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IMMUNOHISTOCHEMICAL STUDY OF THE EXPRESSION OF LINE-1 (ORF2p) AND APOBEC3B PROTEINS IN THE LABIAL MINOR SALIVARY GLANDS OF SJÖGREN'S SYNDROME PATIENTS

ELENI-MARINA KALOGIROU
DDS

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Supervisor of MSc thesis: Evangelia P. Piperi, Assistant Professor, Department of Oral Medicine and Pathology, National and Kapodistrian University of Athens

Three-member Committee for MSc thesis evaluation:

1. Evangelia P. Piperi, Assistant Professor, Department of Oral Medicine and Pathology, National and Kapodistrian University of Athens

2. Alexandra Sklavounou-Andrikopoulou, Professor and Head of the Department, Department of Oral Medicine and Pathology, National and Kapodistrian University of Athens

3. Konstantinos I. Tosios, Assistant Professor, Department of Oral Medicine and Pathology, National and Kapodistrian University of Athens

To my family

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1 INTRODUCTION

When someone starts studying about Sjögren's syndrome (SS), they will soon have to admit themselves the truth: Never is enough, considering that approximately 500-600 articles on SS appear annually in the international scientific database Pubmed. However, despite the mounting knowledge about SS, the pathogenetic background of the disease has still to be elucidated.

Current knowledge suggests that the interplay between immunological, genetic, epigenetic and environmental factors triggers the autoimmune response, resulting in a wide range of glandular and systemic manifestations in the affected patients. The salivary gland tissues are among the main targets of SS and are the best evaluated histopathologically. The minor salivary glands (MSGs) biopsy is among the criteria required for SS diagnosis and has contributed significantly to the identification of several molecules that participate in the autoimmune responses in SS. Among these, a key role has been attributed to the activation of type I Interferon (IFN), a pro-inflammatory cytokine that has been recognized to be stimulated in viral infections. A potential trigger factor of type I IFN pathway is the Long Interspersed Nuclear Element (LINE-1, L1), which belongs to the superfamily of transposable elements and encodes two proteins, the L1 ORF1p and ORF2p. The inappropriate expression of L1 elements may be the result of inadequate epigenetic regulation, mediated by various factors, including the APOBEC3 enzymes.

The present thesis provides evidence on the immunohistochemical expression of the L1 ORF2p and APOBEC3B proteins in the labial MSGs of SS patients.

In *Chapters 2-4* the pertinent literature regarding SS, the L1 elements and the APOBEC3 enzymes is reviewed. In particular, in *Chapter 2*, current data about the epidemiological, clinical, histological and serological features, as well as the diagnostic criteria, prognostic parameters and treatment modalities of SS are summarized, accompanied by a comprehensive review on SS pathogenesis. *Chapter 3* reviews the data concerning the structure, the life cycle, the expression and the regulation of of L1 elements expression, as well as their effect on human genome, and their potential implication in SS. In Chapter 4, the literature regarding the structure, expression and functional role of the APOBEC3 subfamily, with special emphasis on the APOBEC3B member is reviewed.

Chapter 6 presents the material of the present study, which was designed in the Department of Oral Medicine and Pathology, the immunohistochemical protocol and the method of the results evaluation. The immunohistochemical results and their statistical analysis are presented in *Chapter 7*. Finally, the findings of the present study are discussed in the *Chapter 8* and the main conclusions are summarized in *Chapter 9*. The main points of the thesis are summarized in an English and a Greek abstract, followed by the list of references and a list with the abbreviations found in the thesis text.

Approaching the completion of a three year postgraduate program, I wish to acknowledge all the people that made these years special to me.

First of all, I wish to express my most sincere gratitude to *Alexandra Sklavounou, Professor and Head of the Department* for the opportunity that she offered to me to study Oral Medicine and Pathology. Her trust is always a great motivation to achieve a future goal that will make her proud.

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I wish to end my acknowledgments by expressing my deepest gratitude to my family, *my grandmother Elli, my uncle George*, who is the reason why I became a dentist, *my mother Katerina, my aunt Eleni and my uncle Alkis*, to whom I owe what I have achieved until today. All my efforts aim to make them proud.

Finally, since best *friends* are more like sisters, I am grateful that I have all these friends supporting me throughout my life.

REVIEW OF THE LITERATURE

2. Sjögren's syndrome

3. Long Interspersed Nuclear Element 1 (LINE-1)

4. Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3B(APOBEC3B)

2 SJÖGREN'S SYNDROME

2.1 An overview

Sjögren's syndrome (SS) is a chronic autoimmune disease that predominantly affects the salivary and lacrimal glands resulting in oral and ocular dryness respectively. The syndrome was designated by the Swedish ophthalmologist Henrik Samuel Conrad Sjogren in 1933, who reported the whole disease spectrum in his doctoral thesis *"Zur Kenntnis der keratoconjunctivitis sicca"*. However, three cases of salivary gland atrophy accompanied by ocular, oral and genital dryness had also been described by the French dermatologist Henri Gougerot in 1925, while the coexistence of keratoconjunctivitis sicca and arthritis was commented by the Dutch ophthalmological surgeon Adriaan Willem Mulock Houwer in 1927.[1] The bilateral, chronic, painless hypertrophy of the lacrimal and salivary glands had also been reported by the Polish-Austrian surgeon Jan Mikulicz-Radecki in 1888 as a disorder that was later named after him.[2] Although Mikulicz's disease was considered identical to SS for years, it is now believed to be a distinct disorder that probably represents an early description of the recently reported IgG4-related disease.[3]

SS involves approximately 0.5-1% of the general population, with a clear predilection for middle-aged female patients and a 9:1 female to male ratio.[4, 5] A wide variability (0.1-4.8%) of the disease incidence has been noted in a recent comprehensive review of SS geoepidemiology, though, possibly due to geographical differences, as well as to the diagnostic criteria and the study methods (sample size, gender distribution, etc) used.[4] The methodological deviations were evident not only between studies of different ethnic groups but also in studies derived from the same country, such as Greece.[4]

SS may present alone as a single autoimmune disorder ***(primary SS, pSS)*** or in the context of an underlying connective tissue disorder, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and systemic sclerosis ***(SS associated with other diseases)***.[4, 6] Until nowadays autoimmune rheumatic patients manifesting sicca symptoms have also been described with the term "*secondary SS"*. This term, though, is collective and does not emphasize the distinct histopathological features of

SS and the significantly higher frequency of sicca symptoms among SS patients in comparison to SLE or RA patients.[7]

The basic characteristics of SS as an autoimmune disorder are: **a)** the **focal lymphocytic infiltration** of the **exocrine glands**, mainly the **salivary** and **lacrimal glands**, by autoreactive T cells and B cells that results in glandular destruction and **b)** the B cell hyperactivity and the production of **circulating autoantibodies** against various autoantigens.[8] As a result, SS involves patients with a **wide clinical spectrum** including glandular and extraglandular manifestations.[5]

The most important complication of SS is the development of a **non-Hodgkin B-cell lymphoma**, affecting **5-10%** of SS patients.[9] Several clinical, laboratory, histopathological and genetic risk factors have been described for lymphoma development in the context of SS, including the low C4 levels and the palpable purpura, which will be discussed later in detail.[7] Ioannidis et al[10] proposed the classification of SS patients into those with low C4 levels and/or palpable purpura (***type I-high risk***) and those without any of these two characteristics (***type II-low risk***). The practical clinical value of this classification is obvious, as it can determine a more appropriate treatment approach and shorter follow-up intervals in the type I SS subgroup.[7]

2.2 Clinical manifestations

The main targets of SS are the exocrine glands, mainly the salivary and lacrimal glands, which leads to oral and ocular dryness in 90-95% of SS patients.[5] In approximately 25–66% of SS patients the parotid or submandibular glands are enlarged, often bilaterally,[11] and this sign is considered a major clinical prognostic factor for lymphoma development.[9] The ***glandular involvement*** in SS may also cause dryness of the skin, the upper respiratory system or the vagina, resulting in pruritus, bronchitis sicca and cough, and dyspareunia respectively. Additionally, SS may involve several parenchymal organs, including the kidneys, the lungs and the liver, with a broad range of ***systemic (extraglandular) clinical features*** (Table 1).[5]

Table 1. Systemic (extraglandular) manifestations in SS.(adapted by[5])	
Non specific	• Musculoskeletal manifestations (Jaccoud arthropathy) • Raynaud's phenomenon • Fatigue
Periepithelial	• Lung (bronchial/bronchiolar primarily, interstitial lung disease rarely, dry cough, breath shortness during exertion) • Liver (hepatomegaly, primary biliary cirrhosis, autoimmune hepatitis) • Kidney (glomerulonephritis, interstitial nephritis-tubular dysfunction, renal tubular acidosis) • Endocrine glands (autoimmune thyroid disease, autoimmune adrenal disease, autoimmune ovarian disease)
Immunocomplex-related disease	• Cutaneous vasculitis (palpable purpura) • Peripheral neuropathy (sensory axonal neuropathy, small fibre neuropathy resulting in painful or burning paresthesia, sensorimotor neuropathy) • Glomerulonephritis • Central nervous system vasculopathy
Lymphoma	• Minor or major salivary glands, stomach, lungs, nodes, bone marrow (rarely)

Xerostomia, corresponding to the decrease of the salivary flow rate to less than 50%, induces changes in the saliva protein composition, i.e.the levels of secretory IgA, lactoferrin, β2-microglobulin, sodium, lysozyme C and cystatin C are elevated and the salivary amylase and carbonic anhydrase are reduced. These alterations in protein content predispose to changes in oral microflora and, consequently, to susceptibility to oral infections.[11-13] Table 2 summarizes the oral complications in SS.[5, 12, 14]

Table 2. Oral complications in SS.[5, 12, 14]	
Objective signs	*Subjective symptoms*
• Dental decay • Dental erosions • Periodontal disease • Teeth loss • Fungal infection (pseudomembranous or erythematous candidiasis, denture-associated stomatitis, angular cheilitis) • Atrophy of filiform papillae • Fissured tongue • Dry, cracked, peeled lips • Oral ulcers • Salivary glands swelling	• Difficulty in chewing • Difficulty in swallowing • Sensitivity to spicy foods • Need for increased water intake • Dysgeusia (absent, diminished or altered taste perception) • Oral discomfort • Burning sensation • Difficulty in speaking • Voice disturbances • Pain in salivary glands

2.3 Autoantibody production

As has already been mentioned, SS is primarily characterized by the presence of various autoantibodies that target organ or non-organ systemic autoantigens. Most of these autoantibodies are found in increased frequency among SS patients and have been associated with several clinical parameters, as well as prognostic factors of lymphoma development (Table 3).[9, 15] However, several may be also found in patients suffering from other autoimmune diseases, including RA and SLE.[15]

The major autoantibodies identified in SS patients are those directed against the intracellular ribonucleoproteins Ro/SS antigen A (Ro/SSA) and La/SS antigen B (La/SSB), the detection of which in the serum is one of the diagnostic criteria of SS.[16, 17] The Ro/SSA autoantigens consist of the 52 kDa-Ro (Ro52) and the 60 kDa-Ro (Ro60) proteins that are encoded by the tripartite motif-containing protein 21 (TRIM21) and the TROVE domain family, member 2 (TROVE2) mRNAs respectively.[15] The Ro/SSA and La/SSB autoantigens are usually part of a heterogeneous antigenic complex, consisting of three different proteins and four small hY RNAs particles, found predominantly in a nuclear location.[15] They are released by almost all cell types, while an antigen driven autoimmune response against them occurs within the involved SGs in SS patients.[15]

Although the exact factors promoting the overexpression of these autoantigens in the SS autoimmune lesions remain obscure, several studies suggest an altered expression and localization of the Ro/SSA and La/SSB autoantigens within the salivary glands epithelial cells (SGECs) of SS patients.[15] Proposed mechanisms of autoantibodies production against these autoantigens include the apoptosis of the SGECs that results in the exposure of the Ro/SSA and La/SSB autoantigens on the cell surface and the apoptotic blebs,[18] as well as the release of exosomes containing these autoantigens.[19] Exosomes are structures that will be discussed in detail later.

Table 3. Autoantibodies in SS patients.(adapted by[15])

Autoantigen-target	Prevalence	Clinical association
Ro52/TRIM21	66.7%	• Younger age at diagnosis and longer disease • Exocrine gland hypofunction
Ro60/TROVE2	57.2%	• Severe infiltration of salivary glands • Salivary gland enlargement • Extraglandular manifestations
La/SSB	49%	• Hypergammaglobulinemia • Cryoglobulinemia • Neonatal lupus-congenital • Heart block
Rheumatoid factor (RF)	36-74%	• Younger age at diagnosis • Extraglandular manifestations
Cryoglobulins	9-15%	• Younger age at diagnosis • Salivary gland enlargement • Extraglandular manifestations • Hypocomplementemia • Hypergammaglobulinemia • Mucosa-associated lymphoid tissue (MALT) lymphoma
Centromere (ACA)	4-17%	• Overlap with systemic sclerosis • Milder disease • Negative correlation with • anti-Ro/SSA and anti-La/SSB antibodies

Mitochondria (AMA)	1.7-27%	• Primary biliary cirrhosis
Smooth muscle	30%	• Autoimmune hepatitis
Cyclic citrullinated peptides (CCP)	3-10%	• Articular manifestations
Muscarinic 3 receptor	11%	• Sicca symptoms
Carbonic anhydrases	12.5-20.8%	• Renal tubular acidosis
U1RNP	2%	• Overlapping syndrome with MCTD
Calreticulin	20%	

2.4 Histopathological features

The histopathological hallmark of SS is the **focal mononuclear cell (MNC) infiltration** of the exocrine glands, predominantly the salivary and lacrimal glands, that results in gradual destruction of the glandular parenchyma.[20] Moutsopoulos in 1994[21] was the first to point out that the MNC infiltration is developed in proximity to the glandular parenchyma of the involved tissue, i.e. the acinar or ductal epithelium of salivary glands, the conjunctival epithelium of lacrimal glands,[22] the tubular epithelium of kidney,[23] the bronchial epithelium of lung[24] and the cholangial epithelium of liver.[25] Based on his observations, Moutsopoulos proposed the term ***"autoimmune epithelitis"***, to indicate that the epithelium is the major affected tissue in SS.[21]

Due to the easier access to the minor salivary glands (MSGs) through the normal-appearing labial mucosa in comparison to other affected tissues, and the relatively easy performance of MSGs biopsy without serious postoperative complications,[26] the MSGs have been the tissues best evaluated histopathologically in SS patients. Moreover, the histopathological features of the MSGs are among the required criteria for SS diagnosis[16, 17] that will be presented later in detail. The MSG tissue biopsy is suggestive of SS when the **focus score**, i.e., **the number of lymphocytic foci/4 mm^2 area in a minimum of four representative lobules**, is **≥1**, according to Chisholm and Mason criteria,[27] who defined the term "focus" as a cluster of at least 50 lymphocytes. Moreover, according to the classification by Tarpley et al[28] the MSG lesions are characterized by variable grades of infiltration severity, extending from focal mild to intermediate and, finally, to diffuse severe MNCs infiltrates accompanied by the loss of tissue architecture.

T cells and B cells are the major cell populations,[29-33] comprising more than 90% of the MSG cellular infiltrate,[29] but plasma cells, a few macrophages and dendritic cells may also be found in the MSG biopsies of SS subjects. A distinguishing feature between T-cells and B-cells is their distribution in MSG lesions of SS patients. **T cells** are mainly observed in the *periphery* of MNC infiltrates, in contrast to the *centrally* located **B cells**. This pattern is more prominent in severe lesions, especially in those with organized **ectopic germinal center (GC)-like structures** (fig. 1). Signs of lymphoid neogenesis with the formation of functional ectopic lymphoid-like structures have been described in a subset of SS patients.[32, 34] Salomonsson et al[32] found GC-resembling structures, defined as aggregates of CD3+ T cells, CD20+ B cells, Ki-67+ proliferating cells, CD35+ follicular dendritic cells (FDCs) and CD31+ activated endothelial cells, e.g. high endothelial venule (HEV) in 28 out of 165 (17%) SS patients. FDCs do not derive from the bone-marrow hematopoietic stem cells but are stromal cells found in the follicles of the secondary lymphoid organs and exert a crucial role in the shaping of B-cell responses.[35] The histopathological features combined with the immunohistochemical expression of markers of the FDC networks, including CD21[33] or/and CD38[34] and fascin,[29, 36] in the center of the lymphoid aggregates, are nowadays used for the identification of the ectopic GC-like structures that are usually[29, 33] but not always[35] found in SS MSG biopsies of advanced infiltration grade and a higher focus score. The enzyme Activation-induced cytidine deaminase (AID), which is responsible for the class switch recombination (CSR) and the somatic hypermutation of the Ig genes have been detected within the FDC networks in SGs with GC-like structures.[37] Furthermore, lymphoepithelial lesions, ductal dilatation, hyperplasia or metaplasia of the ductal epithelium, areas of lipoid degeneration and periductal or interstitial fibrosis are commonly observed in SS MSG biopsies.[38]

A significant correlation between the MNC infiltrate content and the **severity of the histopathological lesion**, determined by the **infiltration grade** and the **biopsy focus score**, has been observed.[29, 33] **CD3+ T cells** predominate in *mild lesions* and **CD20+ B cells** in *advanced* ones while the CD3+/CD20+ ratio is negatively associated with the lesion's severity. Both these cellular populations have also been found to penetrate the salivary ducts *(lymphoepithelial lesions)*. CD4+ T cells constitute the majority of T cells and their proportion follows the severity-dependent pattern, thus, they are increased in mild lesions. Since the number of the CD8+ T cells has been

observed to remain unchanged between mild and severe lesions, the CD4+/CD8+ ratio has been found to be inversely correlated with the infiltration grade. A particular CD4+T-cell subset, the transcription factor forkhead box p3 positive (**Foxp3+**) **T regulatory cells (Tregs)** (Tregs) have been found to prevail in *intermediate lesions*,[29, 39] while their number declines in severe cases, probably indicating an inefficient control of the local immune mechanisms that results in the dysregulation of the in situ immune system response.[29] The so-called professional antigen-presenting cells (APCs), the macrophages and the dendritic cells (DCs), have also been detected in the MSG biopsies of SS patients, while they were absent in the MSG tissues from healthy controls. The **CD68+ macrophages** have been found in elevated proportions in advanced SS lesions characterized by dense MNCs infiltrations, usually in proximity to the ductal epithelium, while their incidence is positively correlated to the infiltration grade.[29, 40, 41] **Myeloid DCs (mDCs)** and **plasmacytoid DCs (pDCs)**, the two main DC subtypes, have also been identified in the MSGs of SS patients. The accumulation of DCs in mild MSGs lesions of pSS[42, 43] or SS related with other diseases,[42] and their concurrent decrease in the peripheral blood of SS patients implies an situ role of DCs, possibly in promoting the T cell-mediated initial immune response.[29] **CD56+-Natural Killer (NK) cells** have also been observed in MSGs biopsy, but have not been associated with the biopsy focus score.[29]

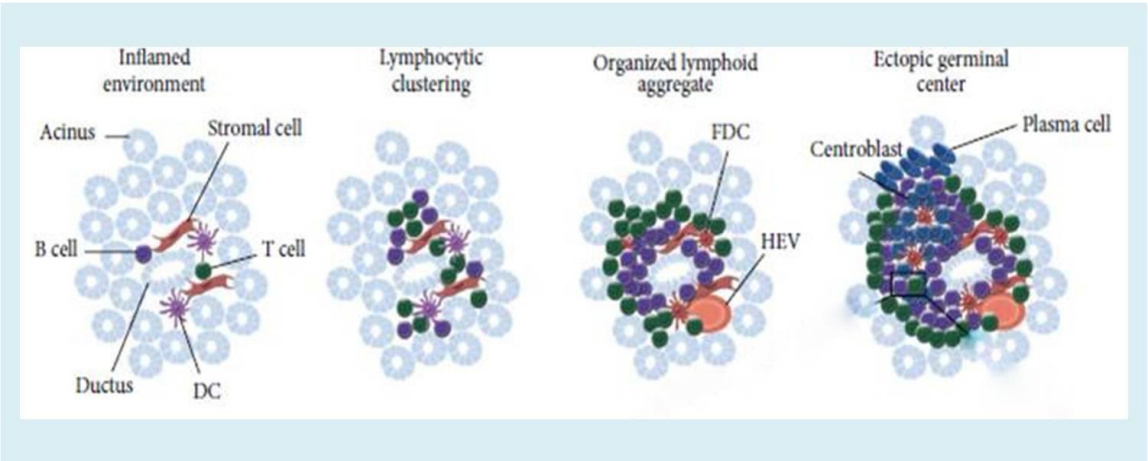


Fig. 1. Graphical summary of the formation of ectopic lymphoid-like structures in exocrine tissues of patients with SS.(adapted by[33])

