Prevalence and Predictive Value of Disease Specific Autoantibodies over 13 Years in a Large Cohort Representative of the General Population of Northern Italy

Thesis

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PREVALENCE AND PREDICTIVE VALUE OF DISEASE SPECIFIC AUTOANTIBODIES
OVER 13 YEARS IN A LARGE COHORT REPRESENTATIVE OF THE GENERAL
POPULATION OF NORTHERN ITALY

ADVANCED BIOMEDICAL RESEARCH

AFFILIATED RESEARCH CENTER: IRCCS ISTITUTO CLINICO HUMANITAS

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

THE OPEN UNIVERSITY

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1. ABSTRACT

Autoimmune diseases represent chronic disorders that affect a significant proportion of the population in Europe, with an impact on patients’ quality of life and socioeconomic costs for the healthcare system and community. Virtually, all autoimmune diseases are associated with the presence of serum autoantibodies. After the preclinical period, clinical manifestations develop, and a delay in the diagnosis and treatment can account for further organ damage, physical and psychological disability that cause a reduction of the working capacity and finally a high economic impact on society. Based on these findings, we aimed to:

1. Estimate the prevalence of disease specific autoantibodies in the general population of an area of Lombardia region by using the most recently developed methods for the detection of autoantibodies.

2. Analyze the autoantibodies predictive value for the development of autoimmune diseases over 15 years of follow-up.

3. Analyze if there are factors at baseline that can predict the development of autoimmune disease over 15 years of follow-up, in order to identify patients at higher risk of developing an autoimmune disease that need a closer follow-up.

4. Analyze the autoantibodies predictive value for the development of malignancies over 15 years of follow-up.

Research design and methodology

The present study was conducted on two different cohorts derived from the general population of Lombardia region in Northern Italy: ISOLA (2,828 subjects) and CA.ME.LI.A. (Cardiovascular risk, Metabolic syndrome, Liver, and Autoimmunity) (1,712 subjects). Serum samples were tested for serum autoantibodies (ANA, anti-ENA, rheumatoid factor, anti-CCP, anti-phospholipid antibodies, AMA, anti-LKM, anti-LC1, anti-SLA, anti-DGP, anti-tTG) using the most innovative techniques. Autoimmune diseases diagnosis were researched analyzing the administrative health databases.
Furthermore, we detected hospitalization, cancer diagnosis, and death. In the CA.ME.LI.A. cohort cardiovascular risk factors were analyzed and 1/3 underwent carotid ultrasonographic analysis.

In summary, we report that:

1. Serum ANA are detected in up to 18% of the general population, being more frequent in women and in elder ages while being ANA associated with an higher risk of connective tissue disease development

2. Serum rheumatoid factor is detected in 8.1% of the general population, while serum anti-CCP are found in 4.8% of the population, the prevalence of double positivity is 0.6%. Anti-CCP are associated with a significant increased risk of RA development, while rheumatoid factor is associated with both HBV and HCV infection. Anti-CCP are associated with an increased risk of cancer and rheumatoid factor is associated with an increased risk of death.

3. Anti-phospholipid antibodies are detected in 15% of an unselected population, especially newly identified autoantibodies not currently included in the classification criteria. Anti-phospholipid antibodies are associated with an increased cardiovascular risk profile and independently with cardiovascular disease, especially subclinical atherosclerosis.

4. AMA are detected in 3.8%, but at high titer in 1% of an unselected population; we identified only two cases of PBC, of which only one was AMA positive therefore we cannot estimate the risk; anti-LKM, anti-SLA, anti-LC1 antibodies are rarely found.

5. Anti-DPG antibodies are detected in 1.85% of an unselected population, while, anti-tTG were found in 2.2%, with no differences between sexes. We identified 7 cases of coeliac disease, of which only one resulted positive for both anti-DPG and anti-tTG antibodies at high-titer.

In conclusion, autoantibodies are frequently found in subjects randomly selected from the general population, while being only seldom associated with an increased risk of developing an overt autoimmune disease. Furthermore, autoantibodies may be associated with predisposing factors, as viral infections or cancer.
2. DECLARATION

The work described in this dissertation was performed at the IRCSS Istituto Clinico Humanitas (ICH), between February 2015 and July 2019. I declare that this dissertation has not been submitted in part or in whole to any other academic institution. The work reported here was entirely carried out by the author, unless otherwise indicated. Part of the results included in this dissertation have been published to the peer-reviewed journal Autoimmunity Reviews.
3. LIST OF PUBLICATIONS

Publications obtained during the course of this thesis:


8. Nailfold videocapillaroscopy and serum VEGF levels in scleroderma are associated with internal organ involvement. De Santis M, Ceribelli A, Cavaciocchi F, Crotti C, Massarotti M,


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5. ABBREVIATIONS

2-OADC: 2-oxoacid dehydrogenase

ACA: anticentromere antibodies

aCL: anti-cardiolipin antibodies

ACR: American College of Rheumatology

AIH: autoimmune hepatitis

AILD: autoimmune liver disease

ALT: aminotransferases

AMA: anti-mitochondrial antibodies

ANA: antinuclear antibodies

ANKRD55: ankyrin repeat domain-containing protein

Anti-CCP: anti-cyclic citrullinated peptides antibodies

Anti-DPG: anti-deaminated gliadin peptide antibodies

Anti-dsDNA: anti-doublestranded DNA

Anti-EMA: Anti-endomysium antibodies

anti-ENA: anti-extractable nuclear antigen antibodies

anti-GPI: anti-β2 glycoprotein I domain I antibodies

Anti-LC1: anti-liver cytosol type 1 antibodies

Anti-LKM1: anti-liver kidney microsomal type 1 antibodies

Anti-RNP: anti-ribonucleoprotein antibodies

Anti-Sm: anti-Smith

Anti-SMA: anti-smooth muscle antibodies

Anti-tTG: anti-transglutaminase antibodies

APC: antigen presenting cells

APCs: antigen presenting cells

aPLs: anti-phospholipid antibodies syndrome
aPS/PT: antibodies targeting prothrombin complexed with phosphatidylserine

APS: anti-phospholipid syndrome

aPT: anti-prothrombin antibodies

aSP: anti-phosphatidylserine/prothrombin

BLK: B lymphocyte kinase

body mass index (BMI)

C1Q: complement component 1q

C4: complement component 4

CA.ME.LI.A.: CArdiovascular risk, MEtabolic syndrome, LIver, and Autoimmunity

CD: cluster of differentiation

CI: confidence intervals

coronary artery disease (CAD)

CRP: C-reactive protein

CTDs: connective tissue diseases

CTLA4: cytotoxic T-lymphocyte-associated protein 4

CV: cardiovascular

DAMPS: damage-associated molecular patterns

DCs: dendritic cells

DKK1: Dickkopf-related protein 1

DNA: deoxyribonucleic acid

E2E3BP: E2 and E3-binding protein

ECM: extracellular matrix

ELISA: enzyme-linked immunosorbent assay

ESPGHAN: European Society for Paediatric Gastroenterology Hepatology and Nutrition

EULAR: European League Against Rheumatism

FcRs: Fc receptors
LE: lupus erythematosus
MHC: major histocompatibility complex
MHz: mega-Hertz
MMP: matrix metalloproteinase
mTOR: mammalian target of rapamycin
NFIA: Nuclear factor 1 A-type
NF-KB: nuclear factor kappa-light-chain-enhancer of activated B cells
NIEHS: National Institute of Environmental Health Sciences
NS: non significant
OCA: obeticholic acid
OR: odds ratio
PAD: peptidyl arginine deiminases
PAH: pulmonary arterial hypertension
PAMPs: pattern-associated molecular patterns
pANCA: perinuclear anti-neutrophil cytoplasmic antibodies
PBC: primary biliary cholangitis
PDE: phosphodiesterase -5 inhibitors
PDGF: platelet-derived growth factor
PRRs: pattern recognition receptors
PSC: primary sclerosing cholangitis
PTM: post-translational modifications
PTPN22: protein tyrosine phosphatase, non-receptor type 22
RA: rheumatoid arthritis
RF: rheumatoid factor
RNA: ribonucleic acid
ROC: receiver operating characteristic
ROS: reactive oxygen species
RR: risk ratio
SGEC: salivary gland epithelial cells
SjS: Sjögren’s syndrome
SLE: systemic lupus erythematosus
SNPs: single nucleotide polymorphisms
SPAG: Sperm-associated antigen
SSc: systemic sclerosis
STAT1: signal transducer and activator of transcription 1
TGF-β: transforming growth factor beta
Th: T helper cells
TLR: Toll-like receptors
TNF: tumor necrosis factor
TNFAIP: tumor necrosis factor, alpha-induced protein
UDCA: ursodeoxycholic acid
UV: ultraviolet
VEGF: vascular endothelial growth factor
VHS: video home system
6. INTRODUCTION

Epidemiology is the study of the distribution and determinants of disease in human populations [1, 2]. This definition is based on two fundamental assumptions: first, that human disease does not occur at random and, second, that human disease has causal and preventive factors that can be identified through systematic investigation of different populations or subgroups of individuals within a population in different places or at different times. As a result, epidemiologic studies include simple descriptions of the manner in which disease appears in a population (i.e., levels of disease frequency as well as incidence and prevalence, mortality, trends over time, geographic distributions, and clinical characteristics) and descriptions of the role of putative risk factors for disease occurrence. Incidence studies include all new cases of a specified condition arising in a defined population over a specified time period, and prevalence studies include all patients with the condition who are present in a population at a particular point in time. Prevalence cohorts exclude patients who died or left the population soon after their incidence date, and they include patients arising in different populations who moved into the cohort after their incidence date. Because of this, there is a greater potential for bias to be introduced in prevalence cohorts as compared to incidence cohorts. Thus, population-based incidence cohorts are superior to prevalence cohorts for descriptive epidemiologic studies.

Epidemiologic studies of risk factors fall into three major categories: prospective cohort studies, retrospective cohort studies, and case-control studies. In a prospective cohort study, a study population is assembled, and is followed forward into the future. People in the cohort are classified according to those characteristics that might be related to outcome, that is, putative risk factors. These people are then observed over time to determine which of them experience the outcome. The analysis addresses the question of whether people who were exposed to the risk factor were more likely to develop the outcome compared with those who were not exposed. In a retrospective cohort study, the cohort of individuals is identified from past records and followed forward up to the present. Data regarding historical exposure to the putative risk factor are collected retrospectively, typically by examination of medical records. As in a prospective cohort study, retrospective cohort studies also
compare the frequency of the outcome in exposed patients as compared to unexposed patients. In a

case-control study, two cohorts are assembled, one that has the outcome of interest and another that

is free of the outcome of interest. Data regarding exposure to the putative risk factor in the two groups

are collected retrospectively so as to determine whether patients with the outcome of interest were

more likely to have had a history of the exposure of interest compared with those who were free of

the outcome of interest. Of these three study designs, prospective cohort studies have fewer potential

biases than the other two; however, they are frequently not feasible because they typically require

extended follow-up, often 5 to 10 years or more into the future.

In this project, we took advantage of two cohort studies to determine the prevalence of serum

autoantibodies in the general population and their predictive value for autoimmune disease

development, associated-risk factors, and cancer and death.
Autoimmune diseases include chronic conditions characterized by the presence of specific serum autoantibodies and a panel of symptoms related to the autoimmune injury of different organs, for examples joints, skin, internal organs, such as lung, kidney, liver, thyroid and others. The epidemiology of autoimmune diseases varies (Table 1), however up to 9% (range 7.6-9.4%) of the European population is affected with a North-South gradient [3], with a higher prevalence in women (Table 2) [4, 5], with different symptom severity, disease course, response to therapy and overall survival between sexes [6]. Furthermore, many autoimmune diseases co-occur in the same patient or in family members [7].

Table 1. Prevalence of various autoimmune diseases in studies from Europe, North America, Australia, New Zealand (adapted from Cooper G.S. et al. [3]).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Rate per 100,000</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addison’s disease</td>
<td>11-14</td>
<td>UK, Italy, Norway</td>
<td>[8-10]</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>180-1900</td>
<td>Greece, Netherlands, Iceland, Italy, Finland</td>
<td>[11-15]</td>
</tr>
<tr>
<td>Type I diabetes</td>
<td>87-355</td>
<td>Spain, Germany, US, New Zealand</td>
<td>[16-19]</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>11-17</td>
<td>Spain, Sweden, Norway</td>
<td>[20-22]</td>
</tr>
<tr>
<td>Primary biliary cholangitis</td>
<td>15-40</td>
<td>Norway, Finland, Spain, UK</td>
<td>[22-26]</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>46-358</td>
<td>US, Canada, Italy, Greece, Norway, Ireland, Portugal</td>
<td>[27-35]</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>310-810</td>
<td>France, Spain, Hungary, Turkey, Greece, UK</td>
<td>[36-41]</td>
</tr>
<tr>
<td>Sjogren’s syndrome</td>
<td>93-600</td>
<td>UK, Denmark, Greece</td>
<td>[37, 42-47]</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>5-34</td>
<td>France, Greece, Spain, Italy, US</td>
<td>[48-53]</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>34-150</td>
<td>US, Spain, Greece, Canada</td>
<td>[54-57]</td>
</tr>
<tr>
<td>Granulomatosis with polyangiitis</td>
<td>2-10</td>
<td>France, Australia, New Zealand</td>
<td>[58-62]</td>
</tr>
</tbody>
</table>
Table 2. Female to male ratio of autoimmune diseases (adapted from Cincinelli G. et al. [63]).

<table>
<thead>
<tr>
<th>Autoimmune disease</th>
<th>Ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiphospholipid antibody syndrome</td>
<td>5:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>7:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>1:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Grave’s disease</td>
<td>7:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Hashimoto’s disease</td>
<td>5-18:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>2:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>3:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Primary biliary cholangitis</td>
<td>10:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>3:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Sjogren’s syndrome</td>
<td>9:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>9:1</td>
<td>[64, 65]</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>5:1</td>
<td>[64, 65]</td>
</tr>
</tbody>
</table>

Autoimmune disease etiology is complex and multifactorial, particularly since no monogenic mutation has been linked to the development of autoimmune disease. More importantly, there are largely incomplete concordance rates between monozygotic twins (Table 3), which share identical genome, and epidemiological evidence suggesting that infections or chemical agents may trigger autoimmunity in genetically susceptible individuals.
Table 3. Concordance rates in monozygotic and dizygotic twins in autoimmune diseases (*adapted from Generali E. et al. [66]*). (MZ: monozygotic; DZ: dizygotic).

<table>
<thead>
<tr>
<th>Reference</th>
<th>MZ pairwise concordance</th>
<th>MZ probandwise concordance</th>
<th>DZ pairwise concordance</th>
<th>DZ probandwise concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 diabetes mellitus</td>
<td>61%</td>
<td>12%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 [67]</td>
<td>27.3%</td>
<td>42.9%</td>
<td>3.8%</td>
<td>7.4%</td>
</tr>
<tr>
<td>[68]</td>
<td>13%</td>
<td>23%</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td>[69]</td>
<td>28.8%</td>
<td>44.7%</td>
<td>11.6%</td>
<td>20.8%</td>
</tr>
<tr>
<td>[70]</td>
<td>38.5%</td>
<td>53%</td>
<td>5.8%</td>
<td>11%</td>
</tr>
<tr>
<td>[71]</td>
<td>47.4%</td>
<td>64.3%</td>
<td>7.7%</td>
<td>14.3%</td>
</tr>
<tr>
<td>[72]</td>
<td>30.6%</td>
<td>46.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[73]</td>
<td>45.5%</td>
<td>16.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 2 diabetes mellitus</td>
<td>58%</td>
<td>11%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 [67]</td>
<td>2.1%</td>
<td>1%</td>
<td></td>
<td></td>
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<tr>
<td>[75]</td>
<td>12.3%</td>
<td>22%</td>
<td>3.5%</td>
<td>6.7%</td>
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<tr>
<td>[76]</td>
<td>15.4%</td>
<td>26.7%</td>
<td>3.6%</td>
<td>6.9%</td>
</tr>
<tr>
<td>[77]</td>
<td>9.1%</td>
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<td>[78]</td>
<td>0%</td>
<td>8.8%</td>
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<td>[79]</td>
<td>21%</td>
<td>35.3%</td>
<td>0%</td>
<td>0%</td>
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<td>[80]</td>
<td>11.1%</td>
<td>20%</td>
<td>0%</td>
<td>0%</td>
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<td>Rheumatoid arthritis</td>
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<td></td>
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<td>Systemic lupus erythematosus</td>
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<td>[85]</td>
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<tr>
<td>Primary biliary cholangitis</td>
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<tr>
<td>(female)</td>
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Genome-wide association studies (GWAS) have identified several hundred loci associated with specific autoimmune diseases, with several overlapping across diseases, frequently encompassing immune system related genes, thus suggesting a common mechanistic pathway, which may indicate a genetic susceptibility resulting from the additive effects of several common risk variants [86, 87]. Among known genetic predisposing factors, the strongest association is exerted by major histocompatibility complex (MHC) haplotypes, however several other common genes include PTPN22, CTLA4, IL23R [86]. Moreover, a recent study has identified new loci associated with seropositive inflammatory diseases, systemic sclerosis (SSc), systemic lupus erythematosus (SLE), immune-mediated myositis and rheumatoid arthritis (RA) (Figure 1), with possible therapeutic targets [88].

![Figure 1. Meta-analysis results for systemic sclerosis, systemic lupus erythematosus, immune-mediated myositis and rheumatoid arthritis [88].](image)

*The Manhattan plot displays the −log10 transformed p values (y-axis) by position on each chromosome (x-axis). The red line depicts the genome-wide significance threshold (p=5×10−8). A total of 26 single nucleotide polymorphisms (SNPs) were independently associated with at least 2 systemic immune-mediated inflammatory diseases. Most of the signals map to known susceptibility loci in autoimmunity (eg, PTPN22, STAT4, TNPO3, FAM167A-BLK) and five loci have never been reported before.*

Environmental factors may trigger autoimmunity, and these have been the focus of the National Institute of Environmental Health Sciences (NIEHS) expert panel workshop in 2012, which has
critically evaluated the epidemiology and laboratory studies associated with autoimmunity in the 1980-2010 scientific literature [89]. In this view, the concept of “exposome” has been introduced to collate and, possibly, measure the effects of environmental factors. The exposome includes all the environmental exposures, both exogenous and endogenous [90]. Infectious and non-infectious agents have long been considered important for the development of autoimmunity [91].

Autoimmune diseases overall share common pathogenic mechanisms, mainly involving B and T cells. B cells represent a crucial mediator of autoimmune diseases, as exemplified by autoantibody production, and hypergammaglobulinemia found in SLE, SSc, and primary biliary cirrhosis (PBC). B cells generate their pre-immune repertoire in the bone marrow through a gene recombination process known as V(D)J recombination, that leads to a 107-108 B cells repertoire with each unique surface receptors. This recombination lead to autoreactive B cells, in fact, early immature B cells in 55-75% of cases display auto-reactivity, which, however, decreases leading to 20% autoreactive mature B cells [92]. Several checkpoints exist to ensure that autoreactive B cells are excluded from the immunocompetent peripheral lymphocytes. Despite the checkpoints, poly-specific autoreactive B cells are found in the periphery and produce poly-specific natural autoantibodies [86]. These natural autoantibodies are usually germline encoded, of the IgM isotype, and non-pathogenic. However, poly-specific B cells might undergo somatic hyper-mutation and class switching to produce high affinity IgG pathogenic autoantibodies [93]. Post-translational modifications (PTM) play an important role in autoimmune diseases pathogenesis. It is estimated that 50-90% of the proteins are subject to PTM, and these contribute to tolerance breakdown. PTM include acetylation, lipidation, citrullination, glycosylation among others, and are crucial for specific autoantibodies recognition in autoimmune disease, i.e. RA, Sjögren’s syndrome (SjS), and multiple sclerosis. Conversely, the lack of PTM is capable of eliciting an immune response in PBC, in fact alters protein degradation leading to the accumulation and exposure of large amounts of autoantigens [94]. Autoantibodies directed towards PTM proteins are able to bind both the native and the modified form. In RA, collagen type II is an autoantigen
involved in the pathogenesis, and studies also from our group have shown different B and T cell epitopes on the molecule [95]. The B and T cell epitopes may undergo citrullination and glycosylation in vivo and thus induce the activation of immune cells in genetic predisposed subjects [95].

