Immune response against *Mycobacterium avium* subsp. *paratuberculosis*, Epstein-Barr virus, HERV-K and IRF5 in rheumatoid arthritis

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PhD thesis

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Foreword

This thesis is based on several manuscripts that were published during my PhD.

The work of the present PhD thesis has been completed during my enrolment as PhD student at the Department of Biomedical Sciences, University of Sassari, Italy, in the period from 1th November 2016 to 31th October 2019 under the supervision of Professor Leonardo A. Sechi.

The studies described in this thesis were also conducted at the Kennedy Institute of Rheumatology, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences (NDORMS), Faculty of Medicine, University of Oxford, UK, from October 2018 to April 2019, under the supervision of Professor Udalova Irina and in collaboration with her group – Udalova Group – Genomics of Inflammation.

All the samples used in these studies were from subjects enrolled by the Rheumatoid Arthritis Centre, UOC Reumatologia, Dipartimento di Medicina Clinica e Sperimentale, Azienda-Ospedaliero Universitaria (AOU) di Sassari, Sassari, Italy; by the Department of

Clinical and Experimental Medicine, Neurology section, University of Sassari and Centro Trasfusionale, Azienda Sanitaria Locale (ASL) Sassari, Italy.

Preface

This dissertation is submitted in accordance with the requirements for the PhD degree in *Life Sciences and Biotechnologies*, Department of Biomedical Sciences, Viale San Pietro 43b, University of Sassari, Italy. The work was carried out at the Department of Biomedical Sciences, Sassari, Italy and at the Kennedy Institute of Rheumatology | NDORMS, Faculty of Medicine, University of Oxford, UK.

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Declaration of work done

The research work presented in this thesis entitled "**Immune response against *Mycobacterium avium* subsp. *paratuberculosis*, Epstein-Barr virus, HERV-K and IRF5 in rheumatoid arthritis**" was done under the guidance of Professor Leonardo A. Sechi, Coordinator of the *PhD Course in Life Sciences and Biotechnologies* at the Department of Biomedical Science, University of Sassari, Italy, and under the guidance of Professor Udalova Irina, at the Kennedy Institute of Rheumatology | NDORMS, faculty of Medicine, University of Oxford, UK. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other University or Institution. All contributions of others are indicated in the references to the literature.

Signature of the PhD candidate: **Stamp of the Institution**

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Dr. Bo Marco **Date:** _____

University of Sassari

Reg. No.: _____

I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Signature of the Supervisor: **Stamp of the Institution**

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Prof. Sechi Leonardo Antonio **Date:** _____

University of Sassari

Dedicated to

My family

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Marco Bo

Abstract

Rheumatoid arthritis (RA) is an inflammatory disease characterized by synovitis, systemic inflammation, autoantibodies that causes joint damage, disability, decreased quality of life, and cardiovascular and other comorbidities. Its aetiology as well the exact etiopathogenetic mechanisms are not well clear so far. RA is triggered by an interplay between genes and environmental factors.

Several studies showed that microorganisms play an important role in triggering autoimmunity through different mechanisms of action. Viral and bacterial infections, such as those caused by Epstein-Barr virus (EBV), Human Endogenous Retrovirus (HERVs) and mycobacteria, may play a pathogenetic role in RA through immunological cross-reactivity or molecular mimicry. Sardinians have a peculiar genetic background resulting from a long lasting geographical isolation with a strong incidence and prevalence of different autoimmune disease such as RA, multiple sclerosis (MS) and diabetes.

During this PhD course, I have studied the role of EBV, HERV-K and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in RA pathogenesis and other rheumatic diseases for a better understanding of how these infectious agents can lead to a deregulation of transcription factors such as Interferon Regulatory Factor 5 (IRF5) that is important in the regulation of different cells type like macrophages and neutrophils.

Finally, in order to better understand the etiopathogenesis of RA, an animal model has been used to study the molecular mechanisms involved in the above-mentioned diseases and to better understand the link between the environment and genes in RA with an objective to develop new therapeutic strategies.

Riassunto

L'artrite reumatoide (AR) è una malattia infiammatoria caratterizzata da sinovite, infiammazione sistemica, autoanticorpi che causano danni articolari, disabilità, patologie cardiovascolari comportando una diminuzione della qualità della vita. La sua eziologia e gli esatti meccanismi eziopatogenetici non sono ancora ben chiari. L'AR è innescata da un'interazione tra geni e fattori ambientali.

Diversi studi hanno dimostrato che i microrganismi hanno un ruolo importante nell'innescare l'autoimmunità attraverso diversi meccanismi d'azione. Le infezioni virali e batteriche, come il virus di Epstein Barr (EBV), i Retrovirus Endogeni Umani (HERVs) ed i micobatteri, possono svolgere un ruolo patogenetico nell'AR attraverso la reattività crociata o il mimetismo molecolare. I sardi hanno uno sfondo geneticamente isolato con una forte incidenza e prevalenza di diverse malattie autoimmuni come l'AR, la sclerosi multipla (SM) e il diabete.

Nel presente lavoro è stato analizzato il ruolo di EBV, HERV-K e *Mycobacterium avium* subsp. *paratuberculosis* (MAP) nella patogenesi della AR e di altre malattie reumatiche per capire come questi agenti infettivi possono portare alla deregolazione di fattori di trascrizione come l'Interferon Regulatory Factor 5 (IRF5), quest'ultimo implicato sia nella regolazione della maturazione che nella funzione di diversi tipi di cellule immunitarie come i macrofagi e neutrofili.

Infine, al fine di comprendere meglio l'eziopatogenesi della AR, è stato utilizzato il modello animale perché rappresenta un buon modello per studiare i meccanismi molecolari coinvolti nelle malattie umane, per approfondire i legami tra ambiente e geni nell'artrite con l'obiettivo di sviluppare nuove strategie terapeutiche.

List of abbreviations

Abs	Antibodies
ACPAs	Anti-citrullinated protein Abs
AIA	Antigen-Induced Arthritis
ANOVA	Analysis of Variance
ALS	Amyotrophic lateral sclerosis
ARA	American Rheumatism Association
BSA	Bovine Serum Albumin
BOLF1	Inner tegument protein
CAIA	Collagen antibody-induced arthritis
CFA	Complete Freund's Adjuvant
CIA	Collagen-induced arthritis
Cit	Citrullinated
DMARDs	disease-modifying antirheumatic drugs
D-PBS	Dulbecco's Phosphate Buffered Saline
EBV	Epstein-Barr virus
ELISA	Enzyme-Linked Immunosorbent Assay
ERVs	Endogenous retroviruses
Figure	Fig.
HCs	Healthy control
HDL	High-density lipoprotein
HERV-K	Human endogenous retrovirus-K
HLA	Human leukocyte antigen
IFA	Incomplete Freund's Adjuvant
IgG	Immunoglobulin G
IL	Interleukin
IRF	Interferon Regulatory Factor
IRF5	Interferon Regulatory Factor 5

Irf5-/-	IRF5-knockout
LDL	Low-density lipoprotein
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MAP_4027	Uncharacterized protein
mBSA	Methylated Bovine Serum Albumin
MS	Multiple sclerosis
Mtb	*Mycobacterium tuberculosis*
NMOSD	Neuromyelitis optica spectrum disorders
NSAIDs	Nonsteroidal anti-inflammatory drugs
OCT	CellPath cryo microtomy embedding medium
OD	Optical density
PBS	Phosphate Buffered Saline
PBS-T	PBS-Tween 20
PCR	Polymerase Chain Reaction
PtpA	Protein tyrosine phosphatase A
PknG	Protein kinase G
qPCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid arthritis
ROC	Receiver operator characteristic
SLE	Systemic lupus erythematosus
SSc	Systemic sclerosis
SS	Sjögren's syndrome
T1DM	type 1 diabetes mellitus
TC	Total cholesterol
TNF	Tumor necrosis factor
T_{reg}	T regulatory cell
VLDL	Very-low density lipoproteins
WT	Wild-type

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CHAPTER 1: *Introduction*

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that mainly affects diarthrodial joints. It can potentially involve every compartment in the organism such as skin, blood vessels, heart, lungs and muscles. At the level of the joint the inflammatory process has an erosive character and can lead to the destruction of the juxta-articular bony heads and to the ankylosis (Scott DL *et al*., 2010).

RA was first described by Augustin-Jacob Landré-Beauvais in the early 1800s. The term "rheumatoid arthritis" was coined by Alfred Bering Garrod in 1876 and was definitively adopted at the American Rheumatism Association (ARA) in 1941.

Epidemiology

RA is the most common inflammatory arthritis, affecting from 0.5 to 1% of the general population worldwide. The prevalence ranges from 0.3 to 2% of the population, while yearly incidence is of 2-4 new cases per 10 000 inhabitants (Fig. 1). Females are predominantly affected with a male/female ratio of 1:4. The disease can occur at any age, but it generally occurs in subjects between 40 and 60 years old. In Italy, it was estimated that more than 5 million people suffer from RA with a higher prevalence among women, and that about one fifth have the most disabling and severe forms. If RA is not properly diagnosed and controlled in time, the joints may become permanently deformed resulting in progressive limitation and loss of self-sufficiency that leads in turn to a worsened quality of life, loss of working capacity and high social and economic costs.

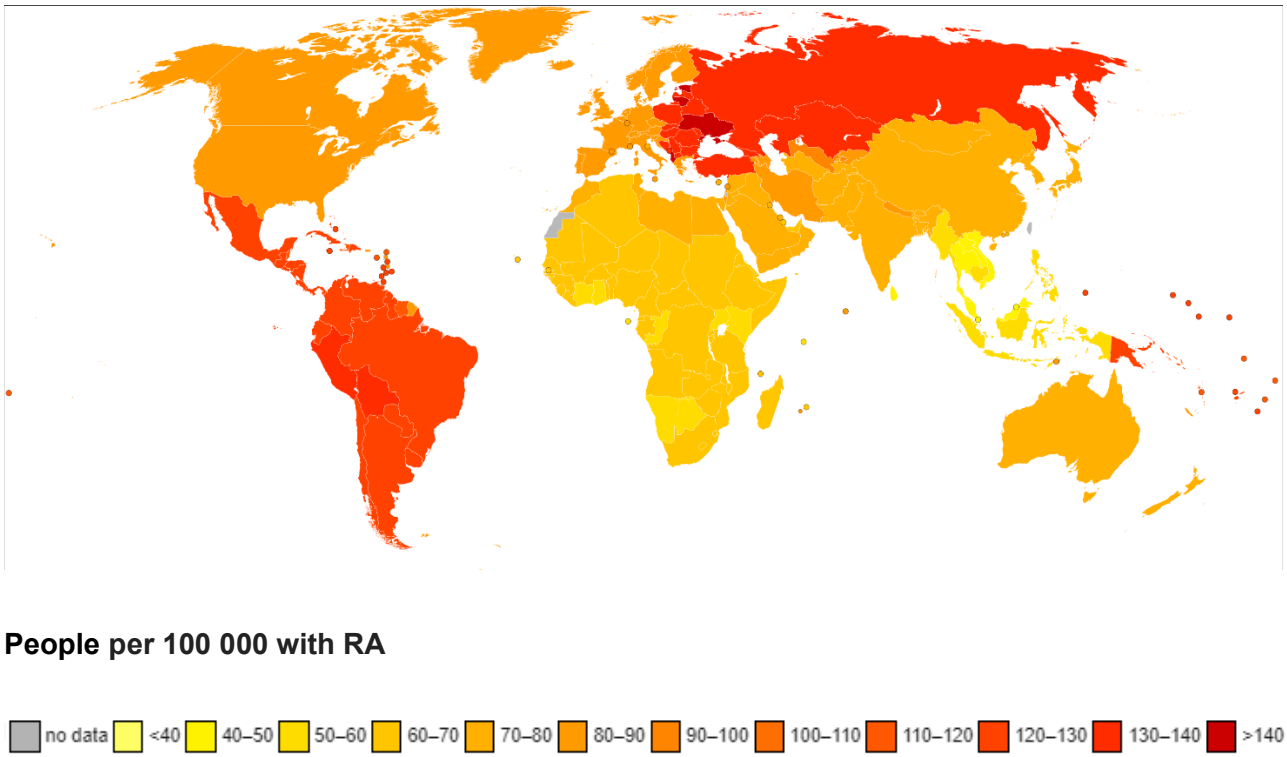

Fig. 1: Global prevalence of RA https://www.who.int/healthinfo/global_burden_disease/estimates_country/en/

Etiopathogenesis

The etiology and pathogenesis of RA are complex and multifaceted. Although RA etiology remains unknown, different studies supported the hypothesis that the interaction of environmental and genetic factors is responsible (Scott DL *et al*., 2010), (Klareskog L *et al*., 2009), (Feldmann M *et al*., 1996). The disease is characterized by an activation of the immune system which, through a complex series of events, involve both humoral and cellular immunity leading to the development of an acute inflammatory process and later to its maintenance and chronicity. As discussed later, this process implicates certain specific genes that can culminate in the breakage of immune tolerance and lead to autoreactivity.

From a genetic point of view, it has been pointed out that class II HLA-DR4 and DR1 antigens are the ones that confer greater susceptibility to the disease. The initiation of RA probably begins years before the onset of clinical symptoms. The loss of tolerance leads to autoreactivity and this process is due to specific genes, including class II major histocompatibility complex (MHC) genes, protein tyrosine phoshatase-22 (PTPN22) (Begovich AB *et al*., 2004), and peptidylarginine deiminases. The first description of a genetic link between HLA-DR and RA has been described in the 1970 century. HLA-DR4 was present in 70% of RA subjects and only 30% of healthy controls (HCs). Other HLA genes such as DRB*1301 that contain, in the third hypervariable region of DRβ-chains, the DERAA sequence, are associated with decreased susceptibility to RA (van der Woude D *et al*., 2010).

In addition, signal transduction gene polymorphisms, cytokine promoter polymorphisms and population-specific genes (e.g., PADI4 in Koreans and Japanese) are involved. PTPN22 and PADI4 increase the risk in some racial and ethnic groups, but not in all. It is likely that the earliest phases are characterized by repeated activation of innate immunity.

A number of environmental factors clearly contribute to RA susceptibility, although no specific exposure has been identified as the pivotal agent. Smoking is the best defined environmental risk factor for RA and the interaction between HLA-DR and tobacco exposure is perhaps the best example of how genes and environment conspire to enhance the risk.

Other triggering factors are represented by bacterial agents (*E. coli*, *Streptococcus*, mycoplasma and mycobacteria), viral agents (EBV, varicella zoster virus (VZV), rubella virus, Herpes Simplex, Parvovirus B19) and superantigens (Arleevskaya MI *et al*., 2016).

These agents can trigger joint inflammation with a direct and/or indirect mechanism. This process occurs often in normal people but is self-limited, while in subjects with a predetermined propensity for an immune hyper-reactivity or autoreactivity it could lead to a different outcome.

The indirect mechanism is the most popular mechanism in which we may observe the triggering of a cross immune response between microbial antigens and articular auto-antigens. In addition, environmental stresses can lead to post-transcriptional modifications of proteins in mucosal surfaces or the synovium, especially carbamylation or citrullination of arginine residues. People with a propensity for RA can develop antibodies (Abs) against these modified proteins with production of rheumatoid factors (RFs) and anti-citrullinated protein Abs (ACPAs).

Pathological anatomy

The most important alterations observed in RA patients are synovitis, nodules and rheumatoid vasculitis. Synovitis is initially characterized by exudation, cellular infiltration (lymphocytes and plasma cells) and proliferation (vessels, synoviocytes, fibroblasts). Proliferation is becoming increasingly important and leads to the formation of the synovial cloth that invades the subchondral bone and destroys the cartilage leading to ankylosis. The nodules are found in 15-30% of the cases, they can be superficial (skin, subcutaneous, tendinous sheaths) or deep (internal organs), single or multiple and their diameter varies from a few millimeters to 2 or more centimeters. Histologically they are characterized by a central zone of fibrinoid necrosis, an intermediate zone formed by one or more layers of cells arranged in a palisade and an external zone of granulation tissue infiltrated by

lymphocytes and plasma cells. Vasculitis is not specific, affects small vessels and can involve any organ.

Diagnostic classification

Different RA diagnostic classifications have been used during the years after the first description (Arnett FC *et al*., 1988), (Kasturi S *et al*., 2014). The most recent diagnostic classification is based on 2010 ACR/EULAR Classification Criteria for RA (criteria of the American College of Rheumatology) (Aletaha D *et al*., 2010).

Therapy

The therapy is multidisciplinary and consists of combining the pharmacological approach [NSAIDs (Nonsteroidal anti-inflammatory drugs), steroids, DMARDs (disease-modifying antirheumatic drugs), biological drugs] with non-pharmacological one (rehabilitation, surgical correction, psychological approach) (Pinals RS *et al*., 1981). The drugs used in RA are aimed at reducing inflammation and consequent structural damage. They are called disease-modifying antirheumatic drugs and can be classified into synthetic DMARDs and biological DMARDs. Synthetic DMARDs are also classified into conventional synthetic DMARDs and target synthetic DMARDs (Smolen JS *et al*., 2014).

Biologic drugs, recently introduced for the treatment of RA, have been very successful; these are molecules (monoclonal antibodies), obtained using recombined DNA technology, which can selectively block a cytokine or a particular cellular interaction identified as key points in the pathogenesis of the disease. Biological drugs allowed clinical remission in a high percentage of patients and proved to slow down (in some cases and conditions blocked) the radiological progression of arthritis. Those currently on the market for RA have as molecular targets the tumor necrosis factor (TNF), the interleukin receptor 1, the IL-6 receptor, the CD-

20, the CD-80 and the CD-86 (also known as B7-1 and B7-2, both of which are fundamental to the co-stimulation of B lymphocytes - T lymphocytes) (Nam JL *et al*., 2014). In Italy, Europe and USA, five anti-TNFα have been approved for the treatment of RA: Infliximab (originator, biosimilar), Etanercept (originator, biosimilar), Adalimumab, Golimumab and Certolizumab. All these drugs have been shown to be more effective on a clinical profile when administered together with methotrexate, especially in the inhibition of radiological damage (Maini RN *et al*., 1998). The biologicals with different mechanisms of action to date prescribed for the treatment of RA are: Abatacept, Tocilizumab, Anakinra and Rituximab (Maini RN *et al*., 1998).

CHAPTER 2: *Infectious agents and RA*

Human endogenous retrovirus-K

For a long time, endogenous retroviruses (ERVs) and other repetitive elements have been considered as junk DNA having no impact on the host. However, over the years, it has been possible to discover the evolutionary interplay between ERVs and host biology, leading to interesting results. ERVs are viral elements that are part of the animal and human genome and that have probably originated from retroviruses. Today we know that ERVs constitute a quantitatively significant component of the human genome; it is estimated that there are roughly 98 000 sequences of complete ERVs and their fragments and that they represent approximately 8% of the whole human genome. If we add other retro elements such as retrotransposons (fragments of DNA that can be independently transcribed in an intermediate to RNA and consequently replicate in different positions of the genome), it is believed that more than 40% of the genome of an individual is of external origin. These sequences of endogenous elements inserted into the seminal line cells by retro-transcription mechanisms are inherited as stable mendelian genes (Temin HM., 1985).

It seems that Human endogenous retrovirus-K (HERV-K) could has a role in the autoimmune process of RA in which the immune system fails to discriminate self from non-self. Several factors can contribute to the phenomenon of molecular mimicry, such as genetic predisposition (HLA genes), environmental factors, age and sex (Freimanis G *et al*., 2010). In RA, it was pointed out that HERV-K-10 has amino acid sequences homologous to IgG1Fc, a target of rheumatoid factor. In patients with RA, it has been observed that there is a differential expression of the HERV-K (HML-2) genes and the transcription of Rec proteins.

Furthermore, it was observed that in the mononuclear cells of synovial fluid of RA patients there is a high expression of the HERV-K type 1 virus encoding a super antigen capable of stimulating a high number of reactive T lymphocytes (Tugnet N *et al*., 2013). The immune response against HERV-K is guided by an antigen and could be an initial event in the autoimmune mechanism that leads to the loss of tolerance to the organism's own antigens. A connection is therefore possible between the activation of HERV-K and the inflammatory process. Moreover, it was observed that in patients with RA there is a statistically significant association between the viral load of HERV-K1 in the plasma and the activity of the disease. This association could reflect the increased cellular activation found in peripheral blood during the period of active disease which, in turn, can facilitate the activation and perhaps the replication of HERV-K type 1 followed by a high viral load (Tugnet N *et al*., 2013). This association has been highlighted only with HERV-K type 1, which encodes the Np9 oncoprotein. In fact, RA is characterized by a chronic inflammation and an incorrect proliferation of invasive synoviocytes, such as the growth of cancer cells. This could suggest that the activation of HERV-K 1 could contribute to the chronicity of the process, where the synoviocytes show defects in apoptosis.

Finally, the differential activation and expression of HERV-K could be induced by environmental signals or by the activation of the immune system following exposure to triggering factors that may include exogenous viruses such as EBV, also involved in RA (Tugnet N *et al*., 2013).

Epstein-Barr virus

Epstein-Barr virus (EBV), known as human gammaherpesvirus 4, is one of the most common viruses in humans identified by Anthony Epstein and Yvonne Barr in 1964 using electron microscopy in biopsies of Burkitt's Lymphoma (BL). The virus belongs to the *Herpesviridae* family, *gammaherpesvirinae* subfamily and genus *Lymphocryptovirus*. EBV is an ubiquitous virus and 90% of the worldwide population is infected. The virus is orally transmitted and infects epithelial cells of the oropharynx (Young LS *et al*., 2004).