The histopathological similarities and differences between pSS and SS associated with other diseases have not yet been adequately identified. Hernández-Molina et al[44] observed a similar incidence of focal infiltrations among 50 pSS subjects and 300 patients with SS accompanied with connective tissue diseases. A significantly higher prevalence of CD20+ cells and a higher B-T cell ratio was found in the pSS study group. Moreover, although the MSG lymphocytic infiltrates are generally considered to progress stepwisely, their progression rate over time has not been elucidated. Jonsson et al[45] found an increase in the focus score of 14/21 (67%) pSS patients and 14/18 (78%) subjects with SS associated with other diseases, who had been followed for 11-112 months (mean follow-up period of 39 ± 20 months). In contrast, no significant change in the biopsy focus score and the Tarpley classification, as well as in several histopathological features (ectopic GC formations, lipoid degeneration areas, interstitial fibrosis areas) was observed in a recent study by Kapsogeorgou et al,[35] who evaluated serial biopsy specimens from 28 pSS patients, with a biopsy interval time of 36 to 110 months (median interval of 55 months). Another question that remains unanswered is whether a correlation exists between the pharmaceutical treatment of SS patients and the histopathologic characteristics in the MSG biopsies. Anderson et al[46] evaluated the two serial biopsies of 14 SS patients, 9 of which were under various drug treatment schemes. With a biopsy interval time of 7-24 months, histological improvement was observed in 2/4 patients receiving cyclophosphamide, while a lymphocytic infiltrate progression was apparent in untreated patients.[46] The predominance of either T cells or B cells in the MSGs of SS patients may point to the appropriate therapy selection, for example a B cell depletion treatment in SS patients with prevailing B cells.[36]

2.5 Pathogenesis

Although the pathogenic mechanisms of SS have been subject of numerous studies for decades, SS aetiopathogenesis has still to be elucidated. The current scientific knowledge suggests a multifactorial pathogenetic model, in which the local immunological factors, i.e. the cellular populations and the produced cytokines and chemokines, interact with various environmental, genetic and epigenetic factors to promote the onset, the perpetuation and the progression of this autoimmune disease.[reviewed in [6, 47]]

2.5.1 The key-role of epithelium

Several lines of evidence in the last 3 decades have supported the crucial interplay between the epithelium and the immune system in SS pathogenesis.[reviewed in [6, 18, 47-50]] In particular, the ability of the SGECs to promote the initiation and the perpetuation of the localized autoimmune inflammatory response in SS has been documented by immunohistopathological studies of MSGs and in-vitro experiments using long-term cultured, non-neoplastic SGECs derived from SS patients and sicca-controls.[47] Several immune-competent molecules, expressed by the epithelial cells, reflect the role of SGECs in SS as non-professional APCs and their contribution to the ectopic germinal-centers formation and the autoantigen release via apoptotic bodies or exosomes.[18]

2.5.1.1.1 Antigen-, co-stimulatory and adhesion signals presentation, TLR receptors expression and proinflammatory cytokines production

The SGECs are able to provide the two essential signals for the naive T-cell activation, as they express various immunoreactive molecules involved in **antigen presentation**, such as the major histocompatibility complex *(MHC) class I and II* (human leukocyte antigen, HLA-DR) antigens and **co-stimulation** peptides *(B7.1/CD80 and B7.2/CD86).*[51-54] The B7 co-stimulatory molecules are mainly expressed by the classic APCs and interact with the *CD28-receptor* or the *CTLA-4-receptor* on the surface of T cells, in order to stimulate or suppress the immune reaction, respectively. The B7 molecules generally display a higher affinity for the T-cell negative CTLA-4. In contrast, the expressed by the SGECs B7.2 molecules in SS exhibit higher affinity for CD28 than CTLA-4, thus promoting the immune response.[51]

The epithelial cells found in the lip MSG biopsies or the cultured SGEC lines from SS patients also express **adhesion molecules**, including the *intercellular adhesion molecule-1 (CD54/ICAM1)*, the *vascular cell adhesion molecule (VCAM)* and the *E-selectin*.[55, 56] Although these molecules are typically produced by the classical APCs, to amplify their synapsis with T cells, the CD54/ICAM-1 has been found throughout the salivary gland tissue of SS patients and is not limited proximal to the lymphoid infiltration.[55]

Epithelial cells are capable of expressing functional **Toll-like receptors (TLRs)** that are implicated in distinct pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns recognition.[57] TLRs are transmembrane receptors on the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6), where they recognize PAMPs, or in endosomal or lysosomal compartments, where they predominantly identify nucleic acids, including the double-stranded RNA (dsRNA) (TLR3), the single stranded RNA (ssRNA) (TLR7) and the dsDNA (TLR9).(summarized in [58]) The immunohistochemical expression of *TLR2, TLR3* and *TLR4* has been found to be more intense in the SGECs (acinar and ductal cells) and the salivary MNC infiltration in 12 SS patients compared to 4 healthy controls. Increased TLR expression in SS subjects was also observed in a human salivary gland cell-line cultured with the *TLR2, TLR3* and *TLR4 ligands*, i.e. peptidoglycan, polyinosinic:cytidylic acid and lipopolysaccharide, respectively.[59] In another study, a significantly higher constitutive expression of *TLR1, TLR2* and *TLR4 mRNA* was found in SGEC lines derived from SS patients compared to SGECs from controls.[60] The SGEC stimulation with the respective *TLR2, TLR3* and *TLR4 ligands* in the same study resulted in an upregulated expression of the epithelial surface antigen-presenting (MHC-I), the co-stimulatory (CD40), the adhesion (CD54/ICAM-1) and the apoptotic molecules (CD95/Fas), thus, providing a link between the innate and adaptive immune response.[60] Ittah et al also reported the expression of *TLR2, TLR3* and *TLR7* mRNA in the ductal epithelial cells derived from minor salivary gland biopsies.[61] More recently, Zheng et al[62] reported a *TLR7* and *TLR9* positive expression in the ductal cells, the epithelial islands and the lymphocytes in the parotid glands of 20 pSS cases, via immunofluorescence and immunohistochemistry respectively. In contrast, *TLR7* and *TLR9* were expressed only in the ductal epithelial cells of the controls' parotid glands, but not in the parotid infiltrating lymphocytes.[62]

Expression of the *CD40 molecule* by the SGECs and the lymphocytic expression of its ligand (CD40L/CD154) further support the interplay between the SGECs and the infiltrating lymphocytes. The CD40-CD40L binding enhances the immune response directly or indirectly, by stimulating T cells or APCs' co-stimulatory and adhesion molecules, e.g. ICAM1, respectively.[63, 64] Kobayashi et al[65] found that the ductal and acinar epithelial cells of SS patients expressed the *PD-1 ligand (PD-L1)*, while the salivary gland infiltrating lymphocytes of SS patients showed immunopositivity for the PD-1. Moreover, the SGECs-lymphoid infiltrates crosstalk in SS is supported by the presence of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 IL-18 and adiponectin, which are produced by the SGECs and promote the inflammatory cell-mediated immune response in SS.[40, 41, 66-68]

2.5.1.1.2 Chemokines expression and germinal-center structures formation

The SGECs of SS patients produce several **chemokines** that induce the lymphoid cells recruitment and homing, thus, regulating the SS autoimmune lesion maintenance, the formation of ectopic GCs and, finally, the clonal B-cell proliferation, differentiation and progression to lymphoma. Among the regulatory chemokines highly expressed by SS-SGECs are the naive B cells- and T cells-chemoattractant *CXCL13 (B-cell attractant, BCA1)*, the T cells- and DCs-chemoattractant *CCL21 (secondary lymphoid tissue/T-cell attractant, SLC/TCA)* and the T cells-chemoattractants *CXCL9 (Mig)* and *CXCL10 (IFN-c inducible 10 kd protein, IP10)*. Furthermore, the T cells-chemoattractant *CXCL12 (stromal-cell derived factor, SDF1)* is expressed by the epithelial cells of both SS subjects and healthy controls.[49]

Another immunoregulatory molecule produced by the SGECs of SS patients is the **B cell activating factor (BAFF)**, a member of the TNF superfamily.[61, 69, 70] BAFF is a cytokine exerting crucial role in B-cell differentiation, survival,[71] ectopic GC-like structure organization[72] and autoantibody synthesis.[73] The link of BAFF to autoimmunity was proven over 15 years ago, when Mackay et al[74] showed that BAFF transgenic mice exhibited manifestations, e.g. elevated levels of RF, circulating immune complexes and anti-DNA autoantibodies, reminiscent of a SLE-like disorder or SS. Polymorphisms in the BAFF 5' regulatory gene region have been related to Ro/SSA and La/SSB autoantibodies seropositivity among pSS patients,[75] while the incidence of

several BAFF polymorphisms has been recently associated with a subgroup of SS patients with higher susceptibility for lymphoma development.[76]

2.5.1.1.3 Autoantigen presentation to the immune system

The ability of the salivary gland epithelium to release intracellular molecules and present them to the immune system is based on two pathways. Firstly, the SGECs express numerous proapoptotic molecules, such as Fas, Fas liganda and Bax, as well as antiapoptotic molecules, including the cellular FLICE-like inhibitory protein (c-FLIP) and B cell lymphoma-2 (bcl-2) protein, which mediate apoptosis and tissue-repair, respectively. The increased **apoptosis** of SGECs and the subsequent exposure of apoptotic bodies containing endogenous nucleic acids or autoantigens might trigger the in-situ immune response.[6, 47] The second mechanism of autoantigen presentation involves the **exosomes**, which are small endosomal vesicles (30-100 nm) that are released in the cellular membrane and transfer the autoantigenic molecules to the APCs.[77] Exosomes secreted from cultured SGECs have been reported to contain well-known protein targets of autoimmune diseases, such as the Ro/SS-A, La/SS-B proteins and serve as an alternative autoantigen presentation pathway to the immune system.[19] Further support of the epithelial-lymphocytic interaction comes from current data that provide evidence of a B cell-induced apoptosis of SGECs via the activation of kinase Cδ.[78]

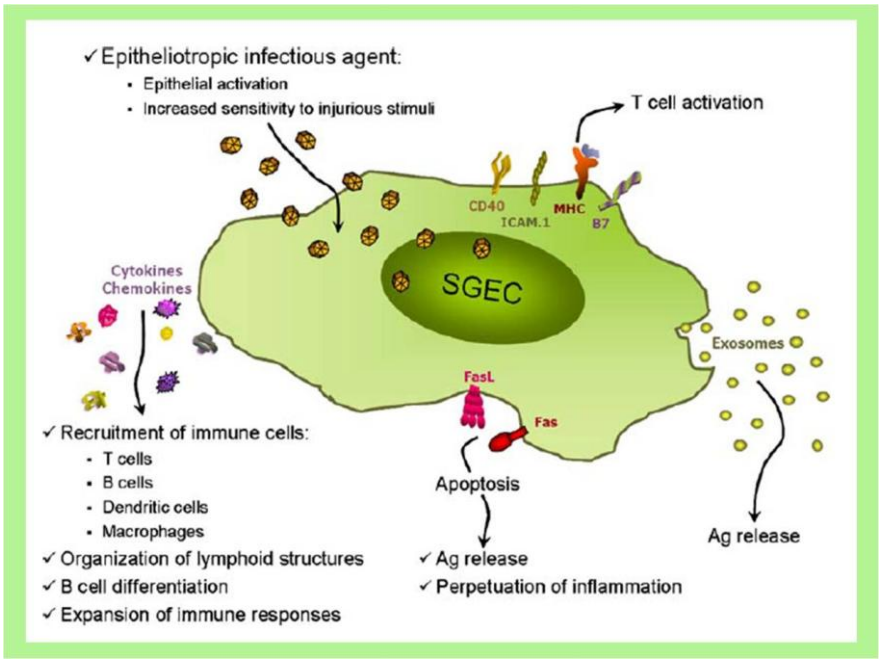


Fig.2 The role of the SGECs in SS pathogenesis.(adopted by[48])

2.5.2 The inflammatory milieu

The immune response in SS patients is mediated by an activated inflammatory milieu that consists of various cellular populations and the cytokines and chemokines produced by them. The major contributors to SS pathogenesis are the self-activated T cells and B cells and their subsets. Macrophages, DCs and NK cells are also a source of various cytokines and chemokines, which are also involved in antigen presentation, inflammatory cells recruitment, T cell activation and cytotoxic effects.[41] An imbalance of the cytokine profile in SS has been observed. As has been shown, pro-inflammatory cytokines, including Interferon (IFN)α and γ, tumor necrosis factor (TNF) α, interleukin (IL-)12 and IL-18, cytokines involved in chronic inflammation, such as IL-1β, IL-17 and IL-23, as well as cytokines inducing T cell and B cell activation and autoantibody production, such as IL-6 and BAFF are overexpressed in SS SGs. On the contrary, anti-inflammatory cytokines, including IL-4 and transforming growth factor (TGF) β, are decreased.[79] Overexpression of several CCL and CXCL chemokines, such as CCL2, CCL3, CCL4, CXCL8, CXCL10 and CXCL11 has been also identified in the SGs of pSS.(reviewed in[80]) Finally, the concomitant activation of the innate and adaptive immune system results in the elevated expression of IFN-regulated genes, immunoglobulins and autoantibodies in the peripheral blood and MSGs of SS patients.[79]

2.5.2.1 T cells

The T cells are mainly classified in T helper 1 **(Th1)** and **Th2 cells**, based on the production of cytokines and chemokines, such as the pro-inflammatory cytokines IFN-γ and TNF-α. An imbalance in the Th1- and Th2-associated cytokines ratio has been closely related to the pathogenesis of SS and other autoimmune diseases.[81] Another CD4+ subtype, the **Th17 cells**, and the cytokines released by them, such as the IL-17, have also been found to be involved in SS lesions, especially of advanced severity. IL-17 is also produced by recently identified T cell subpopulation, the (CD4- and CD8-) **double-negative (DN) T cells**. Additionally, the CD4+T-cell subset that plays a critical role in the formation of GCs in secondary lymphoid organs, the **follicular Th cells (T_{FH})**, has also been found to mediate the GC-like structure forming in the MSGs of SS patients.[33] T_{FH} are the main source of IL-21. Increased levels of circulating IL-21 have been observed to be involved in ectopic GC-like formation and B cell differentiation into plasma cells in SS patients.[6] Several T_{FH} markers have also been identified

immunohistochemically in MSGs lesions of SS patients, especially of advanced severity and higher focus score, including CD84, a member of the signaling lymphocytic activation molecule family that contributes to the adhesion interactions between T cells and B cells; the programmed cell death protein-1 receptor that regulates the B cells' survival within the GCs; and the (Bcl-6) molecule that is predominantly expressed by GCs centroblasts. The synergistic effect of FDCs and T_{FH} cells induce the positive selection and Ig CSR of centrocytes, as well as their differentiation into high-affinity memory B cells and plasma cells with long lifetime.(summarized in[33])

2.5.2.2 B cells

B cells predominate in severe histopathological MSGs lesions in SS patients.[29] The primary role of B-cell hyperactivation in SS pathogenesis is highlighted by the overexpression of autoantibodies, including anti-Ro/SSA and anti-La/SSB, hypergammaglobulinemia, formation of ectopic GC-like structures, as well as by the oligoclonal B cell proliferation and the development of lymphoma.[32] B cell subpopulations are also differentially distributed in the peripheral blood and MSGs of SS patients during B cell maturation. Naïve B cells are driven by Th1 and Th2 to become polarized **in B effector 1 and 2 cells** respectively. Be1 cells produce IFN-γ and IL-2 and Be2 cells are sources of IL-4 and IL-6, while both Be subsets express IL-10, lymphotoxin (LT)-β, TGF-β and TNF-α. Cytokines are also released by the recently identified **B regulatory cells**. Accumulated CD27− **memory B cells** have been observed in inflamed SGs, in contrast to the decreased levels of peripheral CD27− memory B cells, as well as peripheral or salivary CD27+ memory B cells. Moreover, as B cells are organized in a central B zone in advance MSG lesions, CD27+CD138− and CD27+CD138+ **plasma cells** have been identified within or in the periphery of the B cell zone respectively. In contrast to the normal glands, in which IgA immunoglobulins prevail, the activated plasma cells in SS MSGs release predominantly IgG and IgM immunoglobulins.[6, 32, 82]

2.5.2.3 APC cells

Macrophages express several cytokines that have been found to be overexpressed in SS, including IL-10, IL-12, IL-18 and IL-34. Macrophages are mainly found in MSGs lesions of advanced severity, while low levels of IL-12 and high levels

of IL-18 have been associated with adverse prognostic factors for lymphoma development.[6, 29]

The most significant effect of dendritic cells (DCs) in SS is the expression of IFNα by pDCs. All nucleated cells are characterized by an IFNα production capacity, but the **pDCs are the predominant IFNα source**, contributing to a 1000 times more production of type I IFNs compared to the remaining cells. pDCs are considered a hallmark in SS patients, based on immunohistochemical studies that have revealed pDCs' expression in the MSGs of pSS patients but not in controls.[83] It has also been suggested that pDCs may be a sign of local activation within the salivary glands, as they have been found in decreased levels in the peripheral blood of pSS patients.[43] Circulating pDCs in pSS subjects, though, have been found to express elevated levels of the CD40 salivary gland activation marker compared to controls.[84]

2.5.2.4 IFN signature in SS

Mounting evidence during the last decade supports the activation of IFNs as a prevailing event in SS development. In brief, IFNs are proteins that participate in innate *(type I and III IFNs)* and adaptive *(type I, II and III IFNs)* immune defensive mechanisms, induced by endogenous or exogenous trigger factors and most commonly in response to viral infections. The predominant role of ***type I IFNs*** in SS pathogenesis has been well documented in many studies. Current reports suggest that ***type II IFNs*** are also involved in SS, with ***type I*** and ***II IFNs*** being associated with distinct clinical phenotypes among SS patients. The production of type I IFNs, especially **IFNα**, is stimulated by a viral infection through two receptor systems: **a) TLRs** and **b) cytosolic RNA or DNA sensors**.(reviewed in[58, 82, 85])

With regards to ***type I IFNs signaling pathway***, the binding of ***type I IFNs*** to their membrane receptor ***(IFNAR)*** activates the *Janus activated kinases* that further activate the transcription factors of the *signal transducer and activator of transcription* pathway. This leads to the expression of downstream molecules in the cytoplasm, where they form complexes, such as the *IFN stimulated gene factor 3* and the *IFNA activated factor*. These molecular complexes enter the nucleus in order to bind to specific genomic regions within the promoters of the **IFN-inducible genes**, including the ***APOBEC3*** genes, to induce their transcription. Gene expression profile studies have identified the **overexpression of numerous IFN-inducible genes** in MSG tissues and

peripheral blood of SS patients that is broadly known as ***"IFN signature (ISG)"***. Except for the pDCs, the **SGECs of SS patients have also been reported to produce IFNα.**(reviewed in[58, 82, 85]) The exact trigger factor, viral or not, of ***type I IFNs signaling pathway*** in SS, though, remains to be elucidated. Mavragani and Crow[58] have proposed a model of ***type I IFNs signaling pathway*** **activation** by the excessive expression of the viral-like ***Long Interspersed Nuclear Elements (LINEs, L1s)*** in the salivary gland cells, which will be discussed in detail in a separate chapter. As presented in fig. 3, once the type I IFNs are expressed, they may exert their variable immunomodulatory effects, leading to the activation of the inflammatory milieu and the antibody production.[58, 86]