While the study of autoimmune diseases has long been centered on the adaptive immune system, the discovery that innate immune cells express sensors for foreign and self-ligands has shifted the focus toward the first defense from the environment, which precedes the adaptive response [96, 97]. The innate immune system recognizes broad patterns or molecular motifs called pattern-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by germline encoded “common” receptors called pattern recognition receptors (PRRs). This mechanism allows a more rapid screening of self from “non-self” molecules, as lipopolysaccharide, flagellin, peptidoglycan, lipoprotein, or single-stranded RNA and demethylated patterns on DNA such as unmethylated CpG DNA [89]. Toll-like receptors (TLR) are a family of PRRs that recognize PAMPs characteristic of pathogen microorganisms. TLR play a key role in the interplay between the innate and adaptive immune systems, and are linked also to autoimmune diseases [98]. These sensors are both endosomal and cytosolic [99]. The activation of these sensors in dendritic cells (DCs) could represent the initiating factor of some autoimmune diseases, inducing the production of type I interferons and pro-inflammatory cytokines, which can in turn activate T and B cells and autoantibody production [100]. Interestingly, it has been shown that environmental factors, such as viral infections, stress, injury, and UV light are sufficient to expose endogenous PAMPs to the innate immune system through active/passive release, these molecules interact with PRRs such as TLRs to activate the NF-KB-like transcription factors [101]. Ultimately, tissue damage and dysfunctions ensue and present as clinical symptoms. However, the onset of clinical symptoms is often delayed and occurs following irreversible damage to the affected tissues or organ. The clinical expression of autoimmune diseases can be very heterogeneous, with some diseases affecting only specific organs, while others causing multi-
system dysfunction. Autoantibodies may be present years before the development of clinical symptoms [102, 103], and their determination is usually one of the first steps in recognizing an autoimmune disease, although not being pathogenic. In 1901, autoantibodies were first described by Paul Ehrlich, but it took several years and the development of more modern technology (i.e. electrophoresis, radioactivity, chromatography) to identify other autoantibodies [104]. Across the 1930s and 40s, Eric Waaler and Harry Rose simultaneously described what we now call rheumatoid factor (RF) in RA [105], while in patients with SLE “LE cells” were described in 1948 and antibodies directed against cells nuclei in later years. Between the 1960s-70s, rheumatologic diseases were drawn together and autoantibodies were recognized as the main hallmark, while, however, could not explain all the pathogenesis.
6.2. ANTI-NUCLEAR AND ANTI-ENA ANTIBODIES IN CONNECTIVE TISSUE DISEASES

Antinuclear antibodies (ANA) are autoantibodies directed against self-antigens that are produced in response to systemic autoimmune conditions, such as connective tissue diseases (CTDs), or also in the presence of organ-specific autoimmune diseases (i.e. autoimmune hepatitis – AIH – which will be treated in the dedicated section – and thyroiditis), cancer or infections [106-108]. The most widely used test for the identification of ANA is indirect immunofluorescence (IIF) [109], which is routinely performed in laboratories worldwide and allows the identification of the ANA pattern and titer. The most common nuclear ANA patterns reported by routine laboratories are four and they are speckled, homogeneous, nucleolar, and centromere [109], followed by a series of less frequent patterns that can be seen only in a limited number of patients (Figure 2).

**Figure 2.** Antinuclear antibodies patterns (www.anapatterns.org).

**Antinuclear antibodies patterns: nomenclature and classification tree**

As for the titer, ANA positivity ≥1:160 is considered clinically significant by most routine autoimmunity laboratories and strong positivity is associated with higher risk of autoimmune
disorders, while tires ≤1:160 are present in up to 20% of healthy people, mainly in the elderly and in female population [110]. The ANA positivity is poorly specific, and in the suspect of autoimmune diseases, in particular systemic, it is necessary to require further testing for anti-ENA (extractable nuclear antigen) specificities. With this term, we refer to a group of autoantigens originally identified as antibody targets in people with systemic autoimmune diseases that are commonly composed of ribonucleoproteins (RNP) and non-histone proteins, called by the name of the donor who provided the prototype serum (i.e. Sm for Smith), or the name of the disease in which the antibodies were found (i.e. SS-A and SS-B for SjS antigen A and B; Scl-70 for the first identification in a SSc patient) [111].

In the suspect of systemic autoimmune diseases, routine laboratory testing requires both ANA with titer and anti-ENA. As mentioned, autoantibodies are central to the diagnosis of rheumatic diseases and are included in the diagnostic and classification criteria of several diseases.
6.2.1. Systemic Lupus Erythematosus

Systemic lupus erythematosus is a chronic autoimmune disease that predominately affects women, characterized by a broad spectrum of clinical manifestations (Figure 3), however, its course and organ involvement are unpredictable.

**Figure 3.** Clinical manifestations of systemic lupus erythematosus [112].

*Prevalence of systemic lupus erythematosus clinical manifestations.*

SLE is a global disease associated with an increased risk of premature death. The incidence in Western Countries is estimated to range from 2 to 7.6 per 100,000/year, while prevalence varies even more widely from 19 to 159 per 100,000 [112]. Women are more frequently affected, especially
between puberty and menopause [113]. Furthermore, African American have higher incidence and severity [114].

The pathogenesis of SLE is multifactorial, where genetics and environmental factors lead to the breaking of self-tolerance and to the activation/expansion of innate immune cells and autoreactive lymphocytes that can virtually affect any organs and tissues [100]. In brief, environmental factors in genetically predisposed individuals lead to the production of apoptotic cells and/or impaired clearance of apoptotic cells that in turn elicit an innate and adaptive immune response (Figure 4).

Figure 4. Immune dysfunction in systemic lupus erythematosus [112].
Proposed pathogenetic mechanisms involved in systemic lupus erythematosus development.
Key determinants of the progression of the autoimmune process include genetic susceptibility factors that shape immune function, sex and stochastic factors that affect responses to exogenous or endogenous triggers. With regard to genetics, the strongest links to SLE are the rare complement component C1Q and C4 single-gene defects, while association with MHC loci include the HLA-B8 and HLA-DR3 alleles [115], however in most patients several genes contribute to the risk of developing the disease. GWAS identified several candidate loci including interferon (IFN) regulatory factor 5 (IRF5), mutations in which are associated with increases in the levels of the type 1 IFN family of molecules in patients with SLE, but several additional loci are also important [116]. Interestingly, in HLA-DRB1 positive subjects, CD8+ T cells can be induced by IFN-α via STAT1 [117].

Sex hormones contribute to immune system activation. Estrogens can modulate the activation of lymphocytes, and prolactin is expressed at increased levels in serum of patients with SLE compared with controls, but the specific mechanisms by which prolactin might alter immune function in SLE are not clear [118]. Furthermore, environmental factors, such as infections, UV light and certain drugs, are known to trigger the disease [119]. In particular, UV light can induce DNA breaks, causing alterations in gene expression, generate nucleic acids fragments or lead to apoptotic or necrotic cell death. These triggers interact with TLRs on plasmacytoid DCs. In addition, aberrant cytoplasmic nucleic acid-sensing mechanisms in other cells, possibly epithelial cells, may enable direct stimulation of type I IFN release, allowing immune stimulation [120]. Type I IFNs are central to the activation of the innate immune system, and the interaction with their receptors activate JAK-STAT (Janus kinase - signal transducer activator of transcription) pathway and transcription of hundreds of IFN-responsive genes — the ‘interferon signature’ — encoding proteins that are involved in immune function regulation. Activation of antigen-presenting DCs by type I IFNs promotes their capacity to effectively present antigens, including self-antigens, to T cells [120]. The generation of T effector cells results in the production of cytokines and the expression of cell surface molecules that support amplification of a self-directed immune response as well as inflammation. With a steady supply of apoptotic material bound to factors (including nucleosomes), B cells are driven to produce
autoantibodies facilitated by CD40 (also known as TNR5)–CD40 ligand (CD40L) interactions [100]. T cell interactions are important in driving B cell differentiation and autoantibody production, as are B lymphocyte stimulator, TLR ligands and tumor necrosis factor (TNF) secreted by DCs. Normal anergic responses (that is, processes that suppress an immune response against self-antigens) are lost, leading to failure to delete self-reactive clones of T cells and B cells. The generation of immune complexes — containing nucleic acids, nucleic acid-binding proteins and autoantibodies directed against those components — sets the stage for inflammation and organ damage. Perpetuation of damage occurs when the immune complexes are deposited in target tissue with amplification of immune system activation after accessing endosomal TLRs and triggering downstream signals that induce IFNα and other pro-inflammatory mediators [100].

As shown, B cell regulation is impaired in SLE, contributing to the production of autoantibodies, which are traditionally viewed as essential mediators of pathology in SLE, particularly when they form immune complexes. Virtually all patients with SLE are positive for ANAs or other characteristic SLE autoantibodies, and for instance ANA, anti-dsDNA and anti-Sm/RNP are included in the classification criteria (Table 4). The diagnosis of SLE is made based on clinical manifestations and laboratory tests, functional tests and imaging.
### Table 4. 1997 Update of the 1982 American College of Rheumatology Revised Criteria for Classification of Systemic Lupus Erythematosus and 2012 Systemic Lupus International Collaborating Clinics classification criteria [121-123] (Adapted from Hartman et al [124])

<table>
<thead>
<tr>
<th>ACR 1997</th>
<th>SLICC 2012</th>
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<tbody>
<tr>
<td>4 of 11 criteria</td>
<td>4 of 17 criteria, including 1 of 11 clinical criteria and 1 of 6 immunologic criteria OR biopsy-proven SLE nephritis in the presence of ANA or anti-dsDNA antibodies</td>
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</tbody>
</table>

#### Clinical criteria

1. **Malar rash**  
   Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds

2. **Discoid rash**  
   Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions

3. **Photosensitivity**  
   Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation

4. **Oral ulcers**  
   Oral or nasopharyngeal ulceration, usually painless, observed by physician

5. **Non erosive arthritis**  
   Involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion

6. **Pleuritis or pericarditis**  
   Pleuritis: convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion

#### 1. **Acute cutaneous lupus**  
   Lupus malar rash (do not count if malar discoid), bullous lupus, toxic epidermal necrolysis variant of SLE, maculopapular lupus rash, photosensitive lupus rash *in the absence of dermatomyositis*  
   OR **subacute cutaneous lupus** (non indurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with post-inflammatory dyspigmentation or telangiectasias)

#### 2. **Chronic cutaneous lupus**  
   Classic discoid rash (localized (above the neck) or generalized (above and below the neck)), hypertrophic (verrucous) lupus, lupus panniculitis (profundus), mucosal lupus, lupus erythematosus tumidus, chillblains lupus, discoid lupus/lichen planus overlap

#### 3. **Oral ulcers**  
   Palate, buccal, tongue  
   OR **nasal ulcers**  
   *in the absence of other causes, such as vasculitis, Behcet’s disease, infection (herpesvirus), inflammatory bowel disease, reactive arthritis, and acidic foods*

#### 4. **Non scarring alopecia**  
   Diffuse thinning or hair fragility with visible broken hairs *in the absence of other causes such as alopecia areata, drugs, iron deficiency, and androgenic alopecia*

#### 5. **Synovitis**  
   involving 2 or more joints, characterized by swelling or effusion  
   OR tenderness in 2 or more joints and at least 30 minutes of morning stiffness

#### 6. **Serositis**  
   Typical pleurisy for more than 1 day  
   OR pleural effusions  
   OR pleural rub  
   Typical pericardial pain for more than 1 day (pain with recumbency improved by sitting forward)  
   OR pericardial effusion
<table>
<thead>
<tr>
<th><strong>OR pericarditis: documented by electrocardiogram or rub or evidence of pericardial effusion</strong></th>
<th><strong>OR pericardial rub</strong> OR pericarditis by electrocardiography in the absence of other causes, such as infection, uremia, or Dressler’s pericarditis</th>
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<tbody>
<tr>
<td><strong>7. Renal disorder</strong> Persistent proteinuria &gt; 0.5 grams per day or &gt; than 3+ if quantitation not performed, OR cellular casts (may be red cell, hemoglobin, granular, tubular, or mixed)</td>
<td><strong>7. Renal</strong> Urine protein-to-creatinine ratio (or 24-hour urine protein) representing 500 mg protein/24 hours OR red blood cell casts</td>
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<tr>
<td><strong>OR pericarditis by electrocardiography in the absence of other causes, such as infection, uremia, or Dressler’s pericarditis</strong></td>
<td><strong>8. Neurologic disorder</strong> Seizures OR psychosis in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance</td>
</tr>
<tr>
<td><strong>8. Neurologic</strong> Seizures, psychosis, mononeuritis multiplex in the absence of other known causes such as primary vasculitis, myelitis, peripheral or cranial neuropathy in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus, acute confusional state in the absence of other causes, including toxic/metabolic, uremia, drugs</td>
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<tr>
<td><strong>9. Hematologic disorder</strong> Hemolytic anemia with reticulocytosis OR leucopenia: &lt; 4,000/mm3 on ≥ 2 occasions OR lymphopenia: &lt; 1,500/mm3 on ≥ 2 occasions OR thrombocytopenia: &lt;100,000/mm3 in the absence of offending drugs</td>
<td><strong>9. Hemolytic anemia</strong></td>
</tr>
<tr>
<td><strong>10. Leukopenia</strong> &lt;4,000/mm3 at least once in the absence of other known causes such as Felty’s syndrome, drugs, and portal hypertension OR Lymphopenia &lt;1,000/mm3 at least once in the absence of other known causes such as corticosteroids, drugs, and infection</td>
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<tr>
<td><strong>11. Thrombocytopenia</strong> &lt;100,000/mm3) at least once in the absence of other known causes such as drugs, portal hypertension, and thrombotic thrombocytopenic purpura</td>
<td><strong>Immunologic criteria</strong></td>
</tr>
<tr>
<td><strong>10. Immunologic disorder</strong> Anti-DNA: antibody to native DNA in abnormal titer OR anti-Sm: presence of antibody to Sm nuclear antigen OR positive finding of antiphospholipid antibodies on a) an abnormal serum level of IgG or IgM anticardiolipin antibodies, b) a positive test result for lupus anticoagulant using a standard method, or c) a false-positive test result for at least 6 months confirmed by Treponema</td>
<td><strong>1. ANA</strong> level above laboratory reference range</td>
</tr>
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</table>
Table 5. Correlation between systemic lupus erythematosus subtypes and autoantibodies [125].

<table>
<thead>
<tr>
<th>SLE manifestations</th>
<th>Antibody</th>
<th>Target</th>
</tr>
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<tbody>
<tr>
<td>Neuropsychiatric SLE</td>
<td>Anti-ribosomal P</td>
<td>Ribosomal-P proteins and neuronal antigens</td>
</tr>
<tr>
<td>Lupus nephritis</td>
<td>Anti-C1q, anti-dsDNA, anti-Sm</td>
<td>C1q, double strand DNA, Sm1</td>
</tr>
<tr>
<td>Secondary Sjögren’s syndrome</td>
<td>Anti-SSA, anti-SSB</td>
<td>SSA/Ro (proteins 60/52 kD) and SSB/La</td>
</tr>
<tr>
<td>Interstitial lung disease and shrinking lung syndrome</td>
<td>Anti-RNP</td>
<td>U1 ribonucleoprotein (U1-RNP) and SSA/Ro</td>
</tr>
<tr>
<td>Lupus arthritis</td>
<td>Anti-Sm</td>
<td>Sm</td>
</tr>
<tr>
<td>Leukocytopenia</td>
<td>Anti-dsDNA</td>
<td>Double strand DNA</td>
</tr>
<tr>
<td>Secondary antiphospholipid syndrome</td>
<td>LAC, anti-beta2glycoprotein IgM/IgG</td>
<td>Phospholipids</td>
</tr>
</tbody>
</table>

Moreover, autoantibody specificities overlap between the different clinical manifestations of SLE and positivity for a specific antibody does not necessary mean that a certain organ will be affected, however some autoantibodies are associated with peculiar clinical manifestations (Table 5).
The treatment of SLE has been recently addressed in the EULAR (European League Against Rheumatism) recommendations [126]. The goal of treatment is disease remission or low disease activity and prevention of flares. Hydroxychloroquine (5 mg/kg real body weight) is virtually recommended in every SLE patient. Glucocorticoids can be used both in the induction and maintenance phase, in the latter ideally not exceeding 7.5 mg/day of prednisone equivalent. In case of patients not responsive to hydroxychloroquine or glucocorticoids, or who cannot reduce below the accepted dosage, immunosuppressants should be started. Biologics, such as belimumab, can be added in patients with inadequate response to standard of care therapies for extra-renal disease. Interestingly, patients with persistent disease may benefit from belimumab and are more likely to respond if high disease activity, prednisone>7.5 mg/day and serological activity (low C3/C4, high anti-dsDNA titres) [127]. Future therapies include stem cell transplantation, targeted biologic and synthetic therapies.

SLE prognosis has dramatically changed in the last 30 years, however most patients have an impact in their quality of life, observe the development of comorbidities, both related to disease and therapy, and manifest organ damage.
6.2.2 Sjögren’s syndrome

Sjögren’s syndrome is a chronic, systemic autoimmune disease [128], affecting 10.3/10,000 inhabitants in the United States [129], with a female predominance and two peaks of incidence, around 30 and 50 years of age, while being rare in children [130, 131]. SjS can be primary or secondary to other systemic autoimmune diseases, i.e. RA, SLE, SSc, mixed connective tissue disease, inflammatory muscle disease, autoimmune liver disease, and autoimmune thyroid disease [132, 133]. The pathogenesis of SjS is currently based on the concept of “autoimmune epithelitis”, similarly to PBC [134], as discussed later, since in SjS the exocrine glands, especially salivary and lacrimal, are progressively destructed by an immune-mediated process (Figure 5)[135, 136].

Figure 5. Autoimmune epithelitis pathogenesis across Sjögren’s syndrome and primary biliary cholangitis [137].

Proposed pathogenetic mechanisms leading to Sjögren’s syndrome and primary biliary cholangitis development.

As other autoimmune diseases, genetic and environmental factors interact in the development of SjS, which takes several hits. First, when epithelial cells become apoptotic due to various factors, and aberrant autoantigen expression on exocrine gland epithelia may lead to an increased presentation to autoreactive T cells [138]. More importantly, disease specific molecules, i.e. anti-SSA, have been
demonstrated to be expressed in salivary gland epithelial cells (SGEC) from 50% of patients with SjS [139]. Moreover, SGEC have the peculiarity to release after apoptosis intact autoantigens via apoptotic blebs and bodies [140, 141]. While in healthy tissues, apoptotic cells are cleared by antigen presenting cells (APC) and phagocytes, in SjS this capacity is impaired and may account for the prolonged availability of autoantigens to stimulate an immune process [142]. Once the autoantigens have been presented, a multi-lineage T and B cell response develops. CD8+ T cells directly infiltrate the target organs, where also an increased expression of pro-inflammatory cytokines, chemokines, adhesion and co-stimulatory molecules are found. Overall an increased Th1 and Th17 response is observed, and elevated serum levels of IFN-gamma, necessary for Th1 differentiation, are detected [143] [144]. Recently, it has been demonstrated that also Th17 cells are involved in the pathogenesis of SjS, and Th17 related cytokines, i.e. IL-17, IL-6 and IL-12, are elevated in the serum [145], while transforming growth factor β (TGF-β), IL-6 and IL-23 are found in abundance in minor salivary glands. A recent study has further clarified that elevated IL-17F levels are found in SjS sera, and are associated with increased IgG and IgM, higher titers of ANA and anti-SSA antibodies, and reduction of C3 and C4; while serum IL-17A was only increased in patients with longer disease duration and showed few correlation with clinical and laboratory features [146].

B cells are also involved in SjS pathogenesis, as exemplified by the presence of serum autoantibodies and hyper-IgM [147-149]. Indeed, therapeutic B cell depletion therapy may ameliorate the disease and decrease antigen presentation by B cells but data are conflicting or inconclusive [150, 151]. Moreover, B cell hyperactivity in SjS has been associated with extraepithelial immune complex-mediated manifestations such as vasculitis, purpura, glomerulonephritis and peripheral nephropathy and the development of lymphoma. The production of cryoglobulins and the deposition of immune complexes to the affected tissues represent the main mechanisms that drive the pathogenesis of the extraepithelial manifestations [152].

Since SjS is an epithelium-specific disease, the most common clinical manifestations include oral and eye dryness, which are usually present at the time of diagnosis although in some rare cases, systemic
manifestations, such as vasculitis, may precede the classical sicca symptoms. A significant percentage of patients with SjS complain of systemic dryness involving the nose, the trachea, the vagina and the skin, suggesting that other glands are also affected in the context of exocrine involvement [152]. Systemic extraglandular manifestations are present in 10–15% of patients with SjS, and arise from diverse pathogenetic mechanisms, being the result of the typical lymphocytic infiltration around the epithelium of target organs such as the liver, the kidney and the bronchi/bronchioles, or the systemic form of the disease that arises from immune-complex deposition, presenting with the clinical picture of vasculitis [153]. Other extra-glandular manifestations include arthralgia and arthritis, and fatigue. The diagnosis of SjS should be suspected in case of persistent sicca syndrome symptoms, especially in women. The diagnosis can be made in individuals with an objective finding of ocular and/or oral dryness for whom there is substantive evidence of an underlying autoimmune basis for the exocrine glandular dysfunction (Table 6).
Table 6. Sjögren’s syndrome classification criteria [154].

