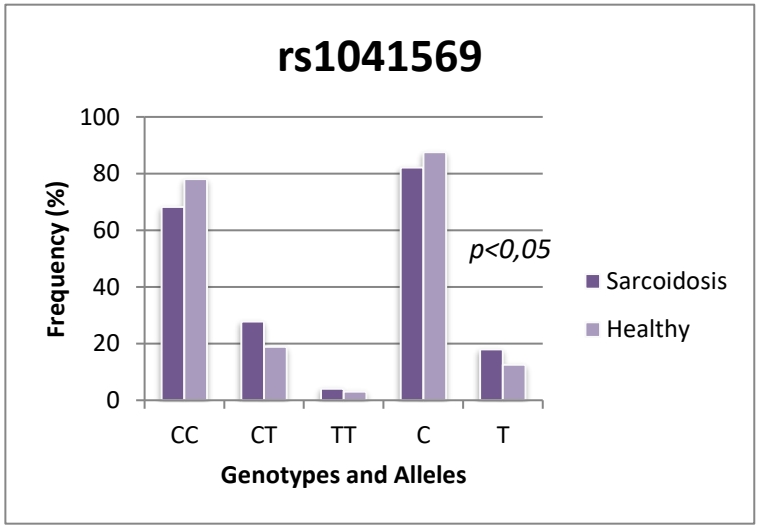
For the second *BAFF* SNP studied, rs9514828, representative products of digestion can be observed in Figure 12.

Similar to rs28932321, no statistically significant relationship can be deduced from the acquired genotypic frequencies. The three genotypes have comparable frequencies, it is noteworthy however, that the TT genotype appears to be slightly more common in sarcoidosis patients (*p=0.08*). When examining allelic frequencies, a statistically significant difference was found for the T allele, which is over-represented in sarcoidosis patients (*p=0.03*).



4.1.3 rs1041569

In the case of rs1041569, again no significant relationship between any of the three genotypes and sarcoidosis was found. On the contrary, comparison of allelic frequencies revealed an association of the T allele with sarcoidosis, which was statistically significant (*p=0.03*).



4.2 Genotypic and allelic frequencies of *BAFF-R* SNP rs61756766

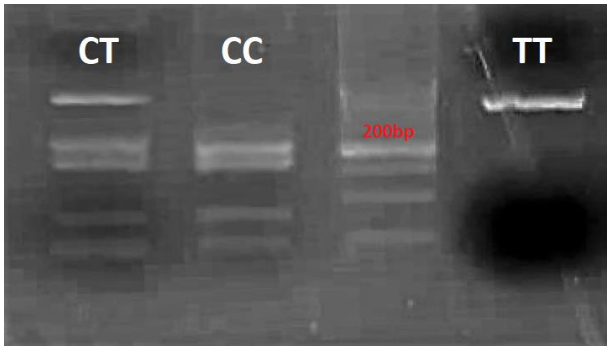
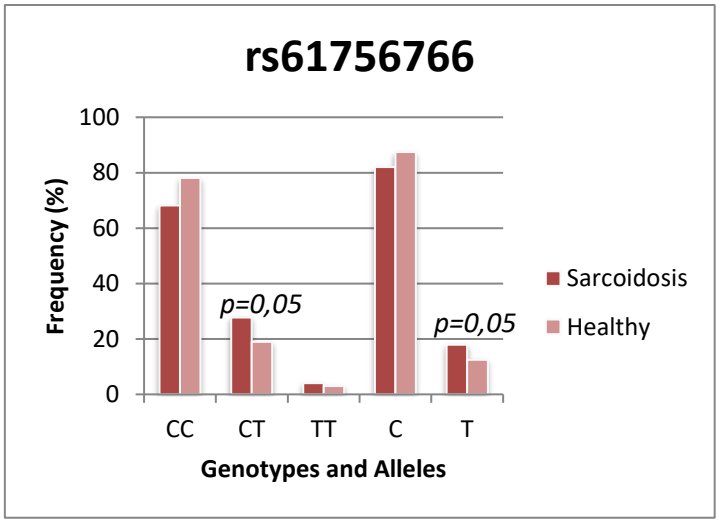


Figure 13: Representative results of rs61756766 genotyping on a 3% agarose gel, stained with Gel Red.