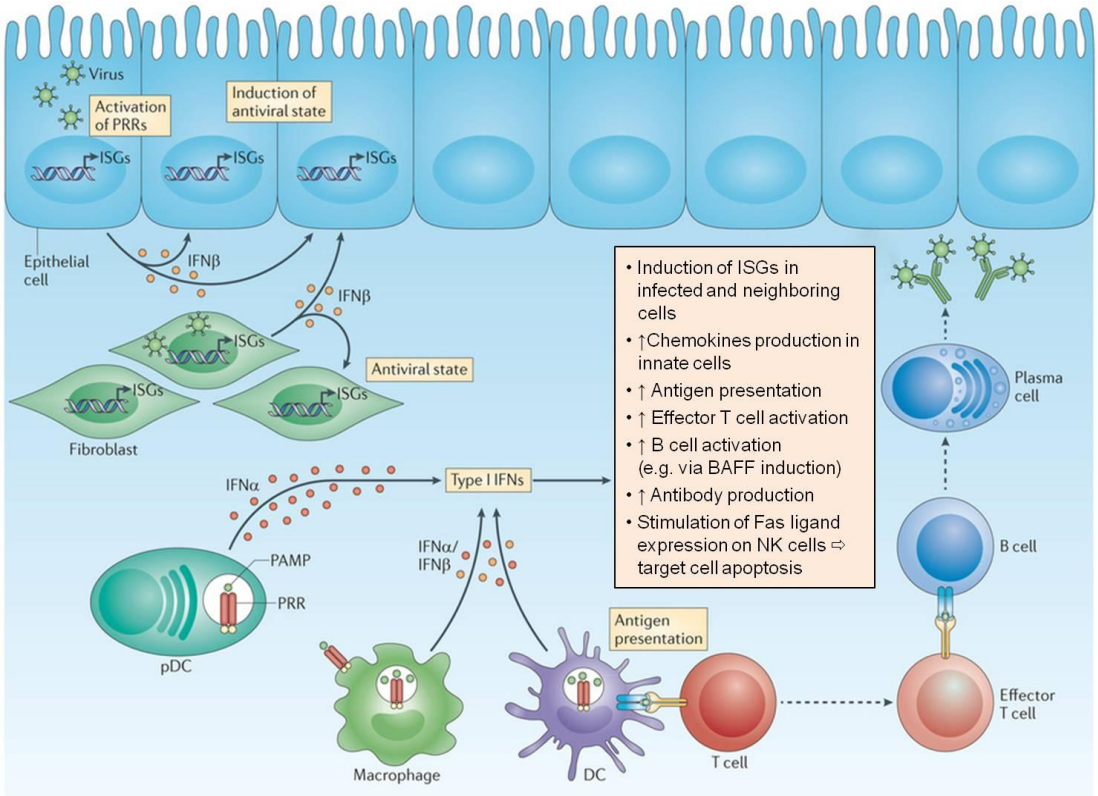


Fig. 3. The immunomodulatory effects of type I IFNs.(adapted by[86])

2.5.3 Genetic factors

The higher incidence of SS and other autoimmune disorders among monozygotic twins or family members compared to healthy controls suggests a genetic background that predisposes to SS. In a recent review of the literature, Nezos and Mavragani[87] summarized the genes that have been reported as predisposing factors for SS development, which can be divided in **a) genes within the MHC/HLA locus** and **b) genes outside the MHC/HLA locus**. Regarding the first category, a significant association with increased susceptibility for SS has been reported for the MHC class II alleles that encode the HLA-DR and HLA-DQ antigens. The genetic variants outside the MHC/HLA locus include gene polymorphisms that are involved in signaling pathways of the innate and acquired immune response, especially in the IFN signaling pathway, implicated in B cell activation, function and autoantibody synthesis or in cellular apoptosis mechanisms (Table 4).[87, 88]

Table 4. Genes associated with increases SS susceptibility.(adapted by[87, 88])	
A) Genes within the MHC/HLA locus	
MHC class I *(HLA-B8)* ***MHC class II*** *(HLA-DR2, DR3, DR5, DR9, DR1, Dw2, Dw3, DRw52, DRw53, Cw7, DQA1∗0501-DQB1∗0201-DQA1∗0301, DRB1∗0803-DQA1∗0103-DQB1∗0601, DRB1∗03-DRB3∗0101-DQB1∗0201-DQA1∗0501, DR3-DQA1∗0501-DQB1∗02, DRB1∗0405-DRB4∗0101-DQA1∗0301-DQB1∗0401, DQA1∗001-DQA1∗0201-DQB1∗0501, DQA1∗0501, DRBI∗1501∗-0301-DQB1∗0201∗-0602, DRB1∗03-DQB1∗02-DQA1∗0501, DRB1∗0301, DRB1∗03, DQB1∗0201-DRB1∗03-DQB1∗0501, DQA1∗0501-DQB1∗0201-DRB1∗0301-DQA1∗0201-DQA1∗0301-DQB1∗0501)*	
B) Genes outside the MHC/HLA locus	
i) Genes associated with the IFN signaling pathway	• *Interferon Regulatory Factor 5 (IRF5)* • *Signal Transducer and Activator of Transcription 4 (STAT4)* • *Interleukin 12A (IL12A)* • *Natural Cytotoxicity Triggering Receptor 3 (NCR3)* • *Protein Tyrosine Phosphatase Nonreceptor 22 (PTPN22)*

ii) Genes associated with B-cell activation and autoantibody production	• *BAFF* • *B-Lymphocyte Kinase (Blk)* • *Chemokine (C-X-C Motif) Receptor 5 (CXCR5)* • *Early B-Cell Factor 1 (EBF1)* • *Ox40 Ligand/Tumor Necrosis Factor Superfamily 4 (Ox40L/TNFSF4)* • *General Transcription Factor 2I (GTF2I)*
iii) Genes associated with the NF-κB signaling pathway	• *Tumor Necrosis Factor-Alpha Induced Protein 3 (TNFAIP3)* • *TNFAIP3-Interacting Protein 1 (TNIP1)* • *Lymphotoxin Gene A (LTA)* • *Chemokine (C-C Motif) Ligand 11 (CCL11)* • *BAFF Receptor (BAFF-R)*

2.5.4 Environmental factors

2.5.4.1 Viruses and viral-like elements

Viruses and viral-like elements have been considered as the potential trigger factors that may act as "intrinsic activators" of the epithelial cells in SS. The viral involvement in the disease pathogenesis is supported by the activated type I IFN pathway and the overexpression of IFN-inducible genes, including the IFN-stimulated transcription factor 3g and the IFN-induced transmembrane proteins, in SS patients. DNA viruses, retroviruses as well as ssRNA viruses have been implicated in SS (Table 5). Among these factors, the ***Epstein–Barr virus (EBV)***, the ***Coxsackieviruses*** and the endogenous ***intracisternal A-type retroviral particles (IAPs)*** have attracted the greatest interest as potential triggers of SS.[89] ***IAPs*** are endogenous non-infectious retrotransposons, antigenically related to human immunodeficiency virus (HIV), that induce the formation of immature, intracellular virus-like particles.[6, 47] Retrotransposons are mobile elements that will be discussed in detail in a separate chapter. Serum antibodies to ***IAP***-associated proteins have been found in patients with SS or other autoimmune diseases.[90] ***IAPs*** have also been observed by electron microscopy in the SGECs of pSS patients,[91] as well as in a T-lymphoblastoid cultured cell-line that had been exposed to salivary gland tissue homogenates derived from pSS patients.[92]

Table 5. Viruses that have been associated with SS pathogenesis.	
DNA viruses	Cytomegalovirus,[93, 94] EBV[95-99]
Retroviruses	Human T lymphotropic Virus Type 1 (HTLV-1),[100] HIV-1,[101, 102], Human Retrovirus-5 (HRV-5)[103]
Retrovirus-like elements	IAPs, L1s[58]
ssRNA viruses	Hepatitis C virus (HCV),[104, 105] Coxsackievirus[106]

2.5.4.2 Hormonal and psychosocial factors

The contribution of ***hormonal factors*** in SS pathogenesis has been hypothesized due to the disease strict predilection for females in the perimenopausal period.[tz, Mav] This hypothesis has further been supported by data from estrogen-deficient mouse models that have developed an SS-resembling autoimmune exocrinopathy,[107-109] as a result of an increased estrogen inadequacy–dependent apoptosis of their salivary gland epithelium.[107] Low levels of androgens, mainly of dihydrotestosterone, also detected in the serum[110, 111] and saliva[111] of SS patients and their deficiency has been correlated with more severe dry mouth manifestations.[110] ***Psychosocial factors*** may also promote SS development, as stressful events have been reported to precede SS onset, especially in patients with a lower adaptability and limited a social support.[112]

2.5.5 Epigenetic factors

The term "epigenetics" describes the inheriTable changes in gene expression that do not presuppose alterations in the DNA sequence. Epigenetic mechanisms are crucial during the cell cycle, differentiation and response to endogenous or environmental factors[113-115] and affect genes at the level of transcription (by ***DNA methylation*** and ***histone modifications***) or regulate the gene expression via posttranscriptional [by the ***microRNAs (miRNAs)*** expression] or posttranslational modifications. ***DNA methylation*** refers to the addition of a methyl-group to the cytosine residues of the cytosine-guanine (CpG) pairs, resulting in changes in the chromatin structure and gene silencing. CpG dinucleotides are accumulated within genes in particular regions termed CpG islands, which are methylated or hypomethylated in silenced or transcriptionally active genes respectively.[113] ***MiRNAs*** are small non-coding

RNA molecules, approximately 20-24 nucleotides in length, which participate in posttranscriptional regulation of genes by promoting mRNA degradation or translation interruption. **Dysregulated epigenetic mechanisms have been associated with the development of autoimmune diseases, including SS**.[reviewed in [114]] Defects in ***DNA methylation*** and ***microRNAs*** expression have been reported in SS (Table 6), whereas the potential role of histone modifications remains unclarified.[113-115]

Table 6. Defective epigenetic mechanisms in SS.

DNA methylation	• DNA ***demethylation*** in **acinar SGECs** in correlation with increased lymphocytic infiltration and anti-La/SSB antibody detection[116] • DNA ***hypomethylation*** in **SGECs** via downregulation of the *PKC-delta/Erk/DNMT1* pathway and upregulation of the *Gadd45alpha*[117] • DNA ***hypomethylation*** of IFN-regulated genes in **CD19+ B cells**[118] • more frequently altered DNA methylation status in blood **CD19+ B cells** than **CD4+ T cells**[119] • DNA ***hypomethylation*** of genes that influence lymphocyte activation, leukocyte differentiation, immune response and chromatin organization; DNA ***hypermethylation*** of genes involved in antigen processing and presentation in **CD4+ T cells**[120] • DNA ***hypermethylation*** of the promoter of the FOXP3 transcription factor in **CD4+ T cells**[121] • DNA ***hypomethylation*** of *CD70(TNFSF7)* in **CD4+ T cells**[122]
miRNA expression	**upregulated miRNAs** (miR-146a, miR-181a, miR-768-3p, mir-200b, mir-223, mir-16), **downregulated miRNAs** (miR-574, miR-17-92, let-7b, miR-155)[123-128]

2.6 Diagnosis

In 2002 the American-European Consensus Group (AECG) revised the classification criteria for SS including two subjective and four objective items.[17] Although in 2012 the Sjögren's International Collaborative Clinical Alliance (SICCA) group proposed a modified set of three criteria that were approved by the American College of Rheumatology (ACR),[16] the AECG criteria continue to be broadly adopted and have been cited more than 1500 times in the pertinent literature.[129] Table 7 summarizes the AECG and ACR criteria. The two sets share almost common exclusion criteria, presented in Table 8.

The newly proposed criteria have omitted the symptoms of ocular and oral dryness that are the basic complaints of sicca patients, as well as the functional and morphological tests for the evaluation of the salivary glands involvement. This modification, though, may underestimate the frequency of SS among patients with subjective and objective dry mouth and consequently exclude them from clinical trials for SS treatment.[129] The salivary gland ultrasonography is also nowadays considered a valuable diagnostic tool for patients suspicious for SS, with a sensitivity and a specificity of 63% and 95% respectively that may contribute to the diagnostic methodology for SS.[130] The ACR criteria also introduced a new ocular staining score (OSS) for the objective assessment of ocular involvement and rejected the widely used tests, especially the simple and of good specificity Schirmer's test.[130] Moreover, in the new set of diagnostic criteria, the RF positivity combined with an ANA titer of less than 1:320 is considered equivalent to the anti-Ro/SSA or anti-La/SSB positivity. However, RF and ANA show a lower specificity and this revised criterion may intensify difficulties in the accurate disease classification, especially among patients with SS associated with other autoimmune diseases.[129, 130] The aforementioned reasons have led to an international debate about which criteria should be applied in sicca patients and warrant the need for the development of new criteria with a broader consensus.[129-132]

Table 7. AECG and ACR-SICCA criteria for the diagnosis of SS.		
	AECG revised criteria[17]	**ACR criteria**[16]
Criteria	I. Ocular dryness symptoms II. Oral dryness symptoms III. Ocular signs 1. Schirmer's test ≤5 mm/5 min **OR** 2. Rose bengal score or other ocular dye score (≥4 according to van Bijsterveld's scoring system) IV. Focus score ≥1 focus/4 mm^2 on minor salivary gland biopsy V. Presence of positive anti-Ro/SSA **OR** anti-La/SSB antibodies in the serum, **OR both** VI. Salivary gland involvement 1. Unstimulated whole salivary flow (≤1.5 ml in 15 minutes) **OR** 2. Parotid sialography showing the presence of diffuse sialectasias, without evidence of obstruction in the major ducts **OR** 3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer	I. Keratoconjunctivitis sicca with OSS ≥3,[1] for subjects that do not currently use daily eye drops for glaucoma and did not have corneal surgery or cosmetic eyelid surgery in the last 5 years II. Focus score ≥1 focus/4 mm^2 on minor salivary gland biopsy III. Positive anti-Ro/SSA **OR** anti-La/SSB antibodies **OR** [positive RF **AND** ANA ≥1:320] in the serum
Rules for SS diagnosis	• 4/6 items or 3/4 objective items (III, IV, V and VI) for ***pSS*** • [item I **OR** II] **AND** 2/3 items (III. IV, VI) for **SS *associated with other diseases***	2/3 items must be fulfilled in a SS suspected patient

Table 8. Exclusion criteria for the diagnosis of SS.	
AECG revised criteria[17]	**ACR criteria**[16]
Past head and neck radiation treatment	history of head and neck radiation treatment
Hepatitis C infection	Hepatitis C infection
Acquired Immunodeficiency Syndrome (AIDS)	AIDS
Sarcoidosis	Sarcoidosis
Graft versus host disease	Graft versus host disease
Pre-existing lymphoma	Amyloidosis
Use of anticholinergic drugs (for a time shorter than 4-fold the half life of the drug)	IgG4-related disease
	participation in SS studies or therapeutic trials because of overlapping clinical features or interference with criteria tests

It is worth noting that the focus score of ≥1 focus/4 mm^2 in the labial MSG biopsy is the only constant item in both AECG and ACR criteria sets. Current literature suggests that the labial MSG biopsy shows high levels of specificity[133, 134] and sensibility,[133] as well as a positive[133, 134] and negative[133] predictive value to diagnose SS. MSG biopsy is also helpful in the differential diagnosis of SS from other disorders, such as IgG4-related disease, while it is of greatest importance for the confirmation of lymphoma development not only at the time of the initial SS diagnosis but also during the follow-up of SS patients.[36, 135] In the study by Kapsogeorgou et al[36] who evaluated the repetitive MSG biopsy specimens from 28 pSS patients, 5/28 (17,9%) developed a MALT lymphoma during a follow-up period of 36 to 64 months. In all these 5 cases, the diagnosis of the B cell malignant transformation was rendered in the second MSG biopsy by the immunohistochemical and/or molecular detection of B cell monoclonality.[36]

2.7 Prognosis

Studies examining the survival rates of SS patients have revealed a benign prognosis in general, with a minimally elevated mortality risk in comparison to the general population.[9] In the largest study of 1045 pSS patients who were followed up for a mean period of 117 months, an overall standardized mortality ratio of 4.66 was observed.[136] However, approximately 1 out of 3 SS patients is expected to develop severe systemic manifestations, with the most important complication being the development of a non-Hodgkin's lymphoma (NHL). Beyond the NHL development, systemic features that increase the mortality rate of SS patients include interstitial lung disease, renal failure, grievous cryoglobulinemic vasculitis, cardiovascular disease and infections.[9] Table 9 summarizes the clinical, hematological, serological, histological and molecular factors that have been reported as adverse predictors of lymphoma development in the context of pSS.(reviewed in[9, 137])

Table 9. Risk factors for lymphoma development in pSS.(adapted by[9, 137])

Clinical factors	• Persistent lacrymal or salivary gland enlargement
	• Skin vasculitis or palpable purpura
	• Peripheral neuropathy
	• Lymphadenopathy
	• Glomerulonephritis
	• Splenomegaly
Serological-hematological factors	• ↓ serum C3 or/and C4 levels
	• CD4 T-cell lymphopenia
	• Neutropenia
	• Monoclonal serum component
	• ↑ serum free light chain ratio
	• ↑ beta2-microglobulin levels
Histopathological factors	• ↑ biopsy focus score
	• Ectopic GC-like structure formation
	• ↑ DCs and macrophages infiltration rate
	• ↑ IL-18/IL-12 expression
	• ↑ AID expression
Molecular-biologic factors	• ↑ Serum BAFF levels

	• BAFF genetic variations • ↑ Serum Fms-like tyrosine kinase 3 ligand levels • A20 genetic variations • p53 mutations • Peripheral B-cell subpopulation disturbances

The two most frequent NHL subtypes among SS patients are the MALT and the diffuse large B-cell (DLBC) lymphoma. SS patients show a 44-time higher relative risk for lymphoma development compared to the general population.[138] It has been estimated that 5–10% of pSS patients may develop NHL.[9] In particular, it may take a median time of 5.2 years for MALT lymphoma and 8.1 years for DLBC lymphoma to occur since the initial pSS diagnosis.[139] It should also be noted that pSS patient exhibit the highest risk for lymphoma development among patients with autoimmune diseases, such as SLE or RA.[140, 141] The outcome of SS-complicated lymphoma has been related to the lymphoma subtype and the disease activity, as it is determined based on the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) score. A worse overall survival has been observed in SS patients with DLBC lymphoma compared to MALT lymphoma, as well as in SS patients with a higher disease activity (ESSDAI score ≥10).[139]

2.8 Treatment

The current treatment approaches for SS patients include topical and systemic agents aiming mainly to alleviate the mucosal dryness and prevent its complications. The administration of saliva substitutes is the first line of treatment for oral dryness. These agents may improve symptoms of oral discomfort, burning sensation and difficulty in mastication and swallowing, without, though, improvement in salivary flow.[14, 142] Systemic therapies have also been developed for the management of the extraglandular manifestations present in a remarkable SS subgroup. These therapies are predominantly based on immunosuppressive agents, while the identification of the B-cell hyperactivity in SS has led to the development of antibodies that act antagonistically to B-cells, such as *rituximab*, a monoclonal antibody against CD20.[14, 142-144] Table 10 summarizes the therapeutic options that have been reported in SS patients with glandular or systemic manifestation.