EBV is about 122-180 nm in diameter and is composed of a linear, double helix of DNA which contains ~172.000 base pairs and 85 genes that codifies for more than 85 proteins. There are six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and EBNA-LP); three latent membrane proteins (LMPs 1, 2A, 2B); small non-polyadenylated RNAs, EBERs 1 and 2; microRNAs (miR-BHRF1 and miR-BART); and several early lytic genes (Young LS *et al*., 2004).

Deoxyribonucleic acid is enclosed within a protein nucleocapsid, which is surrounded by a tegument made of protein, in turn surrounded by an envelope containing both lipids and surface projections of glycoproteins, which are essential to infect the host cell. EBV is usually found in the episomal form inside cells, although, in some cases it has been described as a viral DNA randomly integrating into the host's genome (Young LS *et al*., 2004). Also, EBV has the ability to express different viral proteins according to different cells and differentiation stages of infected cells.

EBV is involved in several diseases, e.g. infectious mononucleosis (IM), BL, Hodgkin's lymphoma, gastric cancer, nasopharyngeal carcinoma and other neoplasms. Recent reports show that EBV has been associated with autoimmune disease including multiple

sclerosis (MS), systemic lupus erythematosus (SLE), Sjogren's syndrome (SS) and RA (Olsson T *et al*., 2017), (Ascherio A *et al*., 2015), (Trier NH *et al*., 2019).

Mycobacterium avium* subspecies *paratuberculosis

Mycobacterium avium subspecies *paratuberculosis* (MAP) belongs to the order of the Actinomycetales and is part of the group Mycobacterium avium complex (Mac) (Harris NB *et al*., 2001). MAP is an optional anaerobic Gram-positive intracellular bacterium, with a small rod shape, ranging from 0.15μm to 1.15μm (Fig. 2). It is strongly acid-alcohol resistant, asporiginal and capsule-free bacterium (Harris NB *et al*., 2001), (Lévy-Frébault VV *et al*., 1992).

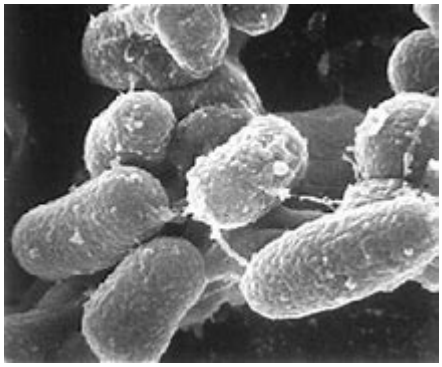

Fig. 2. *Mycobacterium avium* subspecies *paratuberculosis* https://johnes.org/

It is a mandatory pathogen for ruminants that requires the presence of a favorable environment to multiply. MAP has a considerable tropism towards ruminant animal tissue and is the etiologic agent of severe gastroenteritis in ruminants known as Johne's disease or paratuberculosis (Chiodini RJ *et al*., 1984), but other animal can be also affected. Recent research on a survey of 48 countries found paratuberculosis to be very common in livestock representing also a serious problem for public health (Whittington R *et al*., 2019), (Garvey M., 2018).

It is a bacterium that grows very slowly in culture media taking up to 6-8 weeks at 37°C for a visible growth, with duplication times of about 48-72 hours compared to 12-24 hours of *Mycobacterium tuberculosis*. This feature is mostly due to the bacterial wall rich in complex lipids that slows metabolic exchange with the external environment.

MAP may occur in two forms: bacillary or spheroplasty. The presence of a large number of bacteria in a bacillary form is necessary to give rise to overt disease (pluribacillary form) and can be detected with the coloring of Ziehl-Neelsen. Few bacteria in spheroplastic form, on the other hand, are able to cause the disease (paucibacillary form, characterized by the formation of granulomas) (Chiodini RJ *et al*., 1984).

MAP is transmitted via the orofecal route and is localized in the intestine of the host, it is excreted with the stool of the infected animals, it is able to withstand exposure to the environment for a long time, enhancing the risk for the cycles of re-infection and making it difficult to eradicate the disease in endemic areas. The presence of MAP in the environment is influenced by the type of soil, pH and soil moisture, however this microorganism tolerates a wide range of temperature excursions. Through rainwater, MAP can be transferred to waterways, such as lakes and rivers, and some controls have documented its presence in these types of water (Chiodini RJ *et al*., 1984), (Richardson H *et al*., 2019). The water intended for domestic use is subjected to various treatments that make it possible to eliminate the solids suspended in it and reduce the probable presence of MAP; however, its complete elimination is difficult due to the high resistance to chlorine and other biocides, which constitutes a danger of contamination for domestic supplies (Whan LB *et al*., 2001).

In all hosts, the most common route of infection is represented by the ingestion of feces or contaminated foods such as milk, colostrum or water. Once penetrated into the host, MAP

reaches the intestine, where, through transcytosis, it passes through the membrane of the M cells in the Peyer plaques (besides being able to infect the epithelial cells of the mucosa) and into the deep layer of the intestinal mucosa, where it is captured by macrophages. MAP has a marked capacity to resist the enzymatic degradation mechanisms implemented by macrophages during infection; this ability allows MAP not only to survive, but also to grow and multiply, albeit very slowly, within these cells. The mechanism by which the mycobacterium survives and multiplies inside the phagosome is still not clear. Phagosomes constitute an extremely hostile environment to many bacteria due to the rather acid pH, the presence of proteolytic enzymes and different antibacterial molecules such as defensins and toxic intermediates of oxygen and nitrogen. It is probable that MAP inhibits the action of oxygen and nitroxide by the synthesis of oxidase and alkyl hydroperoxidase C and D. It is also known that, in the case of mycobacterial infections, normal phagosome-lysosome fusion does not occur with subsequent acidification and the release of hydrolytic enzymes. Recent studies have shown that the components of the host cell, steroid cholesterol and a surface phagosomal protein called TACO (tryptophane aspartate-containing), promote the establishment of intracellular infection by mycobacteria (Pieters J *et al*., 2002), (Meena LS *et al*., 2010). During growth within the macrophage phagosome, mycobacteria develop a capsule similar to a lamellar lining. The presence of MAP also induces a significant increase in the expression of pro-inflammatory cytokines in the macrophage, such as IL-1α (interleukin-1α), IL-1, IL-6, IL-12 and TNF-α. The latter, together with IL-12, stimulates the natural killer (NK) lymphocytes to produce INF-γ, in turn responsible for the activation of other macrophages which accentuate the inflammatory process (Chiodini RJ *et al*., 1984).

This bacterium has been also associated to Crohn's disease (McNees AL *et al*., 2015), (Kuenstner JT *et al*., 2017), (Feller M *et al*., 2007), (Sechi LA *et al*., 2005). The possible

involvement of MAP has been suggested for the first time in 1984 by Rodrick Chiodini by the close similarity from a clinical, pathological and epidemiological point of view, between Crohn's disease and Johne's disease of ruminants (Chiodini RJ 1984). Another proof was the first isolation of MAP from a Crohn's patient with a further successful cultivation (Chiodini RJ *et al*., 1986).

Following colonisation of the host, the parasitic organism alters the host immune system through molecular mimicry, displaying peptide sequences similar to that of the host cells and causing a disruption of self *vs*. non-self-recognition. Theoretically, the failure to recognize the invading organism from host cells may result in numerous autoimmune conditions.

In addition to Crohn's disease, several studies show that MAP could be another triggering factor in other human autoimmune diseases such as MS (Frau J *et al*., 2013), (Cocco E *et al*., 2011) (Bo M *et all*., 2018b), Type 1 Diabetes (T1D) (Songini M *et al*., 2010) and Hashimoto's thyroiditis (Cossu D *et al*., 2015).

Aim of the study

The goals of my research can be summarized in the following points:

- Deepening knowledge on the role of endogenous retroviruses in RA disease.
- Analysis of the synergistic effect between EBV, HERV-K and MAP in RA disease.
- Investigation of the molecular mimicry mechanism between EBV, MAP and host peptides.
- Starting from previous discoveries of anti-Interleukin-2 (IL-2) Abs present in subjects affected by autoimmune diseases and their possible role in alterations involving regulatory T cell responses, I investigated the immune response against two epitopes of IL-2 in RA Sardinian patients.
- Analysis of reactivity to PtpA and PknG proteins secreted by MAP in the sera of RA patients and correlation analysis with immune response against BOLF1, MAP_4027 and IRF5 peptides.
- Investigation of the correlation between humoral reactivity against MAP and serum lipoprotein levels in subjects at T1D risk (rT1D) grouped by geographical background and in patients affected by MS and RA.
- Considering that anti-CCP antibodies are a biomarker for precocious diagnosis of RA, I've investigated the reactivity against wild type and citrullinated peptides of MAP, EBV and IRF5 in RA, SLE, systemic sclerosis (SSc) and SS patients in order to assess similitudes and/or differences.
- Investigation of the reactivity against three homologous peptides IRF5, BOLF1 and MAP previously analyzed in human, in the serum of three arthritis models (AIA, Collagen-Induced Arthritis (CIA) and Collagen Antibody-Induced Arthritis (CAIA)) and comparison with human results.

CHAPTER 3: *Mouse model of rheumatoid arthritis*

RA is an autoimmune/inflammatory disease that causes joint damage, disability, decreased quality of life, and cardiovascular and other comorbidities. The etiology is unknown and pathogenesis is not clear yet. For this reason, in order to better understand the complexity of the pathogenesis of RA, animal models have been extensively used to understand the molecular mechanisms involved in human diseases with the objective to develop new therapeutic strategies. Obviously, a disease identical to RA cannot be developed in experimental animals because they are different species with different genetics and life environments, as compared with humans.

Many rodent models of arthritis have been developed through the decades of research in the field and, among all animal models, mouse models of RA have many features of human disease. This has contributed to numerous advances to better understand pathogenic pathways in RA and major advances in development of new drugs (Bevaart L *et al*., 2010), (McNamee K *et al*., 2015), (Kollias G *et al*., 2011). This way, by translating results from mouse to human, it has been possible to refine the understanding of molecular pathways involved in disease pathogenesis. These outcomes have been achieved by combining knowledge on RA-associated genes, environmental factors and the presence of serological elements.

Arthritis models can be induced in different ways that can be divided into 3 groups:

1) those elicited by active immunization,
2) those elicited by passive immunization,
3) those elicited by the administration of irritant chemicals resulting in chronic inflammation. CIA is an example of an active immunization strategy, whereas

antibody- induced arthritis models, such as collagen antibody–induced arthritis and K/BxN antibody transfer arthritis, represent examples of passive immunization strategies (Caplazi P *et al*., 2015), (Asquith DL *et al*., 2009).

Induced arthritis models

RA is characterized by immune perturbation in both the innate and adaptive compartments and subsequent chronic inflammation. The etiology is complex and, for this reason, it was indispensable to generate animal models that better represent RA disease for a simplified study of RA immunopathogenesis.

Collagen-induced arthritis

CIA is one of the models used. Arthritis is normally induced in mice by immunization with autologous or heterologous type II collagen in adjuvant. Susceptibility to collagen-induced arthritis is strongly associated with major histocompatibility complex class II genes, and the immunopathogenesis of CIA involved both a T-cell and B-cell specific response to type II collagen. The chief pathological features of CIA include a proliferative synovitis with infiltration of polymorphonuclear and mononuclear cells, pannus formation, cartilage degradation, erosion of bone, and fibrosis (Brand DD *et al*., 2007). As in RA, pro-inflammatory cytokines, such as tumour necrosis factor α (TNFα) and interleukin (IL)-1β, are abundantly expressed in the arthritic joints of mice with CIA, and blockade of these molecules results in a reduction of disease severity.

Antigen-induced arthritis

The above indicated model of arthritis can be developed when mice are primed with an antigen like methylated bovine serum albumin (mBSA) in complete Freund's adjuvant (CFA), and subsequently challenged by intra-articular injection of the same antigen (Asquith DL *et*

al., 2009). Such models are useful in that mice of several strains can be investigated to establish a hierarchical role for given factors in adaptive immune-mediated articular damage. Subsequent pathology comprises immune complex-mediated inflammation with infiltration of polymorphonuclear cells, pannus formation, erosion of cartilage and bone, followed by articular T-cell-mediated responses and in particular is Th17 dependent (Asquith DL *et al*., 2009), (Egan PJ *et al*., 2008). Also, regarding the molecular pathway of immune response, a recent study has shown that synovial macrophages from the AIA joint have high levels of IRF5 that is an important transcription factor implicated in the adjustment of immune response, both innate and adaptive (Weiss M *et al*., 2013). Through the comparison of naïve joints and arthritis inflammed joints, researchers have shown that IRF5 deficient mice (IRF5-/-) had a significantly less swelling at day 2 in comparison with wild-type controls (WT). Reduced swelling corresponded to a decreased number of cells recovered from excised knee joints at that point. Also, IRF5 knockout mice showed a significantly lower synovial membrane thickening score in the mBSA-challenged knees than WT mice and reduced synovial membrane thickening has also been observed at day 7 (Weiss M *et al*., 2013).

Macrophages and neutrophils are the two major types of myeloid cells involved in RA inflammatory process. Considered the heterogeneity of macrophages (Murray PJ *et al*., 2014), (Sieweke MH *et al*., 2013) and the interesting role of neutrophils able to form distinct subsets and extracellular traps (NETs) (Khandpur R *et al*., 2013), a variety of destructive enzymes as well as autoantigens can be generated (Becher B *et al*., 2014), (Ericson JA *et al*., 2014). Further investigations must be carried out to understand the exact nature of the myeloid cells in various inflammatory diseases; this not only will provide other clues about pathogenesis, but can also contribute to the discovery of more effective therapeutics improving the quality of life of RA subjects.

In this research AIA mouse model has been used to analyse the immune response against viral and bacterial epitopes in order to better understand the possible role of some infections in RA pathogenesis and compare these results with those obtained in human.

CHAPTER 4: Investigating the role of viral and bacterial infections in RA and other rheumatic diseases

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Identification of a HERV-K env surface peptide highly recognized in rheumatoid arthritis (RA) patients: a cross-sectional case-control study

Among environmental factors, the role of viruses in autoimmunity has been long postulated and endogenous retrovirus (HERV) is one potential candidate.

The hypothesis of a potential pathogenic link between HERV-K and RA is based on several observations descending from both polymerase chain reaction (PCR)-based and serological studies (Trela M *et al*., 2016). The prevalence of insertionally polymorphic HERV-K was reported increased significantly in the RA population (Krzysztalowska-Wawrzyniak M *et al*., 2011). Moreover, a significantly increased expression of HERV-K genes and transcription of viral proteins has been demonstrated in peripheral blood, synovial fluid and synoviocytes of RA patients compared to HCs (Hohn O *et al*., 2013), (Reynier F *et al*., 2009). In addition, common amino acid sequences between HERV-K and host antigens have been reported in RA, suggesting that a mechanism of molecular mimicry may play a role in disease pathogenesis (Freimanis G *et al*., 2010), (Nelson PN *et al*., 2014).

Considering this background, we performed an *in silico* analysis to predict key antigenic regions of HERV-K on the basis of their putative ability in igniting an RA-specific humoral response. Candidate epitopes were then synthesized as short peptides, and an enzyme-linked immunosorbent assay (ELISA) was used to test the reactivity of RA and control serum to HERV-K peptides. *In silico* analysis performed using IEDB Analysis Resource software, in particular the Antibody Epitope Prediction, Bepipred linear epitope prediction software

(http://www.iedb.org/home_v3.php), allowed us to identify different antigenic peptides derived from HERV-K env surface protein (UniProtKB Accession number: Q69384).

Four peptides were selected for further analysis:

- HERV-K $env_{19–37}$ (VWVPGPTDDRCPAKPEEEG)
- HERV-K $env_{109–126}$(RPKGKTCPKEIPKGSKNT)
- ERV-K $env_{164–186}$ (SGQTQSCPSAQVSPAVDSDLTES)
- HERV-K $env_{205–226}$ (EKGISTPRPKIISPVSGPEHPE)

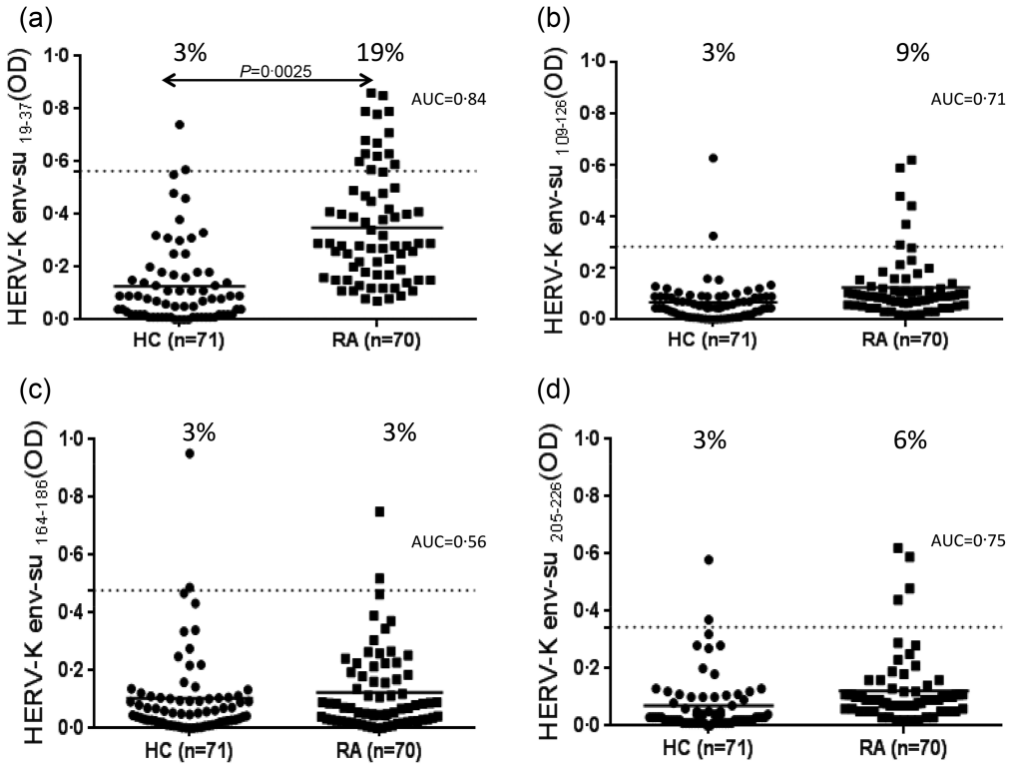
Results

Antibodies against HERV-K env peptides were tested in serum of 70 RA patients and 71 HCs. HERV-K env-$su_{19–37}$ antibodies were above positivity in the sera of 19% RA patients and in only 3% of HCs (AUC=0.84, RA *versus* HCs $p<0.0025$) (Fig. 3a).

Regarding HERV-K env-$su_{109–126}$ peptide, 9% of RA patients and 3% of HCs were positive for antibodies in serum ($p=0.165$, AUC=0.71) (Fig. 3b).

No statistical differences were found regarding positivity against HERV-K env-$su_{164–186}$ in RA patients (3%) *versus* HCs (3%) (AUC=0.56, Fig. 3c). HERV-K env-$su_{205–226}$ peptide was recognized in the sera of 6% of RA patients and 3% of HCs ($p=0.44$, AUC=0.75) (Fig. 3d). No significant difference was found in the proportion of HERV-K env-$su_{19–37}$ antibody positivity according to disease activity (moderate–severe RA *versus* low activity and remission RA) and type of immunosuppressive treatment.

Fig. 3. Prevalence of Abs against peptides derived from HERV-K envelope (env) protein. Scatterplots present median values with interquartile range. Area under receiver-operating characteristic (ROC) curve (AUC) as well as significant *P*-values are displayed.



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No correlation was found between HERV-K env-su$_{19–37}$, antibody levels and RA descriptors [age, DAS-28, HAQ, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)]. The same results were obtained with the other HERV-K env-su peptides tested (data not shown).

Discussion

HERV-K mRNA transcripts and IgG anti-HERV-K Abs have been associated with autoimmunity and cancers, as well as neurological conditions (e.g. MS) and rheumatic diseases (Manghera M *et al*., 2016), (Balada E *et al*., 2010), (Tugnet N *et al*., 2013).

For the first time, a humoral response against selected surface epitopes of HERV-K env-su (HERV-K env-su$_{19–37}$, HERV-K env-su$_{109–126}$ and HERV-K env-su$_{209–226}$) was searched in patients with RA and controls. Among them, the most interesting peptide was HERV-K env-su$_{19–37}$, which was recognized in the sera of 19% of RA patients and in only 3% of controls.

HERV-K env-su$_{19–37}$ has a high homology with different human antigens, including myosin (with 61% of positive amino acids with sequence EAW76415.1) and netrin (73% of positive amino acids with sequence NP_006172.1). Evidence of a molecular mimicry as a pathogenic mechanism linking HERV-K to RA was provided by several groups. Freimanis and co-authors (Freimanis G *et al*., 2010) demonstrated a significant humoral response in RA patients against one HERV-K epitope which shares residues with collagen type II, a common target of pathogenic RA autoantibodies. Similarly, IgGs against antigenic peptide on the matrix segment of HERV-K10 sharing common sequence with IgG1Fc, a target of rheumatoid factor, were demonstrated in RA patients (Nelson PN *et al*., 2014). An intriguing explanation of this result would be that the surface epitope HERV-K env-su$_{19–37}$ may function as an 'autoantigen' driving a secondary immunological response.

Unfortunately, it was not possible to find any statistically significant correlation between antibody positivity and disease activity, systemic inflammation and type of immunosuppressive treatment. It may be possible that therapy could alter the humoral response. Notably, also in the series of RA patients described by Nelson and co-authors, levels of antibodies to HERV-K antigens showed no correlation with RA disease activity (Nelson PN *et al*., 2014).