<table>
<thead>
<tr>
<th align="left">I) OCULAR SYMPTOMS:</th>
</tr>
</thead>
<tbody>
<tr>
<td align="left">• Daily, persistent, troublesome dry eyes for more than 3 months</td>
</tr>
<tr>
<td align="left">• Recurrent sensation of sand or gravel in the eyes</td>
</tr>
<tr>
<td align="left">• Need of tear substitutes more than 3 times a day</td>
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<tr>
<th align="left">II) ORAL SYMPTOMS:</th>
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</thead>
<tbody>
<tr>
<td align="left">• Daily feeling of dry mouth for more than 3 months</td>
</tr>
<tr>
<td align="left">• Recurrently or persistently swollen salivary glands</td>
</tr>
<tr>
<td align="left">• Need of drinking liquids to aid in swallowing dry food</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th align="left">III) OCULAR SIGNS: OBJECTIVE EVIDENCE OF OCULAR INVOLVEMENT DEFINED AS A POSITIVE RESULT FOR AT LEAST ONE OF THE FOLLOWING TWO TESTS:</th>
</tr>
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<tbody>
<tr>
<td align="left">• Schirmer’s test, performed without anesthesia (&lt;5 mm in 5 minutes)</td>
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<tr>
<td align="left">• Rose bengal score or other ocular dye score (&gt;4 according to van Bijsterveld’s scoring system)</td>
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<tr>
<th align="left">IV) HISTOPATHOLOGY</th>
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<tr>
<td align="left">• In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialadenitis, evaluated by an expert histopathologist, with a focus score &gt;1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm2 of glandular tissue.</td>
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<table>
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<tr>
<th align="left">V) SALIVARY GLAND INVOLVEMENT: OBJECTIVE EVIDENCE OF SALIVARY GLAND INVOLVEMENT DEFINED BY A POSITIVE RESULT FOR AT LEAST ONE OF THE FOLLOWING DIAGNOSTIC TESTS:</th>
</tr>
</thead>
<tbody>
<tr>
<td align="left">• Unstimulated whole salivary flow (&lt;1.5 ml in 15 minutes)</td>
</tr>
<tr>
<td align="left">• Parotid sialography showing the presence of diffuse sialectasis (punctate, cavitary or destructive pattern), without evidence of obstruction in the major ducts</td>
</tr>
<tr>
<td align="left">• Salivary scintigraphy showing delayed uptake reduced concentration and/or delayed excretion of tracer</td>
</tr>
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<tr>
<th align="left">VI) AUTOANTIBODIES: PRESENCE IN THE SERUM OF THE FOLLOWING AUTOANTIBODIES:</th>
</tr>
</thead>
<tbody>
<tr>
<td align="left">• Antibodies to Ro(SSA) or La(SSB) antigens, or both</td>
</tr>
</tbody>
</table>

In patients without any potentially associated disease, primary SjS may be defined as follows:

1. The presence of any 4 of the 6 items is indicative of primary SjS, as long as either item IV (Histopathology) or VI (Serology) is positive
2. The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)

The presence of any four of the following six criteria items provides sensitivity 97.4%, specificity 89.4%.
Anti-SSB are usually found with anti-SSA antibodies, while are the only specificity in 25-40% of cases [155], and are not included in the latest classification criteria [156]. ANA, albeit not specific for SjS, are present in up to 90% of cases, while RF is present in up to 70% of cases [155]. Histologic examination remains the gold standard for the diagnosis of SjS. Minor salivary gland biopsy should be performed in those subjects who do not have diagnostic dry eye tests or who have negative autoantibodies, with a high clinical suspicion of SjS (Figure 6)[157].

**Figure 6.** Histopathologic findings characteristics of Sjögren’s syndrome [158].
*Minor salivary gland biopsy of SjS patient, revealing a heavy periductal and interlobular lymphocytic infiltrate. A. Lymphocytic infiltrate of >50 cells is depicted (dotted circle). Original magnification ×10. B. The area in the dotted circle (×20). Note the acinar atrophy and the distorted ducts, changes consistent with SS. The entire specimen had a focus score > 1.*

The treatment of SjS should challenge both symptoms relief, i.e. the management of dry eye and xerostomia, and systemic disease manifestations. Patient education is of primary importance when managing both glandular and extra-glandular manifestations of SjS. New treatments are under investigations, but no biologic therapy has received specific indication for SjS yet [159].
6.2.3. Systemic Sclerosis

Systemic sclerosis is an autoimmune disease of unknown origin characterized by microvascular damage and progressive fibrosis of skin that in severe cases can affect internal organs such as heart, lungs, and kidneys. As most autoimmune diseases, it is more common in female patients (female:male 4:1) with age of onset at 30-60 years. In Italy, it is considered a rare disease due to its low prevalence, which is estimated to be 1/6,500 adults in the general population, with differences linked to ethnicity and geographic areas [51]. One of the typical symptoms at SSc onset is the Raynaud’s phenomenon, expression of severe peripheral vaso-constriction, but the clinical aspects of SSc can be very heterogeneous and they can develop sometimes very quickly and aggressively, and sometimes more slowly and over a period of decades [160]. The etiology and the pathogenesis of SSc are unknown, but two processes, fibrosis and microvascular occlusion, characterize the pathological findings seen in all involved organs in SSc patients [161]. Several mechanisms are proposed in the development of SSc disease (Figure 7), such as (i) activation of the adaptive and immune system and activation of endothelial cells; (ii) release of cytokines (i.e. TGF-β, PDGF, IL-4) from platelets, macrophages, and T-cells; (iii) cytokine activation of fibroblasts to increase extracellular matrix production; (iv) vascular endothelial growth factor (VEGF) altered expression [162, 163]. In this view, we studied levels of serum VEGF165b concentration in our SSc patients, and we could see that this molecule is variably expressed in SSc patients with pulmonary interstitial involvement [164] and it could be considered as a biomarker of this specific clinical feature in SSc.
Figure 7.

Proposed pathogenic mechanisms involved in systemic sclerosis [165].

A proposed pathogenetic hypothesis identifies the *primum movens* in an event at the level of the vasculature, probably the endothelium. This insult could be a viral infection, an attack on the endothelium by autoantibodies, a toxin, or oxidative stress. On turn, endothelial cell damage and apoptosis ensue, leading to the vascular leakiness that manifests in early clinical stages as tissue edema. The vascular compartment is further compromised by impaired angiogenesis and impaired vasculogenesis (fewer endothelial progenitor cells). The release of vasoconstricting agents outweighs the release of vasodilating agents, leading to vascular instability; the damaged endothelium serves as a nidus for platelet activation, leading to thrombi formation that further contributes to ischemia-reperfusion injury and the generation of reactive oxygen species (ROS). The damaged endothelium upregulates adhesion molecules and chemokines to attract leukocytes,
which enables the development of both innate and adaptive immune responses, including loss of
tolerance to various oxidized antigens (such as topoisomerase 1). B cells mature into antibody-
secreting plasma cells, further propagating the autoimmune response. T cells differentiate into
various subsets, including T helper 2 (Th2) lineage cells, which may play an important role in tissue
fibrosis. Anti–topoisomerase 1 antibodies form immune complexes, are taken up via Fc receptors
(FcRs), and activate endosomal TLRs in immune cells, especially plasmacytoid DCs, which leads
to type I IFN production. Finally, fibroblasts are recruited and activated by multiple cytokines and
growth factors to generate myofibroblasts. The presence of stimulatory platelet-derived growth
factor (PDGF) receptor antibodies that could drive ROS production by fibroblasts and fibrosis is a
questionable finding that awaits confirmation. Dysregulated TGF-β signaling in fibroblasts and
myofibroblasts has been observed in multiple studies of SSc patients. Activation of fibroblasts and
myofibroblasts leads to excessive deposition of collagen and other extracellular matrix (ECM)
proteins, which eventually results in the tissue fibrosis observed in SSc [165].

SSc is classified in diffuse or limited according to the extent of skin fibrosis as established by LeRoy
in 1988 [166]. Each of these forms is associated to the presence of a specific autoantibody profile,
and the great importance of autoantibodies in SSc is demonstrated by their inclusion in the recent
diagnostic criteria developed by ACR/EULAR in 2013 (Table 7), as nearly all patients have highly
specific circulating antibodies.
**Table 7. 2013 ACR/EULAR Criteria for the classification of systemic sclerosis [167].**

<table>
<thead>
<tr>
<th>Item</th>
<th>Sub-items</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin thickening of the fingers of both hands extending proximal to</td>
<td>Puffy fingers</td>
<td>2</td>
</tr>
<tr>
<td>the metacarpophalangeal joints (<em>sufficient criterion</em>)</td>
<td>Sclerodactyly of the fingers (distal to the metacarpophalangeal joints, but proximal to the proximal interphalangeal joints)</td>
<td>4</td>
</tr>
<tr>
<td>Skin thickening of the fingers (<em>only count the higher score</em>)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Fingertip lesions (<em>only count the higher score</em>)</td>
<td>Digital tip ulcers</td>
<td>2</td>
</tr>
<tr>
<td>Fingertip pitting scars</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Teleangectasia</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Abnormal nailfold capillaries</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Pulmonary arterial hypertension and/or interstitial lung disease</td>
<td>Pulmonary arterial hypertension</td>
<td>2</td>
</tr>
<tr>
<td>(<em>maximum score 2</em>)</td>
<td>Interstitial lung disease</td>
<td>2</td>
</tr>
<tr>
<td>Raynaud’s phenomenon</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>SSc-related autoantibodies (<em>maximum score 3</em>)</td>
<td>Anticentromere</td>
<td>3</td>
</tr>
<tr>
<td>Anti-topoisomerase I (anti-Scl70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-RNA polymerase III</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The criteria are not applicable to patient with skin thickening sparing the fingers or to patients who have a scleroderma-like disorder that better explains their manifestations (e.g. nephrogenic sclerosing fibrosis, generalized morphea, eosinophilic fasciitis, scleredema diabeticorum, scleromyxedema, erythromyalgia, porphyria, lichen sclerosis, graft-versus-host disease, diabetic chorioarthropathy).

*Patients with a total score ≥ 9 are classified as having definite scleroderma, with a sensitivity of 91%, and specificity of 92.*
The most frequently identified autoantibodies in SSc are anticentromere (ACA) - (30%), anti-topo I (30%), and -RNA pol III (4-20%). However, several other autoantibodies can be identified in SSc patients and they can have an important role for their clinical and prognostic value (Table 8).

**Table 8.** Selected autoantibodies linked to clinical manifestations and complications in systemic sclerosis (Adapted from Allanore et al [168]).

<table>
<thead>
<tr>
<th>Antinuclear antibodies pattern</th>
<th>Primary target of the autoantibody</th>
<th>Clinical association</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diffuse cutaneous systemic sclerosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Speckled</td>
<td>DNA topoisomerase I</td>
<td>Interstitial lung disease</td>
</tr>
<tr>
<td>Speckled</td>
<td>RNA polymerase III</td>
<td>Renal crisis and cancer</td>
</tr>
<tr>
<td>Nucleolar</td>
<td>Fibrillarin (ribonucleolar protein, targets U3 RNP)</td>
<td>Pulmonary arterial hypertension and myositis</td>
</tr>
<tr>
<td><strong>Limited cutaneous systemic sclerosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centromeric</td>
<td>Centromere proteins</td>
<td>Ischaemic digital ulcers and teleangectasia</td>
</tr>
<tr>
<td>Nucleolar</td>
<td>Th/To ribonucleoprotein</td>
<td>Interstitial lung disease</td>
</tr>
</tbody>
</table>

The treatment of SSc presents a clinical challenge, because the various clinical manifestations, the progressive nature of the diseases with a relatively poor prognosis and lack of proven treatment. Current treatment recommendations suggest an approach to the organ involvement [169]. Vascular involvement, i.e. Raynaud’s phenomenon, digital ulcers, and pulmonary arterial hypertension (PAH), benefit most from vasoactive agents, as dihydropyridine-type calcium antagonists, prostanoids, endotelin receptor antagonists and phosphodiesterase -5 (PDE5) inhibitors. Conversely, skin and lung involvement, probably due to the different pathogenic involvement, should be treated with immunosuppressive agents. Furthermore, in severe diffuse rapidly progressive SSc cases at risk of organ failure, hematopoietic stem cell transplantation (HSCT) may improve skin involvement and stabilize lung function.
6.3 ANTI-CYC LIC CIT RULL INATED PEPTIDES AND RHEUMATOID FACTOR IN RHEUMATOID ARTHRITIS

Anti-cyclic citrullinated peptides antibodies (anti-CCP) and RF represent the hallmarks of RA, a chronic autoimmune disease primarily affecting the joints in up to 1% of the general population, more frequently in women >50 years [170]. Several risk factors are known to be involved in the development of RA, including genetics, female sex and environmental factors (Figure 8).

Figure 8. Mechanisms involved in initiation and progression of rheumatoid arthritis [171].
RA has a strong genetic component, twin studies have estimated that the heritability (the proportion of phenotypic variance that is due to genetic variance in a population) of RA to be ~60%, in particular for anti-CCP positive subjects [172], however, disease concordance is of only 12–15% [66]. Several genetic loci have been associated with RA, especially HLA-DRB1*01 and HLA-DRB1*04, which encode the “shared epitope”, are significantly associated with the risk of developing RA [173]. However, other risk loci have been identified, albeit with weaker associations (Figure 9).

Figure 9. Genetic loci associated with rheumatoid arthritis [174].

Manhattan plot showing 106 gene loci identified so far, divided by ethnic region. The significance of each SNP in the rheumatoid arthritis GWAS is indicated on a logarithmic scale (X-axis).
Of interest, genetic differences between autoantibody-positive and negative RA have been shown. For example, variants in HLA-DRB1, PTPN22, BLK, ANKRD55 and IL6ST associate with RA regardless of serological status whereas AFF3, CD28 and TNFAIP3 are found only in seropositive RA and PRL and NFIA are found only in seronegative RA. Indeed, several RA susceptibility genes are also associated with severity (for example, HLA-DRB1, IL2RA, DKK1, GRZB, MMP9 and SPAG16) [175].

Like other autoimmune diseases, RA is influenced by sex hormones, as estrogens elicit a stimulatory effect on the immune system. Interestingly, nulliparity often increases the risk of RA, whereas pregnancy is often associated with disease remission, although disease flares are common in the postpartum period [176]. Conversely, men have a later disease onset and are more likely to have high titers of RF and anti-CCP [177]. Several environmental factors have been linked to RA development, and in particular tobacco smoking raises the risk of RA in a graded fashion.

Infections can trigger or predispose to RA development. Recent studies have suggested that the initial steps of the pathological autoimmune response originate in mucosal sites [178], and intestinal dysbiosis could play a role in RA, as well as other autoimmune diseases [179]. *Prevotella copri* has been identified as highly enriched in the gut microbiota of patients newly diagnosed with RA and an increased immune response to this organism has been demonstrated in patients with RA suggesting a role of *P. copri* in the disease onset. Sequence homology between RA-specific autoantigens and epitopes from proteins of *P. copri* have been reported, supporting the molecular mimicry hypothesis, although exact mechanisms remain uncertain [180]. Furthermore, *Porphyromonas gingivalis*, which is common in periodontal disease, expresses peptidyl arginine deiminases (PAD) and can induce citrullination and thereby promote anti-CCP generation. This mechanism has been demonstrated especially in genetically predisposed individuals, i.e. HLA-DR+4 subjects [95].

Similarly to other autoimmune diseases, autoantibodies in RA (RF and anti-CCP) are present in 70-90% of the subjects [181], and also before the onset of clinical symptoms by several years in a proportion of patients with RA [182, 183]. Interestingly, the autoantibody response evolves in the
pre-clinical phase of the disease, with level increase, epitope spreading, isotype switching, affinity maturation, and change in glycosylation over time [184]. Conversely, autoantibody-positive subjects suffering from arthralgia have higher risk of developing RA if have higher autoantibody levels, more epitope spreading, and more frequently are anti-CCP and RF double positive [185-187]. Remarkably, it is important to note that the introduction of the 2010 classification criteria (Table 9) has profoundly changed the clinical picture of RA [188], in fact in this new setting, the high weigh attributed to autoantibodies in the scoring system has produced the paradoxical effect of making the clinical presentation of autoantibody-positive patients significantly milder compared to autoantibody-negatives [189]. Accordingly, anti-CCP positive patients do not show more severe disease burden in terms of fatigue, pain, well-being, and independence compared to autoantibody negative patients [190].

**Table 9.** 2010 rheumatoid arthritis classification criteria [191].

<table>
<thead>
<tr>
<th>Joint involvement</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 large joint</td>
<td>0</td>
</tr>
<tr>
<td>2-10 large joints</td>
<td>1</td>
</tr>
<tr>
<td>1-3 small joints</td>
<td>2</td>
</tr>
<tr>
<td>4-10 small joints</td>
<td>3</td>
</tr>
<tr>
<td>10 joints</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serology (at least 1 test result is needed for classification)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative RF and negative ACPA</td>
<td>0</td>
</tr>
<tr>
<td>Low-positive RF or low-positive ACPA</td>
<td>2</td>
</tr>
<tr>
<td>High-positive RF or high-positive ACPA</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acute-phase reactants (at least 1 test result is needed for classification)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal CRP and normal ESR</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal CRP or abnormal ESR</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Duration of symptoms</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;6weeks</td>
<td>0</td>
</tr>
<tr>
<td>≥6weeks</td>
<td>1</td>
</tr>
</tbody>
</table>

Target population (Who should be tested?): Patients who
1) have at least 1 joint with definite clinical synovitis (swelling)
2) with the synovitis not better explained by another disease
A score of 6/10 is needed for classification of a patient as having definite RA
Large joints refer to shoulders, elbows, hips, knees, and ankles. Small joints refer to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.
Low-positive refers to IU values that are higher than the ULN but ≤3 times the ULN for the laboratory and assay; high-positive refers to IU values that are >3 times the ULN for the laboratory and assay.
RF: rheumatoid factor; ACPA: anti-citrullinated protein antibody; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.
Anti-CCP and RF are not only biomarkers of RA diagnosis but also reflect the disease prognosis, as RF of the IgA class was regarded as a specific diagnostic marker in early arthritis [192] and, in general, as a marker of more aggressive and refractory disease [193, 194]. Anti-CCP on the other way are associated with more erosive disease, however are less informative in dividing disease phenotypes [195, 196]. More remarkably, double autoantibody-positive patients seem to be characterized by a more inflammatory phenotype, as shown by the elevation of acute phase reactants [197, 198].

Another aspect of the disease which appears to be strongly (and possibly mechanistically) linked to the presence of autoantibodies is extra-articular involvement, including cardiovascular (CV) comorbidity. Several studies have reported higher rates of CV mortality and events in autoantibody-positive patients [199], in particular anti-CCP positive subjects [200]. This fact could be attributed to the higher inflammatory burden associated to autoantibodies, contributing to accelerated atherosclerosis and endothelial dysfunction [201], leading to CV events even in the absence of clinically apparent RA [202].

Interestingly, autoantibody status also drives the therapeutic choice in RA. In fact, biologic agent targeting of autoantibody production, including B cell depletion or inhibition of T cell co-stimulation, are particularly effective in autoantibody-positive patients. This has been shown in a meta-analysis of four randomized placebo-controlled trials from the rituximab clinical program, particularly in patients for whom at least one TNF-α inhibitor had failed, and without significant differences between RF and anti-CCP [203]. More recently, treatment with abatacept, inhibiting T cell co-stimulation, has been shown to be associated with better clinical response in patients with very high levels of anti-CCP at baseline [204-206]. Studies on the prognostic value of autoantibodies for response to therapy to biologic agents targeting different mechanisms of action are less conclusive. No differences in treatment response to anti-TNF have been reported according to RF and/or anti-CCP status [207].
6.4. ANTIPHOSPHOLIPID ANTIBODIES – ANTI-CARDIOLIPIN, ANTI-BETA2GLICOPROTEIN I, ANTI-PHOSPHATIDYL-SERINE/PROTHROMBIN – AND ANTI-PHOSPOLIPID SYNDROME

Anti-phospholipid (aPL) syndrome (APS) is a rare autoimmune disease characterized by vascular (arterial and/or venous) thrombosis and/or pregnancy morbidity in the presence of aPL antibodies [208]. Primary aPL syndrome is a rare disease with a prevalence between 40 and 50 cases /100,000 adults [209], while can be secondary to other autoimmune diseases, particularly SLE [210]. The incidence of APS in around 2 per 100,000 population (age >= 18 years) [210]. The etiology of APS is unknown, however many autoantibodies associated with the disease are directed against a number of plasma proteins and proteins expressed on, or bound to, the surface of vascular endothelial cells or platelets, leading to coagulation abnormalities (Figure 10). Of interest, anti-β2 glycoprotein I domain I antibodies (anti-GPI) have been associated with thrombosis [211]. Furthermore, anti-prothrombin antibodies (aPT) have been associated with prothrombotic effect in APS mouse models [212], however antibodies targeting prothrombin complexed with phosphatidylserine (aPS/PT) have a stronger correlation with thrombosis compared to aPT [213]. The pathogenicity of anti-GPI appears to be prominent in pregnancy morbidity as well. By binding to and disrupting the anticoagulant Annexin A5 shield on endothelial and trophoblast cell monolayers, anti-GPI induce a pro-coagulant state at the level of the placenta leading to thrombosis and infarction [211, 214]. The imbalance between pro-inflammatory and anti-inflammatory processes significantly affects embryo implantation and fetal growth, and acute inflammation initiated by aPLs during the gestation period may lead to fetal loss. Recent studies have also shown that non-thrombotic vascular stenosis and occlusions are reported in aPL-positive/APS patients and attributed to intimal hyperplasia. Furthermore, patients with APS nephropathy show a hyperactivation of mTOR complexes 1 and 2 by IgG, leading to neo-intima formation and vascular stenosis [215].
Antiphospholipid antibodies are produced by B cells, bind to anionic surfaces and thus convert the closed, nonimmunogenic β2 glycoprotein I to the open immunogenic β2 glycoprotein I. Subsequently, antiphospholipid antibodies bind to the immunogenic β2 glycoprotein I, resulting in endothelial-cell, complement, platelet, neutrophil, and monocyte activation, including NETosis. Further, antiphospholipid antibodies promote clot formation, and interfere with trophoblasts and decidual cells. Antiphospholipid antibodies, on the basis of multiple mechanisms, not mutually exclusive, result in inflammation, vasculopathy, thrombosis and pregnancy complications.
APS clinical manifestations include classification criteria, venous and arterial thromboses, and pregnancy morbidity (fetal losses and placental insufficiency) [216], however, due to its vascular nature, various manifestations not included in the criteria may be observed and various organs and tissues may be affected (Figure 11). In particular, cardiac involvement is a peculiar sign of APS. Both thrombosis and immune-mediated injury are present, and the most common clinical manifestations are valvulopathies, including non-bacterial thrombotic endocarditis (Libman-Sacks endocarditis), and coronary artery disease (CAD).