In Figure 13 is a representative gel image of the bands produced for rs61756766 genotyping. Although the homozygous genotypes CC and TT do not seem to be correlated with the disease, the heterozygous genotype was found more frequently in patients. The CT genotype was found in 27.5% of patients and 18.9% of the controls, which shows there is a marginally statistically significant relationship with sarcoidosis (*p=0.05*). The exact statistical significance was found for the association of the T allele and the disease (*p=0.05*).



4.3 Genotypic and allelic frequencies of *APRIL* SNP rs11552708

Due to the lack of clear gel bands and trustworthy data for rs11552708, the *APRIL* polymorphism was not included in the analysis. In a representative image of the agarose below, the multiple unspecific bands that led to the rejection of the data can be seen.

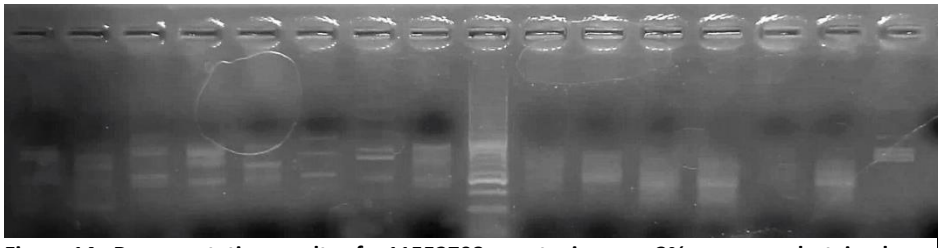


Figure 14: Representative results of rs11552708 genotyping on a 3% agarose gel, stained with Gel Red.

4.4 Genotypic and allelic frequencies of *BAFF* and *BAFF-R* SNPs in patients with cardiac involvement

A second stratification of patients resulted in the splitting of sarcoidosis patients in those with and without cardiac involvement. The genotypic and allelic frequencies that resulted from the analysis imply no significant association between the studied *BAFF* and *BAFF-R* SNPs and cardiac sarcoidosis (Table 6).

Table 6: Genotypic frequencies of rs2893321, rs9514828, rs1041569 (*BAFF*) and rs61756766 (*BAFF-R*) in sarcoidosis patients, with or without cardiac involvement.

SNPs	**Patients with cardiac involvement (n=42)**	**Patients without cardiac involvement (n=131)**	**OR; (95%CI)**
rs2893321 (*BAFF*)			
AA (wt)	20(47.6)	54(41.2)	1
AG	15(35.7)	60(45.8)	0.67; (0.31-1.45)
GG	7(16.7)	17(13)	1.11; (0.40-3.08)
Alleles			
A	55 (65)	168 (64)	
G	29 (35)	94 (36)	
rs9514828 (*BAFF*)			
CC (wt)	4(9.5)	20(15.3)	1
TC	15(35.7)	51(38.9)	0.77; (0.36-1.62)
TT	23(54.8)	60(45.8)	0.52; (0.16-1.69)
Alleles			
C	23(27)	91(35)	
T	61(73)	171(65)	
rs1041569 (*BAFF*)			
AA (wt)	14(33.3)	59(45)	1
AT	19(45.2)	51(38.9)	1.57; (0.72-3.44)
TT	9(21.4)	21(16)	1.81; (0.68-4.79)
Alleles			
A	47(56)	169(65)	
T	37(44)	93(35)	
rs61756766 (*BAFF-R*)			
CC (wt)	28(66.7)	90(68.7)	1
CT	12(28.6)	36(27.5)	1.07; (0.49-2.33)
TT	2(4.8)	5(3.8)	1.29; (0.24-6.99)

Alleles			
C	68(81)	216(82)	
T	16(19)	46(18)	

4.5 Haplotype analysis of *BAFF*

Since three SNPs of the same gene, *BAFF*, were studied, haplotype analysis was conducted using the second grouping of patients, involving those with and without cardiac involvement. Through this analysis 8 haplotypes were formed, of which the ATT, GTA and GTT haplotypes frequencies were significantly higher in patients with cardiac involvement compared to patients without (*p=0.012*, *p=0.013* and *p=0.024* respectively).