Table 10. Treatment of glandular and systemic manifestations in SS.(adapted by[5])	
Clinical manifestation	**Treatment approach**
Glandular	
Oral dryness	• Increased water intake • Stimulation of salivary flow with sugar-free gums or citrus juice • Administration of saliva substitutes • Proper oral hygiene • Regular dental follow-up • Avoidance of smoking, alcohol and drugs, such as diuretics, antidepressants and antihistamines • Pilocarpine (10–30 mg /day) or Cevimeline (30 mg, 3 times/day) in patients with residual salivary gland function
Ocular dryness	• Preservative-free teardrops • Eye lubricants with sodium hyaluronate or hydroxy-propyl methylcellulose • Steroid-containing ophthalmic solutions • Cyclosporine drops (0.05%)
Vaginal dryness	• Vaginal lubricants
Painful salivary gland enlargement	• Local administration of moist heat • Non steroidal anti-inflammatory drugs
Non specific	
Musculoskeletal manifestations	• Non steroidal anti-infammatory drugs • Hydroxychloroquine, • Methotrexate
Raynaud's phenomenon	• Avoidance of cold and stress • Calcium-channel blockers
Fatigue	• Antidepressants • Exercise
Periepithelial	
Interstitial nephritis-tubular	• Oral potassium and sodium carbonate

dysfunction, renal tubular acidosis *Bronchial or bronchiolar Inhaled therapy involvement*	• Inhaled therapy
Interstitial lung disease	• Prednisolone • Azathioprine
Primary biliary cirrhosis	• Ursodeoxycholic acid
Autoimmune hepatitis	• Prednisolone • Azathioprine
Immunocomplex-related disease	
Peripheral neuropathy	• Intravenous gamma globulin • Rituximab • Plasma exchange
Vasculitis	• Prednisolone • Cyclophosphamide • Rituximab • Plasmapheresis
Glomerulonephritis	• Prednisolone • Intravenous cyclophosphamide
Central nervous system vasculopathy	• Pulse steroids • Prednisolone • Cyclophosphamide • Azathioprine

2.9 In summary

- Sjögren's syndrome (SS) is a chronic autoimmune disease, affecting approximately 0.5-1% of the general population, with a clear predilection for middle-aged female patients.
- SS predominantly affects the salivary and lacrimal glands, resulting in oral and ocular dryness, but may also manifest with a wide range of systemic (extraglandular) clinical features.
- The hallmark characteristics of SS as an autoimmune disease are **a)** a focal mononuclear cell infiltration of the exocrine glands in close proximity to the ductal epithelium *(autoimmune epithelitis)*, that results in the destruction of the glandular parenchyma and **b)** B-cell hyperactivity and production of circulating autoantibodies against various autoantigens, such as the ribonucleoprotein complexes Ro/SSA and La/SSB.
- The most important complication of SS is the development of non-Hodgkin lymphoma in about 5-10% of patients. Several clinical, serological, histopathological and molecular risk factors for lymphoma development have been described in the context of SS.
- Topical and systemic agents are prescribed as palliative treatment of the mucosal dryness, while various systemic therapies are used for the management of the extraglandular manifestations.
- A multifactorial pathogenetic model has been proposed for SS, in which local immunological factors, i.e. the epithelial and inflammatory cells and the produced cytokines and chemokines interact with various environmental, genetic and epigenetic factors to induce SS.
- Type I and II Interferons (IFNs) are involved in SS pathogenesis. An overexpression of numerous **IFN-inducible genes**, including the ***APOBEC3*** genes, has been observed in the minor salivary glands and peripheral blood of SS patients ("IFN signature").
- The viral-like ***Long Interspersed Nuclear Elements (LINEs, L1s)*** are considered potential trigger factors of the ***type I IFNs signaling pathway*** in SS.

3 LONG INTERSPERSED NUCLEAR ELEMENT 1 (LINE-1, L1)

3.1 L1, a member of the transposable elements family

The *Long Interspersed Nuclear Elements-1 (LINEs-1* or *L1s)* are highly repetitive DNA sequences found in all mammalian genomes. L1s belong to a wide gene family, termed *mobile* or *transposable elements (TEs)*, due to their capacity to "jump" into various genomic locations, promoting reshaping and diversity of the human genome. TEs constitute approximately 50% of genomic DNA.[145] Barbara McClintock in 1956 was the first to describe TEs as "controlling elements", as they were found to transpose within chromosomes, while their position affected the activity of their neighbor genes.[146] Nowadays, TEs are considered intrinsic mutagenic factors, due to their ability to insert in new genomic locations and induce chromosomal rearrangement through homologous gene recombination.[147]

TEs are roughly classified as DNA transposons and retrotransposons (or retroelements). DNA transposons encompass almost 3% of the human genome[145] and transpose through a *"cut-and paste"* mechanism, which involves the excision of these elements from their initial localization by a DNA intermediate, named DDE transposase, and their insertion in a new genomic site.[148] Retrotransposons represent the largest subcategory of TEs, accounting for about 42% of the human genome.[145] They mobilize through a *"copy and paste"* mechanism facilitated by an RNA intermediate, i.e. a reverse transcriptase that catalyses the reverse transcription of the RNA transcripts into cDNA, to give origin to new retrotransposons. The nascent retroelement duplicate is excised and integrated into a new genomic site, while, in contrast to DNA transposons, the parental retrotransposon remains in its original location in the human genome.[148] Retroelements are subdivided structurally, based on the presence of long terminal repeats (LTR) into LTR- and non-LTR retrotransposons respectively.[147] Human LTR retroelements are retrovirus-like elements (endogenous retroviruses, ERVs), which represent 8% of the human genome and are considered in general retrotransposition-incompetent, although some retrotransposition capacity has been attributed to HERV-K.[145] According to their transposition mode, human non-LTR retrotransposons are further distinguished into the ***autonomous LINEs*** that encode the necessary proteins for their retrotransposition, and the *short interspersed nuclear elements (SINEs)*, including Alu element, SVA, MIR and MIR3, that have no protein

coding capacity ***(non-autonomous retrotransposons)***.[145, 149] Accounting for about 21% of the human genome,[145] LINEs drive SINEs retrotransposition *in trans*,[150, 151] while their interaction also results in the formation of non-autonomous pseudogenes.[152, 153] Among human LINEs, **L1 is the most abundant (~20% of the human genome)** and the only retrotransposition-competent element, while LINE-2 (L2) and LINE-3 (L3) are inactive.[145] The human genome of elements comprises numerous L1 subfamilies, some of which have been found active, e.g. pre-Ta (i.e. transcriptionally active), Ta-0, Ta-1d, Ta-1nd, resulting in the generation of more than 35.000 L1 copies.[154, 155] Fig. 4 presents examples of the various subtypes of transposons.[145, 156, 157]

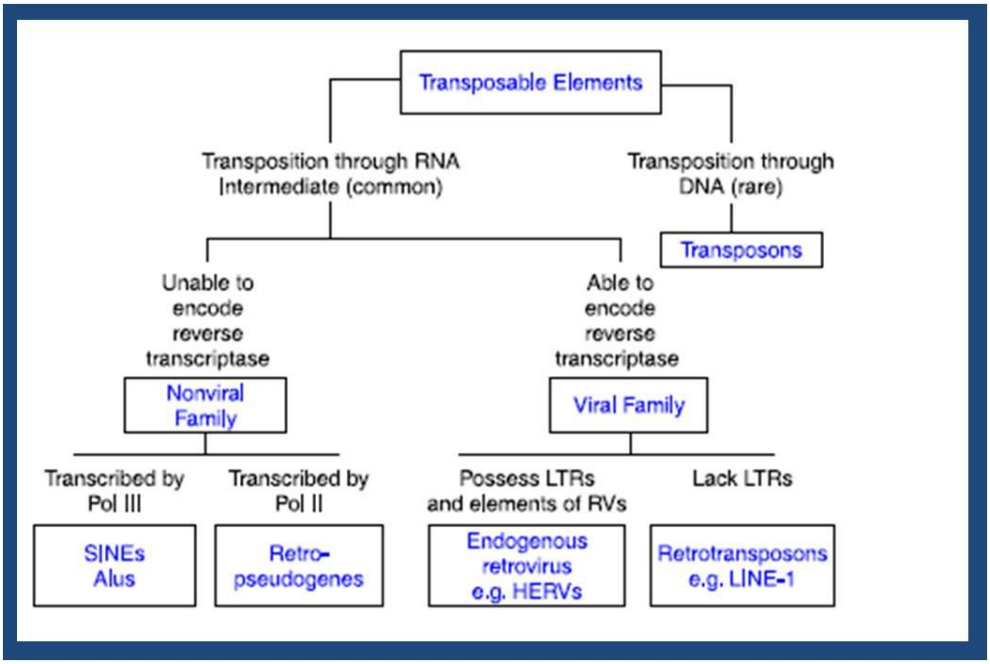


Fig. 4. Classification of transposable elements.(adapted by[157])

3.2 L1 structure

Although human L1 elements have been evolving for approximately 100 million years,[158] they were initially identified by Adams et al. in 1980 near the human β-globin gene.[159] L1 elements constitute a large subclass of about 516000 copies found within the human genome, accounting for nearly 20% of the genomic DNA.[145] Active human L1 is approximately 6.0 kb in full length and consists of a 5'-untranslated region (UTR), two non-overlapping open reading frames (ORF1 and ORF2), separated by an intergenic ORF region, and a 3'-UTR, terminating in a adenine-rich tail *(poly-A-tail)*,[160],[161]. Additionally, L1 is flanked by short direct repeated sequences of up to 20 bp in length, termed *target site duplications (TSDs*) (fig.5).[160]

Human **L1 5' UTR** is about 910 bp in length and harbors within its first 100–150 bp an internal sense promoter with RNA polymerase II activity that contains the essential genomic sequences to drive the initiation of L1 full-length mRNA transcription, either at the level of the first L1 nucleotide or upstream to this,[162] in sites between nucleotide -9 and nucleotide +4.[163] Apart from that internal promoter, the CpG-rich human L1 5' UTR includes around nucleotide +500 an antisense promoter that also exhibits RNA polymerase II activity and, working into the opposite direction to the sense promoter, mediates the transcription of several genes adjacent to L1.[164-166]

The **first human L1 ORF** encodes the multidomain polypeptide **ORF1p**, (also termed p40) a ~40 kDa protein of 338 amino acids.[167-169] ORF1p consists of three distinct domains:[170] **a)** an ***amino (N)-terminal domain*** with a leucine zipper motif-containing alpha helical *coiled coil (CC),*[171] which facilitates ORFp1 trimerization[168] and ORFp1-ORFp1 protein interplay;[169] **b)** a ***central non-canonical RNA recognition motif (RRM)***;[170] and **c)** a basic, conserved ***carboxyl (C)-terminal domain (CTD)*** showing the nucleic acid binding- and nucleic acid chaperone activities that characterize ORF1p.[167] The RRM and the CTD domain attach to the N-terminus, so that the coordinated trimeric structure mediates retrotransposition.[168] The interaction of ORF1p with its encoding RNA *(cis preference)* results in cytoplasmic foci formation, regardless the ORF2p presence.[172] The CTD domain activity induces the generation of these cytoplasmic foci, while mutations in the RRM domain residues influence unfavorably the L1 retrotransposition.[173]

The **intergenic ORF region** of 63 bp length includes 33 nucleotides and at least two termination codons, being able to code for a third ORF of 6 amino acids,[160, 174] while it is not implicated in ORF2 translation.[174]

The **second human L1 ORF** encodes the protein **ORF2p**, a ~150 kDa protein of 1275 amino-acids. ORF2p constitutes of three domains: **a)** a ***N-terminal domain*** (1-239 amino acids) with endonuclease (EN) activity; **b)** ***a central domain*** (~480-773 amino acids) supplying the enzymatic activity of reverse transcriptase (RT),[175] and a **c)** a ***C-terminal cysteine-rich zinc knuckle domain*** (~1130-1275 amino acids) with elusive properties, but, though, essential for retrotransposition,[176-178] possibly via its RNA-binding activity.[179] A Z domain (~380-480 amino acids) is also interposed between EN and RT domains, while mutations in that domain lead to restriction of

reverse transcriptase activity *in vitro*.[178] Based on a sequence similarity between the L1 EN and RT and the apurinic/apyrimidinic EN[180] and RT[181] of the ancestral eukaryotic host cells, respectively, a possible origin of the ORF2p domains from these cells has been hypothesized.

Mutations to the ORF2p EN domain adversely affect the ability of ORF2p to cleave DNA, thus rendering L1 retrotransposition-incompetent, while mutations on the ORF1p CC and RRM domains and the ORF2p cysteine-rich motif ($CX_3CX_7HX_4C$, where *X* indicates any amino acid residue) reduce ORF1p and/or ORF2p concentration in RNPs.[173, 180] In particular, ORF2p cysteine-rich domain mutations probably modify the ability of ORF2p to interplay with its mRNA and/or other factors in order to form the **L1 ribonucleoprotein particles (RNPs)**,[173] causing inhibition of L1 retrotransposition.[177] L1 RNA, ORF1p and ORF2p co-localize in RNPs, in where ORF2p is associated more strongly with its encoding RNA than ORF1p.[173, 182] Both ORF proteins have also been found to be related to the Ago2-GFP fusion protein of P-bodies and stress granules, as well as to the stress granule marker G3BP-GFP.[172, 173]

The **L1 3' UTR** is ~206 bp in length and is located between the termination codon of ORF2and the A-replete region at the 3' end of L1 element.[160, 183] The 3' UTR possesses a polyadenylation signal, which leads the RNA polymerase II to finish L1 mRNA transcription in any appropriate position, independently of the site of L1 insertion.[184] The native L1 polyadenylation signal, though, is not necessary for L1 retrotransposition, as L1 mutants with deletions in the 3' UTR region remain retrotransposition-competent.[177, 185]

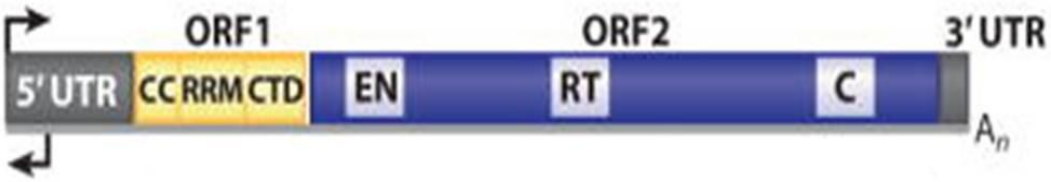


Fig.5 L1 structure.(adapted by[186])

3.3 L1 life cycle

L1 life cycle includes the sequential phases of the L1 DNA transcription, the L1 mRNA translation into ORF1p and ORF2p and the formation of L1 RNPs and, finally, the L1 retrotransposition and integration into a new genomic site (fig. 6).[187] Although retroelements are ubiquitous within the human genome, only a minority of them retains the functional activity of retrotransposition, while the remaining retrotransposons, similarly to DNA transposons, are immobilized.[154] Most L1s copies within the human genome are 5'UTR-truncated, rearranged or significantly mutated, thus they are retrotransposition-incompetent.[145] It has been estimated that about 4000 human L1 are composed of a full-length genomic sequence with intact 5'- and 3'-UTRs and absence of significant internal rearrangements,[164] 150 and 100 human L1s contain both ORFs and solely ORF2, respectively,[188] while only about 80-100 human L1 elements per individual are retrotransposition-competent.[189]

3.3.1 L1 transcription

By utilizing the 5' UTR internal promoter, L1 DNA is transcribed in the nucleus, producing a *bicistronic* L1 mRNA.[147] L1 transcription starts at base 1[162] via catalysis by the RNA polymerase II,[190] while within the 5' UTR have been recognized various binding sites of transcription factors, including the SRY-family-binding sites,[191] the RUNX3-binding site[192] and the YY-1-binding site.[193]

3.3.2 L1 translation

The L1 mRNA transcript is exported to the cytoplasm, where ORF1 and ORF2 encode for the ORF1p and ORF2p proteins, respectively.[147] Translation of ORF1p initiates from the first methionine (AUG) codon of ORF1 frame,[171, 194] while ORF2 translation is mediated via an *unconventional termination/reinitiation mechanism*, during which the ribosome continues to work beyond the ORF1 termination codon and restarts translation at the first AUG codon in ORF2 frame.[195] Both ORFs exhibit a pronounced *cis-preference*,[182] showing a predilection for association with their own encoding mRNA transcript to form the RNPs.[196]

3.3.3 RNPs formation

Human RNPs were initially recognized in teratocarcinoma cell lines[169] and are now considered a requisite RNA intermediate for L1 retrotransposition.[153, 182, 196, 197]

ORF1p is the prevailing molecule within RNPs, while ORF2p is translated in comparatively lower levels. Given that the translation of each L1 mRNA generates one or two ORF2p molecules, the difficulty to detect ORF2p in engineered L1 elements in cell cultures could be expected.[174, 195, 198] Recent studies, though, have managed to overcome this obstacle by constructing epitope-tagged engineered L1 plasmids that revealed the physical interplay between L1 RNA, ORF1p and ORF2p, thereby enhancing the hypothesis about the ORF2p presence within L1 RNPs.[173, 199] Both ORFs[177] and RNPs[196] are necessary for L1 activity, although the formation of the latter is not adequate for L1 retrotransposition.[197] Lately, Taylor et al.[200] proposed that at least three distinct types of functional L1 RNPs exist, i.e. a cytoplasmic in cells expressing only ORFp1, a cytoplasmic or nuclear in cells expressing both ORF proteins and a third one that may result from RNP content change during the L1 retrotransposition.

3.3.4 L1 retrotransposition

In cases of active L1 retrotransposons, the L1 RNP components are imported into the *nucleus*, where retrotransposition takes place by a process termed *target-site primed reverse transcription (TPRT).*[180] The nuclear transport of RNPs occurs irrespective of the cell division or the nuclear envelope breakdown[201] and has been recently assumed to be induced by a nuclear or nucleolar signal assigned to the ORF2p N-terminus.[198] During TPRT, the ORF2p EN domain nicks the first strand of the target DNA in Thymine (T)-rich consensus sequences, i.e. 5' TTTT/A 3', 5'-TCTT/A, 5'-TTTA/A etc., where the "/" corresponds to the cleavage point site, to liberate a 3′-hydroxyl residue (OH).[163, 180] This free 3′-OH is used by the ORF2p RT domain as a primer for the reverse transcription of L1 mRNA and the synthesis of the L1 complementary DNA (cDNA).[180, 182, 202] Based on models studying the R2 element from Bombyx mori,[203] the opposite strand of genomic DNA is cleaved by ORF2p EN 7 to 20 bp downstream from the first strand cut, without any sequence predilection, to provide the template for the second-strand cDNA production.[163, 202]

3.3.5 L1 integration

Retrotransposition ends up with the integration of the nascent L1 element at the target genomic site, generally flanked by TSDs. TSDs are considered structural hallmarks of the L1 insertion process, showing variable length and sequences, depending on the distance between the two cleavage positions of ORF2p EN.[163, 204] This

L1 copy may be either full-length, 5′-truncated, spliced or partially rearranged and, as a result, unable to further replicate.[155, 205]

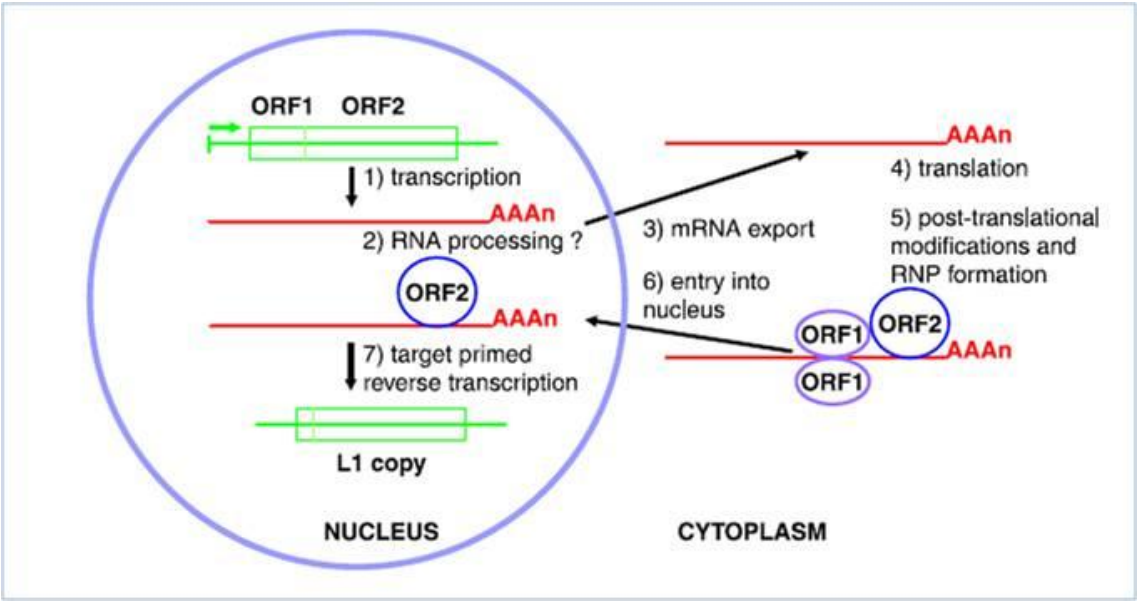


Fig. 6. L1 element life cycle.[157]

3.4 Human L1 expression

In an attempt to gain better understanding of the potential role of the L1 elements, a considerable number of studies have focused on their spatiotemporal expression in human and rodent (rat and mouse) genome. According to the review of the pertinent literature by Rosser and An [206], the experimental evidence derives mostly from research on the expression of **endogenous L1** RNA, ORF1p and ORFp2 or their overexpression within human genome (Table 11), while several studies have also included **L1 transgenic mouse models**.[207, 208]

Human L1 expression has been analyzed predominantly in experiments not only in *cell lines* or *bioptic material* from **benign or, mainly, malignant tumors,** but also in **normal human somatic or germ cell tissues**.[206] Studies aiming at the quantification of human L1 RNA used mostly the reverse transcriptase-polymerase chain reaction (RT-PCR) technique, while Northern blot analysis was preferred in surveys focusing on the detection of full-length L1 transcripts.[206] The profile of the human L1 encoded ORF1p and ORFp2 has been detected with Western blot analysis, immunostaining and immunoprecipitation studies, and more recently with epitope tagging techniques.[209] Moreover, advanced techniques implemented in engineered wild-type and mutant

human L1 elements, including enzymatic assays and epitope-tagging systems,[182, 197] as well as bioinformatics-based studies have also shed light on the wide distribution of L1 elements within the human genome.[209]

Table 11. Endogenous L1 expression within human genome.