However, it should be remembered that a number of conditions, both genetic and environmental, may modulate HERV-K env protein transcription contributing to changes in

the amount of immunological response. HERV-K transcription exhibits a significant increase in the presence of proinflammatory cytokines [tumour necrosis factor (TNF)-α and interleukin (IL)-6] in RA with regard to the general population (Freimanis G *et al*., 2010). Exogenous viruses such as EBV, reported to be linked to RA (Erre GL *et al*., 2015), may transactivate and stimulate HERV-K expression (Nelson PN *et al*., 2003). Moreover, differential HERV-K transcription has been shown to be regulated epigenetically by the degree of DNA methylation in RA (Neidhart M *et al*., 2000).

In conclusion, a specific humoral response against a superficial envelope epitope of HERV-K was demonstrated in RA patients. Results of this study clearly add to the evidence for a possible role of HERV-K in RA pathogenesis.

Interferon regulatory factor 5 is a potential target of autoimmune response triggered by Epstein-Barr virus and *Mycobacterium avium* subsp. *paratuberculosis* in rheumatoid arthritis: investigating a mechanism of molecular mimicry

As discussed above, 50% of the risk for RA is attributable to environmental contributors such as microbial infections (Duarte JH*.,* 2015). EBV and mycobacteria were among pathogens reported to be involved in RA pathogenesis (Song YW *et al*., 2010), (Adtani P *et al*., 2015) (Ball RJ *et al*., 2015), (Erre GL *et al*., 2015), (Westergaard MW *et al*., 2015), (Rao DA *et al*., 2017).

In recent years, the potential role of IRF5 in the pathogenesis of RA has been frequently reported (Adtani P *et al*., 2015), (Eames HL *et al*., 2016), (Negi VS *et al*., 2014). IRF5 is a key transcription factor of the immune system, playing an important role in modulating inflammatory immune responses in many cell types including dendritic cells and macrophages by driving them toward a proinflammatory phenotype in concert with cytokines and chemokines expression, and by regulating B cell maturity and antibody production (Popko K *et al*., 2015), (Lino AC *et al*., 2016), (Dekkers J *et al*., 2016), (Olalekan SA *et al*., 2015), (Fillatreau *et al*., 2015), (Mahabadi M *et al*., 2016).

Recent *in vivo* studies identified the importance of IRF5 as a new link between the pathogenic activation of RNA-sensing Toll-like receptors and proinflammatory cytokine production in inflamed joints of arthritic mice (Duffau P *et al*., 2015).

Intriguingly, the presence of a specific seroreactivity against EBV and MAP epitopes cross-reacting with homologous human antigens such as IRF5 have been recently demonstrated in various autoimmune diseases (Cossu D *et al*., 2015), (Mameli G *et al*., 2015). Here it was supposed that significantly increased antibody titers following infection with MAP and EBV

may cross-react with homologous peptides of the transcription factor IRF5 leading to modulation of its expression.

Based on the above-mentioned preliminary findings, our study aimed at evaluating the presence of a cross-reactivity between three homologous peptides derived from EBV tegument protein BOLF1 (BOLF1$_{305-320}$), the MAP_4027 antigen (MAP_4027$_{18-32}$) (Eames HL *et al*., 2016), (Negi VS *et al*., 2014) and the human interferon regulatory factor 5 (IRF5$_{424-434}$) in a group of RA patients and HCs.

Results

71 RA patients (11 males, 60 females; median age 56.18) and 60 HCs (23 males, 37 females; median age 46.1) were enrolled in the study; demographic and clinical features of RA patients and HCs are summarized in the Table 1.

Table 1. Demographic, clinical and laboratory features of RA patients and HCs.

	RA n=71	HCs n=60	p value
Age, yrs	56 (17)	46 (15)	0.0001
Female sex, n (%)	37 (61.7)	59 (83.1)	0.01
HAQ (0-3)	1 (1.37)	-	-
DAS-28	3.32 (2.32)		
CRP, mg/dL	0.5 (1)		
ESR, mm/h	18 (28)		
Steroids use, n (%)	31 (43.7)		
DMARDs, n (%)	43 (60.6)		
Anti-TNF, n (%)	22 (31)		
Tocilizumab, n (%)	12 (16.9)		

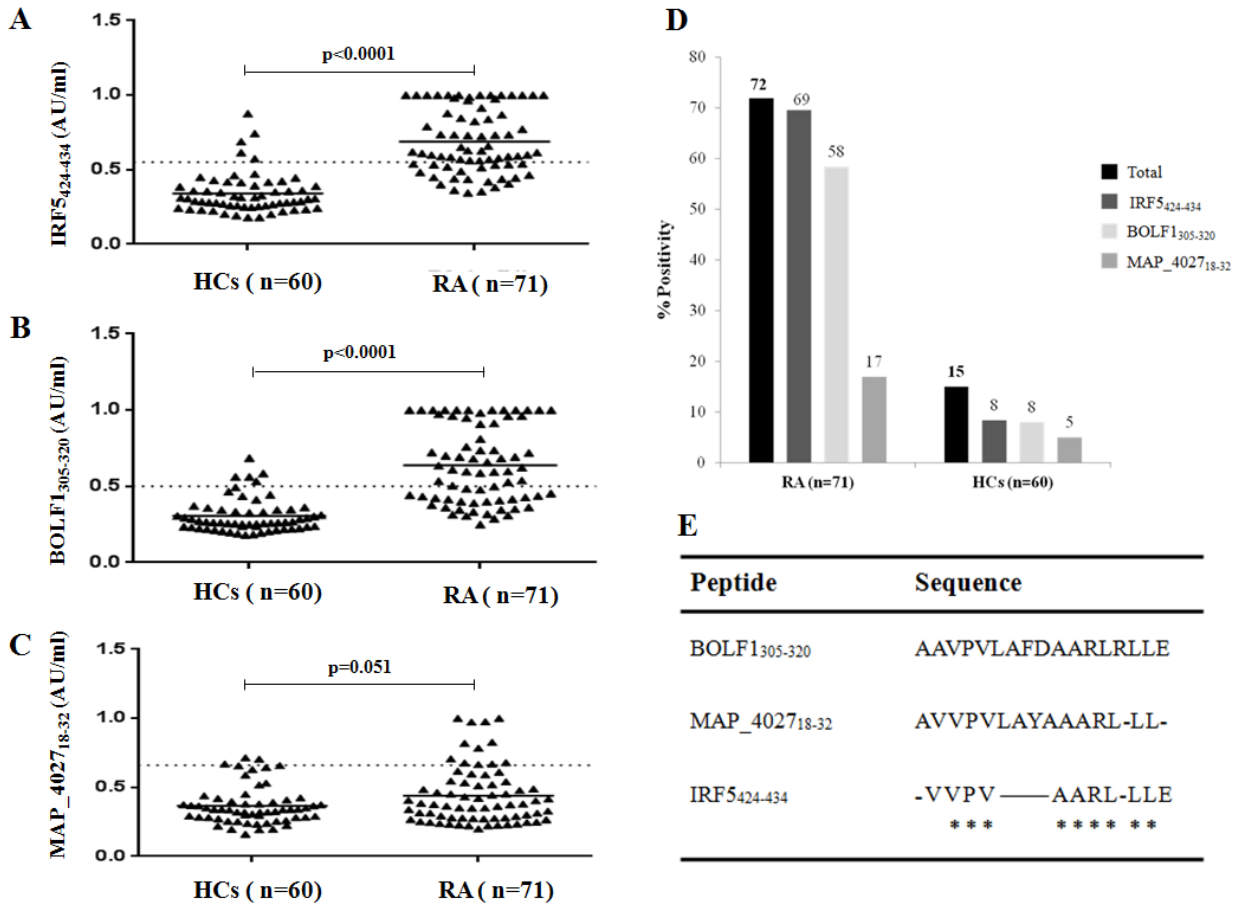
Non normally distributed data are expressed as median (IQR). Health Assessment Questionnaire (HAQ); Disease Activity Score-28 (DAS-28); C-reactive protein (CRP); Erythrocyte sedimentation rate (ESR); Disease modifying anti-rheumatic drugs (DMARDs); Anti-Tumor Necrosis Factor alpha (Anti-TNF).

IRF5$_{424-434}$ was found to have the highest Abs seroreactivity among the selected antigens being positive in 49 out of 71 RA patients (69%) and only in 5 out of 60 HCs (8%; AUC=0.9; p<0.0001; Fig. 4A and Fig. 4D).

Abs against BOLF1$_{305-320}$ were observed in 41 out of 71 RA patients (58%) and in 5 out of 60 HCs (8%; AUC=0.9; p<0.0001; Fig. 4B and Fig. 4D). Humoral responses against MAP_4027$_{18-32}$ was detectable in 12 out of 71 RA patients (17%), whereas HCs showed Abs prevalence accounting for only 5% giving a p value close to the threshold of statistical significance (AUC= 0.6; p=0.051; Fig. 4C and Fig. 4D).

Globally, RA subjects displayed much more increased positivity to at least one of the assessed epitopes compared to HCs with a high degree of statistical significance (71% *vs.* 15%, respectively; p<0.0001; Fig. 4D).

Fig. 4: A-C) ELISA-based analysis of Abs reactivity against the homologous epitopes derived from human, EBV and MAP in RA patients and HCs. The sera were tested against plate-coated $IRF5_{424-434}$ (A), $BOLF1_{305-320}$ (B) and MAP_4027_{18-32} (C) peptides. Bars represent the median ± interquartile range. Cutoff values for Abs positivity are indicated by dashed lines. P-values, respective to each epitope showed a statistical difference between RA patients and HCs and are reported above the distributions. **D) Prevalence of Abs directed against $BOLF1_{305-320}$, MAP_4027_{18-32} and $IRF5_{424-434}$ epitopes in RA patients and HCs.** Total percentage of Abs positivity to at least one peptide is represented by the first bar in each group. Other bars correspond to a single-peptide positivity relative to each epitope. **E) Peptides alignment obtained using Clustal W2.** The results show the region of identity with a star (*), strong similar amino acid with a dot (.) and a missing region in dashes (-).



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A good correlation was found for all the homologous pairs with the highest coefficient observed for BOLF1$_{305\text{-}320}$ and IRF5$_{424\text{-}434}$ peptides (R^2=0.68; Fig. 5A) and slightly lower for BOLF1$_{305\text{-}320}$ and its MAP_4027$_{18\text{-}32}$ homolog (R^2=0.66; Fig. 5B). MAP_4027$_{18\text{-}32}$ and the human-derived IRF5$_{424\text{-}434}$ peptides correlated to a somewhat lower extent than those described, however it still permitted us to hypothesize a cross-reactivity between the two epitopes (R^2=0.61; Fig. 5C).

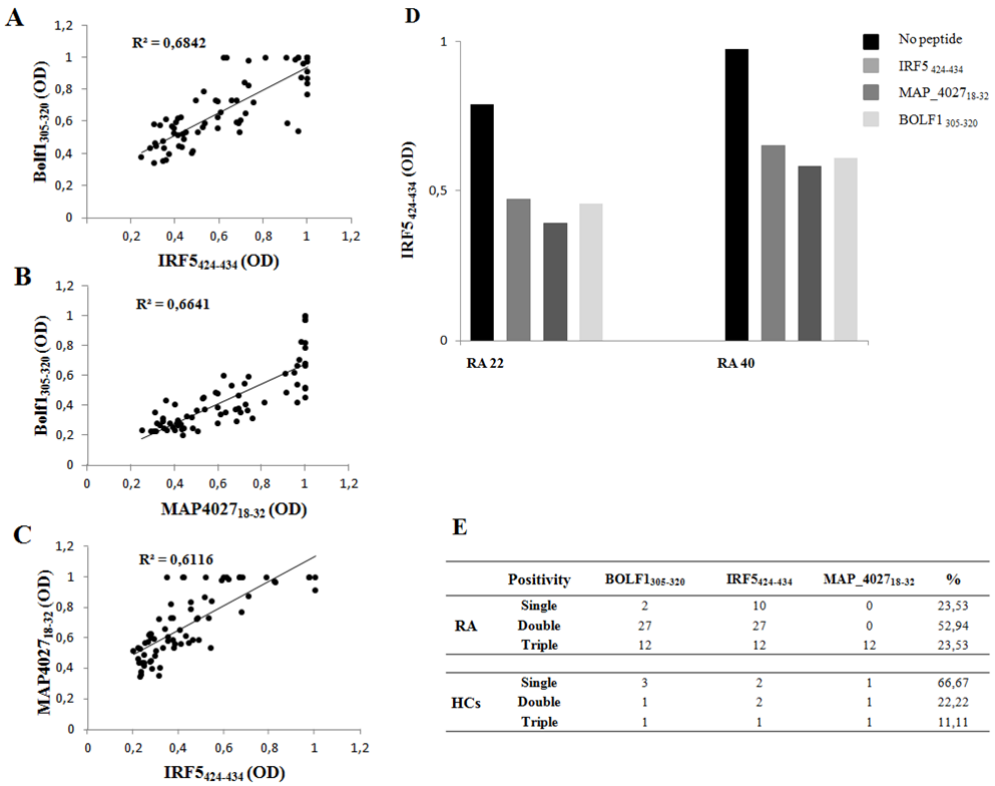
To further demonstrate the presence of a cross-reactivity between MAP/EBV and self epitopes, ELISA-based competitive inhibition assays have been performed selecting IRF5$_{424–434}$ as antigen representative of the highest Abs responses induced in RA patients. Of the three inhibiting concentrations tested, incubation with 10µg/ml peptide solution yielded the most accurate results with the lowest variability among replicates. The low OD values obtained for sera of two RA patients (RA22 and RA40) pre-incubated with BOLF1 and MAP_4027 peptides indicate that binding of Abs specific for the plate-coated IRF5$_{424–434}$ was efficiently inhibited by its homologs (Fig. 5D). Most likely anti-BOLF1 and anti-MAP_4027 Abs are cross-reactive and target the same IRF5 conformational epitope. Moreover, the double IRF5-BOLF1 positivity pattern clearly visible among RA patients was changed in HCs that displayed a case of reactivity to IRF5-MAP_4027 and a single Abs response against MAP (Fig. 5E).

When Fisher's exact test was repeated excluding the two HCs with single and double anti-MAP positivity, statistical significance relative to MAP_4027 greatly improved (p<0.0061). This picture may indicate the presence of Abs specific for the extracellular MAP phenotype that entered in contact with the HCs not MAP susceptible host conferring a natural protection against mycobacterial infection. On the other hand, a higher efficiency of the MAP-derived antigen to inhibit binding of anti-IRF5 Abs coupled with a simultaneous

reactivity to the other two homologous epitopes in cases with the triple-peptide positivity may be indicative of the synergistic role attributed to EBV and MAP in RA.

Analysis of correlation between RA features and positivity of Abs demonstrated a close link between Abs and the burden of systemic inflammation. In particular multivariate regression analysis show that levels of CRP are the main predictors of high levels of Abs against BOLF1$_{305-320}$ and IRF5$_{424-434}$.

Fig. 5: A-C) Scatter plot showing correlations between Abs titers recognizing (A) BOLF1$_{305-320}$ and IRF5$_{424-434}$, (B) BOLF1$_{305-320}$ and MAP_4027$_{18-32}$ and (C) MAP_4027$_{18-32}$ and IRF5$_{424-434}$ in 71 RA patients. Person's correlation was calculated through Graphpad Prism 6.0 software. **D) Competitive inhibition assay in IRF5-coated ELISA plate.** Sera of two RA patients were selected randomly among subjects highly positive for Abs against the three homologous epitopes. Bars indicate OD levels relative to the sera pre-incubated with single peptides or with no peptide. **E) Coincidence of seroreactivity to the homologous peptides among Abs-positive RA and HCs subjects.**



E

	Positivity	BOLF1$_{305-320}$	IRF5$_{424-434}$	MAP_4027$_{18-32}$	%
RA	Single	2	10	0	23,53
	Double	27	27	0	52,94
	Triple	12	12	12	23,53
HCs	Single	3	2	1	66,67
	Double	1	2	1	22,22
	Triple	1	1	1	11,11

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Discussion

The currently accepted theory of RA pathogenesis assigns the main role in autoimmune reactions to different viral or bacterial infections leading to joint inflammation and chronic anatomical damage (Arleevskaya MI *et al*., 2016). Although activation of the T-cell response has a crucial role in RA pathogenesis, the B-cell response is equally responsible for the development of the disease due to the enhanced synthesis of immunoglobulins, usually IgGs (Rao DA *et al*., 2017).

Within pathogenetic hypothesis, the theory of the "molecular mimicry" is based on amino acid sequence or structural motif homology between microbial epitopes and self proteins: due to this homology the immune response against microbial epitopes could also induce undesired humoral and/or cellular immunity against host proteins.

Data from our study demonstrated a remarkable reactivity of RA sera against IRF5 supporting the potential role of IRF5 as an important target in RA specific autoimmunity. Moreover, we also demonstrated a high grade of cross-reactivity between IRF5, EBV and MAP epitopes.

A possible explanation for these results could be that in RA patients past EBV and/or MAP infection may induce specific humoral immunity reacting against IRF5 host protein. This secondary response may contribute, through the epitope-spreading phenomenon, to synovial tissue destruction with the production of Abs against previously “sequestered” antigens and amplification of autoimmune cascade.

Study of genome-wide analysis demonstrated association between IRF5 polymorphisms and anti-CCP negative RA (Dieguez-Gonzalez R *et al*., 2008), (Stahl EA *et al*., 2010) suggesting that RA associated IRF5 polymorphism may lead to higher level of IRF5

expression by promoting stability of IRF5 messenger RNA. In addition, it has been recently demonstrated that IRF5 may act as a crucial mediator of joint inflammation, through Toll-like receptors signals activation in synovial macrophages cells promoting a vicious loop of proinflammatory cytokine and chemokine production and release in articular microenvironment (Duffau P *et al*., 2015).

In this scenario, the cross-reactivity mounted against $IRF5_{424–434}$, $BOLF1_{305–320}$ and $MAP\_4027_{18–32}$ may alter the function of the IRF5 protein, modulating or inhibiting its activity. Penetration of Abs into cell cytosol by endocytosis, a mechanism that has been demonstrated in some cells (Geis C *et al*., 2010), (Magrys A *et al*., 2007) may "regulate" IRF5 expression in a pro-inflammatory manner. The presence of a strong correlation between CRP levels and Abs titer in our series of patients further supports the hypothesis that autoantibodies against IRF5 may increase TLR-regulated production of pro-inflammatory cytokine boosting systemic and local inflammation in RA. Shortcomings of the present study were mainly related to small sample size and a cross-sectional design including RA patients under immunosuppressive and anti-inflammatory treatment at the moment of study enrollment.

In conclusion, this data suggests for the first time that IRF5 may be a target of the immune response in RA. Moreover, molecular mimicry involving EBV and MAP is proposed as a potential mechanism of anti-IRF5 autoimmune response. While association between anti-IRF5 autoantibodies and RA is strongly significant, the functional capacity of these Abs to modulate IRF5 activity remains to be assessed.

Further analysis of T cell responses will provide additional indices on cross-reactivity between BOLF1, MAP and IRF5 epitopes in a similar fashion to previously described EBV gp110 and *E. coli* dnaJ (Toussirot E *et al*., 2000), (Albani S *et al*., 1992).

Rheumatoid arthritis patient antibodies highly recognize IL-2 in the immune response pathway involving IRF5 and EBV antigens

Results

IRF5 is known to mediate virus-induced immune responses including expression of proinflammatory cytokines and its pro-apoptotic effect is activated by EBV in transformed cells (Xu D *et al*., 2011), (Cham CM *et al*., 2012). In SLE, IRF5 was found to negatively regulate the expression of IL-2 (Guo Q *et al*., 2016). IL-2 is crucial for function, expansion and survival of regulatory T cells (T_{reg}) and balance within this pathway is disrupted in Th1-mediated autoimmune diseases such as RA, SLE or T1D (Bayer AL *et al*., 2005), (Thornton AM *et al*., 2004), (Niu Q *et al*., 2012). Recently, the loss of self-tolerance to IL-2 has been described in T1D subjects whose peripheral blood mononuclear cells yielded high quantities of INF-γ upon stimulation with IL-2-derived peptides (Pérol L *et al*., 2016). Similarly, RA patients displayed raised levels of anti-IL-2 Abs supposed to affect IL-2 bioavailability necessary for T_{reg} homeostasis.

In the present study, humoral responses to synthetic IL-2 peptides have been evaluated in a larger cohort of Sardinian RA patients. A correlation analysis with seroreactivity to HERV-K and homologous EBV, MAP and IRF5 epitopes permitted to assess a possible cross-reactivity of the antigens supposedly involved in RA pathogenesis.

Human autoantigens along with EBV elicited the highest responses, while the strongest correlation was found between IL-2 and HERV-K pointing at a potential pathway that links EBV-induced transactivation of retroviral proteins and the subsequent cytokine secretion mediated by IRF5.

The potential to raise Ab responses differed between the two analysed IL-2 peptides. IL-2_{6-20KK} elicited a higher Ab seroreactivity accounting for 39% (n= 55) among RA patients and 7% (n= 10) in HCs (p< 0.0001, Fig. 6A), while Abs against IL-2_{56-70} were detected in 23% (n= 32) of RA subjects and 8% (n= 13) of HCs (p=0.0031, Fig. 6B).