Table 7: *BAFF* haplotypes and their frequency in patients with or without cardiac involvement.

Haplotype	**Polymorphism**			**Cardiac Involvement** (n=42)	**No cardiac Involvement** (n=131)	**OR** (95% CI)	**P-value**
	rs2893321	rs9514828	rs1041569				
1	A	T	A	0.22	0.31	1.00	
2	A	C	A	0.17	0.19	0.70(0.40-1.23)	0.21
3	A	T	T	0.18	0.12	0.43(0.23-0.83)	0.012
4	G	T	A	0.18	0.12	0.44(0.23-0.84)	0.013
5	A	C	T	0.07	0.10	1.06(0.51-2.18)	0.88
6	G	C	A	0.05	0.09	1.25(0.58-2.68)	0.57
7	G	T	T	0.09	0.04	0.39(0.17-0.88)	0.024
8	G	C	T	0.04	0.04	0.67(0.23-1.92)	0.46

5. DISCUSSION

The aim of this work was to uncover the putative associations among SNPs of 3 innate immune system genes, *BAFF*, *BAFF-R* and *APRIL*, with sarcoidosis. The calculation and comparison of genotypic frequencies showed no correlation between the studied *BAFF* SNPs, rs2893321, rs9514828 and rs1041569 and the disease. When allelic frequencies were compared however, an interesting finding was that the T allele in both rs1041569 and rs9514828 appeared more frequently in the group of patients, compared to healthy controls. The examination of the *BAFF-R*, rs61756766, led to the discovery of an over-representation of the CT genotype, as well as of the T allele in sarcoidosis cases, which was of marginal significance. As for rs11552708, a polymorphism of *APRIL*, the data generated were not clear enough, hence the exclusion of rs11552708 from the analysis. The patient cohort was then further separated into groups of patients with and without cardiac involvement, though no correlation of any SNP and cardiac sarcoidosis arouse. Lastly, the acquisition of allelic frequencies of 3 *BAFF* SNPs allowed the conduction of haplotype analysis, the results of which showed an increased frequency of haplotypes ATT, GTA and GTT in patients with the cardiac manifestation of the disease.

All the SNPs examined herein, have never been studied for their association with sarcoidosis before. They have, on the other hand, been associated with several autoimmune disorders in previous studies. Although in this cohort rs2893321 is not associated with sarcoidosis, this SNP has been linked to the development of Myasthenia Gravis[61] and Grave's disease[59]. It should be noted though, that in both cases rs2893321 affected disease development in a sex-specific manner, and most importantly, both studies sampled Chinese populations.

Continuing with the *BAFF* SNPs, the T allele of rs9514828 has been found to be significantly associated with sarcoidosis in the present study. This allele has been previously identified as a characteristic of a high risk group for B-cell lymphoma development in Sjogren's syndrome[77]. It is noteworthy that the T allele has been suggested to increase susceptibility in a cohort of Greek SLE patients[44], thus confirming the potential significance of this SNP's T allele in autoimmune and auto-inflammatory disorders in the Greek population. Nevertheless, when the same polymorphism was

studied in a Mexican SLE cohort neither genotypic nor allelic associations were detected[68]. These contradictions highlight the importance of investigating the effect of genetic variants in the same disease in a variety of ethnic groups, and the possibility that rs9514828 could serve as a risk factor for sarcoidosis in other ethnicities, as well.

In a similar fashion, the T allele of rs1041569 has been found to be significantly associated with sarcoidosis in the present study. Faustova et al., have reported a significantly higher frequency of the particular allele in patients with Idiopathic Inflammatory Myopathies. More specifically they reported that the allele was present in 18% of patients and 12% of healthy controls with a p-value of 0.029, which is extremely similar to the statistical significance in this study (*p=0.03*) [80]. In the same study, an increased frequency of the AT genotype was observed[80]. The same heterozygous genotype appears to have a strong correlation with CD[43], as well as a protective role in CLL[68]. This comes in contrast with the findings of this study, since the AT genotype of rs1041569 is clearly not associated with sarcoidosis, while the genotype homozygous for the T allele tends to show a more marginally significant relationship.