Figure 11. Non-criteria clinical manifestations associated with anti-phospholipid syndrome [217].
The diagnosis of primary aPL syndrome is based on the combination of clinical features, such as arterial/venous thrombosis and/or recurrent miscarriages/placental insufficiency, and the detection of circulating aPLs, according to Miyakis classification criteria (Table 10) [218].

Table 10. Classification criteria for the anti-phospholipid syndrome [218]

<table>
<thead>
<tr>
<th>Clinical criteria</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vascular thrombosis:</td>
<td></td>
</tr>
<tr>
<td>One or more clinical episodes of arterial, venous, or small vessel thrombosis, in any tissue or organ. Thrombosis must be confirmed by objective validated criteria (i.e. unequivocal findings of appropriate imaging studies or histopathology). For histopathologic confirmation, thrombosis should be present without significant evidence of inflammation in the vessel wall.</td>
<td></td>
</tr>
<tr>
<td>2. Pregnancy morbidity:</td>
<td></td>
</tr>
<tr>
<td>a. One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, with normal fetal morphology documented by ultrasound or by direct examination of the fetus, or</td>
<td></td>
</tr>
<tr>
<td>b. One or more premature births of a morphologically normal neonate before the 34th week of gestation because of: (i) eclampsia or severe pre-eclampsia defined according to standard definitions, or (ii) recognized features of placental insufficiency</td>
<td></td>
</tr>
<tr>
<td>c. Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.</td>
<td></td>
</tr>
<tr>
<td>In studies of populations of patients who have more than one type of pregnancy morbidity, investigators are strongly encouraged to stratify groups of subjects according to a, b, or c above.</td>
<td></td>
</tr>
<tr>
<td>Laboratory criteria</td>
<td></td>
</tr>
<tr>
<td>Lupus anticoagulant (LA) present in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis (Scientific Subcommittee on LAs/phospholipid-dependent antibodies) [219, 220]</td>
<td></td>
</tr>
<tr>
<td>Anticardiolipin (aCL) antibody of IgG and/or IgM isotype in serum or plasma, present in medium or high titer (i.e. &gt;40 GPL or MPL, or &gt;the 99th percentile), on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA [221-223]</td>
<td></td>
</tr>
<tr>
<td>Anti-β2 glycoprotein-I antibody of IgG and/or IgM isotype in serum or plasma (in titer &gt;the 99th percentilc), present on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA, according to recommended procedures [224]</td>
<td></td>
</tr>
</tbody>
</table>

Anti-phospholipid syndrome is present if at least one of the clinical criteria and one of the laboratory criteria that follow are met.

Classification of APS should be avoided if less than 12 weeks or more than 5 years separate the positive aPL test and the clinical manifestation. Coexisting inherited or acquired factors for thrombosis are not reasons for excluding patients from APS trials. However, two subgroups of APS patients should be recognized, according to: (a) the presence, and (b) the absence of additional risk factors for thrombosis. Indicative (but not exhaustive) such cases include: age (>55 in men, and >65 in women), and the presence of any of the established risk factors for cardiovascular disease (hypertension, diabetes mellitus, elevated LDL or low HDL cholesterol, cigarette smoking, family history of premature cardiovascular disease, body mass index ≥30 kg m−2, microalbuminuria, estimated GFR <60 mL min−1), inherited thrombophilias, oral contraceptives, nephrotic syndrome, malignancy, immobilization, and surgery. Thus, patients who fulfill criteria should be stratified according to contributing causes of thrombosis. A thrombotic episode in the past could be considered as a clinical criterion, provided that thrombosis is proved by appropriate diagnostic means and that no alternative diagnosis or cause of thrombosis is found. Superficial venous thrombosis is not included in the clinical criteria. Generally accepted features of placental insufficiency include: (i) abnormal or non-reassuring fetal surveillance test(s), e.g. a non-reactive non-stress test, suggestive of fetal hypoxemia, (ii) abnormal Doppler flow velocimetry waveform analysis suggestive of fetal hypoxemia, e.g. absent end-diastolic flow in the umbilical artery, (iii) oligohydramnios, e.g. an amniotic fluid index of 5 cm or less, or (iv) a postnatal birth weight less than the 10th percentile for the gestational age.
Nevertheless, aPLs are not specific to aPL syndrome and can be detected in different clinical settings, including other autoimmune diseases (secondary aPL syndrome), non-autoimmune diseases (cancers, infections), and healthy individuals [208]. Other aPLs, defined as non-criteria aPLs, include anti-phosphatidylserine/prothrombin (aSP) [225], anti-phosphatidic acid, anti-vimentin/cardiolipin complex, anti-protein C/S, anti-factor XII, anti-factor X, anti-annexin A5/A2, anti-D1 [226], but their prevalence and clinical significance are not well established. Interestingly, aPS/PT antibodies have been shown to have a better overall agreement and higher test reproducibility when compared to lupus anticoagulant (LAC), especially during treatment with vitamin K antagonist [227]. Furthermore, “seronegative” APS cases have been reported, however this could be due to several factors, i.e. negativities of previously positive aPLs, new aPLs yet to be discovered [228].

Although the definition of criteria for classification of the antiphospholipid, clinically significant antiphospholipid-antibody positivity is not well established. The frequency of aPL positive subjects outnumbers the clinically overt disease. Despite the standardization and the universal diffusion of ELISA tests for anti-cardiolipin (aCL) and aGPI detection [229], there is no accurate estimate of aPL prevalence or sex predominance in the general population. APLs have been detected in ~45% of patients with SLE [230], 7% of women with recurrent miscarriages [231, 232], and 8% of blood donors [208, 233], but all these estimates are poorly representative of aPL prevalence in the general population.

The major causes of mortality in aPL syndrome are thrombotic events, but a number of non-thrombotic events are frequently reported in association to aPLs, such as migraine, livedo, ulcers, and heart valve diseases [210]. These manifestations are thought to be associated to the aPL-mediated endothelial activation [210]. To date, it has not been investigated if such endothelial activation could be also implicated in the development of atherosclerosis or contribute to atherothrombosis in patients with aPLs.

The treatment of patients with aPLs in the absence of thrombosis is currently risk stratification: based on age, aPLs profile, concomitant risk factors for thrombosis, and other systemic autoimmune
diseases. A moderate-to-high risk aPLs profile warrants avoidance of estrogen supplements when possible and aggressive postoperative prophylaxis against thrombosis if possible [208]. For primary thrombosis prevention, the use of low-dose aspirin is controversial, given the low quality of evidence and lack of prospective data. In secondary arterial thrombosis prevention, low-dose aspirin is indicated if patient has other risk factors for cardiovascular disease, or prevention of pregnancy complications in pregnant patients with obstetric or thrombotic APS or both. Anticoagulation represent the cornerstone therapy in case of secondary arterial and venous prevention. The role of hydroxychloroquine is controversial, but can be used as a potential add-on treatment for recurrent thrombosis despite therapeutic dose anticoagulant therapy. Immunosuppressive agents are used in cases of catastrophic APS, and are an option in cases of severe thrombocytopenia, hemolytic anemia, or both [208].
6.5. ANTI-MITOCONDRIAL, ANTI-LKM, ANTI-LC1, AND ANTI-SLA AUTOANTIBODIES AND AUTOIMMUNE LIVER DISEASE

Autoimmune liver disease (AILD) includes three main distinct clinical entities: primary biliary cholangitis (PBC), previously known as cirrhosis, autoimmune hepatitis (AIH), and primary sclerosing cholangitis (PSC). However, they differ according to the focus of autoimmune injury, the pattern of inflammation and the clinical phenotype. In particular, in PBC, the small, interlobular bile ducts are affected, causing the typical appearance of non-suppurative, destructive cholangitis. In AIH the autoimmune injury primarily affects the hepatocytes, leading to interface hepatitis. Conversely, in PSC, an immune-mediated injury affects the medium-sized intra-and extrahepatic bile ducts, causing concentric and obliterator fibrosis and multifocal bile duct stricturing [234].

6.5.1. Primary biliary cholangitis

PBC is a chronic cholestatic autoimmune liver disease characterized by the destruction of intrahepatic bile ducts, progressive fibrosis that can, in turn, cause cirrhosis [136, 235, 236]. PBC presents a geographical pattern with widely variable prevalence and the highest rate was described in the north, with a point prevalence of 402 per million in the general population of Minnesota [237, 238]. Similar to other autoimmune diseases, PBC most commonly affects women, with a 1:9 male:female ratio [136], and the average age at PBC diagnosis is within the 5th and 6th decades of life [136]. Similarly to other autoimmune diseases, PBC etiology is multifactorial. Several studies have reported a strong link with HLA alleles, in particular DRB1*08, DR3, DPB1*0301, DRB1*08-DQA1*0401-DQB1*04, whereas DRB1*11 and DRB1*13 seem to be protective [239]. Furthermore, non-HLA genes have been associated with PBC development, i.e. genes involved in the regulation of the immune system and antigen presentation, such as IL-1RL1, STAT4, STAT1, IL12A.

Environmental factors have been extensively studied in PBC. Infections are thought to increase the susceptibility to PBC based on molecular mimicry which ensues when an infectious agent, bacterial or viral, presents antigens with a significant amino acid similarity to self-proteins [134, 240]. Furthermore, smoking, the use of hormonal replacement therapy and history of recurrent urinary tract
infections have been associated with an increased risk of PBC development. Moreover, xenobiotics, such as nail varnish, hair dye and cleaning chemicals can replace the PDC-E2 domain and break self-tolerance. Once the immune response is established, aberrant autoantigens are expressed on biliary epithelial cells, which may lead to an increased presentation to autoreactive T cells. Once the autoantigens have been presented, a multi-lineage T- and subsequently B-cell response develops. CD4+ and CD8+ T cells directly infiltrate the target organs, as well as B cells and macrophages, where also an increased expression of proinflammatory cytokines, chemokines and adhesion and costimulatory molecules is found. Proinflammatory cytokines play a crucial role in promoting systemic inflammation and affect the proliferation and maturation of the infiltrating lymphocytes. Overall, an increased Th1 and Th17 response is observed. T cells likely contribute both directly and indirectly to tissue damage and systemic manifestations through production of cytokines (Th1, Th17 cells) and maintenance of B cell-mediated responses (Tfh cells). It has recently been demonstrated that Th17 cells are also involved in the pathogenesis of PBC, and correlate with disease severity. Altered B-cell function is also present, as exemplified by the presence of serum autoantibodies and hyper-IgM levels [134].

Early PBC symptoms are fatigue and pruritus, whereas physical findings may include skin hyperpigmentation, hepatosplenomegaly, and (rarely) xanthelasmas. Fatigue and pruritus are nonspecific symptoms present in 70% of patients with PBC. In contrast, end-stage symptoms are secondary to the complications of liver cirrhosis, including ascites, jaundice, hepatic encephalopathy, and upper digestive bleeding. Portal hypertension is frequently found in patients with PBC and, importantly, does not imply the presence of liver cirrhosis. Metabolic bone disease is increased in PBC compared with sex-matched and age-matched healthy individuals. Similar to other types of cirrhosis, end-stage PBC can be complicated by the occurrence of hepatocellular carcinoma (HCC). The progression of PBC varies widely, and the factors influencing the severity and progression of the disease are largely unknown. However, the presence of symptoms at presentation are a major factor
determining PBC survival rates; asymptomatic PBC produces 10-year survival rates lower than those in the general population, but symptomatic PBC produces even lower survival rates [241].

The diagnosis of PBC is generally based on the presence of two of the following three criteria (Table 11): (1) biochemical evidence of cholestasis with elevation of alkaline phosphatase activity over six months; (2) presence of serum anti-mitochondrial antibodies (AMA) at significant titers; and (3) histological non-suppurative cholangitis and destruction of small or medium-sized bile ducts when a biopsy is performed (Figure 12). The differential diagnosis includes a cholestatic drug reaction, biliary obstruction, sarcoidosis, AIH, and PSC.

**Table 11. Diagnostic criteria for Primary Biliary Cholangitis.**

<table>
<thead>
<tr>
<th>Elevated ALP &gt; 2 x ULN or GGT &gt; 5 x ULN</th>
<th>AMA positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic granulomatous cholangitis at liver biopsy</td>
<td></td>
</tr>
</tbody>
</table>

*Diagnosis is made in the presence of at least 2 out of 3 of the criteria. ALP: alkaline phosphatase; ULN: upper limit of normal; GGT: γ-glutamyltransferase; AMA: anti-mitochondrial antibodies.*

**Figure 12.** Histology and immunohistochemical staining in primary biliary cholangitis [242].

*Liver histology in primary biliary cirrhosis (PBC). (a) Photomicrograph of hematoxylin and eosin stain (x200 magnification) from a percutaneous liver biopsy demonstrating a classic florid duct lesion in a patient with PBC. (b) Immunostaining of the portal tract in a with anti-cytokeratin-7 highlighting the bile duct that is infiltrated with lymphocytes.*

AMA are the hallmark of PBC, being present in 90-95% of patients when sensitive techniques are used [243], and less than 1% of healthy subjects [136, 244]. Similarly, to other autoimmune diseases, AMA positivity arises years before the development of PBC [245], and are included in the internationally accepted criteria for PBC diagnosis [136].
AMA are directed against components of the 2-oxoacid dehydrogenase (2-OADC) family of enzymes within the mitochondrial respiratory chain, most frequently the E2 and E3-binding protein (E3BP) components of the pyruvate dehydrogenase complex and the E2 components of the 2-oxo glutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes [246]. In all three antigens epitopes contain the motif DKA, with lipoic acid covalently bound to the lysine (K) residue [247]. Similar to AMA, ANA have also been identified in 52% of patients, with the most specific patterns being “nuclear-rim” and “multiple nuclear dots”, produced by antibodies directed against the nuclear membrane gp120 and nucleoporin 62, and the nuclear body sp100, sp140 and promyelocytic leukaemia proteins, respectively. ANA-positive patients are more frequently AMA-negative, possibly because of the lack of a masking effect of these latter antibodies in such sera. Furthermore, AMA are also detectable in other autoimmune diseases, such as SSc, which can be associated with PBC [248].

PBC treatment is currently based on ursodeoxycholic acid (UDCA), which represents the only approved drug. During the early disease, short-term glucocorticoids might be effective; however, prolonged use raises safety concerns. Budesonide, because of its high first-pass metabolism, has minimum systemic adverse effects and, at 6 to 9 mg daily, has been shown to be superior to UDCA in terms of both histology and biochemical markers. Other immunosuppressants, such as methotrexate and azathioprine, have also been suggested and there is evidence supporting the use of the latter in PBC with AIH overlap syndrome. The use of biologics targeting TNF-alpha has been reported in few cases of overlap syndromes with rheumatic diseases. When the disease has already progressed and bile has accumulated, obeticholic acid (OCA), an analogue of chenodeoxycholic acid with a much higher affinity to the farnesoid X receptor, has been shown to decrease bile synthesis, promote secretion, and induce liver regeneration in animal models. Furthermore, a recent phase III trial of OCA administered with UDCA or as monotherapy for 12 months showed decreases in alkaline phosphatase and total bilirubin levels compared with placebo. Ultimately, UDCA represents the cornerstone therapy for PBC and doses ranging from 13 to 15 mg/kg lead to optimum bile enrichment,
with 50% of patients normalizing their alkaline phosphatase levels. Other immunosuppressive treatments should be started only in combination with UDCA. Liver transplant may be necessary for end-stage PBC, with survival rates of 92% and 85% at 1 and 5 years after transplant, respectively. Recurrence is common and seems to be influenced by immunosuppressants, whereas the use of UDCA for recurrence is safe and recommended [241].

6.5.2. Autoimmune hepatitis

Autoimmune hepatitis is a chronic inflammatory disease of unknown etiology resulting from the immune-mediated destruction of hepatocytes with autoimmune features [249, 250]. AIH is characterized by the presence of typical but non-specific findings on liver biopsy, serum autoantibodies and elevated serum aminotransferases and gamma-globulins [251]. The incidence cannot be accurately estimated, and is thought to be approximately 1 per 100,000 person-years, supposedly with higher incidence in Scandinavia [252]. AIH most commonly affects women, with a male:female ratio of 1:4 [252], and manifests a two-peak incidence during adolescence and at 30–45 years of age [249, 253]. The onset of AIH is most frequently insidious, with 20–30% of patients presenting with an acute icteric hepatitis, constantly associated with hyper-gammablubulinemia. The etiology of AIH is unknown, but genetic and environmental factors are likely to play an important role (Figure 13). Among the predisposing factors, GWAS have identified HLA and non-HLA genes in the pathogenesis of the disease, furthermore female sex influences the development of AIH, which presents a female-to-male ratio of 4:1. The precipitating factors include several environmental exposures that are described as either protective factors, as tobacco, alcohol and vitamin D, or as risk factors as exposure to drugs, hormones, diet and pathogens as viruses, parasites and bacteria. The influence of precipitating factors on susceptible individuals leads to the pre-clinical stage in which APCs recognizing antigens or self-antigens activate Th0 cells who give rise to Th1, Th2 or Th17 cells depending on the cytokine environment. Th2 cells secrete IL-4, IL-10 and IL-13, which stimulate the maturation of B-cells into antibody producing plasma cells which produce ANA and/or anti-smooth
muscle antibodies (anti-SMA), anti-liver kidney microsomal type 1 antibody (anti-LKM1) and anti-liver cytosol type 1 antibody (anti-LC1) depending on the subtype of AIH. Antibodies bind to liver cells and contribute to natural killer (NK) and complement-mediated cytotoxicity. Th1 cells secrete IL-2 and IFNγ stimulating CD8+ cells, expression of HLA class I, expression of HLA class II on hepatocytes. Th17 cells secrete proinflammatory cytokines. Th0 cells differentiate into Treg cells under the stimulus of TGF-β, this process is mediated by IL-21 secreted by NKT cells. The histological plaque shows an inflammatory cell infiltrate, mainly cytotoxic T-cells and plasma cells, around the portal tracts characteristic of interface hepatitis in AIH [254].
Figure 13. *Pathogenesis of autoimmune hepatitis* [254].

Clinical manifestations are unspecific and include hepatosplenomegaly, progressive jaundice, anorexia and fatigue [251, 255]. The most common extrahepatic manifestations are arthralgia and skin rash.

Two types of AIH are distinguished primarily based on the autoantibody patterns; i.e. AIH type 1 with ANA and/or anti-SMA, and AIH type 2 with anti-LKM1 and/or for anti-LC1. Type I AIH (AIH-1) can affect people of any age and sex, and patients with HLA DRB1*0301 AIH-1 are more likely to be male, to present with high IgG levels, and to be ANA/anti-SMA positive to deteriorate despite corticosteroid treatment and to progress more frequently to liver transplantation. Type II AIH (AIH-2), primarily affects girls and young women, and has been linked to alleles encoding the DR3 (DRB1*0301) and DR7 (DRB1*0701) molecules [250], and positivity for anti-LKM antibodies [251, 256, 257]. The diagnosis of AIH is defined as definite or probable, based on the codified Diagnostic Criteria of the International Autoimmune Hepatitis Group (Table 12) [258, 259].
Table 12. Revised Original Scoring System of the International Autoimmune Hepatitis Group [259].