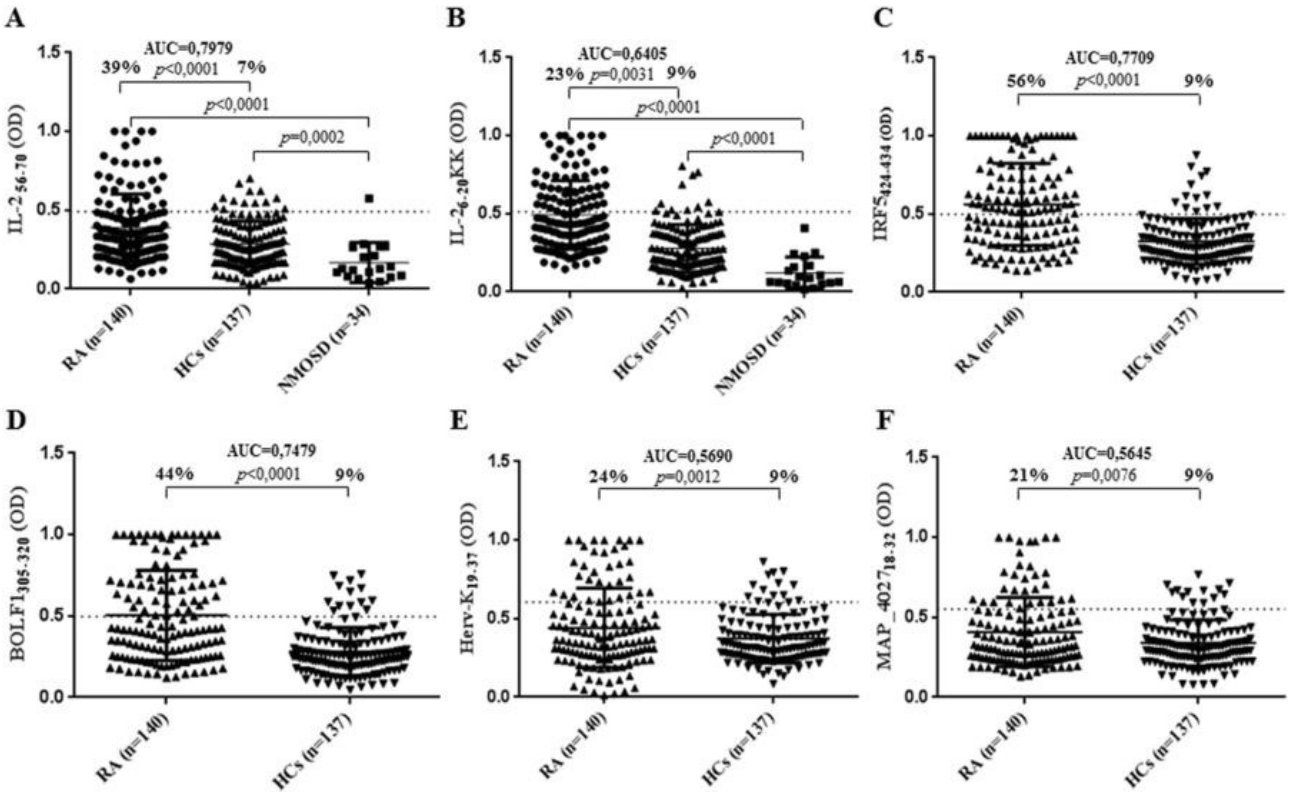
However, the highest levels of autoreactive Abs were directed against IRF$5_{424-434}$ observed in 56% (n= 79) of RA patients and only 9% (n= 13) of HCs (p<0.0001, Fig. 6C).

Slightly lower prevalence was observed for anti-BOLF$1_{305-320}$Abs found in 44% (n= 61) of RA subjects and 9% of HCs (p<0.0001, Fig. 6D).

Responses against Herv-K-env_{19-37} and MAP_4027_{18-32} were maintained at the same levels (9%) among HCs, while seroreactivity of RA patients equalled 24% (n= 34, p=0.0012, Fig. 6E) and 21% (n= 30, p=0.0076, Fig. 6F), respectively.

Despite antigen-related differences in single-type Abs prevalence, all results attained statistical significance with the highest AUC values for IL-2_{6-20KK} and IRF$5_{424-434}$.

Fig. 6. ELISA-based analysis of Abs reactivity against human, viral and MAP-derived peptides in RA, NMOSD and HCs. The sera were tested against plate-coated IL-$2_{6–20KK}$ (**B**), IL-$2_{56–70}$ (**A**), $IRF5_{424–434}$ (**C**), $BOLF1_{305–320}$ (**D**), Herv-$Kenv_{19–37}$ (**E**) and $MAP\_4027_{18–32}$ (**F**) peptides. Bars represent the median ± interquartile range. Thresholds for Abs positivity are indicated by dashed lines. Percentages of Abs prevalence respective to each group, AUC and *P*-values are indicated above the distributions.



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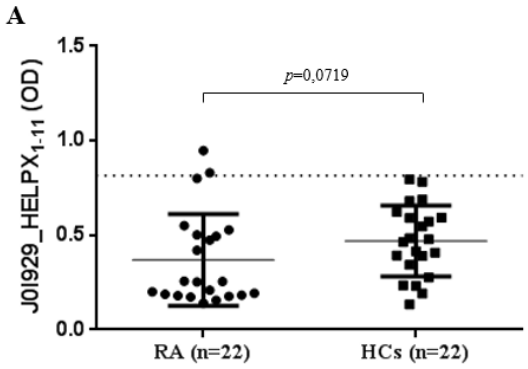
The prevalence of Abs against both IL-2 epitopes was additionally assessed in 34 samples of patients affected by neuromyelitis optica spectrum disorder (NMOSD). Only one patient displayed values above the established cut-off for IL-$2_{56–70}$ (2.9%, Fig. 6A) whereas lower means obtained for IL-$2_{6–20KK}$ were mirrored by the absence of positive cases (Fig. 6B). Moreover, the responses of NMOSD patients were markedly lower compared not only to RA subjects, but also to HCs.

To test the specificity of humoral responses mounted against the selected peptides, 22 HCs and 22 RA patients were randomly selected from the study population and tested for seroreactivity against J0I929_HELPX$_{1–11}$ control peptide derived from *Helicobacter pylori* homologous to human ZnT8 (Masala S ***et al.***, 2015). In both groups, half number of samples tested positive to at least one (HCs) or all (RA) of the previously assessed peptides. The observed mean values were slightly higher for HCs and corresponded to the absence of positive subjects compared to 9% (n=2) among RA patients, however statistical significance was not attained (p=0.07). Interestingly, RA individuals with multiple Abs positivity presented lower mean values compared to HCs with single-peptide positivity (Fig. 7).

Fig. 7. Abs reactivity against the antigenic peptide derived from *H. pylori* in RA patients and HCs. Bars represent mean value ± interquartile range, while dashed lines indicate the positivity threshold. Despite sequence homology to human ZnT8 protein fragment, no significant differences in Abs levels were detected.



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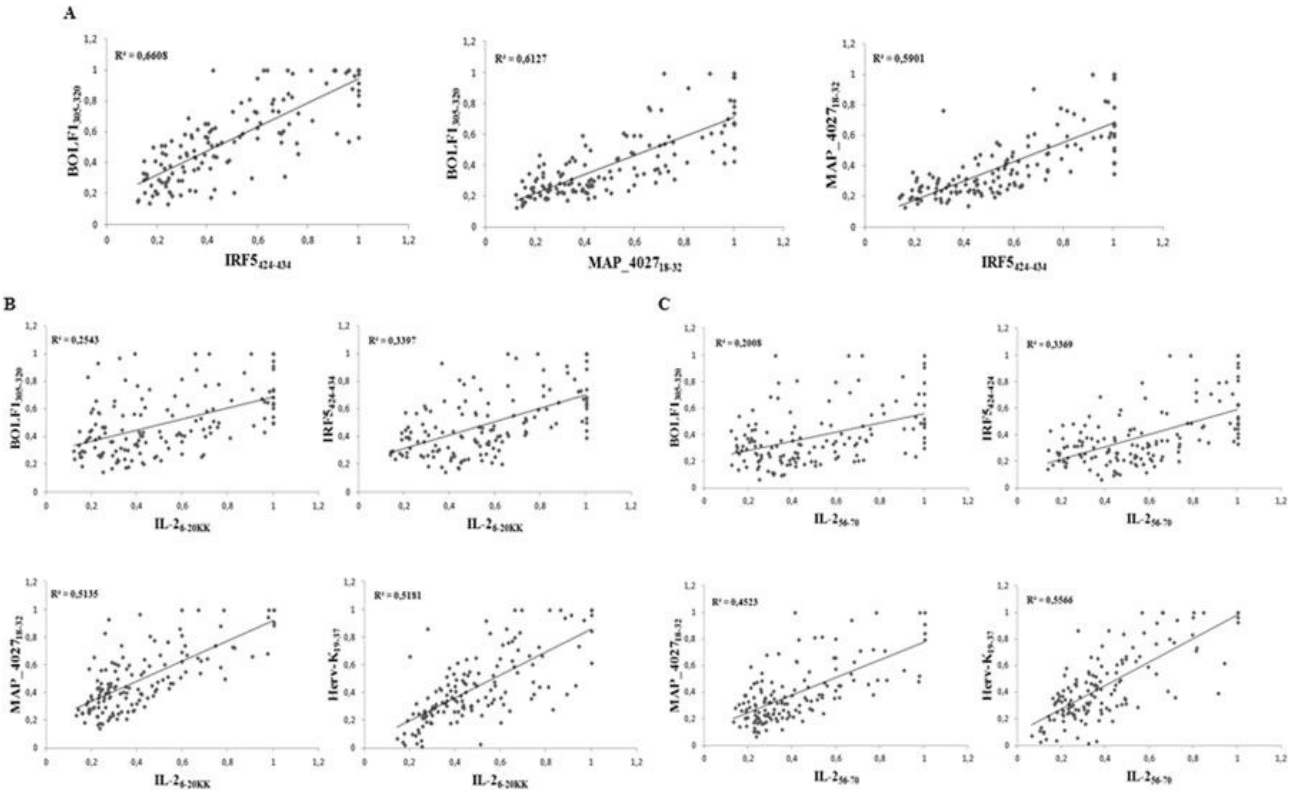
To define associations between the antigenicity of the assessed peptides, correlation analyses of Abs positivity values among RA patients was performed (Fig. 8). The highest coefficients were obtained for the homologous epitopes BOLF1$_{305–320}$, MAP_4027$_{18–}$

$_{32}$ and IRF5$_{424–434}$ in pairwise plots (Fig. 8A) pointing at cross-reactivity due to shared amino acid sequence.

Correlation trends of both IL-2 peptides were similar with respect to the other antigens (Fig. 8B and Fig. 8C): Herv-Kenv$_{19–37}$ and MAP_4027$_{18–32}$ correlated moderately with either IL-2$_{6–20KK}$ or IL-2$_{56–70}$, however IL-2$_{56–70}$/Herv-Kenv$_{19–37}$ distribution corresponded to a slightly higher R^2 value (Fig. 8B). Unexpectedly, weak to modest correlations were found between IL-2, IRF5$_{424–434}$ and BOLF1$_{305–320}$ (Fig. 8C).

Fig. 8. Scatter plots showing correlations between Abs titers in RA patients. Pairwise distributions are classified for homologous peptides (**A**), IL-2$_{6–20KK}$ (**B**) and IL-2$_{56–70}$ (**C**). Each dot correspond to OD values obtained for a single patient.



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Further evaluation was performed to investigate multiple positivity against the assessed peptides in order to verify whether correlations between Ab titers find correspondence with the overlap in seroreactivity against IL-2 (Table 2). In contrast to mild correlations of both IL-2 epitopes plotted against IRF5$_{424–434}$ or BOLF1$_{305–320}$, double or triple humoral responses to these antigens were detected in a major number of subjects with distinctly higher percentages for IL-2/IRF5 reflecting single-peptide Abs prevalence. On the other hand, responsiveness to Herv-Kenv$_{19–37}$ overlapped well with the presence of anti-IL-2$_{56–70}$ Abs as expected from the correlation analysis and stood out also in triple positivity with positivity to IRF5$_{424–434}$. Even though multiple responses among HCs are low in general, they visibly tend to diminish for Abs against at least three antigens.

Table 2. Multiple Abs prevalence in RA patients and HCs. Seroreactivity against IL-2 antigens was compared with humoral responses to MAP, EBV, HERV-K and human IRF5 peptides. The numbers of subjects positive for anti-IL-2$_{6–20KK}$ and/or anti-IL-2$_{56–70}$ Abs are reported with relative percentages in brackets. Horizontal bars indicate Abs against at least two antigens identified in the samples with IL-2 referred to as both IL-2$_{6–20KK}$ and IL-2$_{56–70}$. p<0.0001 for all values except [a]IL-2$_{56–70}$ (p<0.0002) and [b]IL-2$_{56–70}$ (p<0.0003).

IL-2$_{56-70}$		IL-2$_{6-20\,KK}$						
HCs	RA	HCs	RA	IL-2	BOLF1$_{305-320}$	IRF5$_{424-434}$	MAP_4027$_{18-32}$	Herv-K$_{19-37}$
[b] 5 (3.65)	24 (17.14)	5 (3.65)	36 (25.17)	———	———			
2 (1.46)	27 (19.28)	4 (2.92)	43 (30.71)	———		———		
[b] 3 (2.19)	20 (14.28)	4 (2.92)	28 (20.00)	———			———	
[a] 5 (3.65)	25 (17.86)	0	29 (20.71)	———				———
2 (1.46)	20 (14.28)	0	24 (17.14)	———	———			———
1 (0.73)	20 (14.28)	2 (1.46)	27 (19.28)	———		———	———	
1(0.73)	24 (17.14)	3 (2.19)	33 (23.57)	———	———	———		
0	19 (13.57)	2 (1.46)	27 (19.28)	———	———		———	
1 (0.73)	17 (12.14)	0	19 (13.57)	———			———	———
1 (0.73)	23 (16.43)	0	27 (19.28)	———		———		———
0	19 (13.57)	1 (0.73)	26 (18.57)	———	———	———	———	
0	19 (13.57)	0	23 (16.43)	———	———	———		———
1 (0.73)	17 (12.14)	0	20 (14.28)	———		———	———	———
0	19 (13.57)	0	19 (13.57)	———	———		———	———
0	16 (11.43)	0	19 (13.57)	———	———	———	———	———

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Dr. Bo Marco – "Immune response against *Mycobacterium avium* subsp. *paratuberculosis*, Epstein-Barr virus, HERV-K and IRF5 in rheumatoid arthritis" – PhD thesis in *Life Sciences and Biotechnologies* – University of Sassari, Italy

Upon sex-related screening of RA samples, females showed higher mean Abs values and positivity prevalence for all peptides compared to males, however statistical significance was reached only for IRF5$_{424–434}$ (p=0.034). After classification of RA patients and HCs in three age groups (≤49, 50–59 and ≥60), the highest responsiveness was observed for BOLF1, IRF5 and IL-2$_{6–20KK}$ (Table 3). This trend was clearly visible in the youngest RA group regardless of sex, however females maintained it more stably until the age of 59. In contrast to men, humoral responses of RA and HCs women were not significant in the oldest group but this could be affected by a small number of elderly HCs in our study population. A general decrease in Ab positivity proportional to age was common to either male or female patients. Importantly, seroreactivity to IRF5 exceeded 93% in the youngest females and reached a 100% in ≤49 year-old males (one patient).

Table 3. Age and sex related Abs prevalence in RA patients and HCs. The numbers of individuals responsive to single antigens are provided with relative percentages. Statistically significant values are highlighted in bold.

Gender	Age (y)	N	Subjects	BOLF$_1$	*P*	IRF$_5$	*P*	MAP_4027	*P*	IL-2 $_{56-70}$	*P*	IL-2 $_{6-20KK}$	*P*	Herv-K$_{19-37}$	*P*
Females	≤49	16	RA	12 (75%)	**<0,0001**	15 (93,75%)	**<0,0001**	5 (31,25%)	**0,0063**	5 (31,25%)	0,088	8 (50%)	**<0,0001**	5 (31,25%)	0,38
		65	HCs	8 (12,30%)		6 (9,2%)		5 (7,6%)		9 (13,8%)		6 (9,2%)		9 (13,8%)	
	50-59	41	RA	21 (51,21%)	**<0,0001**	23 (56,09%)	**<0,0001**	8 (19,51%)	0,51	10 (24,39%)	**0,045**	20 (48,78%)	**<0,0001**	9 (21,95%)	0,13
		19	HCs	2 (10,52%)		1 (5,2%)		3 (15,78%)		1 (5,2%)		1 (5,2%)		1 (5,2%)	
	60-82	49	RA	17 (34,69%)	0,17	25 (51,02%)	**0,024**	11 (22,44%)	0,92	12 (22,44%)	0,61	17 (34,69%)	0,081	15 (30,61%)	0,17
		6	HCs	1 (16,66%)		1 (16,66%)		0		1 (16,66%)		1 (16,66%)		0	
Males	≤49	6	RA	4 (66,66%)	**0,0046**	6 (100%)	**0,0001**	3 (50%)	0,094	3 (50%)	0,302	4 (66,66%)	**0,0449**	2 (33,33%)	0,34
		15	HCs	2 (13,33%)		1 (6,6%)		4 (13,3%)		1 (6,6%)		2 (13,33%)		1 (6,6%)	
	50-59	11	RA	2 (18,18%)	**0,0023**	3 (27,27%)	0,535	1 (9%)	0,43	2 (9%)	**0,015**	1 (18,18%)	**0,0006**	2 (18,18%)	0,198
		23	HCs	0		3 (13,04%)		1 (4,3%)		1 (4,3%)		0		1 (4,3%)	
	60-87	17	RA	5 (29,41%)	**0,0035**	7 (41,17%)	0,104	3 (17,64%)	**0,0246**	1 (5,8%)	**0,0211**	4 (23,52%)	**0,0005**	1 (5,8%)	0,652
		9	HCs	0		1 (11,1%)		0		0		0		1 (11,1%)	

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PCA analysis permitted to identify relationships between clinical variables and the selected epitopes with 79.17% of cumulative variation describing four principal components (Table 4).

Table 4. **Correlation coefficients between inflammation measures, demographic data and seroreactivity relative to the selected antigens**. All correlations are expressed as squared cosines of the variables.

	PC1	PC2	PC3	PC4
BOLF1	**0,629**	0,017	0,063	0,003
IRF5	**0,732**	0,014	0,037	0,004
MAP_4027	**0,820**	0,000	0,000	0,005
IL-2_{56-70}	**0,685**	0,042	0,012	0,008
IL-2_{6-20KK}	**0,726**	0,047	0,028	0,001
Herv-K	**0,596**	0,099	0,037	0,000
ESR	0,044	0,281	**0,475**	0,007
CRP	0,023	**0,660**	0,002	0,120
Age	0,041	0,065	**0,590**	0,015
Sex	0,054	0,206	0,004	**0,727**

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Correlation between Ab values and two inflammatory parameters was low but attained a statistical significance that differed based on the analysed measure: ESR correlated to Ab values towards IL-2 and HERV-K, while CRP yielded higher coefficients in plots with the homologous MAP, EBV and IRF5 antigens. No correlation with other clinical data was found.

Discussion

Recent reports on the loss of self-tolerance to IL-2 in autoimmune diseases provided basis to evaluate the presence of anti-IL-2 Abs in Sardinian RA patients in association to antigens most frequently described as possible contributors to RA progression. The current results confirm the involvement of IL-2 in RA at higher rates compared to a French cohort (39% *vs.* 15%, respectively) (Pérol L *et al*., 2016) and is mirrored by a concomitant positivity to peptide antigens derived from EBV, HERV-K, MAP or human IRF5. The latter

has been linked to acute inflammation as a factor promoting polarization of macrophages towards an inflammatory phenotype in antigen-induced RA mouse models and driving Th1/Th17 responses (Krausgruber T *et al*., 2011), (Weiss M *et al*., 2015), (Weiss M *et al*., 2013).

In the present study, IRF5, together with the EBV surface tegument protein BOLF1 and IL-2, triggered the greatest response even though devoid of a good correlation with IL-2.

This suggests that the association between the two human autoantigens may not be proportionally dependent on Ab titers but favour autoimmunity when a tolerance threshold is surmounted. In contrast, IRF5 correlated well with homologous BOLF1 and MAP epitopes pointing at molecular mimicry that leads to a probable cross-reactivity with the assessed environmental agents to which humans are constantly exposed. This was recently confirmed by the competitive inhibition assay in our previous study (Bo M *et al*., 2018a).

For IL-2, the best correlation was obtained in the plot with HERV-K. While reactivation of endogenous retroviral protein expression may elicit serological and cell-mediated responses, an uncontrolled expansion of T_{reg} cells in subjects who lost self-tolerance to IL-2 or IRF5 may explain the development of autoimmunity. Interestingly, over 30% of the assessed RA cohort displayed anti-IL-2/IRF5 Abs in a highly significant double positivity ($p<0.0001$) and a more frequent multiple seroreactivity was observed in RA patients compared to HCs (Table 2).

Major prevalence of Abs directed against all single peptides and higher mean Abs values obtained for RA females in a sex-related analysis point at a more grave disease course proper to women and highlight the involvement of IRF5.

This was mirrored by a strikingly high prevalence of Abs against IRF5 in the ≤49 year-old group independently of patients' sex. An elevated general seroreactivity observed in the youngest group that decreases with age points at a strong immune responses accompanying early disease onset.

A significant correlation between levels of anti-IL-2 Abs and measures of systemic inflammation (Table 4) is supportive of the hypothesis that anti-IL-2-driven impairment of T_{reg} activity may alter autoimmune processes and inflammatory burden. Other than expected, no significant correlations have been found between disease severity, immunosuppressive treatment, RF and ACPA status with levels and positivity of anti-IL-2 Abs. It should be acknowledged that all patients were under different immunosuppressive drugs at the moment of sample collection. The heterogeneity of treatment across subjects may have biased interpretation and significance of associations between humoral responses and inflammatory measures.