As for rs61756766, the *BAFF-R* SNP, the CT genotype and the T allele are suggested to have a correlation with sarcoidosis, as their frequency was found to be higher in patients compared to controls, with marginal significance (*p=0.05*). Other studies which align with these results have detected a strong link of rs61756766 with Sjogren's syndrome [70], and of the CT genotype with higher susceptibility to CLL[81]. The significance of this variant lies on the fact that it is hypothesized to induce the continuous activation of NF-κB pathways, by altering the *BAFF* and *BAFF-R* mediated signaling[69]. A common characteristic of active sarcoidosis is the elevation of *BAFF* serum levels[48] which, when combined with aberrant *BAFF-R* signaling, could make this mutation a contributor to the defective B cell signaling of the disease.

This research also focuses on the cardiac involvement of sarcoidosis, for which little is known, especially on the effect of genetic variations to this type of manifestation. An examination of genetic markers across the HLA region has revealed an association between the HLA-DQB1*0601 allele and cardiac sarcoidosis in a Japanese group of patients[82], while similar conclusions were made for TNFa SNPs[83]. In a cohort of Greek patients, it was previously shown that TNFa gene polymorphisms

could be involved in the genetic susceptibility of cardiac involvement [84]. Such a conclusion cannot be made for the *BAFF* and *BAFF-R* SNPs, since the stratification of patients into those with and without cardiac involvement, and the comparison of genotypic frequencies showed no association. What was found however, was a higher prevalence of 3 *BAFF* haplotypes (ATT,GTA,GTT) in cardiac sarcoidosis patients, which indicates that the possibility of *BAFF* influencing the genetic predisposition of cardiac sarcoidosis development is not to be excluded.

In conclusion, the results of this study make it evident that *BAFF* and *BAFF-R* could be utilized as biomarkers for sarcoidosis. Although each SNP on its own doesn't seem to have a predictive value for cardiac involvement, the significant association of 3 *BAFF* haplotypes with cardiac sarcoidosis suggests that when in combination, *BAFF* SNPs could be used as predictors. Undoubtedly, genotyping a larger cohort would provide with even more trustworthy results, and could shed light into the marginal associations that were found with the samples available.

6. REFERENCES

1. Gaddam, M. *et al.* Sarcoidosis: Various Presentations, Coexisting Diseases and Malignancies. *Cureus* **13**, (2021).

2. Arkema, E. V. & Cozier, Y. C. Epidemiology of sarcoidosis: current findings and future directions. *Therapeutic Advances in Chronic Disease* **9**, 227–240 (2018).

3. Hunninghake, G. W. *et al.* Statement on sarcoidosis. *American Journal of Respiratory and Critical Care Medicine* **160**, 736–755 (1999).

4. Karakatsani, A. *et al.* Epidemiology of interstitial lung diseases in Greece. *Respir. Med.* **103**, 1122–1129 (2009).

5. Sanchez, M., Haimovic, A. & Prystowsky, S. Sarcoidosis. *Dermatol. Clin.* **33**, 389–416 (2015).

6. Lill, H., Kliiman, K. & Altraja, A. Factors signifying gender differences in clinical presentation of sarcoidosis among Estonian population. *Clin. Respir. J.* **10**, 282–290 (2016).

7. Grunewald, J. *et al.* Sarcoidosis. *Nat. Rev. Dis. Prim.* **5**, (2019).

8. Bonifazi, M. *et al.* Sarcoidosis and cancer risk: Systematic review and meta-analysis of observational studies systematic review and meta-analysis of observational studies. *Chest* **147**, 778–791 (2015).

9. Spector, W. G. EPITHELIOID CELLS, GIANT CELLS, AND SARCOIDOSIS. *Ann. N. Y. Acad. Sci.* **278**, 3–6 (1976).

10. Sakthivel, P. & Bruder, D. Mechanism of granuloma formation in sarcoidosis. *Curr. Opin. Hematol.* **24**, 59–65 (2017).