		L1 RNA or DNA	L1ORF1p	L1 ORF2p
Normal cell lines	embryonic stem cells (ESCs)	210-213	210-212	
	mesenchymal stem cells (MSCs)	214		
	induced pluripotent stem cells (iPSCs)	211, 215, 216	211	
	fibroblasts (fetal, dermal, fetal lung)	214, 215, 217		
	immature diploid oocytes	218		
	peripheral blood lymphocytes	219, 220		
	embryonic kidney cell line	219		
	immortalized mammary epithelial cells	205		
	neuroblastoma cells	221		
	germ cells (fetal gonocytes, adult secondary spermatocytes and immature spermatids)		222	222
	Leydig cells		222	222
	Sertoli cells		222	222
	testicular covering cells		222	222
	vascular endothelial cells		222	222, 223
	placental syncytiotrophoblasts		222	222
	epididymal columnar epithelial cells		222	222
	esophagus	214	224	
	placenta	214		
	testis	214	225	223
	mammary gland		226, 227	227
	brain (fetal and adult)	212, 214, 216		

Normal tissues	skin	212	224	
	liver	212, 214		
	heart	212, 214, 216		
	synovial tissue	228		
	skeletal muscle	214		
	lung	214		
	adrenal	214		
	cervix	214		
	prostate	214		223
	bladder			223
	stomach	214		
	thymus	214		
	pancreas	214		
	spleen	214		
	ovary	214		
	kidney	214		
Cancer cell lines	immortalized T lymphoblastoid cell line (Jurkat cells)	229		
	teratocarcinoma cell lines	211, 213, 230, 231	174, 194, 211, 232-234	174
	choriocarcinoma cell line		194	
	immortalized human cervical adenocarcinoma cell line (HeLa)	205, 214, 219	173, 197, 198, 227, 235	173, 227
	bladder carcinoma cell lines	194	236	
	T-cell lymphoma cell line	194		
	breast cancer cell lines	205, 214	227, 232, 236, 237	227
	colon carcinoma cell		236	
	melanoma cell line	217, 238	236	
	fibrosarcoma cell lines		219, 236	
	osteosarcoma cell lines		173, 198, 219	173, 198
	medulloblastoma cell line	217	232	
	glioblastoma cell line	217		

	prostate cancer cell line	[238]
	pancreatic cancer cell line	[233]
	lung cancer cell line	[233]
	hepatocellular cancer cell line	[239]
Cancers	breast carcinoma	[226, 227, 233, 237, 240] [227]
	testicular carcinoma	[225, 241, 242]
	prostate carcinoma	[223]
	ovarian cancer	[225, 240, 242]
	extragonadal carcinoma	[225, 242]
	colorectal carcinoma	[233, 240]
	bladder carcinoma	[233, 240]
	pancreatic carcinoma	[233, 240]
	ileal carcinoid	[233]
	esophageal carcinoma	[224, 240]
	lung carcinoma	[240]
	head and neck carcinoma	[240]
	endometrial carcinoma	[240]
	hepatocellular carcinoma	[240]
	renal carcinoma	[240]
	cervical carcinoma	[240]
	biliary tract carcinoma	[240]
	B-cell lymphoma	[240]
	glioblastoma	[240]
	sarcoma	[240]
	astrocytoma	[240]

The wide spectrum of cell lines and tissues, in which L1s have been identified, represents potential sites for retrotransposition events. For example, the well-documented L1 expression in human germ cells coincides with the retrotransposition events occurring very early during embryogenesis,[243] even before the maternal meiosis I end.[244] In addition, concurrent expression of L1s in epithelial carcinomas and their

normal counterparts suggest that retrotransposition takes place possibly prior to malignant transformation.[245-249]

Although the L1 expression has been reported in several human exocrine glands e.g. breast[226] and prostate,[214] data regarding the salivary glands remain scarce. Sirivanichsuntorn et al.[250] found significantly lower L1 hypomethylation levels in 24 cases of mucoepidermoid carcinomas (MECs) arising in minor (21 cases) and major (3 cases) salivary glands in comparison to 14 normal salivary gland samples. This study[250] provides indirect evidence of the L1 presence in salivary glands, as L1 promoters' hypomethylation has been correlated with the expression of L1 ORF1p in several human cell lines of different origin.[232] Previously, Mavragani et al[251] had reported increased full-length L1 mRNA levels in the MSGs of low-risk for lymphoma development pSS patients and sicca controls with autoimmune disease manifestations in comparison to non-autoimmune sicca controls and healthy controls via real-time PCR.

As it can be noted in Table 11, the co-expression of both L1 ORF proteins has been analyzed only in few a studies, most of which reported lower levels of ORF2p compared to ORF1p.[173, 174, 198, 222, 227] The difficulty in ORF2p detection may be attributed to the unconventional ORF2p translation mechanism, which is characterized by a ribosome reinitiating mode of low frequency that results in low ORF2p levels (~one ORF2p molecule per L1 mRNA).[209] A very small number of immunohistochemical studies that have identified L1 ORF2p (Table 12).[198, 222, 223, 227] In these studies, L1 ORF2p has been found predominantly in the cytoplasm, although a supposed nucleolar localization has also been reported. [198, 222, 223, 227] Lately, Sokolowski et al.[252] used a bacterial cells-depleted, recombinant human L1 EN to construct a monoclonal antibody specific for an epitope on the amino acid 205 of the human ORF2p EN. The discovery of a monoclonal anti-ORF2p antibody looks promising in that more studies may be able to analyze successfully human L1 expression and retrotransposition and possibly sets the groundwork for the development of antibody-based inhibitors against L1 deleterious effect.[252]

Table 12. Human L1ORF2p immunohistochemical/immunocytochemical studies						
L1ORF2p antibody (epitope)	**Normal/cancer cells**	**Normal tissues**	**Carcinomas/ neoplasia (% of positive immunostaining)**	**Positive control**	**Expression**	**Ref.**
polyclonal antibody (amino acids 48-63 and 152-166 of ORF2p) raised by the authors	germ cells (fetal gonocytes, adult secondary spermatocytes and immature spermatids), Leydig cells, Sertoli cells, testicular covering cells, vascular endothelial cells, placental syncytiotrophoblasts, epididymal columnar epithelial cells	-	-	NA*	mainly cyt	[222]
α-ORF2-N (amino acids 154-167) and α-ORF2-C (amino acids 1259-1275)	143B human osteosarcoma cells	-	-	NA	cyt (full-length ORF2p), cyt and nuc (truncated ORF2p)	[197]
polyclonal antibody (amino acids 48-63 and 152-166 of ORF2p), same as [222]	-	testis, prostate, urinary bladder (epithelium, interstitial cells and endothelial cells)	prostate carcinoma (epithelium, interstitial cells) bladder carcinoma (epithelial cells)	NA	nuc and/or cyt	[223]
anti-ORF2p antibody raised by the authors (NA)	T47D and MDA-MB-231 breast cancer cell lines	negative normal breast	breast fibroadenomas, in situ ductal carcinomas, invasive breast carcinoma	NTera.2 D1 cells	cyt and/or nuc	[227]

*NA, not available; cyt, cytoplasmic; nuc, nuclear; Ref., reference

3.5 Impact of L1 elements on human genome

Although TE had been initially characterized as "selfish or junk DNA" [253, 254] or "genomic parasites",[255] their multifunctional role was soon recognized. TE active elements can integrate into the human genome either as full-length or as individual fragments such as the L1 internal promoter, the L1 5′-UTR antisense promoter and various L1 internal splice sites.[164, 256] Focusing on human L1 elements, it has been estimated that L1 insertion events may be present up to 5% of newborns.[257] These events exert neutral, advantageous or harmful influence on human genome.[258] Numerous studies indicate L1 impact on gene expression, as well as on shaping, evolution and diversification of human genome, while a considerable amount of evidence also suggests a variety of L1-driven diseases.[149, 186]

3.5.1 Gene expression

Based on the different modes of their effect on human genes, L1 elements have been characterized as "molecular rheostats".[186, 258, 259] L1s can **promote the transcription of** several **genes** adjacent to them, for instance, via the L1 antisense promoter.[164, 166] In contrast, L1s are also capable of **gene silencing**, through different mechanisms, including polyadenylation within the ORFs region[260] and premature interruption of the RNA polymerase II-mediated transcription,[261] the so-called "gene breaking", i.e the production of distinct transcriptional fragments of L1 target genes.[262] Alternatively L1 may also act through the epigenetic regulation of various target genes in either germ cells,[263] for example in X-chromosome,[264] or in somatic cells[212] during or immediately after their insertion or over time.[186]

3.5.2 Genomic diversification

It seems plausible that L1 **insertions** may disrupt human genome at the target sites, thus inducing various genomic structural changes. These insertions are mostly the result of L1 TPRT-mediated retrotransposition, but occasionally they may originate from an alternative mechanism, termed endonuclease-independent L1 retrotransposition. The latter integration mode is preferred in genomic locations lacking the canonical L1 EN splitting action and TSDs. These locations usually present 5' and 3' flanks truncation.[reviewed in [186]] L1 insertions, derived from either TPRT or ENi retrotransposition, may also promote genomic variation indirectly, by mediating **deletions** of approximately 18kb of human genomic sequences.[187, 265] Another L1

mechanism that facilitates human genomic diversity includes **postintegration recombination events**, such as the nonallelic homologous recombination (NAHR) between several L1s.[266] Last but not least, a dominant mechanism of boosting human genome diversification is the L1-induced mobilization of non-L1 DNA sequences.[summarized in [186]] L1s can mobilize the upstream and more commonly the downstream flanking sequences near their 5′ and 3′ ends, respectively, via the so-called **L1-mediated transduction**. Besides 5' and 3' flanking sequences, L1-encoded proteins can also **mobilize** non-autonomous retroelements ***in trans***, retrotransposition-incompetent L1s and cellular full-length or partial mRNAs, leading to **exon shuffling** and **gene** or **chimeric pseudogene formation** in a new chromosomal site.[summarized in [186]]

3.5.3 Normal development

L1 retrotransposition has been found to be at various stages of germline or somatic cells development. Human genome analysis has proved that L1 insertions take place at the early stages of human **embryonic stem cells** growth, before germline partition, and are transmitted from male and female fetal germ cells into the zygote.[210, 243] Among human somatic tissues, L1 retrotransposition has been also found to be involved in normal **neuronal development**, leading to nervous system mosaicism and neuronal alterability among different brain regions of the same person.[212] Variable levels of L1 insertions have been noted in the different brain areas of the same individuals, including hippocampus,[267] cerebral cortex[268] and caudate nucleus,[267, 268] while significantly higher levels of L1s DNA has been identified in the brain of several patients in comparison to their cardiac[212, 216] and liver tissue.[212]

3.5.4 Disease

L1 mutagenesis is mediated predominantly via **insertions** either alone or related to **deletions**[269-274] or **3' transductions**,[244, 249, 275-280] while mutagenic effects may also be achieved via the **postintegration recombination** between L1s, such as NAHR,[269, 273] and the production of **double-stranded DNA breaks** (DSBs) by L1 EN, which are potential trigger factors for cell apoptosis or cell cycle arrest.[281, 282]

As already mentioned, L1 retrotransposition events may adversely affect the human genome, leading to various non cancerous diseases and to tumorigenesis (Table 13).[258]

Table 13. Human non-cancerous disease-inducing L1 insertions.

Disease	Gene	CHR	SF	E/I	Ref.
Hemophilia A	VIII	X	preTa, Ta, Na	E14 E15-20	[283]
Hemophilia A	VIII	X	Ta	E14	[283]
Hemophilia A	VIII	X	NA		[274]
Hemophilia B	IX	X	Ta	E5	[284]
Hemophilia B	IX	X	Hs	E7	[285]
Familiar polyposis coli (colon Cancer)	APC	5	Ta	E16	[277]
Duchenne muscular dystrophy	DMD	X	Ta	E44	[286]
Duchenne muscular dystrophy	DMD	X	Ta	E48	[275]
Duchenne muscular dystrophy	DMD	X	Ta	E	[287]
Duchenne muscular dystrophy	DMD	X	Ta	E67	[288]
Duchenne muscular dystrophy	DMD	X	Ta	E67	[279]
X-linked dilated cardiomyopathy	DMD	X	Ta	E1	[289]
X-linked retinitis pigmentosa	RP2	X	Ta	I1	[278]
X-linked retinitis pigmentosa	RP2	X			[290]
Beta-thalassemia	HBB	11	Ta	I	[291]
Beta-thalassemia	HBB	11	Ta	I2	[290]
Phosphorylase kinase deficiency	PHKB	16?	N/A	E8	[269]
Alport syndrome and associated diffuse leiomyomatosis	COL4A5, COL4A6	X	N/A	I1, I2	[273]
Fukuyama-type congenital muscular dystrophy	FKTN	9	Ta	I7	[270]
Fukuyama-type congenital muscular dystrophy	FKTN	9	Ta?	3'UTR ?	[270]

Chronic granulomatous disease	CYBB	X	Ta	I5	[276]
Chronic granulomatous disease	CYBB	X	Ta	E4	[244]
Choroideremia	CHM	X	Ta	E6	[280]
Coffin–Lowry syndrome	RPS6KA3	X	Hs	I3	[292]
Pyruvate dehydrogenase complex deficiency	PDHX	11	Hs	E	[271]
Ataxia with oculomotor apraxia 2	SXT	9	Hs	E12	[293]
Branchio-oto-renal syndrome	EYA1	8	Hs	E	[272]
Chanarin–Dorfman syndrome	ABHD5	3	Hs	I3	[294]
Neurofibromatosis type 1	NF1	17	preTa	E23	[295]
Neurofibromatosis type 1	NF1	17	Ta	E39	[295]

Table x abbreviations: CHR= involved chromosome, SF= L1 subfamily, E= disrupted exon, I= disrupted intron, VIII = Coagulation factor VIII, IX= Coagulation factor IX, APC= Adenomatous polyposis coli, DMD= Dystrophin, RP2= Retinitis pigmentosa 2, HBB= beta hemoglobin, PHKB= gene encoding the b subunit of phosphorylase kinase, COL4A5= collagen type IV, alpha 5, COL4A6= collagen type IV, alpha 6 FKTN= Fukutin, CYBB= Cytochrome b-245, beta polypeptide, CHM= Choroideremia (Rab escort protein 1), RPS6KA3= Ribosomal protein S6 kinase, PDHX= Pyruvate Dehydrogenase Complex, Component X, STX= Senataxin, EYA1= EYA transcriptional coactivator and phosphatase 1, ABHD5= Abhydrolase domain containing 5, NF1= Neurofibromin 1, N/A= not available, Ref=Reference

As it can be noted in Table 13, most of the L1 insertions have been found on the X chromosome, which coincides with the higher reported content of L1s in this chromosome,[145] while all diseases have been mediated by active members (i.e. Ta, pre Ta, Hs)[154] of the L1 subfamily. L1 full-length or truncated transcripts are integrated in various intragenic locations, while more than one insertion can occur in the same genomic site.[295] Human non cancerous disease-inducing L1-insertions disrupt the protein-coding exons or the regulatory introns, resulting in the latter case in exon skipping or partial exonization, false transcript onset, incorrect splicing, early transcript truncation or limited transcript elongation, and, ultimately, in impaired mRNA

stability.[256, 278, 280, 286, 288, 293-296] The effect of these insertions on human genome depends on both the target genomic site and the L1 inserted sequence itself.[296]

Although the challenging question *"whether L1 elements represent passenger or drivers on human neoplasms"*, posed recently by Rodić and Burns,[297] remains to be answered, mounting evidence support the potential impact of L1s on tumorigenesis. Lately, advanced techniques, including whole-genome and exome sequencing, enhanced retrotransposon capture sequencing and L1-targeted resequencing as well as computational methods of mechanism detection, frequency and precise localization of TE, i.e. the *TE analyzer* and the *Transposome Finder in Cancer*, have revealed various L1 somatic retrotransposition events in colorectal,[245, 246, 248, 277] lung,[245, 298] prostate and ovarian tumors[246] as well as in head and neck carcinomas, endometrial carcinomas[245] and in one case of hepatocellular carcinoma.[247] Morse et al..[299] has reported a structural difference between the myc loci of a breast ductal adenocarcinoma and the normal breast tissue of the same patient that was attributed to an insertional genomic sequence, completely homologous to L1; although doubt remains whether this insertion represented a true somatic L1 retrotransposition event.[298] However, an active L1 locus in breast cancers has been detected in a recent whole-genome sequencing study.[249]

L1 retrotransposition shows a cancer type-specific pattern.[245-248, 298] Tubio et al.[249] found evidence of at least one L1 retrotransposition event in 53% of cancer subjects, especially in those with colon or lung carcinomas, using a bioinformatics method on whole-genome sequencing data from 244 patients presenting with 12 different cancer types,. In the same study, a tumor-specific L1 activity was observed, with lung carcinomas exhibiting the greatest number of active L1 copies,[249] while the number of L1 retrotransposition events was highly variable among individuals with the same tumor type.[245]

3.6 Regulation of human L1 expression

The human L1 expression is regulated at various **transcriptional and posttranscriptional levels**.[reviewed in [206]] **Transcriptional regulation** includes the interaction of human L1 5′ UTR promoter with transcription factors, the methylation of the L1 promoter and the activation of the PIWI/piRNA pathway. The expression of several ***transcription factors, such as YY1, RUNX3, SOX proteins and p53*** and the mutations on their binding sites affect the L1 transcription initiation and activation as well as the L1 retrotransposition.[reviewed in [206]]

DNA methylation is a heritable, epigenetic modification that alters the genomic sequence mainly in the context of CpG dinucleotides (and less frequently at non CpG sites)[300] by addition of a methyl group at the 5th position of carbon of the C base ring.[230] As the human L1 5′ UTR promoter is repleted of CpG dinucleotides,[183, 301] methylation has been a dominant transcriptional silencing mechanism of L1s, leading to the impending of L1 expression or integration through the following ways: **a)** connection with the members of the methyl CpG binding domain family of proteins, **b)** inhibition of transcription factors binding and/or **c)** mobilization of histone deacetylases and chromatin structure modification.[246, 302, 303] Methylation affects a specific genomic region including the promoter of the target L1 element, rather than a wider chromosomal region,[249] and, based on studies with mouse models, is mainly mediated by the DNA methyltransferases (DNMT) DNMT1 and DNMT3a/DNMT3b. Moreover, the methyl-CpG-binding protein 2 (MECP2) participates in the human L1 regulation via identification of genomic DNA epigenetic modifications, such as methylation.[300]

Studies on cell lines and tumor samples have revealed a correlation between human L1 5′ UTR promoter hypomethylation and L1 RNA transcripts and/or ORF proteins overexpression.[204, 211, 215, 218, 226, 230, 232, 298, 304, 305] Moreover, the decrease in L1 methylation has been reported to be a common finding in benign and malignant hematologic disorders[306-311] as well as in various cancer types,[230, 247, 298, 312-315] in some of which L1 hypomethylation is considered an early event.[312, 313] The level of L1 hypomethylation in cancer is tissue-specific[312] and in several cancers, including hepatocellular carcinoma, non-small cell lung carcinoma and prostate adenocarcinoma, L1 hypomethylation has been regarded as a poor prognostic factor.[314, 316-322] Using a niche molecular biology technique *(Combine Bisulfite Restriction Analysis, COBRA)* for

the quantification of L1 methylation level in 24 MECs, Sirivanichsuntorn et al.,[250] found that L1 hypomethylation was significantly lower in MECs than in 14 normal controls. In an attempt to associate L1 hypomethylation levels with MEC histological grading, they also analyzed the L1 hypomethylation profile in three different cell types of MEC. Interestingly, a statistically significant gradual decrease in L1 methylation level was observed between normal salivary gland cells adjacent to MECs, intermediate, mucous and squamous cells of MECs,[250] a finding correlating L1 hypomethylation levels with a higher histological grade of MEC,[250] which is a known poor prognostic factor.