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0</td>
</tr>
<tr>
<td>Female</td>
<td>+2</td>
</tr>
<tr>
<td><strong>Ratio of ALP vs. AST/ALT</strong></td>
<td></td>
</tr>
<tr>
<td>&gt;2.0</td>
<td>+3</td>
</tr>
<tr>
<td>1.5-2.0</td>
<td>+2</td>
</tr>
<tr>
<td>1.0-1.5</td>
<td>+1</td>
</tr>
<tr>
<td>&lt;1.0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Autoantibodies (ANA, SMA, LKM1) titer</strong></td>
<td></td>
</tr>
<tr>
<td>&gt;1:80</td>
<td>+3</td>
</tr>
<tr>
<td>1:80</td>
<td>+2</td>
</tr>
<tr>
<td>1:40</td>
<td>+1</td>
</tr>
<tr>
<td>&lt;1:40</td>
<td>0</td>
</tr>
<tr>
<td><strong>AMA</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>-4</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td><strong>Seropositivity for other autoantibodies</strong></td>
<td>+2</td>
</tr>
<tr>
<td><strong>Viral hepatitis markers</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>+3</td>
</tr>
<tr>
<td><strong>History of drug use</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>-4</td>
</tr>
<tr>
<td>No</td>
<td>+1</td>
</tr>
<tr>
<td><strong>Average alcohol consumption (g/day)</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>+2</td>
</tr>
<tr>
<td>&gt;60</td>
<td>-2</td>
</tr>
<tr>
<td><strong>Presence of genetic factors (HLA, DR3 or DR4)</strong></td>
<td>+1</td>
</tr>
<tr>
<td><strong>Presence of other autoimmune disorders (thyroiditis, colitis, others)</strong></td>
<td>+2</td>
</tr>
<tr>
<td><strong>Liver histology</strong></td>
<td></td>
</tr>
<tr>
<td>Interface hepatitis</td>
<td>+3</td>
</tr>
<tr>
<td>Predominant lymphocytic infiltrate</td>
<td>+1</td>
</tr>
<tr>
<td>Rosetting of liver cells</td>
<td>+1</td>
</tr>
<tr>
<td>None of the above</td>
<td>-5</td>
</tr>
<tr>
<td>Biliary changes</td>
<td>-3</td>
</tr>
<tr>
<td>Other changes</td>
<td>-3</td>
</tr>
<tr>
<td><strong>Response to therapy</strong></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>+2</td>
</tr>
<tr>
<td>Relapse</td>
<td>+3</td>
</tr>
</tbody>
</table>

A score > 15 or > 17 indicates a definite diagnosis of AIH pre- or post-treatment, respectively. On the other hand, scores between 10–15 and 12–17 indicate a probable diagnosis, pre- or post-therapy, respectively. AMA, anti-mitochondrial autoantibodies; LKM-1, anti-liver–kidney microsomal antibodies; SMA, anti-smooth-muscle antibodies.
The clinical criteria for the diagnosis are sufficient to make or rule out a definite or probable AIH in the majority of patients, while the revised scoring system was developed as a research tool by which to ensure the comparability of study populations in clinical trials, and can be used to assess treatment response (Table 13), similar to classification criteria utilized in rheumatology [259]. A pretreatment score of 10 points or higher, or a post-treatment score of 12 points or higher, indicate ‘‘probable’’ AIH at presentation, with a sensitivity of 100%, a specificity of 73%, and diagnostic accuracy of 67%. A pretreatment score of 15 points, indicative of ‘‘definite AIH’’ has a sensitivity of 95%, a specificity of 97%, and a diagnostic accuracy of 94% [260].
Table 13. Codified Diagnostic Criteria of the International Autoimmune Hepatitis Group [259].

<table>
<thead>
<tr>
<th>Features</th>
<th>Definite</th>
<th>Probable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver histology</td>
<td>Interface hepatitis of moderate or severe activity with or without lobular hepatitis or central portal bridging necrosis, but without biliary lesions or well defined granulomas or other prominent changes suggestive of a different etiology</td>
<td>Same as for “definite”</td>
</tr>
<tr>
<td>Serum biochemistry</td>
<td>Any abnormality in serum aminotransferases, especially if the serum alkaline phosphatase is not markedly elevated. Normal serum concentrations of alpha antitrypsin, copper and ceruloplasmin</td>
<td>Same as for “definite” but patients with abnormal serum concentrations of copper or ceruloplasmin may be included, provided that Wilson disease has been excluded by appropriate investigations</td>
</tr>
<tr>
<td>Serum immunoglobulins</td>
<td>Total serum globulin or gamma globulin or IgG concentrations greater than 1.5 times the upper normal limit</td>
<td>Any elevation of serum globulin or gamma globulin or IgG concentrations above the upper normal limit</td>
</tr>
<tr>
<td>Serum autoantibodies</td>
<td>Seropositivity for ANA, SMA or anti-LKM1 antibodies at titers greater than 1:80. Lower titers (particularly of anti-LKM1) may be significant in children. Seronegativity for AMA</td>
<td>Same as for “definite” but at titers of 1:40 or greater. Patients who are seronegative for these antibodies but who are seropositive for other antibodies may be included.</td>
</tr>
<tr>
<td>Viral markers</td>
<td>Seronegativity for markers of current infection with hepatitis A, B and C viruses</td>
<td>Same as for “definite”</td>
</tr>
</tbody>
</table>
| Other etiological factors | Average alcohol consumption less than 25g/day  
No history of recent use of known hepatotoxic drugs | Alcohol consumption less than 50g/day and no recent use of known hepatotoxic drugs  
Patients who have consumed larger amounts of alcohol or who have recently taken potentially hepatotoxic drugs may be included, if there is clear evidence of continuing liver damage after abstinence from alcohol or withdrawal of the drug |
In 2004, the International Autoimmune Hepatitis Group established procedures and reference guidelines for more reliable serum autoantibody testing to overcome the lack of standardization [261]. Not only ANA, anti-SMA, and anti-LKM [262] should be evaluated and other sets of autoantibodies should also be tested in suspected cases, including anti-LC1, perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), SLA/LP, and the anti asialoglycoprotein receptor antibodies [263]. Finally, other less specific autoantibodies are also detected in a subgroup of patients, i.e., aCL, anti-chromatin, anti-dsDNA, RF, antibodies to histones, anti-Ro/SSA, and anti-CCP antibodies. ANA were the first autoantibodies observed in AIH sera over 50 years ago and remain the most sensitive marker of AIH [264], most frequently producing a homogeneous or speckled pattern. However, the test is not specific for AIH, since ANA positivity is not uncommon in sera from patients with viral or other autoimmune liver diseases as well as in as many as 15% of healthy subjects, especially in older age groups [265]. Anti-SMA are autoantibodies reacting with different proteins (actin, tubulin, vimentin, desmin, cytokeratins) of the cytoskeletal components (microfilaments, microtubuli, intermediate filaments). Their presence characterizes both autoimmune (AIH-1, coeliac disease - CD) and viral diseases (chronic hepatitis C, infectious mononucleosis). When detected at high titers (>1:80), they are considered a sensitive marker for AIH-1, being found in up to 80% of cases. A recent study showed that anti-SMA-T/G positive subject with normal liver function are at low risk of progression to AIH, while positive anti-SMA and raised ALT (>55IU/L) are at higher risk, with a positive predictive value of 22% [266]. Autoantibodies against LKM-1 are the main serological markers of AIH-2 and recognize the proximal renal tubule and hepatocellular cytoplasm. Anti-SLA/LP antibodies are occasionally found in patients with AIH who are negative for ANA, anti-SMA, or anti-LKM and are cumulatively detected in 10–30% of cases of AIH-1 and -2. Anti-SLA/LP antibodies are detectable by radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) but not by immunofluorescence and are directed against different epitopes of a UGA tRNA suppressor. Anti-LC1 antibodies are detected by indirect immunofluorescence in sera from up to 50% of patients with type 2 AIH and less frequently in type 1 AIH or chronic hepatitis C. Importantly, however, anti-LC1
are the only detectable markers in 10% of AIH cases. Interestingly, anti-LC1 antibodies correlate with AIH severity and progression. Antibodies to the asialoglycoprotein receptor are observed in up to 90% of patients with AIH and often coexist with other autoantibodies while lacking specificity for the disease. Similar to anti-LC1, however, anti-asialoglycoprotein titers are associated with a more florid inflammatory disease activity and may monitor treatment response.

Immunosuppressants represent the treatment of choice for AIH, based on the good biochemical and histologic response, and survival. Glucocorticoids, in particular prednisone, in monotherapy or in combination with azathioprine are the first-line treatment and induce remission (ie, normal ALT and IgG) in more than 80% of the patients, regardless of the presence of cirrhosis. Once achieved, remission can be maintained with azathioprine alone after steroid tapering. Relapses following steroid discontinuation are common, because only 20% of patients remain in sustained remission. However, subgroups of patients manifest disease progression (approximately 10%) or are intolerant to standard therapy (13%). In such patients, other drugs have been anecdotally tried, including methotrexate, cyclophosphamide, tacrolimus, UDCA, cyclosporine, and mycophenolate mofetil, the last 2 constituting the most frequently reported alternatives. Liver transplant is the most definitive treatment of patients with AIH presenting with acute liver failure or end-stage chronic liver disease and for those with HCC who meet the transplant criteria. Although liver transplant for these patients is very successful, AIH may recur after transplant. Patients with AIH undergoing liver transplant have overall 5-year and 10-year survival rates of 90% and 75%, respectively, although infectious complications and disease recurrence are common.
6.6. ANTI-TRANSGLUTAMINASE AND ANTI-DEAMINATED GLIADINE PEPTIDE ANTIBODIES IN COELIAC DISEASE

Coeliac disease is an autoimmune disease that occurs in genetically predisposed individuals who develop an immune reaction to gluten. It affects primarily the small intestine, and occurs in about 1% of the population, especially in children [267]. Diagnosis rates are increasing, and this seems to be due to a true rise in incidence rather than increased awareness and detection. The clinical manifestations are broad, with both intestinal and extra-intestinal symptoms and ranging from severe malabsorption to minimally symptomatic or non-symptomatic presentations. Classical CD refers to a disease course presenting with signs and symptoms of malabsorption, which include diarrhea, malnutrition, and growth failure. In contrast, patients with non-classical CD present without symptoms and signs of malabsorption.

CD develops in genetically susceptible individuals (almost all patients with CD possess HLA-DQ2, HLA-DQ8, or half HLA-DQ2) who, in response to unknown environmental factors, develop an immune response that is subsequently triggered by the ingestion of gluten (Figure 14).

**Figure 14.** Coeliac disease pathogenesis [268].
The major environmental factor responsible for the development of CD is gluten. Gluten (from the Latin “glue”) is the term for the prolamin storage proteins of the cereal grains wheat, rye, and barley. Gluten is favored in breadmaking for its elasticity; however, it is enriched in glutamines and prolines and, as a result, is incompletely digested by gastric, pancreatic, and brush border peptidases, leaving large peptides up to 33 aminoacids long [269]. These peptides enter the lamina propria of the small intestine via transcellular or paracellular routes where, in affected individuals, an adaptive immune reaction occurs that is dependent on deamidation of gliadin molecules by the enzyme tissue transglutaminase, the predominant autoantigen of coeliac disease. Deamidation increases the immunogenicity of gliadin, facilitating binding to the HLA-DQ2 or HLA-DQ8 molecules on antigen presenting cells. Gliadin peptides are then presented to gliadin-reactive CD4+ T cells [270]. During this process, antibodies against transglutaminase (anti-tTG), gliadin, and actin are made through unclear mechanisms.

Anti-gliadin antibodies were the first serologic test to inform the need for intestinal, gold standard, diagnostic sampling. The accuracy of the antibodies against native gliadin was limited. Their sensitivity and specificity are generally less than 90% with positive predicted values much less than 50%, being present in normal subjects, increase with age, and are genetically determined, gluten dependent, and finally are shared between multiple gastrointestinal and autoimmune diseases [271]. Anti-endomysium (anti-EMA) antibodies belong primarily to the IgA class and are directed against the intermyofibril substance of smooth muscle. Anti-EMA antibodies have higher specificity in children, but low sensitivity. The disadvantages are their higher cost and being labor-intensive to perform and nonobjective with a significant interobserver and intersite variability. Despite those pitfalls, they were included on the revised diagnostic flow chart of ESPGHAN 2012 [272]. Currently, they are less performed and have been replaced by more reliable, reproducible, and objective ones.

IgA to tissue transglutaminase is the most used and studied marker in CD and is primarily recommended by ESPGHAN [272]. It is directed against the ubiquitous enzyme tTg, the identified
autoantigen of CD. It is a reliable, inexpensive, and reproducible test, performed by ELISA. IgA-tTg sensitivity and specificity are generally considered greater than 90–95%, but have a wide range between 74% and 100% depending on performing laboratory and kit used. IgA-tTg antibodies reflect the CD intestinal damage and can predict the enteric pathology [271]. Patients with IgA deficiency do not produce IgA- anti-TTG or IgA- anti-EMA antibodies, and could have a false negative result. Therefore, total IgA concentration should be measured in conjunction with serology. For patients with IgA deficiency, IgG- anti-TTG, IgG- anti-EMA, and IgG anti-deaminated gliadin peptide antibodies (DGP) can be tested instead. A 2012 meta-analysis showed that IgG-DGP had a pooled sensitivity ranging from 80.1% to 98.6% [273]. However, a combination of CD serology testing and duodenal biopsy sampling is required for the diagnosis of CD in adults. Histology remains the gold standard for CD diagnosis. Typical findings include patchy—villous atrophy in areas that are adjacent to non-atrophic villi (Figures 15-16).
Figure 15. Histology findings of coeliac disease [274].
Sections from normal duodenal mucosa (A) and from a mucosal biopsy with coeliac disease (B). In contrast to the long villi with only minimal numbers of intraepithelial lymphocytes, panel B shows an epithelium studded with lymphocytes and a lamina propria obliterated by a mixed inflammatory infiltrate consisting of lymphocytes, plasma cells, eosinophils, and rare neutrophils. The normal mucin content of the normal goblet cells, evident in panel A, is completely depleted in the mucosa depicted in panel B.

Figure 16. Immunohistochemical staining for CD3+ lymphocytes in coeliac disease [274].
Normal gastric mucosa stained with hematoxylin and eosin (A) and an anti-CD3 immunohistochemical stain (B). The normal gastric epithelium contains no intraepithelial lymphocytes; the rare CD3-positive cells seen in panel B are in the lamina propria. Panel C shows transitional gastric mucosa from a patient with lymphocytic gastritis. Although intraepithelial lymphocytes can be seen, particularly in the surface epithelium, their large numbers are best appreciated in sections stained with an anti-CD3 immunohistochemical stain (D).
To optimize the likelihood of a histological diagnosis, a specific duodenal biopsy sample strategy should be used. New evidence supports the usefulness of a duodenal bulb biopsy in diagnosis of CD and requires a minimum of four further biopsy samples from the second part of the duodenum.

However, other diagnostic challenges exist: HLA genotyping, although not required as a routine test in all patients with suspected coeliac disease, can be valuable in equivocal diagnoses, patients who are already on a gluten-free diet who are unwilling or unable to undergo a gluten challenge, and those who refuse a gastroscopy. HLA testing can also be useful when assessing family members of patients with CD, because the absence of HLA-DQ2 and HLA-DQ8 almost always excludes CD, with a negative predictive value of more than 99%. Among patients who are unwilling or unable to undergo gastroscopy, video capsule endoscopy can also provide helpful information, because mucosal changes indicative of coeliac disease can be identified.

Though increased awareness and the introduction of serologic testing have improved disease detection, CD remains significantly underdiagnosed worldwide and symptomatic individuals commonly experience considerable diagnostic delays, often of several years. Current medical literature suggests that between 5% and 13% of patients with diagnosed CD have had a previous endoscopy with either no biopsy or an inadequate biopsy resulting in a delay in diagnosis [266]. Under diagnosis and long diagnostic delay have substantial implications, in terms of morbidity, mortality, and economic burden. Gluten-free diet was shown to reduce complications, realize potential growth and weight parameters, improve pregnancy outcome, enhance bone density, decrease co-occurrence of several autoimmune conditions, shorten needless suffering, prevent malignancies, and overall improve life quality and expectancy.

The mainstay of treatment of CD remains adherence to a gluten-free diet. Improvement and resolution of symptoms typically occurs within days or weeks, and often precedes normalization of serological markers and of duodenal villous atrophy [275]. Patients with newly diagnosed coeliac disease should be referred to an expert dietitian, because the gluten-free diet requires knowledge not only of hidden sources of gluten, but also of healthy gluten-free substitute grains that provide adequate fiber and
nutrients. Upon diagnosis, patients should be tested for micronutrient deficiencies, including iron, folic acid, vitamin B12, and vitamin D [276].
7. AIMS OF THE STUDY

The aims of our study are:

1. To estimate the prevalence of disease specific autoantibodies (ANA, anti-ENA, anti-CCP, RF, aPLs: aCL, aGPI, aSP, AMA, anti-LKM, anti-LC1, anti-SLA, anti-DGP, anti-tTG) in the general population of two areas of the Lombardia region by using the most recently developed methods for the detection of autoantibodies.

2. To analyze the autoantibodies predictive value for the development of autoimmune diseases over follow-up.

3. To analyze if there are factors at baseline (demographic, biochemical, clinical) that can predict the development of autoimmune disease over time, to identify patients at higher risk of developing an autoimmune disease that need a closer follow-up.

4. To analyze the autoantibodies predictive value for the development of malignancies over time.
8. PATIENTS AND METHODS

8.1. Patients

The present study was conducted on two different cohorts derived from the general population of Lombardia region in Northern Italy: the ISOLA and the CA.ME.LI.A. (CArdiovascular risk, MEtabolic syndrome, LIver, and Autoimmunity) cohorts.

The ISOLA cohort derives from an area called Isola Bergamasca, in the Lombardia region (Figure 17) and was originally selected in 1998 to study the prevalence of hepatitis B (HBV) and hepatitis C (HCV) infections.

![Localization of the ISOLA cohort.](image)

Figure 17. Localization of the ISOLA cohort.

The 1998 population of four cities (Bonate Sotto, Ponte San Pietro, Presezzo, Terno d'Isola) included 15,907 subjects with ages between 18 and 75, which were randomly selected 1:4 for participation (n=3,977). 2,828 subjects agreed to participate to the study (Table 14), and we had access to 2,685
sera for ANA, anti-ENA, anti-CCP, AMA, anti-LKM, anti-LC1, anti-SLA, anti-DGP, anti-tTG, and 2,196 were also available for RF analysis, without significant differences between groups (data not shown).

Table 14. Demographic characteristics of the subjects included in the ISOLA cohort.

<table>
<thead>
<tr>
<th></th>
<th>Bonate</th>
<th>Presezzo</th>
<th>Ponte San Pietro</th>
<th>Terno d’Isola</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=622</td>
<td>n=536</td>
<td>n=1,159</td>
<td>n=472</td>
</tr>
<tr>
<td>Women</td>
<td>334 (50.45%)</td>
<td>291 (54.3%)</td>
<td>633 (54.6%)</td>
<td>253 (53.6%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;35 years</td>
<td>215 (35.2%)</td>
<td>177 (33.3%)</td>
<td>293 (25.3%)</td>
<td>151 (32%)</td>
</tr>
<tr>
<td>35-64 years</td>
<td>444 (67.1%)</td>
<td>352 (65.7%)</td>
<td>818 (70.6%)</td>
<td>311 (65.9%)</td>
</tr>
<tr>
<td>&gt;65 years</td>
<td>3 (0.45%)</td>
<td>7 (1.3%)</td>
<td>48 (4.1%)</td>
<td>10 (2.1%)</td>
</tr>
</tbody>
</table>
The CA.ME.LI.A. study included 2,555 subjects (age 18-75 years), randomly selected from the active electorate of the Northern Italian city of Abbiategrasso (Milan area, population 32,000) in 2009 (Figure 18).

![Map of Italy showing Abbiategrasso](image)

**Figure 18. Localization of the CA.ME.LI.A. cohort.**

The present study was performed on a subgroup of 1,712 subjects randomly selected from the original cohort. The selected subjects did not differ significantly from the original cohort in terms of demographic and clinical characteristics (Table 15). The sample size is sufficient to identify an estimated aPLs prevalence of 7.5%, using an alpha error of 0.05, and a power of 80%. Local institutional review board approved the present study and all subjects signed an informed consent.
Table 15. Demographic characteristics of the subjects included in the CA.ME.LIA. cohort.