11. Tana, C. *et al.* Challenges in the diagnosis and treatment of neurosarcoidosis. *Annals of Medicine* **47**, 576–591 (2015).

12. Ungprasert, P. *et al.* Epidemiology of Sarcoidosis 1946-2013: A Population-Based Study. *Mayo Clin. Proc.* **91**, 183–188 (2016).

13. Ungprasert, P., Crowson, C. S., Carmona, E. M. & Matteson, E. L. Outcome of pulmonary sarcoidosis: A population-based study 1976-2013. *Sarcoidosis Vasc. Diffus. Lung Dis.* **35**, 123–128 (2018).

14. Nery, P. B. *et al.* Atrioventricular block as the initial manifestation of cardiac sarcoidosis in middle-aged adults. *J. Cardiovasc. Electrophysiol.* **25**, 875–881

(2014).

15. Iwai, K., Takemura, T., Kitaici, M., Kawabata, Y. & Matsui, Y. Pathological studies on sarcoidosis autopsy. II. Early change, mode of progression and death pattern. *Pathol. Int.* **43**, 377–385 (1993).

16. Sève, P. *et al.* Sarcoidosis: A clinical overview from symptoms to diagnosis. *Cells* **10**, (2021).

17. Ohira, H. *et al.* Myocardial imaging with 18F-fluoro-2-deoxyglucose positron emission tomography and magnetic resonance imaging in sarcoidosis. *Eur. J. Nucl. Med. Mol. Imaging* **35**, 933–941 (2008).

18. Ungprasert, P., Ryu, J. H. & Matteson, E. L. Clinical Manifestations, Diagnosis, and Treatment of Sarcoidosis. *Mayo Clin. Proc. Innov. Qual. Outcomes* **3**, 358–375 (2019).

19. Mañá, J. *et al.* Lofgren's syndrome revisited: A study of 186 patients. *Am. J. Med.* **107**, 240–245 (1999).

20. Ungprasert, P., Tooley, A. A., Crowson, C. S., Matteson, E. L. & Smith, W. M. Clinical Characteristics of Ocular Sarcoidosis: A Population-Based Study 1976–2013. *Ocul. Immunol. Inflamm.* **27**, 389–395 (2019).

21. Bargagli, E. & Prasse, A. Sarcoidosis: a review for the internist. *Intern. Emerg. Med.* **13**, 325–331 (2018).

22. Calender, A., Weichhart, T., Valeyre, D. & Pacheco, Y. Current insights in genetics of sarcoidosis: Functional and clinical impacts. *Journal of Clinical Medicine* **9**, 1–21 (2020).

23. Valentonyte, R. *et al.* Sarcoidosis is associated with a truncating splice site mutation in BTNL2. *Nat. Genet.* **37**, 357–364 (2005).

24. Tong, X. *et al.* The BTNL2 G16071A gene polymorphism increases granulomatous disease susceptibility: A meta-analysis including FPRP test of 8710 participants. *Med. (United States)* **95**, (2016).

25. Rossman, M. D. *et al.* HLA-DRB1*1101: A Significant Risk Factor for Sarcoidosis in Blacks and Whites. *Am. J. Hum. Genet.* **73**, 720–735 (2003).

26. Fischer, A. & Rybicki, B. A. Granuloma genes in sarcoidosis: What is new? *Current Opinion in Pulmonary Medicine* **21**, 510–516 (2015).

27. Newman, L. S. *et al.* A case control etiologic study of sarcoidosis:

Environmental and occupational risk factors. *Am. J. Respir. Crit. Care Med.* **170**, 1324–1330 (2004).

28. Esteves, T., Aparicio, G. & Garcia-Patos, V. Is there any association between Sarcoidosis and infectious agents?: A systematic review and meta-analysis. *BMC Pulm. Med.* **16**, 165 (2016).

29. Facco, M. *et al.* Sarcoidosis is a Th1/Th17 multisystem disorder. *Thorax* **66**, 144–150 (2011).

30. Miyara, M. *et al.* The immune paradox of sarcoidosis and regulatory T cells. *J. Exp. Med.* **203**, 359–370 (2006).