Further analysis of IL-2 levels, quantification of INF-γ upon stimulation with the analysed peptides and T_{reg} activity are needed to complete our observations. More numerous groups of the youngest patients at RA onset and elderly HCs would additionally permit to associate the efficacy of therapy in modulating serological and cell responses.

PtpA and PknG proteins secreted by *Mycobacterium avium* subsp. *paratuberculosis* are recognized by sera from patients with rheumatoid arthritis: a case-control study

The aim of this work was to evaluate the presence of Abs directed against two proteins of MAP in sera of RA subjects, which are crucial for the survival of the pathogen within macrophages. Moreover, we analyzed the correlation of immune response to both proteins with the following homologous peptides: $BOLF1_{305-320}$, MAP_4027_{18-32} and $IRF5_{424-434}$ to understand how the synergic role of EBV and MAP infection in genetically predisposed subjects may lead to a possible deregulation of IRF5.

Results

84 RA patients (19 males, 65 females; median age 59.0 years) who met the 2010 ACR/EULAR Classification Criteria for RA (criteria of the American College of Rheumatology) (Aletaha D *et al*., 2010) were enrolled at the Outpatient Clinic of the Rheumatology Unit, Department of Clinical and Experimental Medicine, University Hospital of Sassari, Italy.

A total of seventy-nine HCs (25 males, 54 females; median age 46.7 years) were recruited at the Blood Transfusion Centre of Sassari, Italy. Demographic, clinical and laboratory features of RA patients and HCs are summarized in Table 5.

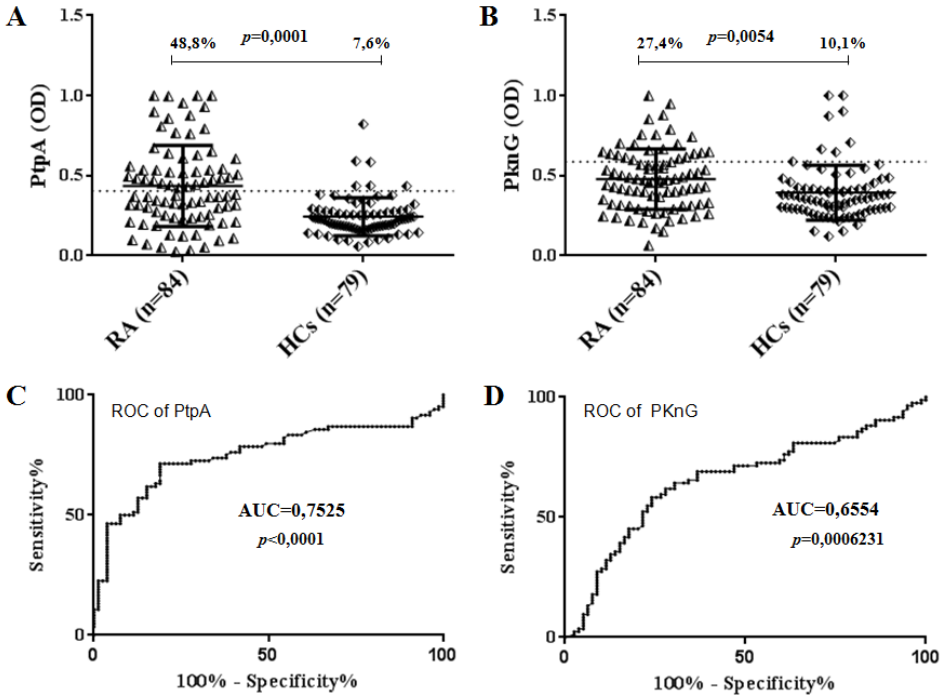
Table 5. Demographic, clinical and laboratory features of RA subjects and HCs

	RA n=84	HCs n=79
Age, years	59.0 ± 9.9	46.7 ± 11.6
Female sex, n (%)	65 (77.38)	54 (68.35)
Disease duration, months	145 ± 134	-
ACPA positivity, n (%)	68.6	-
RF positivity, n (%)	74.5	-
HAQ (0-3)	0.92 ± 0.73	-
DAS-28	3.72 ± 1.38	-
CRP, mg/dL	0.88 ± 0.96	-
ESR, mm/h	30.7 ± 25.8	-
Steroids use, n (%)	43.4	-
DMARDs use, n (%)	66.3	-
Anti-TNF use, n (%)	24.1	-
Tocilizumab use, n (%)	12	-
Abatacept use, n (%)	4.9	-

Production of recombinant PtpA and PknG proteins is described in the materials and methods chapter.

Abs against PtpA were found to be above positive level in 41 out of 84 (48.8%) RA patients, whereas 6 out of 79 (7.6%) HCs were positive in serum (AUC=0.7525, *p*=0.0001, Fig. 9A and Fig. 9C). Regarding PknG, 23 out of 84 (27.4%) RA patients, 8 out of 79 (10.12%) HCs were positive in serum (AUC=0.6554, *p*=0.0054, Fig. 9B and Fig. 9D).

Fig. 9. ELISA-based analysis of antibody reactivity against two proteins of MAP in RA subject and HCs. The sera were tested against plate-coated PtpA (A) and PknG (B) proteins.



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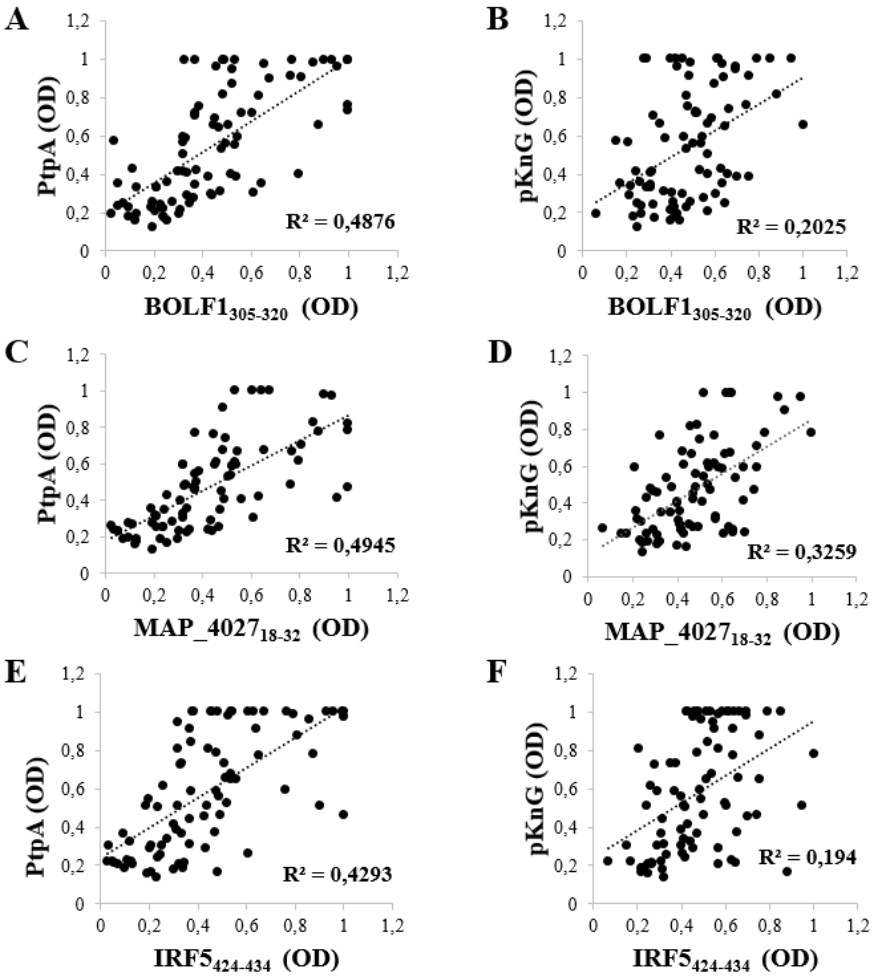
A noteworthy correlation was found between PtpA and all homologous pairs (BOLF1, MAP_4027 and IRF5). The highest correlation was found between PtpA and MAP_4027$_{18-32}$ (R^2=0.4945; Fig. 10C) followed by BOLF1$_{305-320}$ (R^2=0.4876; Fig. 10A). IRF5$_{424-434}$ results showed the lowest correlation with PtpA, compared to MAP_4027 and BOLF1$_{305-320,}$ but it is still well-founded to hypothesize a cross-reactivity between the two epitopes (R^2=0.4293; Fig. 10E). On the other hand, lower correlations between PknG and all three homologous pairs was found compared to PtpA, i.e., (R^2=0.2025; Fig. 10B) with BOLF1$_{305-320}$, (R^2=0.194; Fig. 10F) with IRF5$_{424-434}$ and (R^2=0.3259; Fig. 10D) with MAP_4027$_{18-32}$. The correlation analysis showed that EBV and MAP have a synergic effect in the pathogenesis of RA.

Globally, RA subjects displayed a significantly increased positivity to PtpA protein compared to HCs with a high degree of statistical significance (Fig. 9A and Fig. 11A). However, a lower

reactivity was found against PknG (Fig. 9B and Fig. 11A). Finally, a lower reactivity against MAP_4027$_{18\text{-}32}$ was observed when compared to that against PtpA and PknG, but with a statistical significance vs. HCs (22.6% vs. 3.8%, p=0.0004, Fig. 11B).

No correlation was detected between anti-PtpA/PknG Abs, serological and clinical features of RA, including disease activity and the type of immunosuppressive treatment, with the exception of a marginal correlation between Abs against PtpA serum concentration and a number of tender and swollen joints (r=0.224, p=0.042 and r=0.279, p=0.010, respectively). In the linear regression analysis, performed adjusting for age and sex, a significant linear correlation between the number of swollen joints and the serum concentration of Abs against PtpA was found (B (95% CI) = 0.025 (0.004-0.045), p=0.018).

Fig. 10. A-F) Scatter plot showing correlations between Abs titers recognizing (A) BOLF1$_{305-320}$ and PtpA, (C) MAP_4027$_{18-32}$ and PtpA, (E) IRF5$_{424-434}$ and PtpA, (B) BOLF1$_{305-320}$ and PknG, (D) MAP_4027$_{18-32}$ and PknG, (F) IRF5$_{424-434}$ and PknG in 84 RA patients and 79 HCs. Person's correlation was calculated through Graphpad Prism 6.0 software.



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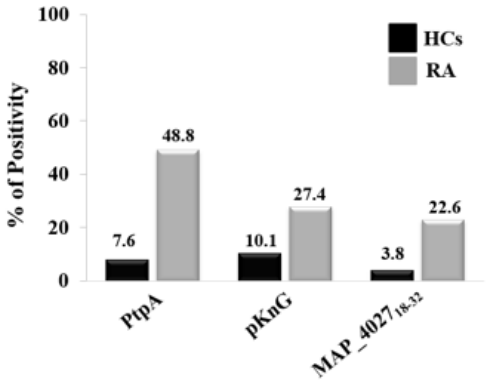
Analyzing single, double, and triple positivity for PtpA, PknG and MAP_4027$_{18-32}$ peptide, we observed that 11 RA patients tested positive for all antigens, while no positivity was found in HCs (p=0.0007, Fig. 11A). These data showed that, in attempt to delete the pathogen, the immune system triggers an immune reaction against different portions of MAP but with a major reactivity against PtpA.

Fig. 11. A) Coincidence of seroreactivity to the PtpA, PknG proteins and MAP_4027$_{18-32}$ peptide among Abs-positive RA and HCs subject. B) Prevalence of Abs directed against PtpA, PknG and MAP_4027$_{18-32}$ in RA patients and HCs.

A

	Positivity	PtpA	P	PknG	P	MAP_4027	P
RA	Single	13	0.0054	2	NS	1	NS
HCs		2		4		3	
RA	Double	17	0.0045	10	NS	7	0.0140
HCs		4		4		0	
RA	Triple	11	0.0007	11	0.0007	11	0.0007
HCs		0		0		0	

B



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The results showed that, generally, PtpA and PknG proteins are highly recognized in RA patients, which may be useful to analyze the molecular mechanisms in macrophages after MAP infection. It is thus helpful to understand how MAP grows inside the macrophage to understand its implication in RA pathogenesis.

Discussion

In this study, the link between MAP and macrophages in RA was further investigated, taking into consideration that different cell populations are involved in the autoimmune process. However, it became increasingly clear that macrophages are very important in the pathogenesis of RA, as they generate cytokines that enhance inflammation and contribute to destruction of bone and cartilage. Bacterial and viral infections and other environmental factors can modify the function of macrophages by modulating the expression of transcription factors. MAP is an intracellular bacterium that grows into the macrophages, and, for this reason, it may be suspected that this infection can lead to a dysregulation of these cells.

Analysing the immune response against PtpA and PknG, which are crucial proteins necessary for the survival of the pathogen within macrophages, in RA patients and HCs, it has been found that the Abs level against both MAP proteins is significantly higher in RA patient sera than HCs sera. Moreover, it may be supposed that these patients have been either exposed or infected with MAP, which supports the theory on the impact of MAP infection on the development of RA. In addition, the existence of a correlation among immune responses against MAP_4027, BOLF1, and IRF5 was evaluated. This is helpful for understanding how the synergic role of EBV and MAP infection in genetically predisposed subjects can lead to a possible deregulation of IRF5.

The present results are in line with corresponding studies that showed that sera of sheep previously infected with MAP, as well as in Crohn's disease and MS have a significantly higher levels of reactivity against PtpA (McNees AL *et al*., 2015), (Kuenstner JT *et al*., 2017), (Feller M *et al*., 2007), (Slavin YN *et al*., 2018). Similarly, a strong immune response found in RA patients compared to HCs points at PtpA as a candidate for the potential detection of humoral immune responses in human. Among different mechanism involved in RA pathogenesis, an interesting role is played by molecular mimicry and citrullination (Trouw LA *et al*., 2017). In fact, the presence of a statistically significant antibody response between RA and HCs against the citrullinated peptide of MAP_4027$_{18\text{-}32}$Cit must be highlighted. Using the Fisher's exact test, MAP_4027 peptide and citrullinated peptide in positive patients were compared. Results showed a statistical difference in RA patients (26% *vs.* 43%, p=0.0170) supporting the hypothesis of the involvement of MAP in RA. These two virulence factors interfering with signal transduction in the host and are secreted proteins necessary for the survival of the pathogen in the harsh environment presented by the macrophage (Bach H *et al*., 2006), (Bach E *et al*., 2018).

The noteworthy result towards MAP citrullinate peptide suggests that it is likely that MAP infection may trigger a citrullination process in the attempt to eliminate the pathogen by self-feeding the autoimmune process. Significantly, by carrying out a bioinformatics analysis, PtpA and PknG proteins present in their primary sequence could be potentially citrullinated from the macrophage PAD after bacterial infection, triggering the inflammatory response (PtpA (Protein tyrosine phosphatase A; UniProtKB Accession number: A0A200GPK8) and PknG (Protein kinase G; UniProtKB Accession number: A0A202FS53)). It has now been established that citrullination, together with carbamylation, is a process that, although physiologically present in nature, is more exacerbated in autoimmune diseases. Furthermore, they are a predictive marker of the onset of the disease. This is significant because the silent stage of MAP when infecting cells in which the citrullination may occur, even the disease develops much later. Therefore, the next step in this research is to citrullinate these proteins and to evaluate the Ab response in subjects with active arthritis and those at onset in order to understand if citrullination is part of a process generated by the host to eliminate the pathogen. In future work, the reactivity towards PtpA and PknG in the different rheumatic diseases should be tested in order to analyse similarities and/or differences.

Moreover, to understand the binding of MAP infection with RA in detail, it is planned to analyze the molecular pathways of macrophages *in vitro* and in mouse models of arthritis following MAP infection. This helps to understand what kind of molecules (cytokines and chemokines (McInnes IB 2007) are produced and how these can influence the activity of the other immune cells as the same macrophages and neutrophils (O'Neil LJ *et al*., 2019), (Weiss M *et al*., 2015), (Wright HL *et al*., 2014). Furthermore, the analysis of expression of some important transcription factors in the regulation of the immune response, such as IRF5

(Almuttaqi H *et al*., 2018), (Khoyratty TE *et al*., 2018), JUNB (Moon YM *et al*., 2017), with the RNA sequencing (RNA-seq) technique and qPCR will allow for a better understanding for how MAP is involved in the etiopathogenesis of RA.

Association between Lipoprotein Levels and Humoral Reactivity to *Mycobacterium avium* subsp. *paratuberculosis* in Multiple Sclerosis, Type 1 Diabetes Mellitus and Rheumatoid Arthritis

A recent study in sheep and cattle showed that MAP uses cholesterol as a primary carbon-based energy source during early stages of infection (Johansen MD *et al*., 2018). The uptake and trafficking of MAP in human cells seems to be favored in cholesterol-rich compartments which are slow to acidify (Keown DA *et al*., 2012). It has also been demonstrated that MAP, similar to other pathogenic mycobacteria (Mattos KA *et al*., 2014), (Brzostek A *et al*., 2009), (Pandey AK *et al*., 2008), (de Chastellier C *et al*., 2006), (Gatfield J *et al*., 2000), is able to manipulate host lipid metabolism and accumulate cholesterol within macrophages to establish infection (Johansen MD *et al*., 2019).

In addition to that, as before mentioned, MAP has been associated with MS, T1DM, and RA through a molecular mimicry mechanism.

Here, the correlation between humoral reactivity against MAP and serum lipoprotein levels has been investigated in subjects at T1DM risk (rT1DM) grouped by geographical background and in patients affected by MS and RA.

Results

In the present study, the following groups were formed based on the pathological condition: 22 MS patients (1:1.4 male/female ratio; 40 years median age), 22 patients at T1DM risk (1:1.6 male/female ratio; 4 years median age), 22 RA subjects (1:2.7 male/female ratio; 49 years median age) and 22 healthy controls (HCs; 2.7:1 male/female ratio; 37 years median age).

MS patients diagnosed according to the revised McDonald diagnostic criteria (Polman CH *et al*., 2011) were enrolled at the Neurological Clinic of the University Hospital of Cagliari, Italy. At the time of the study, 19 patients were diagnosed as relapsing remitting MS and 3 as secondary progressive MS. The expanded disability status scale (EDSS) values ranged from 0 to 7.0 with the average of 1.93 average. Demographic, clinical and laboratory features of MS and HCs are summarized in Table 6.

Table 6. Demographic and clinical characteristics of MS patients and HCs

	MS n=22	**HCs n=22**
Age, years	39.77 ± 12.53	36.72±11.59
Female, n(%)	13(59.09)	16(72.72)
Cortisone	12	
No cortisone	10	
EDSS	2.53 ± 2	
Relapse, n(%)	13(59.09)	
RRMS, n(%)	19(86.36)	
SPMS, n(%)	3(13.63)	

MS, multiple sclerosis; HCs, healthy controls; EDSS, Expanded Disability Status Scale; RRMM, Relapsing-Remitting MS; SPMS, Secondary progressive MS.

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Subjects at T1DM risk were recruited in Sardinia at Diabetology ward, St. Michele Hospital of Cagliari and in mainland Italy at the Tor Vergata University Hospital of Rome. T1DM risk was intended as disease familiarity between first-degree relatives, detection of high-risk HLA alleles and/or presence of diagnostic autoantibodies (ZnT8, GADA, IA2A, IAA and/or ICA). All subjects were free from therapy.

RA patients diagnosed according to the 2010 ACR/EULAR classification criteria were enrolled at the outpatient clinic of the Rheumatology Unit, Department of Clinical and

Experimental Medicine, University Hospital of Sassari, Italy. Collected data included: duration of RA, therapy (steroids, Tocilizumab, DMARDs and/or anti-TNF-α), levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) levels, positivity to rheumatoid factor and anti-cyclic citrullinated peptide (anti-CCP), Disease Activity Score-28 (DAS-28) and grade of disability defined through the health assessment questionnaire. Demographic, clinical and laboratory features of RA and HCs are summarized in Table 7.

Table 7. Demographic and clinical characteristics of RA patients and HCs

	RA n=22	HCs
Age, years	49.3 ± 8.5	36.72±11.59
Female, n(%)	16(72.7)	16(72.72)
ESR, mm/h	18.9 ± 15	
CRP, mg/dL	1.01 ± 0.9	
DAS28 score	3.15 ± 1.3	
HAQ score	0.7 ± 0.6	
ACPA positivity, n(%)	12(54.5)	
RF positivity, n(%)	13(59)	
Steroid use, n(%)	12(54.5)	
Steroid dose, mg/day	1.58± 2.6	
DMARDs use, %	14(63.6)	
TNFi use, %	4(3.3)	
Tocilizumab use, %	7(58.3)	

RA, rheumatoid arthritis; HCs, healthy controls; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS-28, disease activity score-28 joints; HAQ, health assessment questionnaire; ACPA, anti-citrullinated peptide antibodies; RF, rheumatoid factor; DMARDs, disease-modifying anti-rheumatic drugs; TNFi, tumor necrosis factor-alpha inhibitors.