<table>
<thead>
<tr>
<th>Subjects n=1,712</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>&lt;35 years</td>
</tr>
<tr>
<td>35-64 years</td>
</tr>
<tr>
<td>&gt;65 years</td>
</tr>
</tbody>
</table>
8.2. Methods

Serological testing

ISOLA

Serum samples were tested in 2012 by IIF for ANA and by ELISA for anti-ENA in ANA-positive sera (AESKU Diagnostics, Wendelsheim, Germany). Anti-ENA included Ro/SSA, La/SSB, Scl70, Sm, Cenp-B, nucleosome, dsDNA, Jo1, PM/Scl, RibP, and histone. Anti-ENA were tested only if ANA resulted positive, accounting for 273 sera tested.

ELISA was used for RF check as screening test and anti-CCP IgG (AESKU Diagnostics, Wendelsheim, Germany). Samples were defined as being RF-positive if titers >16 IU/mL (high titer >50 U/mL, low titer 16-24 U/mL), whereas the cut-off for anti-CCP was 18 IU/mL (high titer >30 U/mL), as recommended by the manufacturer. RF check-positive sera were further tested by ELISA for IgM, IgG, and IgA isotypes (AESKU Diagnostics). We calculated the normal cut-off value testing 20 RA patients, with known RF positivity, versus 20 healthy controls. Reference ranges for RF isotypes were defined by receiver operating characteristic (ROC) curve, with serum samples considered positive if IgG titers > 33.6 IU/mL (sensitivity 88.2%, specificity 95.6%), IgM titers >18 IU/mL (sensitivity 88.2%, specificity 95.6%), IgA titers >18 IU/mL (sensitivity 70.6%, specificity 100%).

Furthermore, AMA, anti-LKM, anti-LC1, anti-SLA, anti-DGP, anti-tTG were also tested by ELISA. Serum samples had been tested in 1998 for the surface antigen of HBV (HBsAg), HBV core antibodies (HBcAb) and anti-HCV antibodies (Chemiluminescent microparticle immune assay, Roche).

CA.ME.LI.A.

The sera of the 1,712 selected subjects were tested for aCL, aGPI, and aSP antibodies using commercially available ELISA tests (AESKU diagnostic, Wendelsheim, Germany) on frozen sera to identify IgG, IgM, and IgA isotypes for each autoantibody. For all aPLs, results were expressed as
units and cut-off values were established at 15 UI, as recommended by the manufacturer instructions, according to the Clinical and Laboratory Standards Institute guideline for reference intervals. The range of results was 0-300 UI and values >40UI were considered as high titers. Lupus anticoagulant could not be tested since fresh samples were not available.
8.3 Outcomes

ISOLA

We performed a retrospective analysis of administrative databases using ICD-9-codes from the copayment exemptions register (i.e. the Italian legal mechanism that allows subjects with a chronic condition to waive copayments for visits, medications, and blood tests; these are assigned by specialists usually at the diagnosis of chronic diseases) for cases of CTDs, RA, autoimmune liver diseases, and celiac disease updated as of 31st December 2013. Furthermore, we detected hospitalization, cancer diagnosis, and death.

CA.ME.L.I.A.

All the subjects underwent physical evaluation, abdomen ultrasound, and blood draw. Blood samples were used for the determination of routine laboratory tests including C-reactive protein (CRP), total cholesterol, triglycerides, homocysteine, glucose levels and complete blood count. Medical history was assessed with a physician-assisted questionnaire.

Carotid ultrasonographic analysis. Quantitative evaluation of carotid intima-media thickness (IMT) was performed by trained sonographers unaware of clinical data using a 7.5 MHz probe (11) on 1:3 randomly selected subjects (563, no significant differences compared to the cohort tested for aPLs in terms of demographic and clinical characteristics; data not shown). Briefly, the far walls of the left and right common carotids, bifurcations, and internal carotids were visualized in anterior, lateral, and posterior angles and recorded on VHS videotapes. Carotid IMT measurements were performed in a centralized laboratory (Centro Cardiologico Monzino IRCCS, Milan, Italy) using a dedicated software. The ultrasonographic variables used in the statistical analyses were the mean maximum intima-media thickness (IMTmean-max), the atherosclerotic plaques (defined as maximum IMT (IMTmax) >1.5 mm) (11), and the inter-adventitia common carotid artery diameters (ICCAD).

CV risk factors and events
CV risk factors (arterial hypertension, age, active smoking, body mass index (BMI) >25 kg/m2, physical inactivity, diabetes, high levels of CRP, hypercholesterolemia, hypertriglyceridemia, hyperhomocysteinemia) and CV clinical events (history of acute myocardial infarction, stroke, peripheral limb arteriopathy, such as obliterans vasculopathy requiring surgery or prostanoid therapy) were recorded for each subject by the study physicians. Specific CV risk factors were used to estimate the overall Framingham risk score [277].
9. STATISTICAL ANALYSES

Determining the prevalence of autoantibodies in the general population was the aim of the primary analysis. We calculated prevalence rates taking into account the number of subjects enrolled as well as the number of tested samples. Data were analyzed using descriptive and inferential methods. We used both parametric ($\chi^2$) and non-parametric (Mann-Whitney) tests, when appropriate.

The secondary analyses were performed to determine the predictive value of autoantibodies of developing an autoimmune disease over 13 years. The predictive value of autoantibodies was calculated in terms of risk ratio (RR), odds ratio (OR), and hazard ratio (HR), as well as for cancer.

The association of autoantibodies with mortality risk was assessed using Cox proportional hazards models, both crude and adjusting by sex. Results are shown as HR and 95% confidence intervals (CI), calculated using Cox regression test; when appropriate the analysis were adjusted for confounding elements.

To assess the association of aPLs with carotid ultra-sonographic measurements and CV events, univariate and multivariate analyses were performed, after adjusting for pre-specified confounders including CV risk factors: age ≥ 50 years, smoking habit (>1 cigarette/day for at least one year), overweight (BMI>25 kg/m2), physical inactivity (no physical activity except work-related activity), diabetes (glycemia >126 mg/dL), high levels of total cholesterol (>200 mg/dL), high levels of triglycerides (>150 mg/dL), high levels of CRP (>0.5 mg/dL), or high levels of homocysteine (homocysteine >15umol/L). Results were expressed as OR 95% CI for binary, and as beta coefficient (95% CI) for continuous outcomes.

All statistical analyses were conducted with Stata 13.1 (StataCorp, College Station, TX) for Macintosh and P-values <0.05 were considered statistically significant.
10. RESULTS

10.1. Aim #1 To estimate the prevalence of disease specific autoantibodies (ANA, anti-ENA, anti-CCP, RF, aPLs: aCL, aGPI, aSP, AMA, anti-LKM, anti-LC1, anti-SLA, anti-DGP, anti-tTG) in the general population.

10.1.1 ANA and anti-ENA

Serum ANA were detected in 18% (483/2,685, 95% CI 16.7%-19.6%) at titers ≥ 1:80 and 6.1% (163/2,685) at titers ≥ 1:160 (Table 16).

Table 16. ANA and anti-ENA prevalence in the ISOLA cohort.

<table>
<thead>
<tr>
<th></th>
<th>n=2,685</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA &gt;1:80</td>
<td>18.1%</td>
</tr>
<tr>
<td>ANA &gt;1:160</td>
<td>6.1%</td>
</tr>
<tr>
<td>anti-SSA-Ro</td>
<td>0.60%</td>
</tr>
<tr>
<td>anti-SSB-La</td>
<td>0.38%</td>
</tr>
<tr>
<td>anti-Scl70</td>
<td>1.46%</td>
</tr>
<tr>
<td>anti-Sm</td>
<td>0.19%</td>
</tr>
<tr>
<td>anti-CenB-P</td>
<td>0.30%</td>
</tr>
<tr>
<td>anti-Nucleosome</td>
<td>1.95%</td>
</tr>
<tr>
<td>anti-ds-DNA</td>
<td>0.38%</td>
</tr>
<tr>
<td>anti-Jo1</td>
<td>0.08%</td>
</tr>
<tr>
<td>anti-PM-Scl</td>
<td>1.54%</td>
</tr>
<tr>
<td>anti-RibP</td>
<td>0.38%</td>
</tr>
<tr>
<td>anti-Histone</td>
<td>1.58%</td>
</tr>
</tbody>
</table>

Sera positive at titer ≥ 1:160 were further characterized for immunofluorescence pattern, with the most common pattern being classified as speckled in 116/163 (71% of ANA positive at titers ≥ 1:160) of cases, and a minority of other patterns, including nucleolar in 36/163 (22%), homogeneous in 13/163 (8%), centromeric in 11/163 (6.7%), cytoplasmic in 9/163 (5.5%), mitochondrial 8/163 (5%), while 45/163 (28%) sera had more than one immunofluorescence pattern. ANA prevalence rates at all titers were significantly higher (p<0.0001) in women compared to men (Figure 19 A) and increased with the age of subjects at enrollment in 1998, arrayed in four intervals (≤ 34 years; 35-49
years; 50-64 years; ≥ 65 years), despite not reaching statistical significance (Figure 19 B). When age and sex variables were combined, we confirmed both trends with ANA prevalence being highest in women between the ages of 50 and 64 years (136/320, 42.5%; p<0.0001) (Figure 19 C).
Figure 19. Prevalence of ANA according to sex and age.
Of the 237 sera tested for anti-ENA, prevalence rates of specific anti-ENA were below 2.5% of subjects in all cases and are illustrated in Figure 20. Anti-ENA specific for systemic sclerosis, i.e. anti-Scl70 and anti-Cenp-B, were identified in 1.45% and in 0.3% of subjects, respectively. In all specific anti-ENA, antibody prevalence rates were higher in women compared to men, with female to male ratios below 4 in all cases (Figure 20).

Figure 20. Anti-ENA prevalence in the ISOLA cohort.
10.1.2 Anti-CCP and RF

RF screening was positive in 430/2,196 sera (19.6%) and confirmed by ELISA in 177/2,196 cases (8.1%). The ELISA confirmation test was positive in 89/112 (79.5%) subjects with high-titer RF at screening test and in 30/153 (19.6%) with low titers (p<0.0001) (Table 17). Among the 177 RF positive subjects, 123 (69.5%) had IgM isotype, 117 (66.1%) IgG, and 42 (23.7%) IgA. RF IgM coexisted with IgG isotype in 64 (36.2%) cases; IgM with IgA in 15 (8.5%); IgG with IgA in 25 (14.1%) while all isotypes were positive in 12 (6.8%) subjects. RF IgM and IgG had similar frequencies in both sexes, while IgA was more frequently positive in men (p =0.006). High titers of RF were found in 89/177 (50.3%) cases and in 38/42 (90.5%) IgA (versus IgM and/or IgG; p=0.03); and subjects with RF were cumulatively older than the seronegative group (p=0.0047) (Table 17).

Anti-CCP were detected in 121/2525 (4.8%) subjects (57% women, 20.7% at high titers) and, similarly to RF, anti-CCP -positive subjects were significantly older than the seronegative population (p=0.0006) (Table 17).

RF and anti-CCP were concomitantly positive in 8/2196 (0.4%) subjects tested for both autoantibodies with 6 subjects having RF IgM + IgG, one RF IgG and one RF IgA (Table 17).

ANA were positive in 150/2525 (5.9%) subjects, of these 13/150 (8.7%) had anti-CCP and 3/150 (2%) had RF, with one subject having RF IgM, IgG, and IgA, and 2 subjects RF IgG (Table 17).
Table 17. Prevalence of rheumatoid factor, anti-cyclic citrullinated peptides (anti-CCP) in the study population.

<table>
<thead>
<tr>
<th></th>
<th>RF negative</th>
<th>RF positive</th>
<th>RF IgM</th>
<th>RF IgG</th>
<th>RF IgA</th>
<th>Anti-CCP negative</th>
<th>Anti-CCP positive</th>
<th>Anti-CCP + RF 8/2.196</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>1,759 (80)</td>
<td>177 (8.1)</td>
<td>123 (5.6)</td>
<td>117 (5.3)</td>
<td>42 (1.9)</td>
<td>2,404</td>
<td>121 (4.8)</td>
<td>(0.4)</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>1,109 (63)</td>
<td>90 (50.8)</td>
<td>68 (55.3)</td>
<td>60 (51.3)</td>
<td>15 (35.7)**</td>
<td>1,110 (46.2)</td>
<td>69 (57)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>Age, median years (IQR)</td>
<td>43 (32-54)</td>
<td>48 (35-59)*</td>
<td>49 (37-59)*</td>
<td>45 (32-58)</td>
<td>54 (33-56)*</td>
<td>43 (32-54)</td>
<td>47 (38-58)**</td>
<td>54 (42-62)</td>
</tr>
<tr>
<td>Age &gt;60 years, n (%)</td>
<td>347 (19.7)</td>
<td>40 (22.6)</td>
<td>28 (22.8)</td>
<td>25 (21.4)</td>
<td>11 (26.2)</td>
<td>337 (14)</td>
<td>26 (21.5)**</td>
<td>3 (37.5)</td>
</tr>
<tr>
<td>High titer (%)</td>
<td>-</td>
<td>89 (50.3)</td>
<td>63 (51.2)</td>
<td>57 (48.7)</td>
<td>38 (90.5)**</td>
<td>-</td>
<td>25 (20.7%)</td>
<td>0</td>
</tr>
<tr>
<td>Low titer (%)</td>
<td>-</td>
<td>22 (12.4)</td>
<td>17 (13.8)</td>
<td>13 (11.1)</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

RF: rheumatoid factor; n: number; Ig: immunoglobulin; anti-CCP: anti-cyclic citrullinated peptides; IQR: interquartile range.

* p<0.05 vs RF negative subjects; ** p<0.05 vs RF IgM/IgG; *p<0.05 vs anti-CCP negative subjects.
10.1.3 APLs

The overall prevalence of any aPL (defined as at least one positive aPL) was 15.1% (95% CI 13.4-16.8%) with the highest frequency observed in older subjects (18.1%; p<0.002). High-titer aPL were found in 3.3% of subjects, multiple specificities in 2% (Table 18).

ACL were detected in 1.5% of the subjects with 1% at high titer (Table 18).

AGPI were positive in 4.3% of the subjects with 1.2% at high titer and the highest prevalence in older subjects (6.7%; p<0.001). AGPI IgA were more frequently observed in women (3.1%, p=0.040) and in older subjects, independently of sex (4%, p<0.001; Table 18).

ASP were the most frequently detected aPL, being found in 11.7% of the cases with 1.7% at high titer and in 9.8% of the cases as the only positive test. ASP IgA were not detected (Table 18).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Total</th>
<th>Women</th>
<th>Men</th>
<th>Age&lt;50</th>
<th>Age≥50</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPL negative</td>
<td>1,712</td>
<td>852 (49.8%)</td>
<td>860 (50.2%)</td>
<td>956 (55.8%)</td>
<td>756 (44.2%)</td>
</tr>
<tr>
<td>aPL positive</td>
<td>258 (15.1)</td>
<td>137 (16.1)</td>
<td>121 (14.1)</td>
<td>121 (12.7)</td>
<td>137 (18.1)*</td>
</tr>
<tr>
<td>&gt;1 aPLs</td>
<td>35 (2.0)</td>
<td>17 (2)</td>
<td>18 (2.1)</td>
<td>14 (15)</td>
<td>21 (2.8)</td>
</tr>
<tr>
<td>aPL HT</td>
<td>56 (3.3)</td>
<td>28 (3.3)</td>
<td>28 (3.2)</td>
<td>21 (2.2)</td>
<td>35 (4.6)*</td>
</tr>
<tr>
<td>aCL</td>
<td>26 (1.5)</td>
<td>10 (1.2)</td>
<td>16 (1.9)</td>
<td>10 (1)</td>
<td>16 (2.1)</td>
</tr>
<tr>
<td>aCL HT</td>
<td>17 (1)</td>
<td>6 (0.7)</td>
<td>11 (1.3)</td>
<td>5 (0.5)</td>
<td>12 (1.6)*</td>
</tr>
<tr>
<td>aCL IgG</td>
<td>15 (0.9)</td>
<td>7 (0.8)</td>
<td>8 (0.9)</td>
<td>10 (1)</td>
<td>5 (0.7)</td>
</tr>
<tr>
<td>aCL IgM</td>
<td>18 (1.1)</td>
<td>5 (0.6)</td>
<td>13 (1.5)</td>
<td>5 (0.5)</td>
<td>13 (1.7)*</td>
</tr>
<tr>
<td>aCL IgA</td>
<td>4 (0.2)</td>
<td>2 (0.2)</td>
<td>2 (0.2)</td>
<td>1 (0.1)</td>
<td>3 (0.4)</td>
</tr>
<tr>
<td>aGPI</td>
<td>73 (4.3)</td>
<td>44 (5.2)</td>
<td>29 (3.4)</td>
<td>22 (2.3)</td>
<td>51 (6.7)*</td>
</tr>
<tr>
<td>aGPI HT</td>
<td>20 (1.2)</td>
<td>12 (1.4)</td>
<td>8 (0.9)</td>
<td>8 (0.8)</td>
<td>12 (1.6)</td>
</tr>
<tr>
<td>aGPI IgG</td>
<td>20 (1.2)</td>
<td>11 (1.3)</td>
<td>9 (1)</td>
<td>7 (0.7)</td>
<td>13 (1.7)</td>
</tr>
<tr>
<td>aGPI IgM</td>
<td>28 (1.6)</td>
<td>13 (1.5)</td>
<td>15 (1.7)</td>
<td>11 (1.2)</td>
<td>17 (2.2)</td>
</tr>
<tr>
<td>aGPI IgA</td>
<td>35 (2)</td>
<td>26 (3.1)*</td>
<td>9 (1)</td>
<td>5 (0.5)</td>
<td>30 (4)*</td>
</tr>
<tr>
<td>aSP</td>
<td>201 (11.7)</td>
<td>103 (12.1)</td>
<td>98 (11.4)</td>
<td>104 (10.9)</td>
<td>97 (12.8)</td>
</tr>
<tr>
<td>aSP HT</td>
<td>29 (1.7)</td>
<td>15 (1.8)</td>
<td>14 (1.6)</td>
<td>12 (1.3)</td>
<td>17 (2.2)</td>
</tr>
<tr>
<td>aSP IgG</td>
<td>157 (9.2)</td>
<td>80 (9.4)</td>
<td>77 (9)</td>
<td>78 (8.2)</td>
<td>79 (10.4)</td>
</tr>
<tr>
<td>aSP IgM</td>
<td>65 (3.8)</td>
<td>34 (4)</td>
<td>31 (3.6)</td>
<td>36 (3.8)</td>
<td>29 (3.8)</td>
</tr>
</tbody>
</table>

Data are presented as n (%). aPL: antiphospholipid antibodies; HT: high titer; aCL: anti-cardiolipin antibodies; aGPI: anti-β2-glycoprotein antibodies I; aSP: anti-serin/prothrombin antibodies. * p<0.05.
10.1.4 AMA, anti-LKM, anti-LC1 and anti-SLA

AMA were more frequently observed in women compared to men, both at low (65 vs. 36; p=0.03) and high titer (26 vs. 6; p=0.01) (Table 19). AMA at low titers were more prevalent in subjects younger than 50 years (76 vs. 25; p=0.001), while at high titers was not significant (p=0.83) (Table 19).

Anti-LKM antibodies were detected in 3/2,685 subjects, for an estimated prevalence of 0.1%, with no differences between sexes or age groups (Table 19). In 2/3 subjects we observed a medium titer (>30U/ml), no high titer was observed.

The prevalence of anti-LC1 antibodies was 0.3% (8/2,685), with no differences between sexes (3 vs 5; p=0.3) or age groups (6 vs. 2; p=0.4) (Table 19). In 4/8 subjects we observed a medium titer (>30U/ml), no high titer was observed.

Anti-SLA prevalence was 0.8% (22/2,685), with no differences between sexes (12 vs. 10, p=0.9), or age groups (8 vs. 14; p=0.06) (Table 19). In 10/22 subjects we observed a medium titer (>30U/ml), while high titer (>90U/mL) in 5/22 were observed.

Table 19. Prevalence of liver related-antibodies in the ISOLA cohort.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Total n=2,685</th>
<th>Women n=1,444</th>
<th>Age&lt;50 n=1,596</th>
<th>Age≥50 n=1,089</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMA &gt;24 UI/mL</td>
<td>101 (3.8%)</td>
<td>65 (4.5%)*</td>
<td>76 (4.8%)*</td>
<td>25 (2.3%)</td>
</tr>
<tr>
<td>AMA &gt;40 UI/mL</td>
<td>26 (1%)</td>
<td>21 (1.4%)</td>
<td>16 (1%)</td>
<td>10 (0.9%)</td>
</tr>
<tr>
<td>anti-LKM</td>
<td>3 (0.1%)</td>
<td>2 (0.1%)</td>
<td>3 (0.2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>anti-LC1</td>
<td>8 (0.3%)</td>
<td>3 (0.2%)</td>
<td>6 (0.4%)</td>
<td>2 (0.2%)</td>
</tr>
<tr>
<td>anti-SLA</td>
<td>22 (0.8%)</td>
<td>12 (0.8%)</td>
<td>8 (0.5%)</td>
<td>14 (1.3%)</td>
</tr>
</tbody>
</table>
10.1.5 Anti-DGP and anti-tTG

Anti-DPG Check prevalence was 1.85% (50/2,685), at medium-high titer 0.9% (24/2,685) (Table 20). Anti-DPG IgA were found in 22/2,685 (0.8%), while IgG 38/2,685 (1.4%). No differences between sexes or age groups were observed both at low or medium-high titers (Table 20).