31. Lecossier, D. *et al.* Antigen-induced Proliferative Response of Lavage and Blood T Lymphocytes: Comparison of Cells from Normal Subjects and Patients with Sarcoidosis. *Am. Rev. Respir. Dis.* **144**, 861–868 (1991).

32. Shamaei, M. *et al.* Evidence for M2 macrophages in granulomas from pulmonary sarcoidosis: A new aspect of macrophage heterogeneity. *Hum. Immunol.* **79**, 63–69 (2018).

33. Lee, N. S. *et al.* Disturbed homeostasis and multiple signaling defects in the peripheral blood B-cell compartment of patients with severe chronic sarcoidosis. *Clin. Vaccine Immunol.* **18**, 1306–1316 (2011).

34. Fazel, S. B., Howie, S. E. M., Krajewski, A. S. & Lamb, D. B lymphocyte accumulations in human pulmonary sarcoidosis. *Thorax* **47**, 964–967 (1992).

35. Dasilva, V., Breuil, V., Chevallier, P. & Euller-Ziegler, L. Relapse of severe sarcoidosis with an uncommon peritoneal location after TNFα blockade. Efficacy of rituximab, report of a single case. *Joint Bone Spine* **77**, 82–83 (2010).

36. Schneider, P. *et al. BAFF*, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J. Exp. Med.* **189**, 1747–1756 (1999).

37. Smulski, C. R. & Eibel, H. *BAFF* and *BAFF-R*eceptor in B cell selection and survival. *Frontiers in Immunology* **9**, 2285 (2018).

38. Nicoletti, A. M. *et al.* Unexpected potency differences between B-Cell-Activating Factor (*BAFF*) Antagonist antibodies against various forms of *BAFF*: Trimer, 60-Mer, and membrane-bounds. *J. Pharmacol. Exp. Ther.* **359**, 37–44 (2016).

39. MacKay, F. & Schneider, P. Cracking the *BAFF* code. *Nat. Rev. Immunol.* **9**, 491–502 (2009).

40. Mackay, F. *et al.* Mice transgenic for *BAFF* develop lymphocytic disorders along with autoimmune manifestations. *J. Exp. Med.* **190**, 1697–1710 (1999).

41. Gavin, A. L., Aït-Azzouzene, D., Ware, C. F. & Nemazee, D. δ*BAFF*, an alternate splice isoform that regulates receptor binding and biopresentation of the B cell survival cytokine, *BAFF*. *J. Biol. Chem.* **278**, 38220–38228 (2003).

42. Lahiri, A. *et al.* The complexity of the *BAFF* TNF-family members: Implications for autoimmunity. *J. Autoimmun.* **39**, 189–198 (2012).

43. Andreou, N. P. *et al.* B-cell activating factor (*BAFF*) expression is associated with Crohn's disease and can serve as a potential prognostic indicator of disease response to Infliximab treatment. *Dig. Liver Dis.* **53**, 574–580 (2021).

44. Theodorou, E. *et al.* B-cell activating factor and related genetic variants in lupus related atherosclerosis. *J. Autoimmun.* **92**, 87–92 (2018).

45. Kannel, K. *et al.* Changes in blood B cell-activating factor (*BAFF*) levels in multiple sclerosis: A sign of treatment outcome. *PLoS One* **10**, 1–16 (2015).

46. Ueda-Hayakawa, I. *et al.* Elevated serum *BAFF* levels in patients with sarcoidosis: Association with disease activity. *Rheumatol. (United Kingdom)* **52**, 1658–1666 (2013).

47. Ando, M. *et al.* Significant elevation of the levels of B-cell activating factor (*BAFF*) in patients with sarcoidosis. *Clin. Rheumatol.* **37**, 2833–2838 (2018).

48. Saussine, A. *et al.* Active chronic sarcoidosis is characterized by increased transitional blood B cells, increased IL-10-producing regulatory B cells and high *BAFF* levels. *PLoS One* **7**, (2012).