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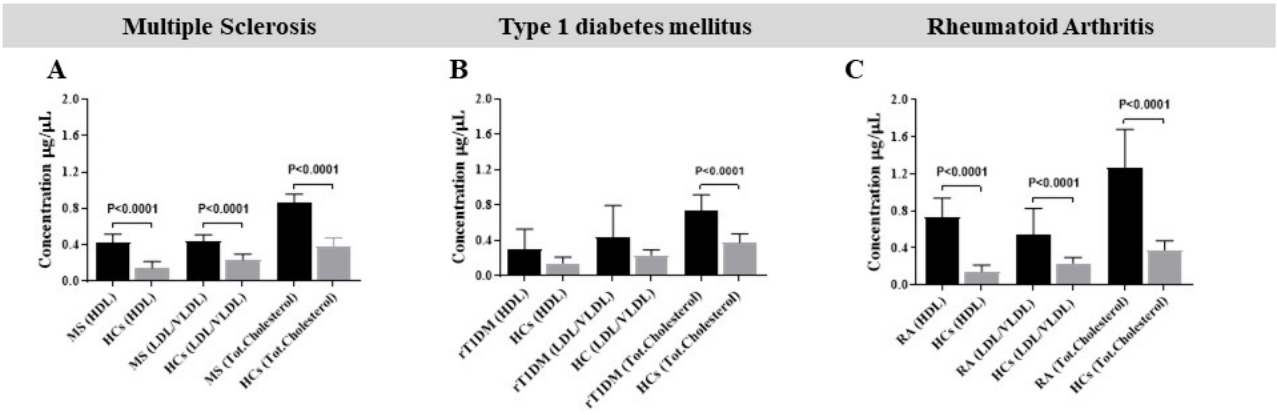
HCs were recruited at the Blood Transfusion Center of Sassari, Italy.

For each disease, 11 patients positive to MAP derived antigens (MAP^+) and 11 MAP seronegative subjects (MAP^-) were selected. The prevalence of MAP-specific Abs was assessed against MAP_4027$_{18\text{-}32}$ peptide highly recognized in MS and RA, and against at least one of the following MAP peptides homologous to zinc transporter 8 (ZnT8) or proinsulin fragments: MAP3865c$_{133–141}$, MAP3865c$_{125\text{-}133}$, MAP2404c$_{70\text{-}85}$ and MAP1,4αgbp$_{157\text{-}173}$.

Indirect ELISA to detect Abs against MAP peptides was performed as described in material and methods. Quantification of lipoproteins (HDL, LDL/VLDL and TC) in serum samples has been analyzed in each disease as in material and methods discussed.

Upon the analysis of differences in lipoproteins levels in each disease group, MS and RA patients showed significantly increased levels of high density lipoprotein (HDL), low density lipoprotein/very low density lipoprotein (LDL/VLDL) and total cholesterol (TC) in comparison with HCs (p<0.0001, Fig. 12A and Fig. 12C). These results are in line with studies in sheep challenged with MAP where total serum cholesterol levels were elevated at 9 weeks post-inoculation (wpi) in comparison to uninfected animals (Johansen MD *et al*., 2018). In contrast, statistically significant difference between T1DM and HC subjects was found only for total cholesterol (p<0.0001, Fig. 12B).

Fig. 12. Levels of serum lipoproteins determined through disease-specific analysis. Bars show values were determined in samples from patients **A)** with MS, **B)** at risk of T1DM (rT1DM) and **C)** affected by RA, each analyzed with reference to concentrations obtained for healthy controls (HCs). *P*-values are specified for each group when statistically significant. Standard deviation is shown for each bar.



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Next, the concentrations of HDL, LDL/VLDL and TC were compared in the sera of MAP+ and MAP- subjects of each pathological condition. The following associations were searched to understand whether anti-MAP Abs indicative of a possible past exposure to the mycobacterium or ongoing silent infection associate with quantity variation of the host lipids: MS MAP+ vs. MS MAP-; MS MAP+ vs. HCs MAP+; MS MAP- vs. HCs MAP-. The same association analysis was done for T1DM and RA subjects.

In MS, a significant difference in HDL levels was found between MAP+ and MAP- patients (p=0.0398, Fig. 12A). MS MAP+ showed high HDL concentrations when compared with HCs MAP+, (p=0.0001, Fig. 12A) and similar trends were observed between MS MAP- and HCs MAP- (p<0.0001, Fig. 12A). It is interesting to note the levels of HDL, LDL and TC were lower in HCs MAP+ than HCs MAP-, although statistical significance was not attained (Fig. 12A-C). The respective analysis performed for LDL/VLDL provided significant results between all groups analyzed (Fig. 12B). Regarding TC concentrations, no difference was

found between MS MAP+ and MS MAP- but significantly higher levels were registered for MS MAP+ comparing to HCs MAP+ (p<0.0001, Fig. 12C) and for MS MAP- vs. HCs MAP- (p<0.0001, Fig. 12C).

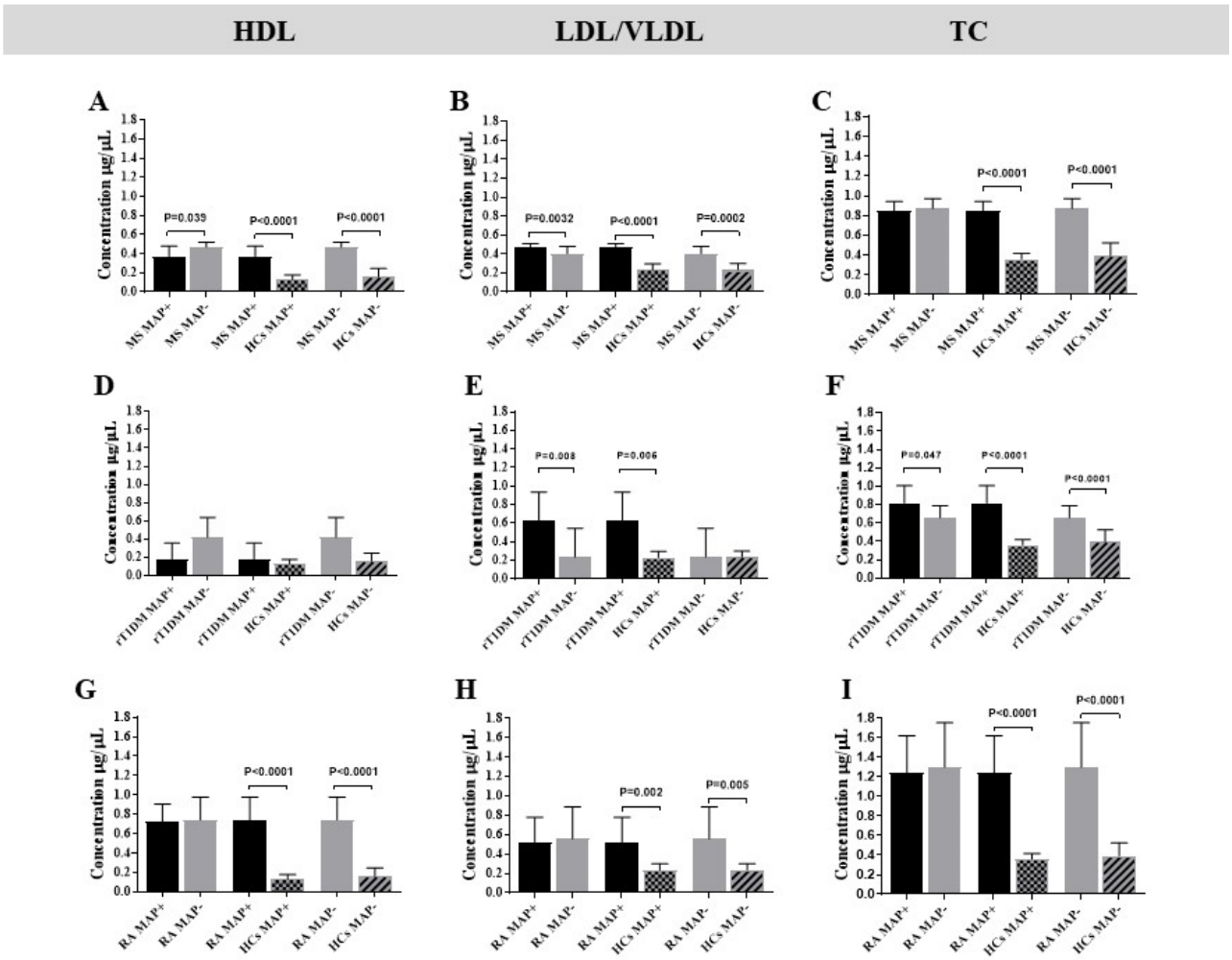
In summary, MS MAP+ subjects are characterized by a significant decrease in HDL levels, an increase in LDL/VLDL (Fig. 12A-B) and no difference in TC levels. It is to be highlighted that the concentrations of HDL, LDL/VLDL and TC in MS MAP+ were markedly elevated in comparison to MAP+ HCs. Following the comparison between MS MAP- and HCs MAP-, it emerged that the overall levels of lipoproteins are remarkably higher in MS MAP- subjects and exceed values registered for MS MAP+. This points at a possible implication of lipids in MAP infection.

Several evidences showed that HDL, LDL and TC variations are associated with MS progression (Zhang Y *et al*., 2019), (Palavra F *et al*., 2013), (Tettey P *et al*., 2014). For this reason, association analysis of serum HDL, LDL and TC levels and disability status in MS MAP+ and MAP- patients were performed. Even though the general association analysis between EDSS and TC levels showed no correlation (R^2 =0.06), it is noteworthy that after subdivision into MAP+ and MAP- groups a higher correlation coefficient between EDSS and TC levels were obtained in MS MAP+ than MS MAP- (R^2 =0.14 vs. R^2 =0.03). Overall, the highest correlation was observed between EDSS and LDL in MAP+ (R^2 =0.55). EDSS correlated well with HDL in MS MAP+ (R^2 =0.34), while low coefficient was obtained for the same variables in MS MAP- (R^2 =0.05). In addition, the correlation analyses were performed between the Abs response to MAP_4027$_{18\text{-}32}$ peptide and TC, HDL and LDL levels, which resulted in higher coefficients for TC and HDL levels in MS MAP+ (R^2 =0.358 and R^2 =0.493, respectively). No correlation with TC, HDL and LDL levels was found in MS MAP- subjects.

Similarly, the assessment of possible lipoprotein variations in patients receiving cortisone therapy revealed no difference.

In subjects at T1DM risk, no difference in HDL levels was observed between the analyzed groups (Fig. 12D). In contrast, significantly lower LDL/VLDL concentrations were found among T1DM MAP- compared with T1DM MAP+ subjects (p=0.0080, Fig. 12E). Statistically significant reduction of TC levels among MAP- subjects were obtained in all association groups: T1DM MAP+ vs. T1DM MAP-, T1DM MAP+ vs. HCs MAP-, T1DM MAP- vs. HCs MAP-, with the respective p-values: p=0.0471, p<0.0001 and p<0.0001 (Fig. 12F).

Fig. 12. Disease-related concentrations of HDL, LDL and TC assessed based on positivity to MAP antigens. The analysis was performed by comparing MAP+ vs. MAP- subjects among patients affected by MS **(A-C)**, at risk of T1DM (rT1DM) **(D-F)** and RA subjects **(G-I)**. Additionally, comparison of between patients and healthy controls (HCs) was carried out for the same MAP-related serological status. Statistical significance is reported above relative bars when attained. For each group, standard deviation is indicated.



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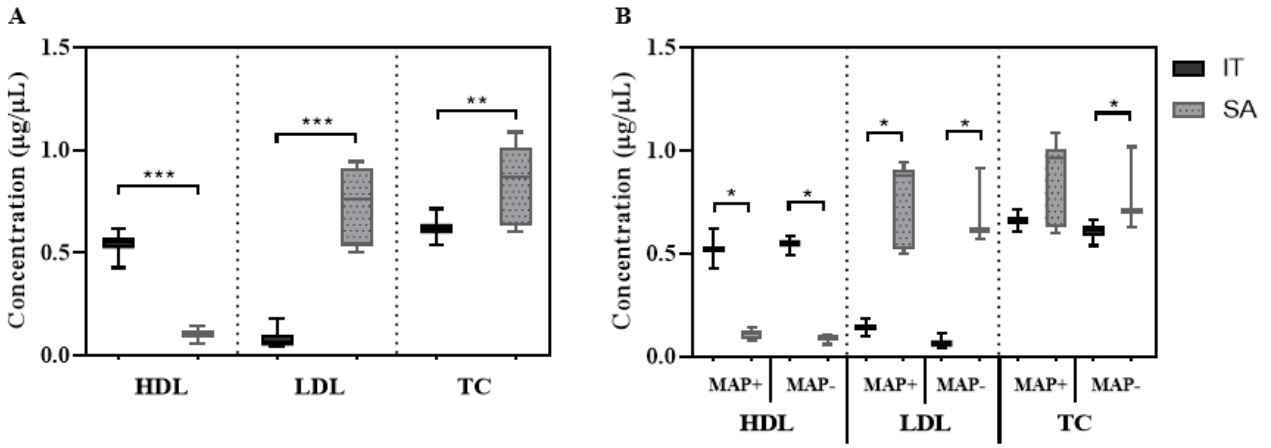
After classifying patients based on their geographic provenience, significantly higher levels of HDL (p<0.0001) and lower LDL (p<0.0001) and TC (p=0.0044) concentrations were observed in the group from mainland Italy compared to samples collected in Sardinia (Fig.

13A), which were mirrored by trends in MAP+ and MAP- subjects considering location of enrollment (Fig. 13B).

Fig. 13. Concentrations of lipoproteins in subjects at risk of T1DM (rT1DM) grouped according to their geographic provenience. A) Comparison between patients enrolled in mainland Italy (IT; n=10) and Sardinia (SA; n=12). B) Differences in lipoprotein levels between IT and SA participants grouped according to their serological response to MAP antigens. Mean and standard deviation are indicated for each bar.



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Upon the analysis of lipoprotein levels in RA, results showed a statistical difference in HDL concentrations between RA MAP+ and RA MAP- and when comparing RA MAP- with HCs MAP- (p<0.0001, Fig. 12G). Insignificantly lower HDL concentrations were found among RA MAP+ respect to RA MAP- (Fig. 12G). Increased levels of LDL/VLDL were observed in RA MAP+ vs. HCs MAP+ (p=0.0024, Fig. 12H) and in RA MAP- vs. HCs MAP- (p=0.0052, Fig. 12G), however comparison between RA MAP+ and RA MAP- showed no difference. Similar results were obtained for TC (Fig. 12I) with strikingly lower levels among HCs regardless of anti-MAP Abs status (p<0.0001, Fig. 12I).

Discussion

The outcomes of this study have shown variations in serum cholesterol levels associated with the presence of anti-MAP Abs.

Genome-wide association study (GWAS) identified over 100 distinct genetic variants associated with MS predisposition (Dendrou CA *et al*., 2015) which genetic modulation of lipid profiles in disability progression. Less is known about environmental MS contributors and their link with genetically conferred susceptibility to infections. Recent evidences associate serum lipid levels and lipid-related polymorphisms with disability progression in MS patients (Polman CH *et al*., 2011). Cholesterol synthesis appears crucial during remyelination of neuroglia (Voskuhl RR *et al*., 2019) while its excess in plasma may aggravate neuronal cell damage (Zhornitsky S *et al*., 2016), thus conflicting results have been obtained for lipoprotein concentrations and degree of disability in this disease (Tettey P *et al*., 2014), (Zhang Y *et al*., 2019), (Voskuhl RR *et al*., 2019). The present results indicated higher levels of serum lipoprotein levels in MS patients, showing lower HDL concentrations among MAP+ subjects which correlated with higher EDSS. It is possible that lipid-related SNPs inducing imbalance of cholesterol favoring thereby mycobacterial survival in macrophages. This hypothesis would need a further screening for relevant gene polymorphisms and assessment to which extent MAP may alter lipoprotein homeostasis. A study in C57BL/6J mice highlights the ability of myelin oligodendrocyte glycoprotein $MOG_{35–55}$ peptide from heat-killed MAP to induce experimental autoimmune encephalomyelitis (EAE) considered a model condition for MS studies (Cossu D *et al*., 2019). In addition, EAE was more severe in MAP-immunized mice than in animals treated with Freud's Complete Adjuvant (CFA) - a nonspecific stimulator

of the immune response. Still, MAP components were able to activate a strong proliferative T cell response.

Differences in lipoprotein levels between patients at T1DM risk with distinct biogeographical background indicating possible genetic and environmental determinants are noteworthy. Low HDL and raised LDL levels are a typical feature of young T1DM patients at high cardiovascular disease (CVD) risk and were detected among Sardinian participants, while opposite trends were displayed by subjects from mainland Italy. The island of Sardinia is characterized by the second highest prevalence of T1DM worldwide (Group. 2006) and the by peculiar genetic heritage of local populations due to long lasting genetic isolation (Lampis R *et al*., 2000). Over 60% of Sardinian livestock herds seem to be infected with MAP (Masala S *et al*., 2011), however these estimates may reach more elevated numbers given the lack of official registers and monitoring strategies. Markedly high concentrations of LDL in Sardinian patients compared to the study group enrolled in Rome reflect observations in MAP-infected animals and may be indicative of past exposure to MAP in combination with gene variants facilitating or enhancing the effects of infection. On the other hand, slightly differing concentrations of serum lipoproteins between MAP+ and MAP- subjects within the same geographically-related group may present a temporary picture of a latent infection when MAP is not actively modulating lipoprotein profiles and should be further investigated in larger cohorts.

Genetic regulation of lipid metabolism, particularly in the context of gene–environment interaction, has not been examined in the RA population. This may be particularly important, since lipid alterations appear to predate the diagnosis of RA (Kitas GD *et al*., 2010) and may be exacerbated during initial phases of MAP infection. Results obtained for RA cohort in this study are discordant compared to literature, although similar differences in lipoprotein levels

have been described for distinct animal species. Upon exposure to different MAP strains, a significant increase in total serum cholesterol has been observed after 9 weeks post infection (wpi) in sheep, while cattle showed an opposite trend at 13 wpi (Johansen MD *et al*., 2018). The way in which cholesterol is exploited by MAP in varying time laps depends therefore on the host and host-specific mycobacterial strains. Lower but not significant lipoprotein levels detected among RA MAP+ patients compared to RA MAP- in our study may reflect MAP infection phase which at the initial stage is silent and difficult to diagnose (Whittington RJ *et al*., 2012). High HDL, LDL and TC levels in RA subject respect to healthy population and elevated values for the latter two in RA MAP+ *versus* HCs MAP+ can be due to different genetic determinants facilitating MAP infection and lipid content in this disease. The evaluation of such interplay may be complex as lifelong therapy administered to RA patients targets elements of the immune system resulting in suppressed responses to antigens in general and may affect cholesterol metabolism (Santosa S *et al*., 2007).

Considering the complicated relationship between MAP and other factors involved in autoimmune processes that lead to an imbalance in lipoprotein levels, follow-up studies and employment of murine models representing the corresponding diseases (MS, T1DM and RA) will allow to monitor the relationship between anti-MAP immune responses and cholesterol levels and to explore mechanisms through which the mycobacterium may favor pathological phenotypes. Supposedly, dependence of MAP survival on cholesterol is not only confined to the first stage of infection but continues later during possibly prolonged silent state which may be dominant in not primary hosts such as humans. A slow release of cholesterol previously accumulated by MAP inside macrophage may be released during gradual killing of the pathogen, thus leading to a variation in lipoprotein

levels. A successful isolation of MAP along with strain characterization would shed light on strain-specific functional differences in lipid regulation and immune responses. This would help to position MAP in complex molecular pathways underlying described autoimmune conditions. For the time being, preliminary results presented here need to be confirmed in a larger cohort and an accurate evaluation of confounding factors such as therapy, genetic predisposition and age/lifestyle-related cholesterol levels.

Antibody response to epitopes of Epstein-Barr virus, *Mycobacterium avium* subsp. *paratuberculosis* and IRF5 in connective tissue diseases

Results

Improve knowledge to different biomarkers is useful for diagnosticate rheumatic diseases early and provide important insights for clinical management. This study evaluates the frequency of reactivity of sera of patients with different connective tissue diseases (CTDs) (RA, SLE, SSc and SSj) to IRF5 and homologous peptides from EBV and MAP. Experiments in mouse models of arthritis, as discussed below, have been performed at the Kennedy Institute of Rheumatology (University of Oxford) for reinforce the idea that Abs against three homologous peptides that we used are cross-reactive. Reactivity against wild-type (wt) and citrullinated (cit) IRF5 ($IRF5_{424-434}$), MAP (MAP_4027_{18-32}) and EBV ($BOLF1_{305-320}$) peptides was tested by indirect ELISA in sera from 100 RA patients, 54 patients with other CTDs (14 SLE, 28 SSc and 12 SSj) and 100 HCs. Sera to mouse models of arthritis have been tested to analyze the antibody response to wt peptides. Demographic and clinical features of all subjects involved in this study are summarized in the Table 8 and Table 9.

Table 8. Demographic and clinical characteristics of groups

	RA n=100	SLE n=14	SSc n=28	SSj n=12	HCs n=100
Age, years	57.6 ± 10.3	36.5 ± 11.2	58.9 ± 13.2	59.5 ± 15.4	45.1 ± 11.7
Female, n(%)	80(80)	14(100)	23(82)	12(100)	74(74)
DAS28	3.45 ± 1.7	/	/	/	/
SLEDAI	/	3.42 ± 4.7	/	/	/
ESCsG-AI	/	/	2.23 ± 2.1	/	/
ESSDAI	/	/	/	2.83 ± 2.16	/

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; HCs, healthy controls. DAS-28, disease activity score-28 joints; SLEDAI, systemic lupus erythematosus disease index 2000; ESCsG-AI, European Scleroderma Research Group Activity Index; ESSDAI, EULAR Sjogren's syndrome disease activity index.