Although the ***P-element induced wimpy testis (PIWI)-interacting RNAs (piRNAs)*** may participate in the transcriptional regulation of the L1 elements by promoting the L1 demethylation, they mainly act as L1 suppressors at a posttranscriptional level. piRNAs are small silencing RNAs that interact with members of the Argonaute protein family (PIWI proteins) leading to piRNA-induced silencing complex (piRISC) generation that mediates L1 mRNA degradation.[323]

Other **posttranscriptional** regulating **mechanisms** include the ***RNA interference pathway***,[324] mediated by microRNAs or small interfering RNAs, several cytoplasmic RNA-binding proteins, e.g. stress granule proteins, and host antiviral proteins. In the latter category belong the proteins of the ***apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like3 (APOBEC3)*** family [reviewed in [325]] and the ***Three Prime Repair Exonuclease 1 (TREX1)***,[326] both of which will be discussed in detail in the following chapters.

The inhibition of L1 retrotransposition has been also attributed to other proteins, including the ***Moloney leukemia virus 10 (MOV10)***[327] and the recently described ***Zinc-finger Antiviral Protein (ZAP)***[328] as well as ***factors participating in DNA repair*** such as the nuclear excision repair endonuclease complex ERCC1/XPF.[329] On the other hand, several environmental factors such as oxidative stress air pollution, ionizing radiation and heavy metals may induce the expression of transposable elements e.g. L1.[221, 239, 259]

3.7 Linking L1 to autoimmunity and Sjögren's syndrome

As it has been analyzed in chapter 2, the crucial role of type I IFN pathway in the initiation and perpetuation of autoimmune diseases and, in particular, in SS has been well documented in the pertinent literature.[58, 82] Although the exact trigger factors of IFNα release in SS patients remain still elusive, evidence exists that human retroelements may represent an endogenous viral-like stimulus adequate to activate, via IRF3, the type I IFN production and subsequently promote autoimmunity.[reviewed in [58, 82]]

Retroelements are not the only endogenous candidates considered to induce autoimmune phenomena via type I IFN activation. Immune complexes containing necrotic or apoptotic particles combined with IgG antibodies,[330] autoantibodies against RNA-binding proteins[331] or with autoantigen-related U1 small nuclear RNA (U1 snRNA) and hY1RNA[332] were the first identified endogenous factors with IFNα inducing capacity in serum of SLE or SS patients. The stimulation of IFNα may lead to the induction and nuclear translocation of the ribonucleoprotein complexes comprising Ro52, a significant autoantigen targeted in both SS and SLE, which have also been found in apoptotic particles.[333, 334] Moreover, a strong positive correlation between the anti-Ro/SSA and anti-La/SSB autoantibodies titers and the type I IFN-inducible genes has been observed in the peripheral blood of SS patients.[335]

So, what has drawn our attention to human L1 retroelements? One possible explanation could be that their molecular structure is considered repleted with *CpG-dinucleotides*.[183, 301] Normal human DNA is comprised of methylated, and thus inactivated, CpG sequences; DNA or RNA motifs enriched with unmethylated CpGs, as are found in bacteria, could activate pDCs via the FcγRIIa receptor and, thus, induce IFNα production through TLR9.[58, 82] Additionally, the significantly elevated levels of hypomethylated CpGs that have been found in cloned plasma of SLE patients have been associated with lupus pathogenesis,[336-338] as they have shown a striking genomic difference compared to that of healthy controls.

Later on, as the CpG-rich motif was found homologous to human retrovirus structure,[339] endogenous human L1s were considered a probable viral-like trigger factor. Human L1 CpG-enriched DNA could stimulate pDCs by binding to TLR9, and/or L1 transcribed mRNA may bind to TLR7 or stimulate TLR-independent mechanisms,

finally leading to type I IFN production.[58] Mavragani et al.[251, 340] found overexpression of L1 mRNA in MSG tissues from pSS patients and sicca controls with autoimmune diseases compared to MSG tissue from healthy controls by quantitive real-time PCR. A significant correlation between L1 and IFNα2, as well as IFNβ mRNA expression in MSG tissue from pSS patients was found as well.[340] In line with the above, a dose dependent elevation of IFNα levels was observed after the in vitro simulation of pDCs with a L1 element-containing plasmid, whereas no L1 stimulation was observed after peripheral blood mononuclear cell (PBMC) treatment with recombinant IFNα.[251, 340] Moreover, the same research group reported the L1 ORF1p overexpression in the MSG ductal epithelial cells of pSS patients compared to healthy, non-autoimmune subjects.[340]

Another connection point between L1 elements and autoimmune diseases has been suggested through the study of two IFN inducible genes, the *(TREX1) gene* and the *SAM domain and HD domain-containing protein 1 (SAMHD1) gene*. [reviewed in [341, 342]] Both genes are mutated in the Aicardi–Goutieres Syndrome, a rare inherited encephalopathy that is characterized by increased levels of type I IFN in the cerebrospinal fluid.[343] TREX1 is the most abundant 3′–5′ exonuclease (DNaseIII) in the mammalian genome and is normally responsible for the adequate clearance of endogenous nucleic acids.[344] Stetson et al.[326] showed that, although functional TREX1 in mice could metabolize reverse-transcribed retroelement DNA, TREX1 mutations resulted in the accumulation of 25 clones of endogenous retroelements ssDNA, 12 of which derived from L1 elements that accumulated in TREX1-deficient mice heart cells. Mutations of the TREX1 gene have also been detected in patients with SLE and SS.[345-347] Thus, it has been hypothesized that dysfunction of TREX1 could promote the accumulation of the otherwise degradated endogenous retroelement nucleic acids, and via a cytoplasmic cell-intrinsic DNA signaling pathway stimulate the inappropriate activation of IRF3 and the increased type I IFN synthesis, leading to autoimmunity, including SS.[326, 348-351] SAMHD1 is an enzyme with phosphohydrolase activity, responsible for blocking HIV replication in DCs, macrophages and monocytes[352, 353] and has been listed among the most differentially expressed IFN-responsive/stimulated genes (IRGs/ISGs) in SS patients.[354] SAMHD1 has been found to inhibit L1 retrotransposition in human embryonic kidney cells (HEK293T), by targeting the ORF2p-mediated reverse transcription in the L1 RNPs, while SAMHD1 silence with siRNAs in the same cell line resulted in a 230% elevation in L1 retrotransposition.[355]

So, similarly to TREX1 dysfunction, SAMHD1 deficiency has been associated to unregulated retroelement cDNA expression and to autoimmune diseases.[342]

As it has already been mentioned, methylation of the L1 promoter inhibits L1 transcription.[249] A countervailing methylation mechanism against L1s in SS has been indirectly highlighted by the strong correlation between the overexpression of the L1 mRNA and the mRNA of methylation mediators (DNMT3B, DNMT1, MECP2) observed in MSG tissue of pSS patients.[340, 356] The sex and tissue specificity of several methyltransferases may be potentially related to the female predilection or/and the several tissue targets that characterize the various autoimmune disorders, respectively. Last but not least, the genetic preponderance of autoimmunity and the coexistence of several autoimmune diseases among the family members might reflect the methylation heritability in the mammalian germ cells, and, perhaps, the polymorphic nature of L1 retroelements.[reviewed in [58, 157]] Taken together, Mavragani and Crow[58] have proposed a model in which L1s overexpressed in the SGECs of SS patients trigger the IFNα production by the epithelial cells themselves, as well as by the pDCs or other IFNα-expressing cells. As it is seen in fig. 7, in ***genetically susceptible*** for increased type I IFNs production SS patients (e.g. patients carrying the IRF5 and/or STAT4 risk alleles or TREX-1 mutations), ***environmental stimuli***, such as viral infection, stressful events or hormonal defects, or ***epigenetic dysregulation***, including demethylation or limited expression of the ***APOBEC3*** inhibitory machinery, may induce the overexpression of endogenous nucleic acids, e.g. ***L1s***, and the type I IFNs production ***by the SGECs***.

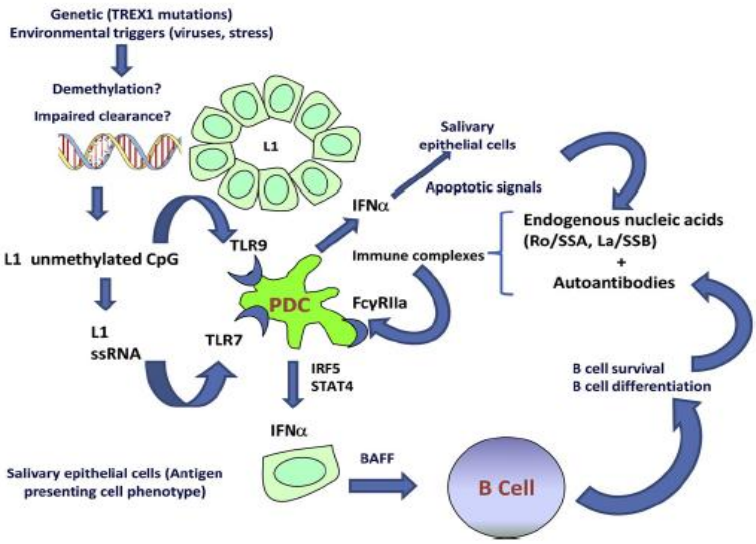


Fig. 7. Activation of Type I IFN in SS.[58]

3.8 In summary

- L1 elements are repetitive, mobile DNA sequences, accounting for nearly 20% of the human genome.
- L1s belong to the *non-LTR retrotransposons* and mobilize through a *"copy and paste"* mechanism facilitated by an RNA intermediate, i.e. a reverse transcriptase that catalyses the reverse transcription of the RNA transcripts into cDNA, to give origin to new retrotransposons.
- L1s are autonomous elements, i.e. they encode the proteins required for their retrotransposition, while they also drive the retrotransposition of non autonomous elements *in trans*.
- Active human L1 elements consist of a 5'-untranslated region (UTR), two non-overlapping open reading frames (ORF1 and ORF2), which are separated by an intergenic ORF region and **encode for L1 ORF1p and ORF2p proteins**, and a 3'-UTR, terminating in a adenine-rich tail (poly-A-tail) that is flanked by short direct repeated sequences, named target site duplications.
- ORF2p is a ~150 kDa protein of 1275 amino-acids that includes three domains: a) a N-terminal domain with endonuclease activity, b) a central domain with reverse transcriptase activity and c) a C-terminal cysteine-rich zinc knuckle domain with elusive properties, which is considered essential for retrotransposition, possibly via its RNA-binding activity.
- ORF2p cysteine-rich domain mutations affect the ORF2p's ability to interplay with its mRNA and/or other factors in order to form the L1 ribonucleoprotein particles (RNPs).
- ORF1p and ORF2p proteins have also been related to a protein of P-bodies and stress granules, as well as to the stress granule marker.
- L1 life cycle includes the L1 DNA transcription into a bicistronic mRNA in nucleus; the L1 mRNA translation into ORF1p and ORF2p and the formation of L1 RNPs in the cytoplasm and, finally, the L1 retrotransposition in the nucleus via the target-site primed reversed transcription and integration into a new genomic site.
- Approximately 80-100 human L1 elements per individual are retrotransposition-competent.
- L1 elements are expressed in normal human somatic or germ cell tissues as well as in benign and malignant cell lines and tumors, including breast carcinoma.

- L1's expression has been reported in normal exocrine glands, such as the breast, the prostate and the salivary glands.
- Several studies have reported lower levels of L1 ORF2p compared to ORF1p, a finding that could be attributed to the unconventional ORF2p translation mechanism or to a lower sensitivity of anti-ORF2p antibodies.
- L1 affects gene expression, evolution and diversification of human genome and has been associated with non-cancerous diseases and tumorigenesis.
- **Human L1 expression is regulated by various transcriptional and posttranscriptional mechanisms, the inhibitory effect of APOBEC3 deaminases included.**
- **L1 elements are considered potential trigger factors of autoimmune diseases, particularly of SS, via the activation of plasmacytoid dendritic cells to produce IFNα.**
- **Increased L1 mRNA levels have been reported in the minor salivary glands (MSG) tissue from pSS patients and other autoimmune sicca controls in comparison to MSG tissue from healthy and non-autoimmune controls, while L1 mRNA levels have been strongly correlated with IFNα mRNA expression.**

4 APOLIPOPROTEIN B MRNA-EDITING ENZYME-CATALYTIC POLYPEPTIDE-LIKE 3B (APOBEC3B)

4.1 APOBEC3B, a member of the AID/APOBEC family

The AID/APOBEC enzymes are part of the large protein superfamily of zinc-dependent deaminases that are capable of modifying RNA and/or ssDNA sequences, by the deamination of cytidine to uridine, and participate in the metabolism of purines [adenine (A) and guanine (G)] and pyrimidines [thymine (T), cytosine (C) and uracil (U)].[357,358] In contrast to other deaminases, such as the cytosine deaminases found in prokaryotes or lower eukaryotes,[359] the AID/APOBEC proteins recognize and bind only to polynucleotides, while they are inactive on free bases or single nucleotides.[360] The AID/APOBEC family, which is restricted to jawed vertebrates, comprises of 11 members: APOBEC1, APOBEC2, AID, 7 APOBEC3 proteins and APOBEC4.[360, 361] All APOBEC enzymes were designated from the apolipoprotein B (*apoB*), a protein synthesized in a shorter *(ApoB48)* and a longer *(ApoB100)* form in the human gut, that participates in the absorption and transport of the diet-derived lipids and the endogenous produced cholesterol and triglycerides.[362] The *apoB* mRNA is the main posttranscriptional editing target of APOBEC1, the founder member of the AID/APOBEC family.[358]

APOBEC1 is located on chromosome 12, displaying an homologous amino acid sequence to *Escherichia coli* cytidine deaminases, and is mainly expressed in the gastrointestinal compartment.[363] Based on the structural similarities with APOBEC1 further studies have found another locus on chromosome 12 where the AID gene is arranged,[363] two loci on chromosomes 6 and 22, corresponding to APOBEC2[364] and APOBEC3[365] respectively, while the APOBEC4 gene has been described more recently to cluster on chromosome 1. It's worth mentioning that, although APOBEC1 was the first enzyme of the AID/APOBEC family to be described, phylogenetic analysis revealed that the ancestral members in the family evolution were AID and APOBEC2.[361]

The AID/APOBEC enzymes belong to the zinc-dependent deaminases and share a common motif of a conserved amino acid sequence [His-Xaa-Glu-$X_{(23-28)}$-Pro-Cys-$X_{(2-4)}$-Cys, where X represents any amino acid].[360, 365] In detail, a zinc atom coordinated

by four ligands i.e. three cysteine (Cys) residues [instead of two Cys residues and one histidine (His) residue found in the rest of deaminases) and a water molecule (H_2O) that mediates the substrate binding, constitute the core of the catalytic domain;[360, 365] there, with the help of a proton shuttling provided by a glutamate (Glu) residue, the cytidine deamination reaction takes place, i.e. the hydrolysis of the amino group at the 4th carbon of a cytidine or a deoxycytidine, leading to the generation of an uridine or a deoxyuridine respectively and the release of ammonia (fig. 8).[366] Curiously, this structure does not resemble that of other cytidine deaminases; instead it is more reminiscent of the structural model of cytosine or dCMP deaminases and, especially, of tRNA adenosine deaminases; thus, an AID/APOBEC family origin from the latter deaminases is speculated.[357]

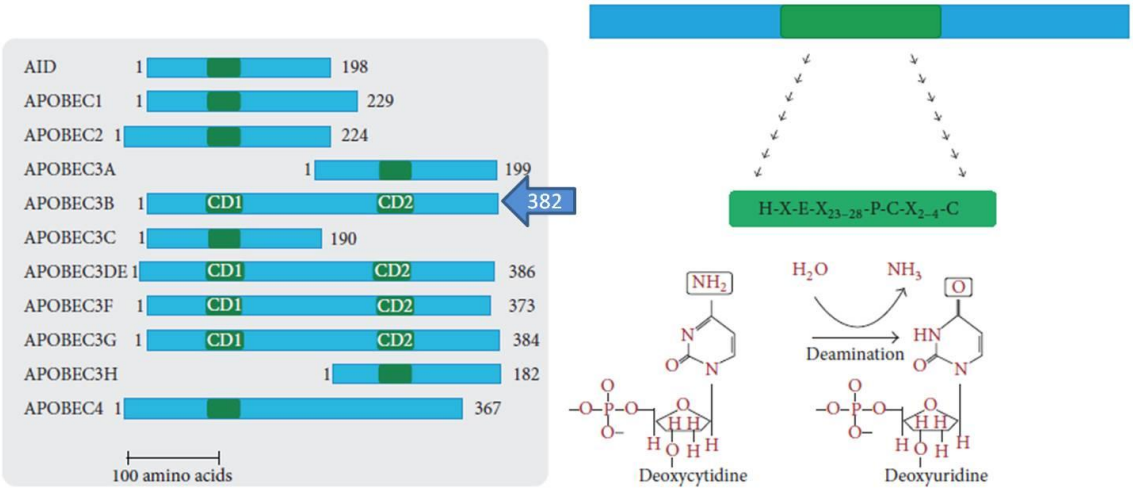


Fig. 8. AID/APOBEC family members and their structure.(adapted by[358])

4.2 Human APOBEC3 deaminases

In the foreground of the AID/APOBEC family stand the APOBEC3 deaminases, a subfamily constituting of seven proteins, alphabetically defined as APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F, APOBEC3G and APOBEC3H, which are identified exclusively in mammals.[361] APOBEC3 proteins are ***IFN-inducible*** antiviral factors that act in the innate immune system, as ***a host defensive mechanism against exogenous viruses and endogenous mobile elements***. This action is accomplished by their ability to bind to RNA and undergo biological changes, including protein–protein interactions, which affect their subcellular

localization.[367-369] The first recognized member of the APOBEC3 enzymes, the APOBEC3G, was initially found in 2002 to block the replication of HIV-1 in macrophages and T-cells, in the absence of the viral infectivity factor (vif).[370] Since then, the seven APOBEC3 genes have been reported to reside tandemly in the same orientation and in head-to-tail configuration within a gene cluster in human chromosome 22, between the conserved flanking genes *CBX6* and *CBX7*.[365, 371] The APOBEC3 genes are considered to be derived from the duplication of a single-copy primordial gene, [365] most probably the AID gene;[361] thus, the several human APOBEC3 mRNA sequences appear 30-100% homologous,[372] but at the same time highly polymorphic.[361]