The overall prevalence of anti-tTG was 2.2% (59/2,685), which decreased to 1.2% for medium-high titer positivity (33/2,685). No differences between sexes or age groups were observed both at low or medium-high titers (Table 20).

Table 20. Prevalence of coeliac disease related -antibodies in the ISOLA cohort.

<table>
<thead>
<tr>
<th></th>
<th>Total n=2,685</th>
<th>Women n=1,444</th>
<th>Age&lt;50 n=1,596</th>
<th>Age≥50 n=1,089</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>anti-DPG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;24 U/mL</td>
<td>50 (1.85%)</td>
<td>28 (1.9%)</td>
<td>27 (1.7%)</td>
<td>23 (2.1%)</td>
</tr>
<tr>
<td>&gt; 40 U/mL</td>
<td>24 (0.9%)</td>
<td>13 (1.8%)</td>
<td>13 (0.8%)</td>
<td>11 (1.0%)</td>
</tr>
<tr>
<td><strong>anti-tTG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;24 U/mL</td>
<td>59 (2.2%)</td>
<td>38 (2.6%)</td>
<td>40 (2.5%)</td>
<td>19 (1.7%)</td>
</tr>
<tr>
<td>&gt; 40 U/mL</td>
<td>32 (1.2%)</td>
<td>19 (1.3%)</td>
<td>19 (1.2%)</td>
<td>11 (1.0%)</td>
</tr>
</tbody>
</table>
**10.2 AIM #2.** To analyze the autoantibodies predictive value for the development of autoimmune diseases over follow-up.

**10.2.1 ANA and anti-ENA**

The absolute and relative risks associated with ANA positivity at different titers are illustrated in Table 21 for CTD. Having detectable serum ANA at any titer in 1998 was associated with a significant HR of developing a CTD between 1998 and 2013 (HR 4.66; 95% CI 1.42-15.27; p=0.011), and was significant also after adjustment for age and sex (3.66, 95% CI 1.10-12.14; p=0.034).

Similarly, ANA titers ≥1:160 were associated with an HR for CTD of 14.19 (95% CI 3.07-65.68; p=0.001), which remained significant after adjustment for age and sex (11.38, 95% CI 2.45-52.97; p=0.002). The median time of development of CTD observed was 9.8 years in all subjects, regardless of ANA status, while in ANA-positive subjects at any titer it was 12.2 years.

**Table 21.** Number of events and 15-year absolute and relative risks associated with the ANA status in 1999. Results are shown with 95% confidence intervals.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Total population n= 2,490</th>
<th>ANA titer n= 518</th>
<th>ANA ≥1:80 n= 483</th>
<th>ANA ≥1:160 n=163</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connective tissue disease (yearly incidence)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute risk in 14 years</td>
<td>11 (2 person time)</td>
<td>6</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Relative risk in 14 years</td>
<td>-</td>
<td>0.01</td>
<td>0.004</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4.59 (1.41-15)</td>
<td>0.92 (0.20-4.38)</td>
<td>8.20 (2.42-27.71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.005</td>
<td>p =0.924</td>
<td>p =0.001</td>
</tr>
</tbody>
</table>

The absence of incident CTD cases and the small number of events (death and cancer) among anti-ENA positive subgroups did not allow calculating the HR in the group of anti-ENA positive subjects.
10.2.2 RF and anti-CCP

We identified 10 incident RA cases over a 15-year follow up (Table 22), 9 of which were women, with a median age at diagnosis of 52 years.

Among RA cases, 2/10 (20%) were previously positive for RF (Table 22), both with IgM isotype, one with low titer RF and coexisting anti-CCP and one with high titer RF, corresponding to a RR of 2.49 (95% CI 0.23-27.21, p>0.05). When taking into account only women, the RR for RA development for RF positive subjects was 1.77 (0.22-14.06, p>0.05).

Among patients with incident RA, 4/10 (40%) were positive for anti-CCP at enrollment, 3 of which with high titer anti-CCP and 1 with ANA (for a coexisting SjS), corresponding to a RR of 13.2 (95% CI 3.8-46.3; p<0.001; Table 22). When including only women in the analysis, the RR for RA development among anti-CCP-positive subjects was 9.38 (2.4-36.7, p<0.001), while only 1 man with anti-CCP developed RA (1/53, 1.9%; p=0.05). The anti-CCP positive predictive value was 3.31% (95% CI 1.54-6.94), while the negative predictive value was 99.75% (95% CI 99.6-99.9).

Table 22. Number of events and 15-year risk ratios and 95% confidence intervals associated with the autoantibodies status.

<table>
<thead>
<tr>
<th></th>
<th>Total population</th>
<th>RF positive</th>
<th>Anti-CCP positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=177</td>
<td>n=121</td>
<td></td>
</tr>
<tr>
<td><strong>Rheumatoid arthritis cases</strong></td>
<td>10</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><strong>Risk ratio</strong></td>
<td>-</td>
<td>2.5</td>
<td>13.2</td>
</tr>
<tr>
<td><strong>95% CI</strong></td>
<td>-</td>
<td>0.2-27.2</td>
<td>3.8-46.3</td>
</tr>
<tr>
<td></td>
<td>p=0.44</td>
<td></td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
10.2.3 APLs

No cases of APL syndrome were detected in the CA.ME.LI.A. cohort. However, CV events were more frequently reported in the aPL positive population (34, 13.2% vs 121, 8.3% in aPL negative subjects, p=0.012; OR 1.67 95%CI 1.12-2.50; data not shown in table); however, only peripheral arteriopathy was associated to anti-PLs independently of other CV risk factors (OR 2.02, 95%CI 1.07-3.64, p =0.013; Table 23).

The atherosclerotic plaque frequency and the mean values of IMT mean-max and ICCAD were generally higher in aPL positive subjects (Table 23). Specifically, aCL IgM were associated to a significantly higher frequency of atherosclerotic plaques (crude OR 5.41, 95%CI 0.61-65.2, p=0.042), while aGPI IgA positive subjects had significantly increased IMT mean-max (beta 0.04, 95%CI 0.004-0.072; p=0.027) and ICCAD (beta 0.02, 95%CI 0.009-0.041; p=0.002; Table 4). Multivariate analyses with adjustment for pre-specified confounders demonstrated that only IgA aGPI were independently associated to increased ICCAD (beta 0.51, 95%CI 0.17-0.84; p =0.003).

Considering sex and age, we observed that aPLs were associated to an increased ICCAD in women < 50 years (6.74 IQR 6.2-7.0 vs 6.47 IQR 6.19-7.75 in women < 50 years without aPLs, at multivariate analysis beta 0.22, 95%CI 0.03-0.41; p=0.025), especially in case of IgG aSP (beta 0.24, 95%CI 0.02-0.46; p=0.03).
Table 23. IMT measurements and clinical cardiovascular outcomes in the cohort studied according to aPL status.

<table>
<thead>
<tr>
<th></th>
<th>IMT Max n (%)</th>
<th>Mean IMT Max m (IQR)</th>
<th>ICCAD Avg m (IQR)</th>
<th>AMI n (%)</th>
<th>Stroke n (%)</th>
<th>PA n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPL negative</td>
<td>106/488 (21.7)</td>
<td>0.98 (1.21)</td>
<td>7.05 (6.66-7.57)</td>
<td>49 (3.4)</td>
<td>23 (1.6)</td>
<td>49 (3.4)</td>
</tr>
<tr>
<td>aPL positive</td>
<td>22/75 (29.3)</td>
<td>1.03 (1.30)</td>
<td>7.12 (6.77-7.66)</td>
<td>10 (3.9)</td>
<td>7 (2.7)</td>
<td>17 (6.6)*</td>
</tr>
<tr>
<td>&gt;1 aPLs</td>
<td>2/16 (12.5)</td>
<td>0.97 (1.04)</td>
<td>6.91 (6.8-7.24)</td>
<td>1 (1.8)</td>
<td>0 (1.8)</td>
<td>0 (1.8)</td>
</tr>
<tr>
<td>aPL HT</td>
<td>1/5 (20)</td>
<td>1.03 (1.04)</td>
<td>7 (6.9-7.22)</td>
<td>0 (1.8)</td>
<td>0 (1.8)</td>
<td>0 (1.8)</td>
</tr>
</tbody>
</table>

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<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>aCL</td>
<td>3/8 (37.5)</td>
<td>1.01 (1.21)</td>
<td>7.03 (6.66-7.30)</td>
<td>1 (3.9)</td>
<td>1 (3.9)</td>
<td>1 (3.9)</td>
</tr>
<tr>
<td>aCL IgG</td>
<td>1/6 (16.7)</td>
<td>0.94 (1.04)</td>
<td>7.26 (6.96-7.38)</td>
<td>0 (1.8)</td>
<td>0 (1.8)</td>
<td>0 (1.8)</td>
</tr>
<tr>
<td>aCL IgM</td>
<td>3/5 (60)*</td>
<td>1.03 (1.39)</td>
<td>7.10 (6.87-7.22)</td>
<td>1 (5.6)</td>
<td>0 (1.8)</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>aCL IgA</td>
<td>0</td>
<td>0.87 (0.98)</td>
<td>7.41 (7.22-7.60)</td>
<td>0 (1.8)</td>
<td>0 (1.8)</td>
<td>0 (1.8)</td>
</tr>
</tbody>
</table>

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<table>
<thead>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>aGPI</td>
<td>7/21 (33.3)</td>
<td><strong>1.12 (1.5)</strong></td>
<td>7.22 (6.84-7.92)</td>
<td>1 (1.4)</td>
<td>2 (2.7)</td>
<td>5 (6.9)</td>
</tr>
<tr>
<td>aGPI IgG</td>
<td>1/6 (16.7)</td>
<td>1 (0.90-1.12)</td>
<td>7.04 (6.67-7.92)</td>
<td>0 (1.8)</td>
<td>0 (1.8)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>aGPI IgM</td>
<td>3/6 (50)</td>
<td>1.12 (1.37)</td>
<td>7.05 (6.84-7.41)</td>
<td>1 (3.6)</td>
<td>1 (3.6)</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>aGPI IgA</td>
<td>3/9 (33.3)</td>
<td><strong>1.22 (1.66)</strong></td>
<td><strong>7.48 (6.92-8.58)</strong></td>
<td>1 (2.9)</td>
<td>1 (2.9)</td>
<td>3 (8.6)</td>
</tr>
</tbody>
</table>

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>aSP</td>
<td>17/59 (28.8)</td>
<td>1.02 (1.27)</td>
<td>7.09 (6.73-7.49)</td>
<td>9 (4.5)</td>
<td>5 (2.5)</td>
<td>12 (6)</td>
</tr>
<tr>
<td>aSP IgG</td>
<td>14/47 (29.8)</td>
<td>0.98 (1.28)</td>
<td>7.18 (6.78-7.72)</td>
<td>6 (3.8)</td>
<td>4 (2.6)</td>
<td>10 (6.4)</td>
</tr>
<tr>
<td>aSP IgM</td>
<td>5/15 (33.3)</td>
<td>1.03 (1.30)</td>
<td>6.9 (6.33-7.26)</td>
<td>3 (4.6)</td>
<td>1 (1.5)</td>
<td>3 (4.6)</td>
</tr>
</tbody>
</table>

Data are expressed as n (%) or median (interquartile range); m: median; aPL: antiphospholipid antibodies; HT: high titer; aCL: anti-cardiolipin antibodies; aGPI: anti-β2-glycoprotein antibodies I; aSP: anti-serin/prothrombin antibodies; IMT: intima-media thickness; avg: average; ICCAD: inter-adventitia Common Carotid Artery Diameter; AMI: acute myocardial infarction; PA: peripheral arteriopathy. *= p<0.05 compared to aPL negative subjects.
10.2.4 AMA, anti-LKM, anti-LC1 and anti-SLA

We identified 2 cases of PBC over a 15-year follow up, one showing AMA positivity (Table 24).

Table 24. Demographic and clinic characteristics of patients with primary biliary cholangitis.

<table>
<thead>
<tr>
<th></th>
<th>Case °1</th>
<th>Case °2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td><strong>AMA status</strong></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Viral hepatitis</strong></td>
<td>HBcAb positive</td>
<td>HBsAg, HBcAb positive</td>
</tr>
<tr>
<td><strong>Liver function tests</strong></td>
<td>High GGT (&gt;2xULN)</td>
<td>Normal</td>
</tr>
</tbody>
</table>

We were not able to identify subjects affected by AIH due to the absence of a disease specific exemption.
10.2.5 Anti-DPG and anti-tTG

Over a 15-year follow up, we identified 7 cases of CD (Table 25), of which only 1 resulted positive for anti-DPG and anti-tTG antibodies.

**Table 25.** Demographic and clinical characteristics of patients with coeliac disease.

<table>
<thead>
<tr>
<th></th>
<th>Case °1</th>
<th>Case °2</th>
<th>Case °3</th>
<th>Case °4</th>
<th>Case °5</th>
<th>Case °6</th>
<th>Case °7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>24</td>
<td>28</td>
<td>23</td>
<td>19</td>
<td>32</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td><strong>Anti-DPG</strong></td>
<td>Negative</td>
<td>Negative</td>
<td>Positive (High-titer)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Anti-tTG</strong></td>
<td>Negative</td>
<td>Negative</td>
<td>Positive (High-titer)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>17</td>
<td>22</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td><strong>Hemoglobin (g/dL)</strong></td>
<td>13.1</td>
<td>15.1</td>
<td>14.2</td>
<td>12.6</td>
<td>12.9</td>
<td>13.3</td>
<td></td>
</tr>
<tr>
<td><strong>Liver function tests</strong></td>
<td>Normal</td>
<td>High GOT (1xULN)</td>
<td>High GOT (1xULN)</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>
10.3 Aim #3. To analyze if there are factors at baseline (demographic, biochemical, clinical) that can predict the development of autoimmune disease over time, to identify patients at higher risk of developing an autoimmune disease that need a closer follow-up.

10.3.1 ANA and anti-ENA
We investigated the prevalence of hepatitis viruses in ANA positive subjects. We observed that ANA positive subjects had HBsAg in 6/150 (4%; p=NS vs. ANA negative), while they had anti-HBc in 73/150 (48.7%; p=0.007 vs. ANA negative; OR adjusted for sex and age 1.11, 95%CI 0.9-1.4). ANA positive subjects had anti-HCV in 10/150 (6.7%; p=NS vs. ANA negative). ANA positive subjects had HBsAg and anti-HCV in 2/150 (1.3%). ANA positive subjects had anti-HBc and anti-HCV in 28/150 (18.7%).

10.3.2 RF and anti-CCP
We investigated the prevalence of hepatitis viruses in RF and anti-CCP positive subjects. HBsAg was positive in 140/2,525 (5.5%) of subjects, anti-HBc in 1,002 (39.8%), and anti-HCV in 109 (4.3%). All the HBsAg positive subjects had also anti-HBc and 8 (0.3%) subjects had both anti-HBc and anti-HCV (Table 26).
RF positive subjects had HBsAg in 18/177 cases (10%; p=0.004 vs. RF negative; OR adjusted for sex and age 1.92, 95% CI 1.12-3.2), with 16 of IgM isotype, 12 of IgG, 7 of IgA (Table 26). RF positive subjects had anti-HBc in 84/177 (47.7%; p=0.003 vs. RF negative; OR adjusted for sex and age 1.17, 95% CI 0.8-1.6), 64 (52.9%) IgG, 50 (42.7%) IgM, and 22 (52.4) IgA (Table 26). RF positive subjects had anti-HCV in 16/177 (9%; p<0.001 vs. RF negative; OR adjusted for sex and age 1.6, 95% CI 0.9-2.7), 15 (12.4%) IgG, 12 (10.3%) IgM, 4 (9.5%) IgA (Table 26). RF positive subjects had both HBsAg and anti-HCV in 1/177 (0.6%), with RF IgG, IgM, and IgA (Table 26). RF positive subjects had both anti-HBc and anti-HCV in 15/177 (8.5%), with 11 RF IgG, 14 IgM, 4 IgA (Table 26).
Anti-CCP positive subjects had HBsAg in 6/121 (5%; p=NS vs. anti-CCP negative; Table 26), while they had anti-HBc in 54/121 cases (44.6%; p=NS vs. anti-CCP negative; Table 26). Anti-CCP
positive subjects had anti-HCV in 7/121 (5.9%; p=NS vs. ACPA negative; Table 26). Anti-CCP positive subjects had HBsAg and anti-HCV in no patient (Table 26). Anti-CCP positive subjects had anti-HBc and anti-HCV in 5/121 (4.1%; Table 26).

Anti-CCP and RF were both positive in 1/8 (12.5%) subjects with anti-HCV, no patient with HBsAg and in 5/8 (62.5%) subjects with anti-HBc (Table 26); RF isotypes were IgM and IgG.

Adjusting the analysis for the significant association between RF and HBsAg, the RR of developing RA if RF positive was 5.7 (95%CI 1.2-26.3; p=0.013). The RF positive predictive value was 1.13% (95% CI 0.33-3.83%), while the negative predictive value was 90.55% (95% CI 98.4-99.7%). None of the patients developing RA had positive viral markers.

Adjusting the analysis for the significant association between RF and HBsAg, the RR of RF positive subject was 5.7 (95%CI 1.2-26.3; p=0.013). The RF positive predictive value was 1.13% (95% CI 0.33-3.83), while the negative predictive value was 90.55% (95% CI 98.4-99.7).
Table 26. Prevalence of hepatitis virus infection according to rheumatoid factor and anti-cyclic citrullinated peptides (anti-CCP).

<table>
<thead>
<tr>
<th></th>
<th>RF negative</th>
<th>RF positive</th>
<th>RF IgM</th>
<th>RF IgG</th>
<th>RF IgA</th>
<th>Anti-CCP negative</th>
<th>Anti-CCP positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>1,759 (80)</td>
<td>177 (8.1)</td>
<td>123 (5.6)</td>
<td>117 (5.3)</td>
<td>42 (1.9)</td>
<td>2,404 (95.2)</td>
<td>121 (4.8)</td>
</tr>
<tr>
<td>HBsAg (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>88 (5)</td>
<td>18 (10)*</td>
<td>16 (13.2)</td>
<td>12 (10.3)</td>
<td>7 (16.7)</td>
<td>134 (5.6)</td>
<td>6 (5.0)</td>
</tr>
<tr>
<td>Anti-HBc (%)</td>
<td>682 (39)</td>
<td>84 (47.7)**</td>
<td>64 (52.9)</td>
<td>50 (42.7)</td>
<td>22 (52.4)</td>
<td>948 (39.4)</td>
<td>54 (44.6)</td>
</tr>
<tr>
<td>Anti-HCV (%)</td>
<td>63 (3.6)</td>
<td>16 (9)***</td>
<td>15 (12.4)</td>
<td>12 (10.3)</td>
<td>4 (9.5)</td>
<td>102 (4.2)</td>
<td>7 (5.9)</td>
</tr>
<tr>
<td>HBsAg+HCV</td>
<td>6 (0.3)</td>
<td>1 (0.6)</td>
<td>1 (0.8)</td>
<td>1 (0.9)</td>
<td>1 (2.4)</td>
<td>8 (0.3)</td>
<td>0</td>
</tr>
<tr>
<td>Anti-HBc+HCV</td>
<td>38 (2.2)</td>
<td>15 (8.5)***</td>
<td>14 (11.6)</td>
<td>11 (9.4)</td>
<td>4 (9.5)</td>
<td>72 (3)</td>
<td>5 (4.1)</td>
</tr>
</tbody>
</table>

RF: rheumatoid factor; Ig: immunoglobulin; anti-CCP: anti-cyclic citrullinated peptides; ANA: antinuclear antibodies; HB: hepatitis B; Ag: antigen; HCV: hepatitis C virus; *p=0.004 vs RF negative subjects; **p=0.03 vs RF negative subjects; ***p< 0.001 vs RF negative subjects.
10.3.3 APLs

We investigated the prevalence of CV risk factors in aPLs positive subjects. We observed that subjects with aPLs had higher prevalence of CV risk as estimated by the Framingham risk score (Table 27).

Table 27. Prevalence of Framingham risk classes according to the anti-phospholipid antibodies status.