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Dr. Bo Marco – "Immune response against *Mycobacterium avium* subsp. *paratuberculosis*, Epstein-Barr virus, HERV-K and IRF5 in rheumatoid arthritis" – PhD thesis in *Life Sciences and Biotechnologies* – University of Sassari, Italy

Table 9. Demographic and clinical characteristics of RA patients and HCs

	RA n=100	HCs n=100
Age, years	57.6 ± 10.3	45.1 ± 11.7
Female, n(%)	80(80)	74(74)
ESR, mm/h	19.5 ± 25	/
CRP, mg/dL	1.34 ± 4.8	/
DAS28 score	3.45 ± 1.7	/
HAQ score	1.04 ± 0.9	/
ACPA positivity, %	65(65)	/
RF positivity, %	73(73)	/
Steroid use, %	64	/
Steroid dose, mg/day	1.5 ± 2.3	/
DMARDs use, %	86	/
TNFi use, %	27	/
Tocilizumab use, %	13	/
Abatacept use, %	4	/
Rituximab use, %	2	/

RA, rheumatoid arthritis; HCs, healthy controls. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS-28, disease activity score-28 joints; HAQ, health assessment questionnaire; ACPA, anti-citrullinated peptide antibodies; RF, rheumatoid factor; DMARDs, disease-modifying anti-rheumatic drugs; TNFi, tumor necrosis factor-alpha inhibitors.

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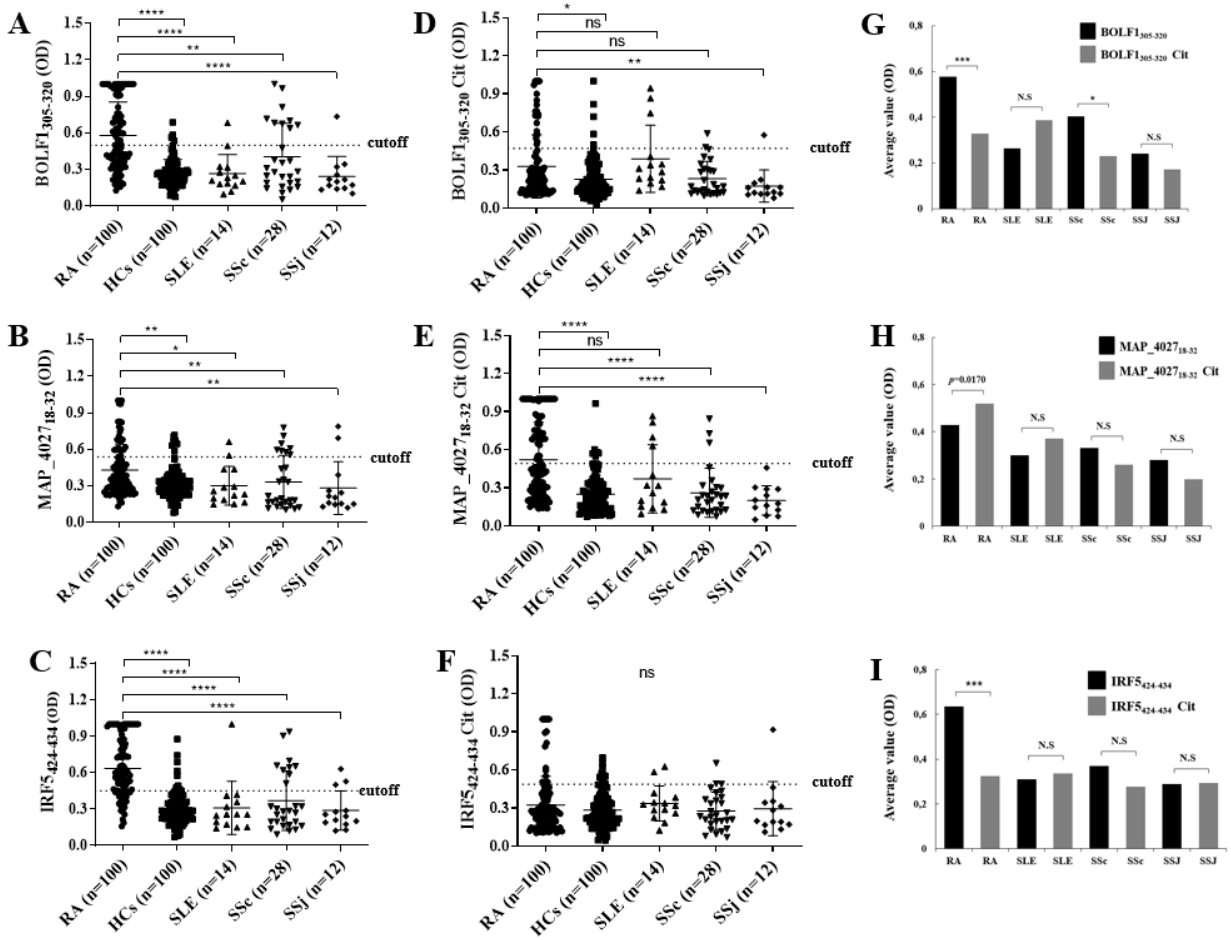
Wt-BOLF1$_{305\text{-}320}$ elicited the highest seroreactivity accounting for 53% (n=53) among RA patients, 7.14% (n=1) in SLE, 32.1% (n=9) in SSc, 7.69% (n=1) in SSJ and 5% (n=5) in HCs (p=0.0001; Fig. 14A), while Abs against cit-BOLF1$_{305\text{-}320}$ were detected in 21% (n=21) of RA subjects, 21.4% (n=3) in SLE, 7.14% (n=2) in SSc and 7.69% (n=1) in SSj and 5% (n=5) of HCs, (Fig. 14D). Also, a statistically significant difference was found between SSc and HCs (32.1% vs. 5%, respectively, p=000.3; Fig. 14A) for BOLF1 that highlights the role of EBV in SSc.

In RA and SSc, the wt-BOLF1$_{305-320}$ elicited a greater reactivity than its citrullinated counterpart (p=0.0001 and p=0.0403 respectively), while no statistically significant difference was attained in SLE and SSj groups (Fig. 14G).

Regarding MAP peptides, wt-MAP_4027$_{18-32}$ elicited the highest seroreactivity among RA patients accounting for 26% (n=26), 14.29% (n=2) in SLE, 28.57% (n=8) in SSc, 15.38% (n=2) in SSj and 8% (n=8) in HCs, (p=0.0001; Fig. 14B), while Abs against cit-MAP_4027$_{18-32}$ were detected in 43% (n=43) of RA subjects, 28.57% (n=4) in SLE, 10.71% (n=3) in SSc, 0% in SSj and 6% (n=6) of HCs (p=0.0001; Fig. 14E). SSc and HCs significantly differed when considering values obtained for MAP_4027$_{18-32}$ (p=0.0076; Fig. 14B). Of note, a statistical difference was registered in RA patients between the proportion of anti-wt-MAP_4027$_{18-32}$ and anti-cit-MAP_4027$_{18-32}$ (26 *vs*. 43%, p=0.0170; Fig. 14H).

IRF5 peptide elicited a higher seroreactivity reaching 73% (n=73) among RA patients, 7.14% (n=1) in SLE, 32.1% (n=9) in SSc, 23.1% (n=3) in SSJ and 9% (n=9) in HCs (p=0.0001, Fig. 14C), while Abs against cit-IRF5 peptide were detected in 14% (n=14) of RA subjects, 14.3% (n=2) in SLE, 10.7% (n=3) in SSc, 7.69% (n=1) in SSj and 10% (n=10) of HCs (Fig. 14F). A significant difference was observed for IRF5$_{424-434}$ between SSc and HCs (p=0.0042; Fig. 14C). We then compared the antibody response against wt-IRF5 peptide versus its citrullinated variant in all disease-specific groups. The proportion of anti-wt-IRF5 vs. anti-cit-IRF5 Abs was statistically significant in RA patients only (73% *vs*. 14%; p=0.0001; Fig. 14I).

Fig. 14. A-F) ELISA-based analysis of Abs reactivity against human, viral and MAP-derived peptides in RA patients, SLE, SSc, SSj and HCs. The sera were tested against plate-coated BOLF1$_{305-320}$ (A), MAP_4027$_{18-32}$ (B) and IRF5$_{424-434}$ (C) peptides. Also, the sera were tested against plate-coated BOLF1$_{305-320}$ Citrullinated (D), MAP_4027$_{18-32}$ Citrullinated (E) and IRF5$_{424-434}$ Citrullinated (F) peptides. Bars represent the median ± interquartile range. Thresholds for Abs positivity are indicated by dashed lines. P-values are indicated above the distributions. (G-I) Mean distribution of OD values and Fisher's exact test. *P<0.05; **P=0.001; ***P=0.0001; ****P<0.0001.



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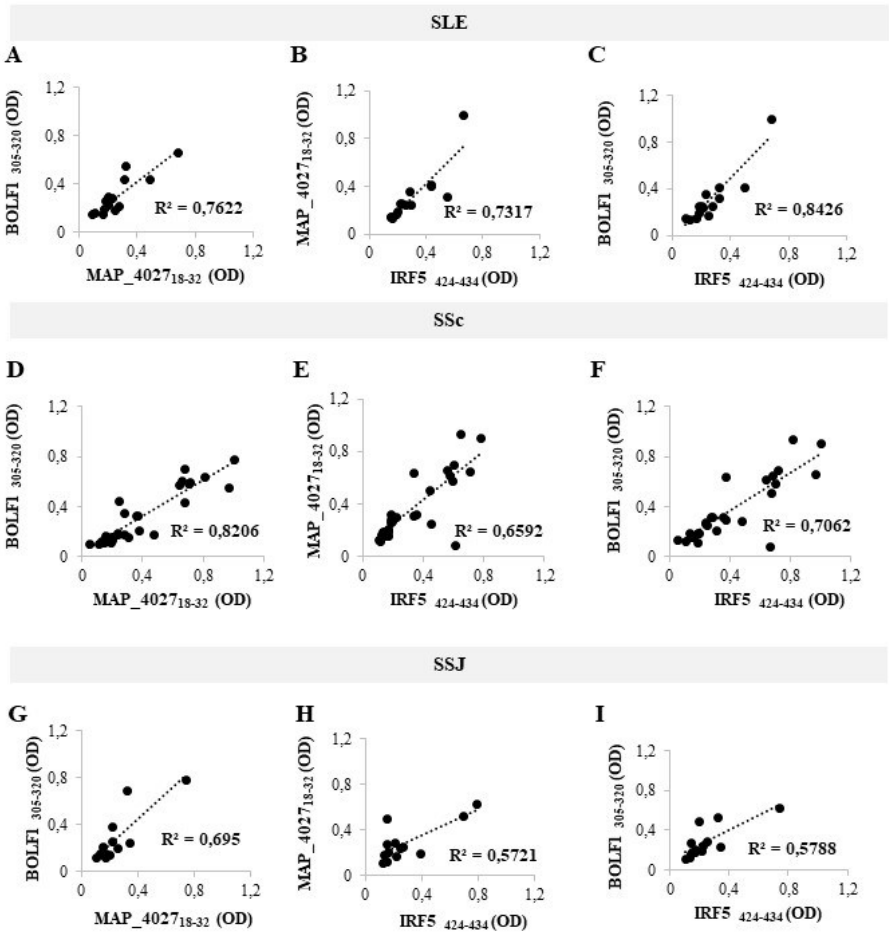
Although there was no statistical significance for the assessed peptides in SLE, SSc and SSJ compared to RA, we performed correlation analyses of Abs positivity values among SLE, SSc and SSJ patients (Fig. 15 and Fig. 16). The highest coefficients were obtained for

the homologous epitopes BOLF1$_{305-320}$, MAP_4027$_{18-32}$ and IRF5$_{424-434}$ in pairwise plots pointing at cross-reactivity due to shared amino acid sequence (Fig. 15).

Fig. 15. A-I) Scatter plot showing correlations between Abs titers recognizing BOLF1$_{305-320}$ and MAP_4027$_{18-32}$, MAP_4027$_{18-32}$ and IRF5$_{424-434}$, BOLF1$_{305-320}$ and IRF5$_{424-434}$ in SLE **(A, B, C),** SSc **(D, E, F)** and SSJ **(G, H, I)** patients. Person's correlation was calculated through Graphpad Prism 8.0 software.



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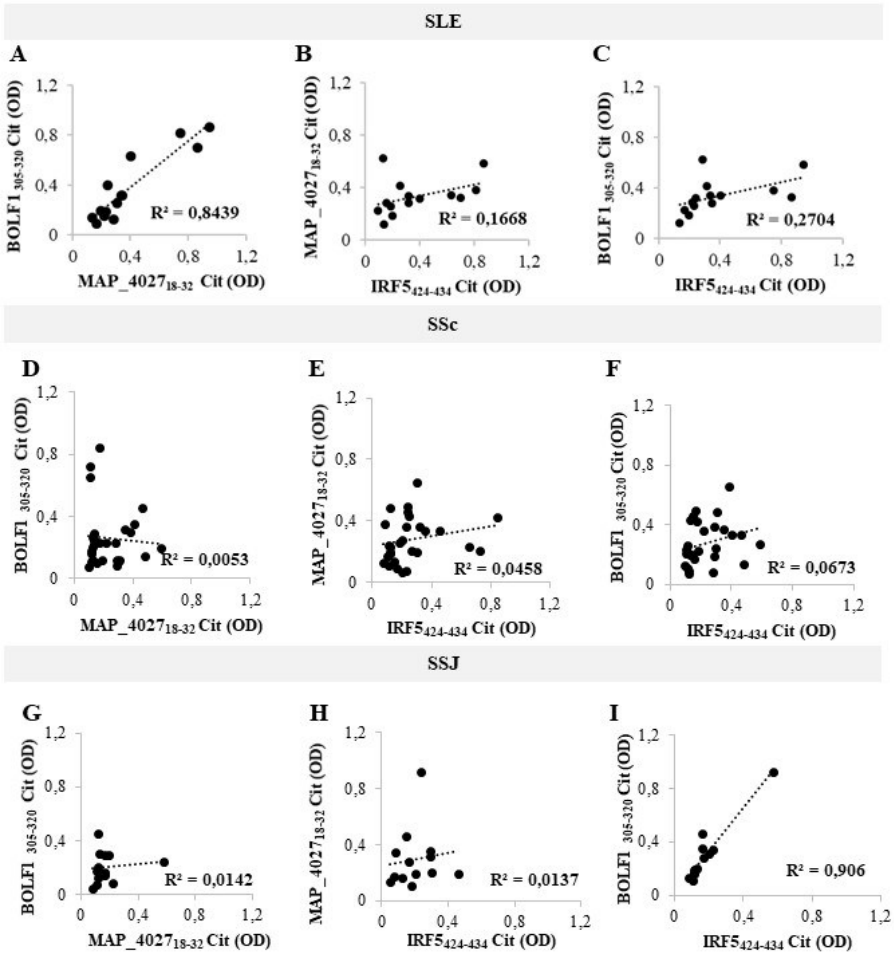
The lack of correlation was found for all homologous pairs of citrullinated peptides, with the exception observed between cit-BOLF1$_{305-320}$ and cit-MAP_4027$_{18-32}$ in SLE (R^2=0.8439; Fig. 16A). Similarly, cit-BOLF1$_{305-320}$ and cit-IRF5$_{424-434}$ highly correlated in SSJ (R^2 =0.906; Fig. 16I).

Fig. 16. A-I) Scatter plot showing correlations between Abs titers recognizing BOLF1$_{305-320}$Cit and MAP_4027$_{18-32}$Cit, MAP_4027$_{18-32}$Cit and IRF5$_{424-434}$Cit, BOLF1$_{305-320}$Cit and IRF5$_{424-434}$Cit in SLE **(A, B, C)**, SSc **(D, E, F)** and SSJ **(G, H, I)** patients. Person's correlation was calculated through Graphpad Prism 8.0 software.



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Discussion

The present study demonstrated that the Abs response to IRF5, EBV and MAP homologous peptides is different across CTDs, with RA sera showing the most significant reactivity against either wild-type or citrullinated peptides. These results confirmed the previous data on RA (Bo M *et al*., 2018a) (Bo M *et al*., 2018c). AIA model has been useful for reinforce the results obtained in humans as discussed below. The antibodies formed after CFA

inoculation could lead to cross-reactions towards MAP antigens triggering a persistent inflammation towards IRF5 and EBV, typical condition of autoimmune diseases. To validate this hypothesis, it is planned to analyze the immune responses in irf5 -/- mice in order to understand if the Abs are able to cross-react at the same way. This could be helpful to broaden the knowledge on IRF5 role and on the base of results understand how is possible intervene for blocking autoantibodies production able to trigger chronic inflammation. In addition, it would be interesting to test the reactivity against the homologues citrullinated peptides in order to compare the results. Citrullination, a fundamental and ubiquitous post-translational modification with potentially relevant effect on the induction of secondary autoimmune responses, may be triggered by various infective agents, mainly at the level of mucosal surface. Therefore, it is plausible that also EBV and MAP may induce citrullination of protein fragments, hence antigenicity of citrullinated homologous peptides derived from IRF5, EBV and MAP was tested. Intriguingly, the response against cit-MAP_4027$_{18\text{-}32}$ was significantly higher than that against its wild-type variant, suggesting the role of MAP citrullinated antigens as possible triggers of autoimmunity in RA supported by the production of specific ACPA. However, with the exception of anti-cit-IRF5, in RA and other CTDs, seroreactivity to the other two citrullinated peptides was similar and in some instances even significantly lower than responsiveness against their wild-types counterparts.

For the first time in this study, a significant Abs response to homologous peptides of IRF5, MAP and EBV was also shown in SSc patients. It has been demonstrated that exposure to EBV is able to infect human dermal fibroblasts *in vitro,* inducing pro-fibrotic phenotypic switching, a relevant pathogenetic pathway underlying skin fibrosis in SSc (Farina A *et al*., 2014). Moreover, EBV viral transcripts and proteins were demonstrated in fibroblasts and endothelial cells in the skin of SSc patients (Farina A *et al*., 2014). EBV chronic replication

in SSc primary monocytes has been proven to activate TLR8 molecular pathway sustaining monocyte-derived inflammation in SSc (Farina A *et al*., 2017)**.** In addition, higher frequency of Abs against EBV has been recently demonstrated in SSc compared to healthy controls**.** Collectively taken, these data suggest that EBV-specific response may be an initiating trigger of SSc, with persistent viral infection-related tissue injury underlying chronic inflammation and fibrosis. It is probable that a defective type I IFN-mediated signaling may blunt anti-viral responses and EBV infection control in patients with SSc, as recently demonstrated in MS (Severa M *et al*., 2019). Interestingly, the number of minor rs4728142 alleles of IRF5 has been described as a predictive factor of a longer survival in SSc patients (Roozbeh Sharif *et al*., 2012). Therefore, it is conceivable that Abs-mediated modulation of IRF expression/function in SSc may have an impact on the pathogenesis and severity of the disease.

The significant reactivity of SSc sera against MAP peptides demonstrated in the present study is intriguing and worth of further investigation. Although preliminary, the obtained data suggest that SSc and RA patients actuate a similar autoimmune response to MAP-derived antigens pointing at MAP infection as a common pathogenetic contributor to various CTDs. Weak or insignificant immune response to the assessed epitopes among patients with SLE and SSj further supports the concept that (auto)immune responses to environmental pathogens are variable across CTDs.

This study has limitation that all patients were subjected to different immunosuppressive therapies at the moment of sample collection, a fact that may have biased the interpretation and significance of humoral responses.

Further analysis of pro-inflammatory cytokine levels and quantification of INF-γ upon stimulation with the analyzed peptides could have strengthen the present observations. In

addition, a study of diagnostic performance of Abs against the selected peptides, has not been performed due to limited sample sizes of non-RA CTDs sera.

Also, analysis of immune response against the above peptides in irf5-/- mice will be useful in order to understand the cross-reactivity among peptides.

The diagnostic performance of antibodies to BOLF1, MAP and IRF5 homologous peptides in differentiating between healthy controls and CTDs and between different CTDs, needs to be tested in larger case-control studies including other autoimmune and chronic inflammatory diseases.

Investigating the immune response against the homologous peptides (BOLF1$_{305-320}$, MAP_4027$_{18-32}$, IRF5$_{434-424}$) in AIA, CIA and CAIA sera of mice

Results

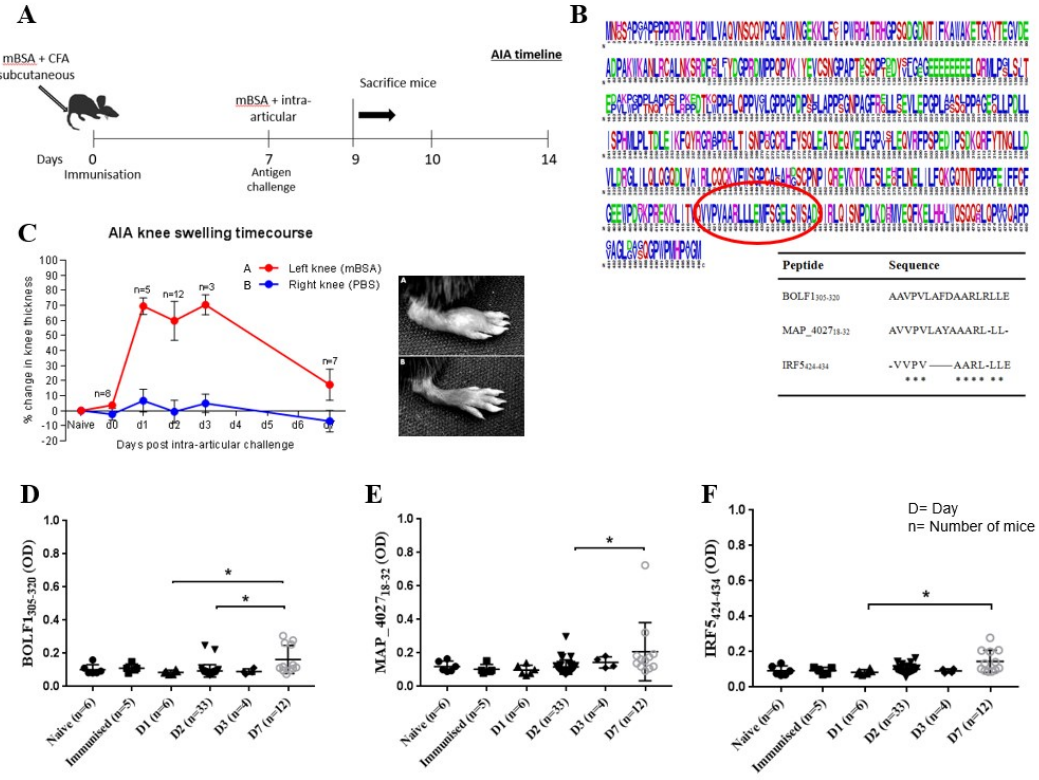
In order to better understand the etiopathogenesis of RA involving largely unknown genetic and environmental factors, animal models have been extensively employed for studies focused on molecular mechanisms underlying human diseases with the objective to develop new therapeutic strategies (Bevaart L *et al*., 2010), (McNamee K *et al*., 2015), (Kollias G *et al*., 2011). A number of rodent models of arthritis have been generated over decades of research in the field and among them mouse models of RA share many features with the relative disease in humans.