APOBEC3 deaminases display a remarkable structural difference; they contain either one (APOBEC3A, APOBEC3C and APOBEC3H) or two (APOBEC3B, APOBEC3DE, APOBEC3F and APOBEC3G) copies of the zinc-binding deaminase catalytic domains (CD), namely CD1 and CD2 near the N- and the C-terminus, resulting in a size of 21kDA or 42 kDa, respectively.[365] In APOBEC3DE, APOBEC3F and APOBEC3G deaminases only CD2 retains its catalytic activity,[summarized in [358]] while **only in APOBEC3B both domains act catalytically**.[373] In general, APOBEC3s' structure is reminiscent of that of AID that lacks a 40-amino acid sequence found in the C-terminus of APOBEC1.[360]

APOBEC3DE, APOBEC3F and APOBEC3G proteins are found in a cytoplasmic localization.[219, 374] The rest of APOBEC3s may be detected in the cell cytoplasm, but they can also enter the nucleus; APOBEC3A, APOBEC3C and APOBEC3H via diffusion and APOBEC3B through a nuclear localization signal.[219, 374] APOBEC3 enzymes also display consensus editing sites within ssDNA.[reviewed in [375]] For example, APOBEC3B and APOBEC3F preferentially deaminate the C of the TpC DNA dinucleotides or the second C of the CpC dinucleotides in case of APOBECB, while the second C of the CpC dinucleotides in DNA is also the favored deamination sites for APOBEC3G.[376, 377]

4.2.1 Human APOBEC3 deaminases against viruses

The antiviral activity of APOBEC3 enzymes primarily originates from their packaging into virions, resulting in the deamination of C bases in the viral reverse transcribed ssDNA and the consequent G to A hypermutation in the provirus.[368, 376] In addition to the deaminase-dependent viral restriction, the APOBEC3 proteins recruit several deaminase-independent mechanisms to inhibit the viral reverse transcription and integration into the host genome.[reviewed in [358]]

Apart from the initially described APOBEC3G, other APOBEC3s, i.e. APOBEC3B, APOBEC3DE, APOBEC3F and APOBEC3H have been reported to restrict HIV-1 strains *in vitro*, with or without the aid of G to A hypermutations and in the absence of vif that normally counteracts the APOBEC3s' antiviral role, while recent studies have deduced the probable *in vivo* HIV-1 blockage via hypermutation.[summarized in [378]] Based on *in vitro* experiments, human APOBEC3 enzymes, except for APOBEC3A and APOBEC3H, are capable of inhibiting other ***retroviruses***, including the (HTLV-1, the equine infectious anemia virus (EIAV), the murine leukemia virus (MLV) and the primate foamy virus (PFV), as well as the wild-type and the Vif-deficient forms of the African green monkey and the rhesus macaque simian immunodeficiency virus (SIVagm and SIVmac respectively).[367, 369, 376, 379-382]

Several APOBEC deaminases also exert an *in vitro* antiviral effect on ***viruses other than retroviruses***. The human deaminases APOBEC3B, APOBEC3C, APOBEC3F and APOBEC3G modify the DNA of hepatitis B virus (HBV) by direct inhibition of its reverse transcriptase. As a result of the APOBEC3s' activity, the serum of HBV-infected subjects contains numerous G to A hypermutations, while the altered HBV DNA is prone to degradation. [383, 384] Another editing target of several APOBEC3 enzymes is the ssDNA derived during the replication and transcription process of the dsDNA human papillomavirus (HPV). The evidence supporting this hypothesis is based on the expression of human APOBEC3A, APOBEC3C and APOBEC3H in keratinocytes, i.e. the HPV replication site, and the identification of hypermutated HPV1a and HPV16 genomes in plantar skin warts and precancerous cervical bioptic material, respectively. Moreover, co-transfection experiments with plasmid-containing HPV DNA have documented an association between the overexpression of these three APOBEC3s and HPV DNA hyperediting.[385] Another DNA virus, the parvovirus adeno-

associated virus (AAV) that replicates as an ssDNA, has also been identified as a potential target of human APOBEC3A, whereas APOBEC3B, APOBEC3C, APOBEC3F and APOBEC3G exert no inhibitory effect.[386]

Investigating the effect of APOBEC3 deaminases on herpes viruses (HSV), Suspène et al[387] found that the *in vitro* overexpression of APOBEC3C in tissue cultures was correlated with a significant reduction in viral titers and infectivity, while hyperedited HSV-1 DNA was revealed in 4 out of 8 buccal lesions. In the same study, the nested PCR/3D PCR analysis of 5 transformed peripheral blood mononuclear cell lines, in which EBV remained in a latent form, revealed an overexpression of APOBEC3C concurrently with EBV DNA hypermutation in 4/5 of them.[387] Finally, although the APOBEC3G has been shown to restrict HCV RNA *in vitro*, no hypermutation has been recognized in the viral genome,[388] a finding that may be attributed to the exclusive predilection of APOBEC3G for ssDNA sequences.

4.2.2 Human APOBEC3 deaminases against retroelements

4.2.2.1 APOBEC3 proteins inhibit L1elements

Independently of their antiretroviral activity, [219] APOBEC3 deaminases provide intrinsic immunity against the L1s, which have also been reported to be suppressed by two other AID/APOBEC family members, APOBEC1[389] and AID.[390] Based on cell culture retrotransposition assays, all members of the APOBEC3 subfamily have been found to be capable of blocking the propagation of these autonomous mobile elements in **varying degrees**.[reviewed in [325, 391]] Stenglein and Harris[392] found that human APOBEC3B or APOBEC3F caused a 5 to 10-fold reduction in the level of L1 retrotransposition. Examining the antiretrotransposon effect of several APOBEC3 enzymes, Kinomoto et al.[219] found a **significant decrease in L1 retrotransposition level**, i.e. to 0.9, **14.0**, 15.6 and 10.2%, by human APOBEC3A, **APOBEC3B**, APOBEC3F and APOBEC3G, respectively. The results of this study are in contrast to the findings of other studies, which showed that APOBEC3G lacks inhibitory potency against L1 retrotransposition. These discrepancies regarding the inhibitory potency of APOBEC3 members found in the literature are most probably due to the discreet different materials i.e. cell lines and experimental methods used in various studies. Despite the diverging rates in the aforementioned studies though, APOBEC3A is

considered the most potent inhibitor of L1 elements,[393, 394] while the similar inhibitory capacity of APOBEC3B and APOBEC3F is probably attributed to their 96% identity between 66–190 and 65–189 amino acid residues respectively.[392]

The exact stage of L1 life cycle that is targeted by the APOBEC3 enzymes has not been clarified yet. **APOBEC3 deaminases might affect various stages of the L1 retrotransposition pathway, irrespectively of their subcellular localization**.[219, 392, 394] It has been hypothesized that when in the cytoplasm, various AID/APOBEC family members might interact with L1 RNPs and influence their intracellular transport and/or their nuclear input, while when in the nucleus, AID/APOBEC proteins could gain direct access to L1 RNA and hamper the synthesis of DNA by inhibiting the required nick generation in the first target DNA strand, thus preventing reverse transcription initiation.[reviewed in [391]]

For several years the antiretrotransposon effect of APOBEC3s was mainly considered **deaminase-independent**.[reviewed in [391]] Stenglein and Harris in 2006[392] demonstrated that the catalytically inactive APOBEC3B retained its L1 inhibition activity, while they failed to detect by a PCR-based assay any cDNA strand-specific C/G to T/A mutations within the retrotransposed L1s that had replicated under the impact of APOBEC3B or APOBEC3F. Their findings are in agreement with other studies regarding the L1 inhibition mechanism by APOBEC3A.[219, 374, 386, 394]] Taken together, these data indicate that the **L1 suppressive potency of APOBEC3s is independent of their enzymatic activity**.[219, 374, 386, 392, 394] Recently, though, the APOBEC3A antiretrotransposon activity was found to **depend, at least partially, on the deamination** of the transiently exposed ssDNA arising during the L1 integration process.[395] On the other hand, Richardson et al[395] found that during TPRT the newly composed L1 ssDNA is prone to APOBEC3A-induced deamination, followed by degradation, while the examination of the nucleotide sequence of 33 L1 retrotransposition events revealed 39 G to A mutations that were considered indicative of APOBEC3A action.[395]

Whether APOBEC3 enzymes target specific L1 RNA or ORFs sequences remains elusive.[325] APOBEC3DE,[396] APOBEC3G and APOBEC3F[397, 398] have been found to accumulate in RNA-binding proteins, such as P-bodies and stress granules, under normal or stressful conditions, respectively. P-bodies are cytoplasmic sites of

RNA metabolism or silencing and are considered cellular pools of suppressed RNA[summarized in [399]] (e.g. originating from endogenous retroelements),[172] although the association between APOBEC3 enzymes and P-bodies is not considered indicative of their L1 inhibition potency.[396] Being part of RNPs, the L1 ORF1p has also been found to localize in relation to P-bodies and stress granules,[172] a finding that raised the probability of APOBEC3s and L1 ORF1p co-localization. To address this issue, Lovsin and Peterlin[399] investigated the interactions between human APOBEC3A or APOBEC3B and L1 ORF1p by several techniques. After confirming the L1 suppression activity of human APOBEC3A and APOBEC3B by a HeLa cell culture based retrotransposition assay, they examined whether these two APOBEC3s and L1 ORF1p affect each other's subcellular localization. In line with a previous study,[172] they verified the distinct intracellular localization of APOBEC3A (nuclear or cytoplasmic), APOBEC3B (nuclear) and L1 ORF1p (cytoplasmic) via immunofluorescence in HeLa cells transfected with plasmids coding for one of these three proteins. Additionally, co-immunoprecipitation of L1 ORF1 and APOBEC3A or APOBEC3B enzymes, followed by RNase treatment and Western blot analysis, showed a co-precipitation of L1 ORF1 with APOBEC3B but not with APOBEC3A, while modified interactions between APOBEC3B and L1 ORF1 observed after the RNase treatment indicated a RNA-dependent binding of APOBEC3B to L1 ORF1. In summary, these results imply that **the inhibitory potency of APOBEC3B against ORF1p does not require their co-localization and is independent of the APOBEC3B to ORF1p interaction**.[399]

On the other Horn et al[393] showed via density gradient centrifugation and coimmunoprecipitation experiments that human APOBEC3C dimerizes to exert its L1 inhibitory activity, while this restriction presupposes a RNA-dependent interaction between APOBEC3C and L1 ORF1p. Finally, the amino acid region of APOBEC3G that targets the HIV-1 Gag protein in order to block HIV-1 displays an almost 50% identity to the corresponding regions of APOBEC3B and APOBEC3F, which are considered responsible for their inhibitory effect.[392, 400] L1 ORF2p resembles HIV-1 Gag protein in its ability to bind both zinc and nucleic acids.[401] Thus, the possible influence of APOBEC3s-L1 ORF2p interaction to L1 inhibition has still to be elucidated,[392] since until recently the cellular detection of L1 ORF2p has remained impossible.[198]

4.2.2.2 APOBEC3 proteins inhibit Alu and LTR retrotransposons

The non-autonomous Alu element constitutes almost 11% of the human genome[145] and its mobilization is L1 ORF2p-induced, without the mediation of L1 ORF1p.[151] APOBEC3 deaminases are capable of restricting Alu retrotransposition.[374] Similarly to L1 restriction, sequence analysis of Alu retrotransposition events in the presence of APOBEC3A, APOBEC3B and APOBEC3C revealed that the inhibition effect of these APOBEC3s was independent of their editing activity.[374]

LTR retrotransposons, also termed endogenous retroviruses or extrachromosomally-primed retrotransposons, account for 10% of human genome and display a different life cycle from non-LTR retroelements, as they form virus-like particles and are reversely transcribed in the cytoplasm of the cell. For years, human endogenous retroviruses, i.e HERVs, were characterized by the accumulation of mutations and were considered replication-incompetent.[summarized in [325, 391]] However, current data suggest that the most recently found endogenous retrovirus HERV-K remains active within the human genome[402] and can be silenced by all human APOBEC3 deaminases, expect for APOBEC3C, with APOBEC3B and APOBEC3F displaying the highest restriction effect.[403]

The IAP and MusD sequences are LTR retrotransposons of murine origin that are absent from the human genome. They retain their retrotransposition capacity in their host genome and have been identified as inhibition targets of the human APOBEC3A,[386, 404] APOBEC3B,[404] APOBEC3F[405] and APOBEC3G[406] in cell culture experiments. APOBEC3-mediated inhibition of the murine IAP and MusD by APOBEC3B, APOBEC3F and APOBEC3G occurs, at least partially, via a deamination-dependent mode[404-406] that leads to the degradation of the nascent cDNA or the insertion of a mutated LTR retroelement.[reviewed in [407]] On the other hand, APOBEC3A is believed to block IAP retrotransposition via a novel, yet unclarified, DNA editing-independent mechanism.[404]

4.3 APOBEC3B

Jarmuz et al[365] were the first to analyze the introns/exons organization of APOBEC3 genes via DNA sequencing. They found that APOBEC3B gene constitutes of a short first exon of 6 amino acids, three exons that are duplicated to produce three additional exons and a last eighth exon, all translated to a 382-amino acid protein.[365] RT-PCR analysis revealed skipping of an exon 1-resembling sequence, located between the fourth and the fifth exon of APOBEC3B.[365] APOBEC3B gene displays significant sequence homology with other APOBEC3 subfamily members' genes, mainly with APOBEC3F (59% identity) and APOBEC3G (57% identity), and to a lesser extent with APOBEC3C and APOBEC3A.[365, 377] As a result of the high degree of homology between the APOBEC3 genes, the production of specific antibodies for each one of the APOBEC3 deaminases has been challenging since recently.[408]

As mentioned previously, APOBEC3B is the only APOBEC3 enzyme with double active site domains, termed CD1 and CD2, which are encoded by exon 3 and 6 respectively.[365] Both CDs enhance the inhibitory capacity of APOBEC3B against retroviruses, although mutations to either one or both of them does not eliminate the APOBEC3B restriction against retroviruses[373] or L1 elements.[373, 392] The two CDs show discrete predilection for their target sequence within ssDNA, i.e. CD1 and CD2 preferentially deaminate C residues flanked by a 5' C or T respectively.[373] These findings led Bogerd et al[373] to propose that the intrinsic preference of APOBEC3B is a composite result of the consensus editing sites of CD1 and CD2.

4.4 APOBEC3B inhibits viruses and retroelements

The restriction effect of APOBEC3 deaminases against exogenous or viruses and transposable elements has been already presented briefly. Table 14 summarizes the APOBEC3B inhibitory repertoire within human genome. APOBEC3B targets retroviruses and DNA viruses, as well as LTR- and non-LTR retroelements, including L1 and Alu sequence. In contrast to APOBEC3G and APOBEC3F, which suppress HIV-1 in the absence of the HIV Vif protein,[376] APOBEC3B's inhibitory function is independent of the Vif deficiency.[376, 377] APOBEC3B is reported to induce a decrease in L1 retrotransposition frequency by 75–90%,(reviewed in[409]) while the shRNA-mediated suppressive effect of APOBEC3B in HeLa and hESCs has been reported to cause a 2–3.7-fold up-regulation in their engineered L1 retrotransposition ability.[410]

Table 14. Antiviral and antiretroelement APOBEC3B functions.	
Retroviruses[219, 376, 377, 379, 382]	HIV-1, SIVagm, SIVmac, HTLV-1, Murine leukemia virus, Rous sarcoma virus
DNA Viruses[411]	HBV
non-LTR retroelements[217, 373, 385, 391, 393, 395, 409]	L1, Alu
LTR retroelements	IAP, MusD, HERV-K[386, 404, 412]

4.5 APOBEC3B expression

Due to the lack of an APOBEC3B-specific antibody, studies elucidating the APOBEC3B expression have been limited at the mRNA level.[365, 372, 374, 413] Studies based on qRT-PCR[372, 413] or Northern blot analysis[365] have revealed a broad expression of the human APOBEC3B in normal or neoplastic human tissues, normal cells or carcinoma-derived cell lines [summarized in Table 15].

In general, APOBEC3 genes display a tissue-specific expression[reviewed in [219]] and are highly expressed in lymphoid organs, where they participate in innate immune responses.[372] In most human cell lines and tissues, APOBEC3B mRNA has been detected in lower levels than other APOBEC3s, such as APOBEC3G and APOBEC3F.[372, 413] The higher expression of APOBEC3G and APOBEC3F, though, may be attributed to their relative strong expression in lymphocytes, which are broadly distributed within tissues.[413] Absolute human APOBEC3B mRNA expression has been found poor[413] to almost absent[372] in freshly isolated PBMCs, i.e. monocytes and lymphocytes. The relative analysis of the mRNA level in each PBMC subset compared to the mRNA level in total PBMCs derived from the same individual revealed that human APOBEC3B is predominantly expressed in B cells.[413] APOBEC3B mRNA levels were found to be 19-fold higher in cell lines resistant to viruses lacking the Vif protein (e.g. CEM and H9 cell lines) than in non-resistant cell lines (e.g. CEM-SS and SupT1),[372] while in another study, APOBEC3B mRNA was slightly detecTable in CEM cells but not in other HIV-permissive (i.e. HeLa, SupT1, and CEM-SS) or non-permissive (i.e H9 and HUT78) cell lines.[376] Low levels of human APOBEC3B mRNA

were also observed in CEM, K562 and SW480 cells by Doehle et al,[377] who failed to detect APOBEC3B mRNA in CEM-SS and H9 cell lines as well as in several human tissues. The discrepancies of the results derived from the aforementioned PCR-based experiments may reflect variations in the primer sets[377] or the cell lines[219] that were used.

Similar to other APOBEC3s, the levels of APOBEC3B may be found up-regulated in some tissues e.g. in the liver, in case of viral infection, as a result of the chronic pro-inflammatory cytokines overproduction.[414] IFN-α is a potential inducer of APOBEC3B mRNA expression in different cell populations, including PBMCs,[372] primary naive CD4+ T cells[413] and hepatocytes.[383] APOBEC3B mRNA levels have also been found up-regulated in CD4+ T cells cultured with the T-cell mitogens (IL-2) and phytohemagglutinin(PHA),[372] while no APOBEC3B mRNA has been detected in PBMCs treated with IL-2/PHA.[376] Moreover, stimulation with IFN-γ, IL-2 or anti-CD3/CD28 alone did not result in significant APOBEC3B mRNA induction in CD4+ T cells.[413]

Interestingly, APOBEC3B mRNA expression has been reported in MSGs from SS patients,[356] as it will be discussed in greater detail later in this chapter, while salivary gland RNA has been used as normal control by Burns et al[415] in order to investigate APOBEC3B levels in head and neck cancer.

The abundance of APOBEC3B in normal cells and tissues might be suggestive of its role in innate immunity mechanisms, while its presence in cancer cells and carcinomas possibly indicates that carcinogenesis might follow APOBEC3B normal expression.[372] In addition, as L1 retrotransposition events occur during the earliest stages of human embryonic development,[243] APOBEC3B expression in early human embryonic tissues may be of major importance in preventing inheritable L1-induced genomic instability.[374]

Table 15. APOBEC3B expression within human genome.	
Normal cell lines [219, 356, 365, 369, 372, 374, 376, 377, 383, 394, 410, 413, 416, 417]	PBMCs (lymphocytes, monocytes), ESCs (e.g. H9p47, BG01p53, and hSF-6p48), human T cells (SupT1H9, CEM cells, CEM-SS), iPSCs, human embryonic kidney cells (293T), hepatocytes, keratinocytes, visceral preadipocytes
Normal tissues [365, 369, 372, 374, 383, 413, 415, 418]	Adipose tissue, bladder, bone marrow, brain, cervix, colon, esophagus, fetal liver, heart, intestine, kidney, liver, lung, lymph nodes, ovary, placenta, prostate, skeletal muscle, spleen, testis, thymus, thyroid, tonsil, trachea
Cancer cell lines [219, 365, 369, 377, 383, 394, 415, 419-421]	B cell lymphoma cells (HT, KIS1, KM-H2, Granta519, SUDHL-6), breast cancer cells, Burckitt's lymphoma Raji cells, chronic myelogenous leukemia cells (K562), colorectal adenocarcinoma cells (SW480), embryonic carcinoma cells (NCCIT), Hela cells, hepatocarcinoma cells (HuH-7, HepG2), lung carcinoma cells, melanoma cells, osteosarcoma cells(143B TK, SW1353, OUMS-27), promyelocytic leukemia cells
Carcinomas [summarized in [422]]	Low grade glioma, prostate adenocarcinoma, thyroid carcinoma, glioblastoma multiforme, renal papillary cell carcinoma, renal clear cell carcinoma, acute myeloid leukemia, ovarian serous cystadenocarcinoma, breast invasive carcinoma, hepatocellular carcinoma, stomach adenocarcinoma, lung adenocarcinoma, rectum adenocarcinoma, colon adenocarcinoma, uterine corpus endometrioid carcinoma, skin cutaneous melanoma, bladder urothelial carcinoma, head and neck squamous cell carcinoma, lung squamous cell carcinoma, cervical squamous cell carcinoma, endocervical adenocarcinoma

APOBEC3B has been considered the sole family member with a constitutively nuclear localization.[219, 374, 383, 392, 394, 399, 423, 424] APOBEC3B's ability of incorporation into cytoplasmic virions of retroviruses,[376, 377, 379] as intended to restrict their replication, prompted researchers to investigate the enzyme's ability to shuttle between the nucleus and the cytoplasm. Indeed, Bogerd et al.[374] showed via immunofluorescence that APOBEC3B contains two distinct signals of nuclear localization (NLS) and export (NES) that render the molecule functional in both subcellular compartments. The existence of APOBEC3B NLS was further confirmed by Lackey et al[425], who found that APOBE3B is excluded from the nuclear DNA during mitosis and returns into nucleus after the end of telophase, although Stenglein et al.[424] supported that APOBEC3B's nuclear entry and residence are independent of its NLS.