49. Hahne, M. *et al. APRIL*, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. *J. Exp. Med.* **188**, 1185–1190 (1998).

50. Mackay, F., Schneider, P., Rennert, P. & Browning, J. *BAFF* and *APRIL*: A tutorial on B cell survival. *Annu. Rev. Immunol.* **21**, 231–264 (2003).

51. Baert, L., Manfroi, B., Casez, O., Sturm, N. & Huard, B. The role of *APRIL* - A proliferation inducing ligand - In autoimmune diseases and expectations from its targeting. *J. Autoimmun.* **95**, 179–190 (2018).

52. Koyama, T. *et al.* Raised serum *APRIL* levels in patients with systemic lupus erythematosus. *Ann. Rheum. Dis.* **64**, 1065–1067 (2005).

53. Jonsson, M. V., Szodoray, P., Jellestad, S., Jonsson, R. & Skarstein, K.

Association between circulating levels of the novel TNF family members *APRIL* and *BAFF* and lymphoid organization in primary Sjögren's syndrome. *J. Clin. Immunol.* **25**, 189–201 (2005).

54. Thangarajh, M. *et al.* Increased levels of *APRIL* (A Proliferation-Inducing Ligand) mRNA in multiple sclerosis. *J. Neuroimmunol.* **167**, 210–214 (2005).

55. Gross, J. A. *et al.* TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. *Nature* **404**, 995–999 (2000).

56. Hsu, B. L., Harless, S. M., Lindsley, R. C., Hilbert, D. M. & Cancro, M. P. Cutting Edge: BLyS Enables Survival of Transitional and Mature B Cells Through Distinct Mediators. *J. Immunol.* **168**, 5993–5996 (2002).

57. Claudio, E., Brown, K., Park, S., Wang, H. & Siebenlist, U. *BAFF*-induced NEMO-independent processing of NF-κB2 in maturing B cells. *Nat. Immunol.* **3**, 958–965 (2002).

58. Otipoby, K. L. *et al. BAFF* activates Akt and Erk through *BAFF-R* in an IKK1-dependent manner in primary mouse B cells. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 12435–12438 (2008).

59. Lin, J. D. *et al.* Analysis of Associations of Human *BAFF* gene polymorphisms with autoimmune thyroid diseases. *PLoS One* **11**, 1–13 (2016).

60. Cheng, C. W., Fang, W. F., Tang, K. T. & Lin, J. D. Possible interplay between estrogen and the *BAFF* may modify thyroid activity in Graves' disease. *Sci. Rep.* **11**, 1–11 (2021).

61. Deng, H. *et al.* Associations of *BAFF* rs2893321 polymorphisms with myasthenia gravis susceptibility. *BMC Med. Genet.* **20**, 168 (2019).

62. Clark, W. B. *et al.* Genetic variation in recipient B-cell activating factor modulates phenotype of GVHD. *Blood* **118**, 1140–1144 (2011).

63. Jasek, M. *et al.* Association of variants in *BAFF* (rs9514828 and rs1041569) and *BAFF-R* (rs61756766) genes with the risk of chronic lymphocytic leukemia. *Tumor Biol.* **37**, 13617–13626 (2016).

64. Novak, A. J. *et al.* Genetic variation in B-cell-activating factor is associated with an increased risk of developing B-cell non-Hodgkin lymphoma. *Cancer Res.* **69**, 4217–4224 (2009).

65. Zhai, K. *et al.* Cytokine *BAFF* gene variation is associated with survival of patients with T-cell lymphomas. *Clin. Cancer Res.* **18**, 2250–2256 (2012).

66. Ruyssen-Witrand, A. *et al.* Association between -871C>T promoter polymorphism in the B-cell activating factor gene and the response to rituximab in rheumatoid arthritis patients. *Rheumatol. (United Kingdom)* **52**, 636–641 (2013).

67. Juge, P. A. *et al.* Variants of genes implicated in type 1 interferon pathway and B-cell activation modulate the EULAR response to rituximab at 24 weeks in rheumatoid arthritis. *RMD Open* **3**, 1–5 (2017).