To validate the hypothesis of cross-reactivity, AIA mouse model was employed in this study for the assessment of immune responses against peptides derived from EBV and MAP sharing sequence homology with IRF5. Mice were grouped in the following treatment conditions: n=6 naïve mice not subjected to immunization, n=5 immunized mice and n=55 AIA mice. Arthritis was induced at ∼12 wk of age as described elsewhere (Egan PJ *et al*., 2008), (Parsey MV *et al*., 1998). Briefly, after sedation with inhaled isoflurane at day zero (D0), mice were immunized with mBSA (Sigma, 100 µg) emulsified in complete Freud's adjuvant (Difco, 100 µg) and subsequently administered subcutaneously at the base of the tail. Seven days later (D7), arthritis was induced in sedated mice by intraarticular injection of mBSA using a sterile 33-gauge microcannula. Treatment with PBS alone was used as a control condition. Spleen, knee joints and blood were harvested on days 9-14 from sacrificed mice (Fig. 17B). Blood was centrifuged at 2000 rpm for 20 minutes to separate serum for serological analysis.

The reactivity to three homologous peptides IRF5, BOLF1 and MAP was tested in serum of three arthritis models (AIA, CIA and CAIA) and revealed a different response.

The results obtained highlight a statistical difference between D1 and D7 and D2 and D7 for BOLF1, between D2 and D7 for MAP_4027 and between D1 and D7 for IRF5 in the AIA model (Fig.17: A, B, C, D, E, F). No statistical difference for the same peptides was found in the CIA and CAIA models (data not shown).

Fig. 17. **A)** Schematic diagram showing treatment and harvest time points in the antigen-induced arthritis model. **B)** Sequence alignment between human and mouse IRF5 protein. The red circle shows the homologue sequence between IRF5, MAP and EBV antigen. **C)** Antigen- Induced arthritis (AIA) knee swelling time course. Comparison of knee swelling between inflamed knees of the AIA and WT animals, expressed as a percentage of swelling of the mBSA-challenged knee compared with the PBS knee. **D-F**) ELISA-based analysis of Abs reactivity against EBV, MAP and IRF5 peptides AIA (Antigen-Induced arthritis) mouse model.



Bo M *et al*. Unpublished data, 2019

Dr. Bo Marco – "Immune response against *Mycobacterium avium* subsp. *paratuberculosis*, Epstein-Barr virus, HERV-K and IRF5 in rheumatoid arthritis" – PhD thesis in *Life Sciences and Biotechnologies* – University of Sassari, Italy

Discussion

AIA model has been useful for reinforce the results obtained in humans. The Abs formed after CFA inoculation could lead to cross-reactions towards MAP antigens triggering a persistent inflammation towards IRF5 and EBV, typical condition of autoimmune diseases. To validate this hypothesis, we plan to analyze the immune responses in irf5 -/- mice in order to understand if the Abs are able to cross-react at the same way. This could be help for broaden the knowledge on IRF5 role and on the base of results understand how is possible intervene for blocking autoantibodies production able to trigger chronic inflammation. In addition, will be interesting test the reactivity against the homologues citrullinated peptides in order to compare the results.

CHAPTER 5: *Conclusions*

RA is a complex chronic inflammatory disease which could be triggered as a consequence of an environmental pressure in genetically susceptible subjects. RA causative agents remain to be determined, despite the fact that a number of studies have been set up in order to discover a putative etiological agent.

For the first time, has been searched a humoral response in patients with RA and controls against four surface epitopes of HERV-K env-su (HERV-K env-su19–37, HERV-K env-su109–126 and HERV-K env-su209–226). Among them, the most interesting peptide was HERV-K env-su19–37, which was recognized in the sera of 19% of RA patients and in only 3% of controls.

Data from this study demonstrated a remarkable reactivity of RA sera against IRF5 supporting the potential role of IRF5 as an important target in RA specific autoimmunity. Moreover, it has been also demonstrated a high grade of cross-reactivity between IRF5, EBV and MAP epitopes. A possible explanation for these results could be that in RA patients past EBV and/or MAP infection may induce specific humoral immunity reacting against IRF5 host protein. This secondary response may contribute, through the epitope-spreading phenomenon, to synovial tissue destruction with the production of Abs against previously “sequestered” antigens and amplification of autoimmune cascade.

In addition, recent reports on the loss of self-tolerance to IL-2 in autoimmune diseases encouraged my studies to evaluate the presence of anti-IL-2 Abs in Sardinian RA patients in association to antigens most frequently described as possible contributors to RA progression. The results confirm the involvement of IL-2 in RA at higher rates compared

to a French cohort (39% vs.15%, respectively) and is mirrored by a concomitant positivity to peptide antigens derived from EBV, HERV-K, MAP or human IRF5.

In addition, research performed on RA Sardinian patients, showed MAP is another one of the candidates that could have a role of the environmental trigger. However, in this scenario, it is still too early to say whether cross-recognition between MAP and EBV antigens and homolog human protein as IRF5 involves an epitopes mimicry phenomenon starting or precipitating RA. For these reasons, to prove that MAP and EBV infection plays a causal role in RA, animal model studies should be performed in order to have a global vision of the situation.

To date, the evidences is not enough in order to prove a causal relationship between MAP, HERV-K, EBV and RA. What we can say is that we showed an evident association of EBV, HERV-K and MAP with RA in Sardinia. It's not unlikely that MAP is one of the environmental factors that trigger the autoimmune process in genetically predisposed people after infection through water or milk ingestion in early life, but other roles are possible.

It will be necessary to perform other studies about the link between HLA genes and infections in order to better understand the progression of infected patients with a chronic autoimmune stage and the containment of the process in HCs. This discover could help the clinicians in order to improve the therapeutic approach to the disease, so will be important to improve diagnostic biomolecular tests for detecting the MAP presence in the early stage of infection and not too late.

Future research will help to discover in which molecular pathways EBV, HERV-K and MAP are involved in RA pathogenesis helping in turn to discover the specific regions

targeted by the immune system. At the same way, it will be important to develop new vaccines based on these regions in order to protect individuals with HLA predisposition to develop RA. Considering the particular background of Sardinian subjects and the complexity of RA disease, the development of alternative approaches, could be useful in order to better take into account the specific need of each patient. The contribute of other researchers around the world will be important to get other steady and consolidate results for support the role of these infectious agents in triggering and/or exacerbate the RA disease.

CHAPTER 6: *Materials and Methods*

Patients and controls

RA, SLE, SSc and SS patients were enrolled at the outpatient clinic of the Rheumatology Unit, Department of Clinical and Experimental Medicine, University Hospital of Sassari, Italy.

Collected data about RA patients included: duration of RA; steroid treatment; DMARDs and/or anti-tumour necrosis factor-alpha therapy, Tocilizumab, Rituximab and Abatacept; levels of C-reactive protein (CRP), mg/dL; erythrocyte sedimentation rate (ESR) levels, mm/h; rheumatoid factor positivity; anti-cyclic citrullinated peptide positivity (anti-CCP); Disease Activity Score-28 (DAS-28) and Health Assessment Questionnaire (HAQ). RA patients met the criteria of the American College of Rheumatology (Arnett FC *et al*., 1988) (Aletaha D *et al*., 2010).

The following disease-specific activity scores were also registered: SLEDAI (Systemic lupus erythematosus disease index 2000, for SLE; (Gladman DD *et al*., 2002), ESCsG-AI (European Scleroderma Research Group Activity Index, for SSc; (Valentini G *et al*., 2001)) and ESSDAI (EULAR Sjogren's syndrome disease activity index, for SSj; (Seror R *et al*., 2010)).

MS patients diagnosed according to the revised McDonald diagnostic criteria (Polman CH *et al*., 2011) were enrolled at the Neurological Clinic of the University Hospital of Cagliari, Italy. At the time of the study, 19 patients were diagnosed as relapsing remitting MS and 3 as secondary progressive MS. The expanded disability status scale (EDSS) values ranged from 0 to 7.0 with the average of 1.93 average. Demographic, clinical and laboratory features of MS are summarized in Table 6.

NMOSD patients (5 males, 29 females; median age 51.32) were enrolled at the Neurology Clinic of the University Hospital of Sassari and at the Department of Neurosciences, Biomedicine and Motion, University of Verona, Italy. All patients were onset free from immunomodulatory/immunosuppressive therapy over the last 12 months. The diagnosis of NMOSD was based on established criteria (Uzawa A *et al*., 2012); all sera were tested for Abs to AQP4 through the commercially available Anti-Aquaporin-4 IIFT screening test (Euroimmun, Germany) (Uzawa A *et al*., 2014) and to myelin oligodendrocyte glycoprotein (MOG) using recombinant live cell-based immunofluorescence assay with HEK293A cells transfected with full-length MOG, as described elsewhere (Di Pauli F *et al*., 2011).

Subjects at T1D risk were recruited in Sardinia at the Department of Diabetes, St. Michele Hospital of Cagliari and in mainland Italy at the Tor Vergata University Hospital of Rome. T1D risk was intended as disease familiarity between first-degree relatives, detection of high-risk HLA alleles and/or presence of diagnostic autoantibodies (ZnT8, GADA, IA2A, IAA and/or ICA). All subjects were free from therapy.

HCs patients were recruited at the Blood Transfusion Centre of Sassari, Italy.

Mice

Antigen-Induced Arthritis (AIA)

Antigen-Induced Arthritis (AIA) mouse model has been induced by a certified competent person at the Kennedy Institute of Rheumatology, University of Oxford with mBSA + Complete Freund's Adjuvant following the instruction in literature (Asquith DL *et al*., 2009) (Egan PJ *et al*., 2008). More precisely, at day 0, mice were sedated using inhaled isofluorane anesthesia and then immunized with 100 µg of mBSA (Sigma) emulsified in 100 µl of

complete Freud's adjuvant (BD Difco), administered subcutaneous at the base of the tail (Fig.18).

Fig. 18. Immunise of mouse

At day 2, 3 and 7, we induced arthritis by means of an intraarticular injection of BSA in sedated animal. Then the mice were killed, the knee joints were excised for the study of cell populations in this site of inflammation through FACS analysis, blood samples were collected for study immunological response against viral and bacterial antigens and other organs for other studies.

Clinical and histological methods

Fix/decalcification/OCT embedding of AIA knees for frozen sectioning

For a good histology analysis and other analysis with confocal microscope is necessary to dissect the knee and trim away as much muscle as possible. The knee is put into the Falcon tube containing paraformaldehyde (PFA) at 4% in D-PBS 1X (Fixation solution) and store at 4˚C in a fridge overnight. The day later discard the PFA and wash the knee with D-PBS 1X for 4 times. Add 10ml decalcification solution (0.5M EDTA, pH7.4) and seal the tube with parafilm and mix on a rotator overnight at 4˚C. The successive step consisted on washing the knee with D-PBS 1X and then it is necessary to add 10ml cryoprotectant solution (20% (w/v) sucrose, 2% (w/v) PVP in PBS: 40g sucrose in 150 ml of PBS + 4g of PVP in 50 ml of

D-PBS 1X) and store at 4˚C in a fridge overnight until the bones sink. Finally, the knee is embedded in OCT (CellPath Innovation in cellular Pathology) block and immersed in methanol-dry-ice bath to snap freeze. Once frozen, the samples are transfered to -80˚C freezer overnight before the section on a cryostat to generate 10μM sections.

Peptides

The following peptides were included in the study:

Peptide	Sequence	Organism
BOLF1$_{305-320}$ wt	AAVPVLAFDAA-L-LLE	EBV
BOLF1$_{305-320}$ Cit	AAVPVLAFDAA-{Cit}-L-{Cit}-LLE	EBV
MAP_4027$_{18-32}$ wt	AVVPVLAYAAA-LLL	MAP
MAP_4027$_{18-32}$ Cit	AVVPVLAYAAA-{Cit}-LLL	MAP
IRF5$_{424-434}$ wt	VVPVAA-LLLE	Human
IRF5$_{424-434}$ Cit	VVPVAA-{Cit}-LLLE	Human
J0I929_HELPX1–11	MIIGGGVSGCA	Helicobacter pylori
IL-2$_{56–70}$	LTEMLTFKFYMPKKA	(Pérol L 2016)
IL-2$_{6–20}$KK	KK-LLSCIALSLALVTNS-KK	(Pérol L 2016)
HERV-Kenv$_{19–37}$	VWVPGPTDDRCPAKPEEEG	HERV-K
HERV-Kenv$_{109–126}$	RPKGKTCPKEIPKGSKNT	HERV-K
HERV-Kenv$_{164–186}$	SGQTQSCPSAQVSPAVDSDLTES	HERV-K
HERV-K env$_{205–226}$	EKGISTPRPKIISPVSGPEHPE	HERV-K

All peptides were synthesized commercially at > 90% purity (LifeTein, South Plainfield, NJ 07080, USA).

Recombinant protein production and plate preparation

Recombinant proteins were produced by Professor Bach Horacio (Division of Infectious Diseases, Faculty of Medicine, The University of British Columbia, Vancouver, BC V6H 3Z6, Canada) according to published protocols. The above proteins were provided thank to collaboration by Professor Bach and Professor Sechi. In brief, PtpA was produced in *M. smegmatis* harboring the *ptpA* gene cloned into the vector pALACE (hygromycin resistance), whereas PknG was produced in *E. coli* harbouring the plasmid *pknG*-pET-30b (kanamycin resistance). Both proteins were purified by affinity chromatography using Ni-NTA resin as published (Bach H *et al*., 2006). Produced proteins were stored at -20°C until used.

ELISA plates were coated with 50 µg/mL of each antigen in PBS overnight at 4°C. The next day, plates were washed with PBS, supplemented with Tween-20 (PBS-T) ×3 and blocked with BSA 3% in PBS overnight at 4°C. The concentration of antigen used in this study was already determined in previous studies as the concentration necessary to obtain a differential change in the readout (Xia A *et al*., 2014), (Gurung RB *et al*., 2014), (Bach E *et al*., 2018). The next day, the blocking solution was discarded, and the plates were dried at room temperature. Plates were then shipped from Professor Bach Horacio to Italy to perform the ELISA. Previous studies performed in Bach laboratory indicated that the shipping of dried plates did not affect the antigen conformation (Gurung RB *et al*., 2014).

ELISA

Blood human samples were collected in Vacutainer tubes for separation of serum and further screening for Abs against BOLF, IRF5 and MAP by indirect ELISA. 96-well plates were coated with the selected peptides dissolved in carbonate/bicarbonate buffer at 10mM concentration and incubated overnight at 4°C. The next day the wells were saturated with

200µl of blocking solution (1xPBS + 0.05% Tween20 containing 5% skim milk) for 1 h at room temperature. Afterwards the plates were washed twice times with 200µl PBS-Tween 20, diluted sera (1:100) were added in duplicate and incubated for 2 h. The plates were subjected to 5 washes with 200µl PBS-Tween with subsequent addition of the alkaline phosphatase-conjugated goat anti-human IgG polyclonal Abs at a dilution of 1:1000 and left to incubate for 1 h at room temperature. Finally, upon another five washes with 200µl PBS-Tween 20, para-nitrophenyl phosphate substrate solution was added to each well and the plates were incubated at room temperature in the dark for 10 min. The optical density (OD) was read at a wavelength of 405 nm using SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). Data was normalized to a positive control serum included in all experiments, the reactivity of which was fixed to 1.0 arbitrary units (AU)/ml.

Competitive inhibition assay

Sera of two RA patients diluted 1:100 in PBS were pre-incubated for 2h at room temperature with MAP_4027, BOLF1 or IRF5 peptide at saturating concentrations of 5µg/ml, 10µg/ml or 50µg/ml for each epitope. The same samples without peptide were treated accordingly as references of seroreactivity. An ELISA was then performed on a plate coated with IRF5 peptide following the protocol described in the previous section.

Quantification of lipoproteins in serum samples

3 mL of peripheral blood were drawn in serum Vacutainer tubes from MS, T1D and RA subjects. Blood was centrifuged at 1500 rpm to separate serum for further quantifications of HDL (high-density lipoprotein), LDL (low-density lipoproteins), VLDL (very-low density lipoproteins) and total cholesterol (TC). Serum was aliquoted and conserved at -20°C for

short-term storage (<5 months) and at -80°C for long-term storage (>5 months). The quantification was performed using HDL and LDL/VLDL Quantification kit (Sigma-Aldrich).

Statistical analysis

The results were expressed as a mean of three separate experiments and the statistical significance of the data was determined using Graphpad Prism 6.0 and 8.0 software (San Diego, CA, USA). Continuous data are expressed as median (IQR) and comparison was made using Mann-Whitney *U* test. Comparison of positivity to the assessed peptides between RA patients and HCs was performed through Fisher's exact test with both Tukey's and Yate's corrections. The significance of differences between the OD values of RA, SLE, SSc, SSj and HCs groups were determined using ANOVA test with multiple comparisons. The same test was employed to assess Abs variations between treatment conditions in mice. The cut-off for positivity was established based on the receiver operating characteristic (ROC) curve at ≥90% specificity and the corresponding sensitivity. Correlation analysis between Abs and RA features, RA activity (DAS-28), systemic inflammation (ESR, CRP) and type of immunosuppressive treatment was explored by bivariate correlation analysis, univariate and multivariate regression analysis. Probability values lower than 0.05 were considered statistically significant.

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List of papers published during the PhD

1) Mameli G, Erre GL, Caggiu E, Mura S, Cossu D, Bo M, Cadoni ML, Piras A, Mundula N, Colombo E, Buscetta G, Passiu G, Sechi LA. **Identification of a HERV-K env surface peptide highly recognized in Rheumatoid Arthritis (RA) patients: a cross-sectional case-control study.** *Clinical & Experimental Immunology*. **189**:127-131 (2017).

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4) Bo M, Niegowska M, Arru G, Sechi E, Mariotto S, Mancinelli C, Farinazzo A, Alberti D, Gajofatto A, Ferrari S, Capra R, Monaco S, Sechi G, Sechi LA. ***Mycobacterium avium* subspecies *paratuberculosis* and myelin basic protein specific epitopes are highly recognized by sera from patients with Neuromyelitis optica spectrum disorder.** *Journal of Neuroimmunology*. **318**:97-102 (2018).

5) Arru G, Mameli G, Deiana GA, Rassu AL, Piredda R, Sechi E, Caggiu E, Bo M, Nako E, Urso D, Mariotto S, Ferrari S, Zanusso G, Monaco S, Sechi G, Sechi LA. **Humoral immunity response to human endogenous retroviruses K/W differentiates**

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6) Yael N. Slavin[#], Bo M[#], Caggiu E, Sechi GP, Arru G, Bach H and Sechi LA. **High levels of antibodies against PtpA and PknG secreted by *Mycobacterium avium* ssp. *paratuberculosis* are present in neuromyelitis optica spectrum disorder and multiple sclerosis patients.** *Journal of Neuroimmunology*. **323**:49-52 (2018). [#] Both authors contributed equally to this work. (2018).

7) Arru G, Sechi E, Mariotto S, Zarbo R, Ferrari S, Gajofatto A, Monaco S, Deiana GA, Bo M, Sechi LA and Sechi GP. **Antibody Response against HERV-W in patients with MOG-IgG associated disorders, Multiple Sclerosis and NMOSD.** *Journal of Neuroimmunology*. (2019). Accepted.

8) Bo M, Erre G, Bach H, Slavin YN, Manchia PA, Passiu G, Sechi LA. **PtpA and PknG proteins secreted by *Mycobacterium avium* subsp. *paratuberculosis* are recognized by sera from patients with rheumatoid arthritis: a case-control study.** *Journal of Inflammation Research*. (2019). Accepted.

9) Bo M, Arru G, Niegowska M, Erre GL, Manchia PA, Sechi LA. **Association between Lipoprotein Levels and Humoral Reactivity to Mycobacterium avium subsp. paratuberculosis in Multiple Sclerosis, Type 1 Diabetes Mellitus and Rheumatoid Arthritis.** *Microorganisms.* 7(10). pii: E423. (2019).

10) Bo M, Eames HL, Niegowska M, Arru G, Erre GL, Passiu G, Udalova IA and Sechi LA. **Antibody response to epitopes of Epstein-Barr virus, *Mycobacterium avium* subsp. *paratuberculosis* and IRF5 in connective tissue diseases.** (Underway to be published)

Dr. Bo Marco – "Immune response against *Mycobacterium avium* subsp. *paratuberculosis*, Epstein-Barr virus, HERV-K and IRF5 in rheumatoid arthritis" – PhD thesis in *Life Sciences and Biotechnologies* – University of Sassari, Italy