<table>
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<tr>
<th></th>
<th>&lt;5%</th>
<th>6-10%</th>
<th>11-15%</th>
<th>16-20%</th>
<th>&gt;20%</th>
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<td>aPL negative</td>
<td>952 (65.5)</td>
<td>186 (12.8)</td>
<td>82 (5.6)</td>
<td>124 (8.5)</td>
<td>110 (7.6)</td>
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<tr>
<td>aPL positive</td>
<td>156 (60.5)</td>
<td>40 (15.5)</td>
<td>16 (6.2)</td>
<td>21 (8.1)</td>
<td>25 (9.7)</td>
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<tr>
<td>&gt;1 aPLs</td>
<td>15 (42.9)</td>
<td>8 (22.9)</td>
<td>5 (14.3)</td>
<td>3 (8.6)</td>
<td>4 (11.2)</td>
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<tr>
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<td>9 (16.1)</td>
<td>1 (1.8)</td>
<td>8 (14.3)</td>
<td>7 (12.5)</td>
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<tr>
<td></td>
<td>10 (38.5)*</td>
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</tr>
<tr>
<td></td>
<td>42 (57.5)</td>
<td>12 (16.4)</td>
<td>5 (6.9)</td>
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<td>0</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td>aSP IgM HT</td>
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<td>2 (16.7)</td>
<td>0</td>
<td>0</td>
<td>3 (25)*</td>
</tr>
</tbody>
</table>

Data are presented as n (%). aPL: antiphospholipid antibodies; HT: high titer; aCL: anti-cardiolipin antibodies; aGPI: anti-β2-glycoprotein antibodies I; aSP: anti-serin/prothrombin antibodies.

*= p<0.05 compared to aPL negative subjects.

CV risk factors had a different distribution between aPLs positive and negative subjects (Table 28).

High-titer aPLs were associated with high cholesterol (69.6% versus 52.4% in aPL negative subjects,
p=0.025 adjusted for sex and age; data not shown in table) and higher LDL levels (142.5 (120-169) vs 133 (113-156), p= 0.021; data not shown in table).

Interestingly, there was a significant association between elevated homocysteine and all aPL specificities: IgG aCL (10, 66.7%; p<0.001), aGPI (27, 37%; p=0.026), and IgG aSP (53, 33.8%; p=0.022; data not shown in table). The presence of more than one aPL was associated to a high homocysteine in 54.3% of cases (19 versus 368, 25.3% in aPL negative subjects, p<0.001 adjusted for sex and age).
<table>
<thead>
<tr>
<th></th>
<th>Age&gt;61.3</th>
<th>BMI&gt;25</th>
<th>Physical inactivity</th>
<th>Smoking</th>
<th>Arterial hypertension</th>
<th>Diabetes</th>
<th>CRP &gt;0.5 mg/dL</th>
<th>Triglycerides &gt;150 mg/dL</th>
<th>Cholesterol &gt;200 mg/dL</th>
<th>Homocysteine &gt;15 μmol/L</th>
</tr>
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<td>aPL negative</td>
<td>336 (23.1)</td>
<td>739 (50.8)</td>
<td>636 (43.7)</td>
<td>373 (25.7)</td>
<td>308 (21.2)</td>
<td>80 (5.5)</td>
<td>173 (11.9)</td>
<td>220 (15.1)</td>
<td>762 (52.4)</td>
<td>368 (25.3)</td>
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<td>91 (35.3)*</td>
<td>136 (52.7)</td>
<td>118 (45.7)</td>
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<td>70 (27.5)</td>
<td>19 (7.4)</td>
<td>36 (14)</td>
<td>48 (18.6)</td>
<td>140 (54.3)</td>
<td>80 (31)</td>
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<td>2 (5.7)</td>
<td>7 (20)</td>
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<td>35 (62.5)</td>
<td>23 (41.1)</td>
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<td>11 (20)</td>
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<td>10 (17.9)</td>
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<td>19 (33.9)</td>
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<td>7 (46.7)</td>
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<td>1 (50)</td>
<td>0</td>
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<td>1 (6.7)</td>
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<td>8 (72.7)</td>
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<td>6 (55)</td>
<td>8 (72.7)</td>
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<td>25 (34.2)</td>
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<td>12 (16.4)</td>
<td>18 (24.7)*</td>
<td>46 (63)</td>
<td>27 (37)*</td>
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<td>8 (40)</td>
<td>7 (35)</td>
<td>4 (20)</td>
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<td>15 (75)</td>
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<td>5 (9.3)</td>
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<td>3 (25)</td>
<td>1 (8)</td>
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<td>8 (42.1)</td>
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<td>26 (40)</td>
<td>17 (26.2)</td>
<td>17 (26.2)</td>
<td>4 (6.2)</td>
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<td>30 (46.2)</td>
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<td>1 (8.3)</td>
<td>8 (66.7)</td>
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When analyzing the prevalence of CV events and CV risk factors in aPLs positive and negative subjects, we observed that subjects with high level of homocysteine had a higher percentages of CV events, independently of aPLs (43, 11.7% versus 60, 5.5%; OR 2.3 95%CI 1.5-3.5, p<0.001), but those with high homocysteine and also aPLs had an even higher risk (12, 15% OR 3.01 95%CI1.4-6.0). Moreover, when analyzing a high-risk population defined by Framingham risk score >20, and/or diabetes, and/or BMI >35, aPL presence was found to be associated with a higher risk for any CV event (OR 2.52, 95%CI 1.24-5.11, p=0.01).
10.3.4 AMA, anti-LKM, anti-LC1, and anti-SLA

We investigated the prevalence of hepatitis viruses in AMA, anti-LKM, anti-LC1, and anti-SLA positive subjects.

AMA positive subjects had HBsAg in 3/101 (3%) of cases (p=NS vs. AMA negative), while HBcAb was positive in 34/101 (33.7%, p=NS vs. AMA negative). HCV-Ab was positive in 4/101 (4%, p=NS vs. AMA negative).

None of anti-LKM positive subjects resulted positive for HBsAg and HCV-Ab, while 1/3 had positive HBcAb (p=NS vs. anti-LKM negative).

None of anti-LC1 positive subjects resulted positive for HBsAg and HCV-Ab, while 3/8 had positive HBcAb (p=NS vs. anti-LC1 negative).

Only 1/22 of anti-SLA antibodies positive subjects resulted positive for HBsAg, 9/22 (41%) tested positive for HBcAb (p=NS vs. anti-SLA negative), while none for HCV-Ab.
10.3.5 Anti-DPG and anti-tTG

Only 1/50 of anti-DPG antibodies positive subjects resulted positive for HBsAg, 19/50 (38%) tested positive for HBcAb (p=NS vs. anti-DPG negative), while 1/50 tested positive for HCV-Ab.

Two/59 (3.4%) anti-tTG positive subjects tested positive for HBsAg (p=NS vs. anti-tTG negative), 24/59 (40.7%) tested positive for HBcAb (p=NS vs. anti-tTG negative), while 3/59 (5.1%) tested positive for HCV-Ab (p=NS vs. anti-tTG negative).
10.4 AIM #4. To analyze the autoantibodies predictive value for the development of malignancies over time.

10.4.1 ANA and anti-ENA

The presence of ANA was not associated with an increased HR of developing cancer (HR 1.03; 95% CI 0.75-1.43; p=NS) (Table 29).

Table 29. Number of events and 14-year absolute and relative risks associated with the ANA status in 1999. Results are shown with 95% confidence intervals.

<table>
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<th>Outcome</th>
<th>Total population n= 2.490</th>
<th>ANA any titer n= 518</th>
<th>ANA ≥1:80 n= 483</th>
<th>ANA ≥1:160 n=163</th>
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<td>Cancer</td>
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</tr>
<tr>
<td>incidence</td>
<td>(yearly) 131</td>
<td>30</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>Absolute risk in 14 years</td>
<td>0.06</td>
<td>0.03</td>
<td>0.04</td>
<td></td>
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<tr>
<td>Relative risk in 14 years</td>
<td>1.13 (0.76-1.68)</td>
<td>0.61 (0.37-1.03)</td>
<td>0.72 (0.32-1.62)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p =0.54</td>
<td>p =0.06</td>
<td>p =0.43</td>
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<tr>
<td>Death</td>
<td></td>
<td>33</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>incidence</td>
<td>(yearly) 122 (0.82 person time)</td>
<td></td>
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<tr>
<td>Absolute risk in 14 years</td>
<td>0.06</td>
<td>0.048</td>
<td>0.061</td>
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<tr>
<td>Relative risk in 14 years</td>
<td>1.50 (1.01-2.21)</td>
<td>1.02 (0.67-1.59)</td>
<td>1.34 (0.72-2.51)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p =0.041</td>
<td>p =0.921</td>
<td>p =0.360</td>
</tr>
</tbody>
</table>

122 subject (33/483, 6.8%, ANA-positive and 89/2.180, 4%, ANA-negative) died between 1999 and 2013, accounting for HR of death associated with ANA of 1.49 (95% CI 1.01-2.22, p=0.047), and 1.40 (95% CI 0.94-2.09, p=0.099) when adjusted for age and sex (Table 29).

The small number of events among the anti-ENA positive subgroups did not allow calculating HR for autoimmune diseases, cancer, or death due to the absence of incident CTD cases in the group of anti-ENA positive subjects and the underpower of the study for survival comparisons.
10.4.2 RF and anti-CCP

We retrieved cancer diagnosis in 4/177 (2.2%), and the presence of RF was not associated with an increased OR of developing cancer (HR for RF 0.42; 95% CI 0.15-1.18; \( p=\text{NS} \)). We did not investigate if any serotype was associated with cancer development. Anti-CCP antibodies positive subjects developed cancer in 10.1% (12/119), accounting for an increased risk of overall cancer (OR 2.12, 95% CI 1.15 -3.93; \( p=0.03 \)), especially for low titer anti-CCP (10/12). The risk was not increased in subjects with RA (OR 0, 95% CI 0 -9.9; \( p=\text{NS} \)).

15/177 (8.5%) of RF positive subjects died between 1999 and 2013, accounting for OR for death associated with RF of 2.17 (95% CI 1.24-3.82, \( p=0.006 \)).

9/121 (7.4%) anti-CCP positive subjects died between 1999 and 2013, accounting for OR for death associated with anti-CCP of 1.89 (95% CI 0.94-3.80, \( p=\text{NS} \)).
10.4.3 APLs

Cancer was diagnosed in 71/1,712 (4.1%) subjects in the CA.ME.LI.A. cohort (Figure 21), of which 11/258 (4.3%, p=NS) were aPLs positive. Cancer risk was not increased in the aPLs positive subjects (OR 1.03, 95%CI 0.54-1.97, p=NS).

Figure 21. Cancer diagnosis in CA.ME.LI.A. cohort, irrespective of antiphospholipid antibodies status.

We were not able to retrieve data about mortality for aPLs positive subjects due to the study design.
10.4.4 AMA, anti-LKM, anti-LC1, and anti-SLA

Cancer was diagnosed in 4/101 (4%) AMA positive subjects. Cancer risk was not increased in the AMA positive subjects (OR 0.82, 95%CI 0.31-2.18, p=NS). None of the anti-LKM, anti-LC1 positive subjects developed cancer. One/22 (4.5%) anti-SLA positive subject developed cancer. Three/101 (3%) AMA positive subjects died during the follow-up, and being AMA positive did not increase the risk of death (OR 0.68, 95%CI 0.23-2.06, p=NS). None of the anti-LKM, anti-LC1 positive subjects died during the follow-up. One/22 (4.5%) anti-SLA positive subject died.
10.4.5 Anti-DPG and anti-\(\tau\)TG

Cancer was diagnosed in 1/50 (2%) anti-DPG and 1/59 (1.7%) anti-\(\tau\)TG positive subjects. Being anti-DPG or anti-\(\tau\)TG did not increase the risk of cancer (OR 0.40, 95%CI 0.232, p=NS; OR 0.34, 95%CI 0.195, p=NS respectively).

Two/50 (4%) anti-DPG positive subjects died during the follow-up, while 3/59 anti-\(\tau\)TG died during the follow-up. Being anti-DPG or anti-\(\tau\)TG did not increase the risk of death (OR 0.89, 95%CI 0.337, p=NS; OR 1.16, 95%CI 0.355, p=NS respectively).
11. DISCUSSION

Serum autoantibodies are being widely used in the diagnostic work-up for autoimmune diseases for decades but their clinical interpretation remains largely subjective as most data on their significance have not been gathered from the general population. Autoimmune diseases affect up to 9% of the European population [3], and serum autoantibodies remain the most used biomarker in the diagnostic toolkit. However, their occasional detection in asymptomatic individuals remains a clinical challenge as serum markers may precede autoimmune disease onset by decades, as illustrated in RA [278, 279] and SLE [102]. The present thesis thus addresses two major questions with a crucial healthcare relevance, i.e. (i) what is the probability of being seropositive for autoantibodies for the general population? and (ii) what should a seropositive subject expect?

In summary, my data herein report that:

1. Serum ANA are detected in up to 18% of the general population, being more frequent in women and in elder ages; ANA are associated with an higher risk of CTD development; ANA are not associated with hepatitis virus infection; ANA do not increase the risk of cancer and/or death.

2. Serum RF is detected in 8.1% of the general population, while serum anti-CCP are found in 4.8% of the population, the prevalence of double positivity is 0.6%; RF is not associated with an increased risk of RA development in an unselected population, while anti-CCP are associated with a significant increased risk of RA development; RF is associated with both HBV and HCV infection, with no association with RA, anti-CCP are not associated with hepatitis virus infections; RF is not associated with an increased risk of cancer, while anti-CCP are associated with an increased risk; RF is associated with an increased risk of death.

3. APLs are detected in 15% of an unselected population, especially new autoantibodies, as aSP, which are not currently included in the classification criteria; no cases of aPL syndrome have been identified, but aPLS are associated with an increased CV risk profile and independently
with CV disease, especially subclinical atherosclerosis; aPLs are not associated with an increased risk of cancer.

4. AMA are detected in 3.8%, but at high titer in 1% of an unselected population; we identified only two cases of PBC, of which only one was AMA positive therefore we cannot estimate the risk; anti-LKM, anti-SLA, anti-LC1 antibodies are rarely found; all of the liver related autoantibodies are not associated with hepatitis virus infection, death or cancer.

5. Anti-DPG antibodies are detected in 1.85% of an unselected population, while, anti-tTG were found in 2.2%, with no differences between sexes. We identified 7 cases of coeliac disease, of which only one resulted positive for both anti-DPG and anti-tTG antibodies at high-titer. We found no association between anti-DPG anti-tTG antibodies and hepatitis virus infection, death or cancer.

Our results provide important information for clinicians. First and foremost, we suggest that the frequency of some serum autoantibodies, in particular ANA, RF, and aSP in the general population could be higher than previously reported. Remarkably, serum ANA positivity has been recently investigated in a large cohort (25,110 subjects) of Chinese heritage with a positive rate of ANA (>1:100) of 14.01% [280]. Interestingly, we observed that the prevalence of IgM RF (5.6%) was similar to that reported elsewhere (2.8-6.6%) [281-283], but slightly higher considering any RF isotype (8.1%). Our data confirm that IgM and IgG (5.3%) represent the main RF isotypes, while IgA are less frequent (1.9%). Furthermore, we confirm that antibodies titers are important in discriminating their association with autoimmune diseases development, in particular ANA 1:160 may represent a more adequate threshold for clinically-relevant positivity. Similarly, Li et al. have shown that ANA at high-titer are detected in more than 5% of subjects [280]. Also, we report for the first time the prevalence in the general population of anti-ENA and confirm that these should be tested only in the presence of a clinical suspicion while not being appropriate for screening purposes. Second, we confirm the sex predominance of serum ANA and AMA although the female to male ratios observed for ANA and ENA are significantly lower compared to what reported for
overt CTD [284, 285]. Third, ageing is associated with significantly higher rates of ANA, aPLs and AMA positivity and this observation is consistent with the proposed models for immunosenescence [284-287], suggesting that ANA positivity in the general population may be indicative of immune dysfunction resulting from advanced cellular aging processes, as recently shown by Maijer et al. Fourth, we observed that serum ANA significantly increases the risk for CTD, serum anti-CCP increases the risk for RA development, confirming that anti-CCP have higher specificity than RF for RA development [288]. Of interest, one (10%) RA incident case was seropositive for both anti-CCP and RF, a status observed in only 0.4% of our population, therefore supporting that the combination of both autoantibodies may represent a major risk factor for RA [288]. RA prevalence in the general population is reported to be 1%, in the present study we have a relatively lower prevalence of double positivity for anti-CCP and RF, however about 10-30% of RA patients are seronegative for known autoantibodies [181]. Based on these observations, we are convinced that no tight follow-up should be sought in asymptomatic autoantibodies positive subjects while the education of subjects with occasional autoantibody positivity to recognize signs and symptoms of autoimmune diseases may prevent the unnecessary use of resources. Fifth, according to the literature, 17-42% of HBV-carrying patients are RF positive [289], and our findings suggest a significant association between RF and HBsAg as a marker of active hepatitis B infection, with a 2-fold increase of RF prevalence among HBsAg positive subjects, but we failed an association with RA disease, such as demonstrated by a previous systematic literature review [290]. This is of particular interest since HBV infection may manifest with extra-hepatic features such as arthralgia, and, in RA with chronic HBV infection, viral particles have been recently demonstrated in the synovia of affected joints, being associated with a more severe inflammatory infiltrate [291]. Of relevance to clinical practice, immunosuppressive treatments for RA in patients with chronic HBV infection should be carefully evaluated due to the risk of viral reactivation [292], particularly in endemic areas [293], such as the Norther Italian area that was investigated herein. Sixth, we report for the first time that aPLs are an independent CV risk factor, therefore should be sought in high
risk populations. ASP were significantly associated with increased subclinical atherosclerosis in childbearing-age women, and one not routinely tested aPL, i.e. aGPI IgA, was found to be more frequent in women and the only aPL significantly and independently associated to increased subclinical atherosclerosis. CV events were overall more frequently found among aPL positive subjects but only peripheral arteriopathy was significantly associated to the aPL, thus suggesting a possible independent role of aPLs in the atherogenesis. Our data, however, suggest also an additive role of aPLs in atherothrombotic events: considering that higher Framingham score, diabetes, higher BMI or hyperhomocysteinemia combined with aPLs increased the risk of CV events by 2.5-3 folds. The association between hyper-homocysteinemia and aPL is of particular interest given the pro-thrombotic action of homocysteine via phosphatidylserine exposure on endothelial cells, myocardiocytes, and red blood cells [294, 295], thus suggesting that the development of aPLs could contribute to the thrombophilic status in hyper-homocysteinemia. These findings would translate clinically in wider testing for aPLs in at risk populations, and potentially prophylactic anti-thrombotic treatment in high-risk patients.

Seventh, the association between autoimmunity and cancer has been proposed by numerous lines of basic and clinical evidence [296-300] but our data demonstrate that autoantibodies-positive and –negative subjects have a similar risk of developing cancer over a 15-year period, except for anti-CCP, which could reflect their association with smoking [199], albeit the causes of death were not recorded in the present study. Finally and possibly most importantly, we did observe a significantly reduced unadjusted survival in subjects with serum ANA but significance was lost after adjusting for age and sex, while RF subjects showed an increased risk of death, maybe due to their association with hepatitis virus infections. Additional confounding factors should necessarily be taken into account for survival analyses and will be the subject of future epidemiological studies. We believe that the ISOLA and CA.ME.LI.A. projects represent a unique opportunity for the study of prevalence and clinical significance over time of serum autoantibodies in an unselected general population of a Northern Italian area thanks to several strengths. First, we had an high response rate
(71%) of the population overcoming the possible limitations in representativeness of this type of studies [301, 302]. Second, the local health system keeps track of all new diagnoses for a chronic disease, hospital admissions and diagnoses or procedures, drug prescriptions, and cases of death thus allowing to determine the occurrence of health-related events over time, as proven in other epidemiological studies [303-305]. Third, the period of time occurring between the ISOLA studies is the longest observational time reported for a population-based longitudinal study in autoimmunity. Fourth, we should note that serum autoantibodies were tested between 2012 and 2017, thus taking advantage of more modern methods compared to what was available in 1999. We are also aware of the limitations of our approach. Indeed, our analysis of administrative databases is limited by the observation that 11% of the target population in 2013 had an exemption due to low income or age over 65 years, and this may potentially limit the emission of clinical exemptions for specific diseases. Second, despite using modern laboratory tests, autoantibodies were tested using IIF and ELISA, while no further testing for rare antigens was performed, as for anti-Th/To or -RNAPIII in scleroderma [306-308]. Third, we acknowledge that the rarity of serum anti-ENA, anti-LKM, anti-LC1, anti-SLA, anti-LC1, anti-DPG, and tTG did not allow estimating the risk for any clinical outcome. Finally, all serum samples were only tested at one timepoint without a subsequent confirmation test on a separate occasion. Analysis of the causes of death in cancer patients would also merit further explorations to better understand their association with anti-CCP.

Future steps in the effort to predict autoimmune disease onset include the use of the most modern technological platforms for high throughput studies of new biomarkers [309] while a better understanding of metabolomics [310, 311] and the microbiota [312] is expected to provide additional candidates.
12. REFERENCES