68. Marín-Rosales, M. *et al.* High *BAFF* expression associated with active disease in systemic lupus erythematosus and relationship with rs9514828C>T polymorphism in TNFSF13B gene. *Clin. Exp. Med.* **19**, 183–190 (2019).

69. Hildebrand, J. M. *et al.* A *BAFF-R* mutation associated with non-Hodgkin lymphoma alters TRAF recruitment and reveals new insights into *BAFF-R* signaling. *J. Exp. Med.* **207**, 2569–2579 (2010).

70. Papageorgiou, A. *et al.* A *BAFF* receptor His159Tyr mutation in Sjögren's syndrome-related lymphoproliferation. *Arthritis Rheumatol. (Hoboken, N.J.)* **67**, 2732–2741 (2015).

71. Russo, R. *et al.* The tnfrsf13c h159y variant is associated with severe covid-19: A retrospective study of 500 patients from southern Italy. *Genes (Basel).* **12**, 1–8 (2021).

72. Koyama, T. *et al.* A novel polymorphism of the human *APRIL* gene is associated with systemic lupus erythematosus. *Rheumatology* **42**, 980–985 (2003).

73. Li, P. *et al.* Association Study of a Proliferation-inducing Ligand, Spermatogenesis Associated 8, Platelet-derived Growth Factor Receptor-alpha, and POLB Polymorphisms with Systemic Lupus Erythematosus in Chinese Han Population. *Chin. Med. J. (Engl).* **129**, 2085 (2016).

74. Namazi, S. *et al. APRIL* gene polymorphism and serum s*APRIL* levels in children with systemic lupus erythematosus. *Clin. Rheumatol.* **36**, 831–836 (2017).

75. Gazouli, M. *et al.* CARD15/NOD2, CD14, and toll-like receptor 4 gene polymorphisms in Greek patients with sarcoidosis. *Sarcoidoisis, Vasc. Diffus. Lung Dis.* **23**, 23–9 (2006).

76. Patel, M. R. *et al.* Detection of Myocardial Damage in Patients with Sarcoidosis. *Circulation* **120**, 1969–1977 (2009).

77. Nezos, A. *et al.* B-cell activating factor genetic variants in lymphomagenesis

associated with primary Sjogren ' s syndrome. *J. Autoimmun.* (2013). doi:10.1016/j.jaut.2013.04.005

78. Kompoti, M. *et al.* Genetic polymorphisms of innate and adaptive immunity as predictors of outcome in critically ill patients. *Immunobiology* **220**, 414–421 (2015).

79. Solé, X., Guinó, E., Valls, J., Iniesta, R. & Moreno, V. SNPStats: A web tool for the analysis of association studies. *Bioinformatics* **22**, 1928–1929 (2006).

80. Faustova, M. *et al.* A7.11 Genetic Variation in Promoter Sequence of B-Cell-Activating Factor of the TNF Family (*BAFF*) in Patients with Idiopathic Inflammatory Myopathies (IIM). *Ann. Rheum. Dis.* **72**, A51.3-A52 (2013).

81. Jasek, M. *et al.* Association of variants in *BAFF* (rs9514828 and rs1041569) and *BAFF-R* (rs61756766) genes with the risk of chronic lymphocytic leukemia. *Tumor Biol.* **37**, 13617–13626 (2016).

82. Naruse, T. K. *et al.* HLA-DQB1*0601 is primarily associated with the susceptibility to cardiac sarcoidosis. *Tissue Antigens* **56**, 52–57 (2000).

83. Takashige, N. *et al.* Genetic polymorphisms at the tumour necrosis factor loci (TNFA and TNFB) in cardiac sarcoidosis. *Tissue Antigens* **54**, 191–193 (1999).

84. Gialafos, E. *et al.* Relationship between tumor necrosis factor-α (TNFA) gene polymorphisms and cardiac sarcoidosis. *In Vivo (Brooklyn).* **28**, 1125–1130 (2014).

85. Denning, D. W., Pleuvry, A. & Cole, D. C. Global burden of chronic pulmonary aspergillosis complicating sarcoidosis. *Eur. Respir. J.* **41**, 621–626 (2013).