For years, the determination of APOBEC3B tissue, cellular or subcellular localization has been hindered by the inability to produce antibodies specific for this protein, due to the high degree of homology between the seven APOBEC3 genes.[372, 413] Table 16 summarizes materials and results of immunohistochemical studies for APOBEC3B found in the pertinent literature. Using a polyclonal antibody targeting the 80-127 amino acids near the N-terminus of human APOBEC3B, Gwak et al.[426] demonstrated a mainly cytoplasmic and/or nuclear APOBEC3B expression in breast, colorectal, gastric and prostate carcinomas, as well as in their corresponding normal glands and in cases of prostate intraepithelial neoplasia. However, as the authors have pointed out that their APOBEC3B antibody is 100% homologous to APOBEC3F; thus, their results may correspond to either one of these two proteins.[426] A predominantly cytoplasmic expression of APOBEC3B was also reported by Xu et al[427], Yan et al[428] and Onguru et al[427] in renal cell carcinoma, lung carcinoma and breast cancer cell lines, respectively. In two other studies about APOBEC3B expression in chondrosarcoma[420] and skin melanoma[429] no information about the immunostaining pattern was provided, while in the study of Kosumi et al,[430] only nuclear APOBEC3B positive staining in esophageal squamous cell carcinoma cases was evaluated. Interestingly, in the last study, the high APOBEC3B immunohistochemical expression was associated significantly with a low L1 methylation status, as determined by bisulfite pyrosequencing analysis.[430] This finding further strengthens the hypothesis of an existing relationship between L1 retroelements and their inhibitor APOBEC3B.

Table 16. Human APOBEC3B immunohistochemical studies					
APOBEC3B antibody (epitope)	**Normal tissues**	**Carcinomas/ neoplasia**	**Positive control**	**Expression**	**Ref.**
NP_004891 (80-127 amino acids)	breast ducts, gastric mucosal glands, colonic mucosal glands, prostate glands	gastric carcinomas, colorectal carcinomas, prostate carcinomas, prostate intraepithelial neoplasia	invasive ductal breast carcinoma	cyt (100% of positive cases), nuc*	[426]
N/A	chondrosarcoma adjacent non-tumor tissue	chondrosarcoma	N/A*	cyt and nuc	[420]
PAB2474 (307-337 amino acids)	-	skin melanoma	N/A	cyt	[429]
sc-130955 (N/A)	-	renal cell carcinoma	N/A	mainly cyt	[427]
ab 191695 (14 amino acids near N-terminus)	noncancerous esophageal mucosa	esophageal squamous cell carcinoma	N/A	only nuc expression was considered as positive	[430]
bs-12494 (20-60 amino acids)	-	drug resistant breast cancer cell lines (doxorubicin, etoposide, paclitaxel and docetaxel resistant MCF-7 cell lines)	breast cancer cell lines (BT474 and MDA-MB231)	mainly cyt	[421]
PAB2474 (307-337 amino acids)	no or low APOBEC3B expression in adjacent normal lung tissue	non-small-cell lung carcinoma	N/A	cyt	[428]

* NA, not available; cyt, cytoplasmic; nuc, nuclear; Ref., reference

4.6 APOBEC3B mutagenesis in cancer

APOBEC3B attracted a great interest early due to its dual mutagenic effect. Besides its well documented role in innate immunity via counteracting with foreign or endogenous pathogens, APOBEC3B may also act as a source of mutations leading in cancer development.[reviewed in [431]] A link between APOBEC3B-induced mutagenesis and cancer was suspected in 2013, when Burns et al.[418] reported the upregulation of APOBEC3B mRNA levels in breast cancer cell lines and tumors. In the same study, the APOBEC3B mutagenic signature was identified in several cancer-related genes, i.e. in the DNA C-to-U conversion that results in C-clustered same-strand mutations (termed *kataegis*), leading to evident chromosomal aberrations, and in a genomic U-abundance, which is considered premutagenic within DNA.[418] Besides breast carcinomas, an APOBEC3B mRNA overexpression has also been implicated in other cancer types,[summarized in [422]] including mainly carcinomas of the bladder, cervix, head and neck, lung and uterus, while a significantly lower APOBEC3B expression has been observed in matched normal tissues of the same origin.[415, 432] In these studies, APOBEC3B has shown a preferred mutation motif, consisting of the C-base replacement within TCA or TCT trinucleotides.[415, 432]

The exact trigger factors of APOBEC3B overexpression in cancers of different origin remains to be elucidated. However, current data suggest that in many HPV-induced cervical or head and neck carcinomas, the elevated transcription of APOBEC3B results indirectly from the inactivation of p53 by the E6 HPV oncoprotein.[433] Since L1 retrotransposition and L1 hypomethylation have been implicated in various cancers, the investigation of their relationship with APOBEC3B levels has been the focus of several studies. Finally, based on studies indicating that high levels of APOBE3B are related to a poorer cancer outcome, such as a shorter disease-free survival or overall survival duration, it seems plausible that APOBEC3B inhibitors may represent a potential therapeutic candidate for several cancers in the future.[422]

4.7 Linking APOBE3B to autoimmunity and SS

Given the well documented contribution of IFN pathway activation in autoimmunity, a link between the IFN-inducible gene APOBEC3B and autoimmune diseases seems plausible. Reviewing the literature regarding the microarray analysis studies in SLE, Crow and Wohlgemuth found that APOBEC3B gene was significantly upregulated among SLE patients.[434] On the contrary, transcriptome comparative analysis of the synvovial fibroblasts derived from two RA patients and two healthy controls via RNA sequencing revealed that APOBEC3B was among the top 10 downregulated genes in RA synovial RNA.[435]

The possible role of APOBEC3B in SS has been previously investigated only by Mavragani et al,[356] who aimed to explore the epigenetic regulation of L1s in pSS. In this study, the mRNA expression of L1 elements, APOBEC3A, APOBEC3B, APOBEC3G, AID, methyltransferases (DNMT3B, DNMT1, MECP2)and lymphoid specific helicase (LSH) were determined via qRT-PCR in SGs tissues from 24 pSS patients and from healthy controls.[356] Of the four members of the AID/APOBEC family, only APOBEC3A and APOBEC3B showed a significant correlation with the elevated L1 mRNA in SS patients, suggesting their possible role as a counterbalancing mechanism against the endogenous L1 enemy.[356]

4.8 In summary

- AID/APOBEC enzymes deaminate cytidine of polynucleotide RNA and/or ssDNA sequences to uridine.
- AID/APOBEC family consists of 11 members: APOBEC1 (in chromosome 12), APOBEC2 (in chromosome 6), AID, 7 APOBEC3 proteins (in chromosome 22) and APOBEC4 (in chromosome 1).
- APOBEC3 subfamily constitutes of 7 proteins: APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F, APOBEC3G and APOBEC3H.
- Significant sequence homology exists between the APOBEC3 members' genes. APOBEC3B gene is 59% and 57% identical to APOBEC3F and APOBEC3G respectively. Due to the high degree of homology between the APOBEC3 genes, the production of specific antibodies for each one of the APOBEC3 deaminases has been impossible since recently.
- APOBEC3 enzymes contain either one (APOBEC3A, APOBEC3C and APOBEC3H) or two (APOBEC3B, APOBEC3DE, APOBEC3F and APOBEC3G) deaminase catalytic domains. APOBEC3B is the only APOBEC3 member in which both domains are catalytically active.
- APOBEC3B preferentially deaminate the C of the TpC DNA dinucleotides or the second C of the CpC dinucleotides.
- APOBEC3 enzymes are highly expressed in lymphoid organs, where they participate in innate immune responses.
- APOBEC3 proteins belong to the IFN-inducible antiviral factors and participate in the innate immune response against exogenous viruses and endogenous mobile elements, including L1 elements, Alu sequence and LTR retroelements such as HERV-K and IAP sequences.
- APOBEC3 proteins inhibit the L1 retrotransposition, independently of their subcellular localization.
- APOBEC3B targets retroviruses, DNA viruses, LTR- and non-LTR retroelements, including L1 and Alu sequence.
- APOBEC3B may decrease L1 retrotransposition frequency by 75–90%.
- The inhibitory potency of APOBEC3B against ORF1p does not require their co-localization and is independent of the APOBEC3B interaction with ORF1p.

- The possible influence of APOBEC3s-L1 ORF2p interaction to L1 inhibition has still to be elucidated.
- APOBEC3B can be found in a cytoplasmic or nuclear localization.
- APOBEC3B is expressed in normal or neoplastic human tissues, such as the breast carcinoma, as well as in normal cells or carcinoma-derived cell lines.
- APOBEC3B mutagenic signature has been identified in several cancer-related genes, as C-clustered same-strand mutations, termed *kataegis*.
- APOBEC3B overexpression has been correlated with unfavorable prognosis of cancer patients.
- Low human APOBEC3B mRNA has been detected in freshly isolated PBMCs, i.e. monocytes and lymphocytes. APOBEC3B is predominantly expressed in B cells in comparison to other PBMC subsets.
- **IFNα is a potential inducer of APOBEC3B mRNA expression in different cell populations, such as the PBMCs, the primary naive CD4+ T cells and the hepatocytes.**
- Most studies regarding the APOBEC3B expression have been limited at the mRNA level.
- Only a few immunohistochemical studies have reported APOBEC3B expression in normal and neoplastic tissues.
- **A cytoplasmic APOBEC3B expression has been observed in the majority of the immunohistochemical studies.**
- **High APOBEC3B immunohistochemical expression has also been associated significantly with a low L1 methylation status.**
- **APOBEC3B gene has been found upregulated among SLE patients, whereas it is among the top 10 downregulated genes in RA synovial RNA.**
- **APOBEC3B mRNA has been significantly correlated with elevated L1 mRNA in SS patients, suggesting their possible role as a defensive epigenetic mechanism against the endogenous L1 enemy.**

5. Aim

6. Materials and Methods

7. Results

8. Discussion

9. Conclusions

5 AIM

The purpose of the present study is to comparatively evaluate the immunohistochemical expression of L1 ORF2 and APOBEC3B proteins in the minor salivary glands of Sjögren's Syndrome patients.

6 MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of the Faculty of Dentistry, National and Kapodistrian University of Athens, Greece *(NKUA code number 284, February 9th, 2016).*

6.1 SS Patients

The material of the present study derived from the Histopathological Archives of the Department of Oral Medicine and Pathology, Faculty of Dentistry, National and Kapodistrian University of Athens, Greece between the years 2003-2014. Among 11.709 cases, **30 (0.26%)** minor salivary gland biopsies with a diagnosis of ***Focal Lymphocytic Sialadenitis (FLS), characteristic of pSS*** were retrieved.

6.1.1.1 Exclusion criteria

Cases with a borderline FLS diagnosis or inadequate tissue for analysis as well as cases with a fixation time of <24h or >48h were excluded. As has been shown, poor or prolonged fixation time may cause impairment of tissue morphology or decreased antigen detection respectively.[436, 437] Additionally, only cases with ≥ 5 MSG lobules and in which biopsies had been performed through normal appearing mucosa were included.[438]

In *5/30* SS cases, the formalin-fixed, paraffin-embedded biopsy tissues were *not available* while *3* SS cases were excluded due to tissue insufficiency. In *five* additional cases re-evaluation of tissue sections resulted in a borderline diagnosis[439] of *Focal Lymphocytic Sialadenitis* (focus score ~1/mm^4) [27] after exclusion of gland lobules with extensive areas of interstitial fibrosis and/or lipoid degeneration.[438]

6.1.1.2 Histologic grading classification

The formalin-fixed, paraffin-embedded biopsy tissues of the remaining **17 cases** were sectioned (3 new slides per case) and the histologic grade of FLS was **evaluated** by two researchers (E-M.K. and E.P.) independently, according to the classification criteria proposed by *Tarpley et al*,[28] resulting in **3, 7** and **7** cases of **Tarpley (T) I, II** and **III categories**, respectively. In order to analyze biopsy tissues from all 4 Tarpley categories, 3 additional cases diagnosed as Tarpley IV before 2003 were included, summing up the **final number of SS cases to 20**. All these patients had been eventually

diagnosed with SS, based on the revised AECG classification and exclusion criteria, as was verified after communication with their attending clinician.[17]

6.2 Sicca controls

The first control group consisted of 5 formalin-fixed, paraffin-embedded tissue biopsies diagnosed as ***"Chronic Sialadenitis (CS), not compatible with SS"***, that were retrieved from the Histopathological Archives of the Department of Oral Medicine and Oral Pathology, Faculty of Dentistry, National and Kapodistrian University of Athens, between the years 2003-2014. These biopsies had been performed in 5 patients that had presented to the Clinic of Oral Medicine of the aforementioned Department with the major complaint of xerostomia, in order to exclude FLS suggestive of SS. None of these patients fulfilled the AECG criteria of SS diagnosis.[17] The diagnosis of CS in these cases was confirmed after re-evaluation of each biopsy by the two researchers (E-M.K. and E.P.) independently.

6.3 Non-sicca controls

The second control group consisted of the normal MSG tissue included in 5 excisional biopsies of ***lower lip extravasation cysts (lower lip mucoceles, LLM)***, that had been submitted for histopathological evaluation in the Department of Oral Medicine and Oral Pathology, Faculty of Dentistry, National and Kapodistrian University of Athens, between the years 2003-2014. According to the referral forms accompanying the biopsies, none of these 5 patients had any signs or symptoms suggestive of SS.[17]

6.4 Clinical characteristics

Information regarding the epidemiological data (gender and age) of the 30 cases included in this study was retrieved from the histopathological records. Nineteen out of twenty (95%) SS patients, all of the CS controls and 3 out of 5 (60%) LLM controls were females, while only 1 Tarpley II SS patient and 2 patients with LLM were males (Table 17 and 18).

Data regarding age were available in 19/20 SS patients (data missing in 1 Tarpley III SS patient) and in all control patients. Ten out of the nineteen (52.6%) SS patients with available data and all of the CS controls were older than 50 years, while only 1 patient presenting with LLM belonged to the ≥50 year-age group (*p=0.037*). A wider age distribution was observed between the SS patients, especially in the Tarpley II group, as well as between the LLM patients in contrast to the CS group, in which all patients were aged between 58-75 years (Diagram 1).

Table 17. Demographics of SS patients.

Study group	No. of cases	Females	Males	Age range (years)	Mean age (years ± SD)
SS TI	3	3	0	29-48	38.67 ± 9.5
SS TII	7	6	1	16-76	58.71 ± 20.94
SS TIII	7	7	0	37-55	45.5 ±6.83
SS TIV	3	3	0	45-75	61.67 ± 15.28
SS total	20	19	1	16-76	51.84 ± 16.49

Table 18. Demographics of sicca (CS)- and non-sicca (LLM) controls.

Study group	No. of cases	Females	Males	Age range (years)	Mean age (years ± SD)
CS	5	5	0	58-75	62.6 ± 6.99
LLM	5	3	2	8-77	33.2 ± 27.42

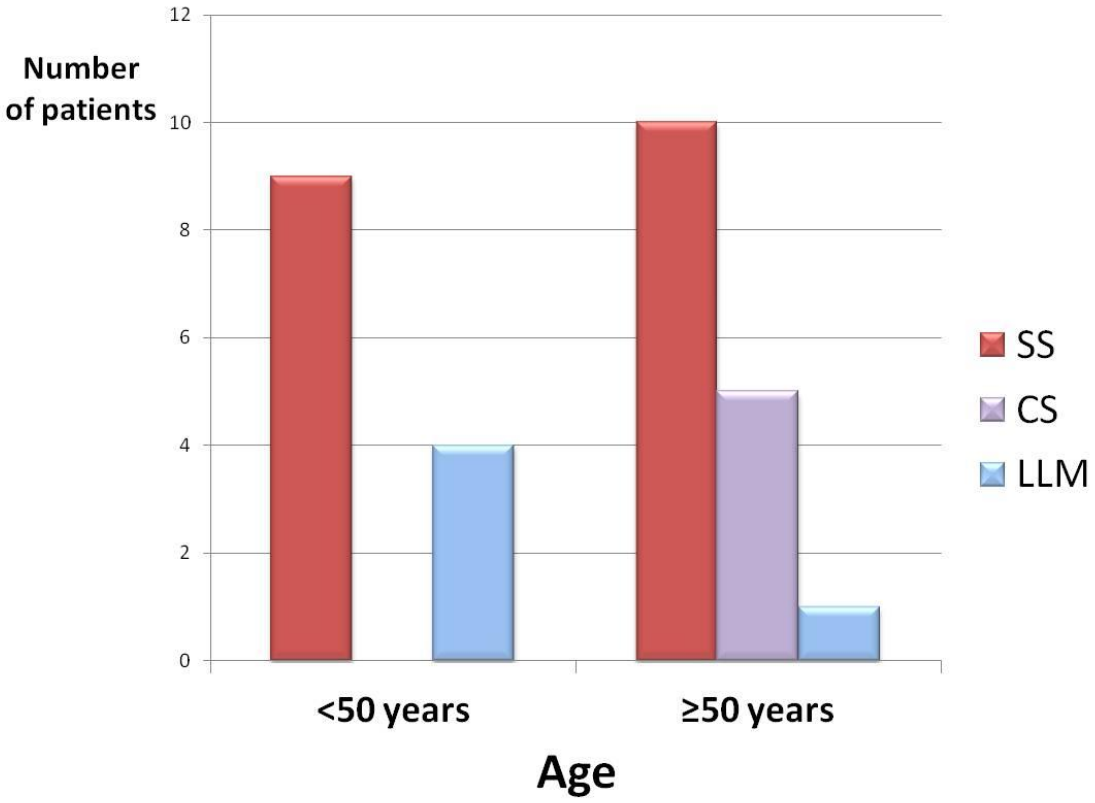


Diagram 1. Age groups in SS patients, sicca (CS) and non-sicca (LLM) controls.

6.5 Histopathological features

The 5-μm, hematoxylin-eosin (H&E) stained slides of the 30 cases containing at least 6 tissue sections per case) were histopathologically evaluated by two examiners (E-M.K and E.P.) with the use of a BX40 microscope (Olympus America Inc., Melville, New York, USA). Tissues from all cases were evaluated for the presence of focal periductal/perivascular or diffuse lymphocytic infiltration. In all specimens, the presence of lymphoepithelial lesions, lymphoid-like structures, interstitial fibrosis and lipoid degeneration as well as of vessels, nerves and striated muscles were also recorded. Focal periductal lymphocytic infiltration was observed in all SS cases (fig. 9-12), while the MSGs in CS (fig. 13) and LLM (fig. 14) cases showed mild diffuse infiltration by lymphocytes and plasma cells.

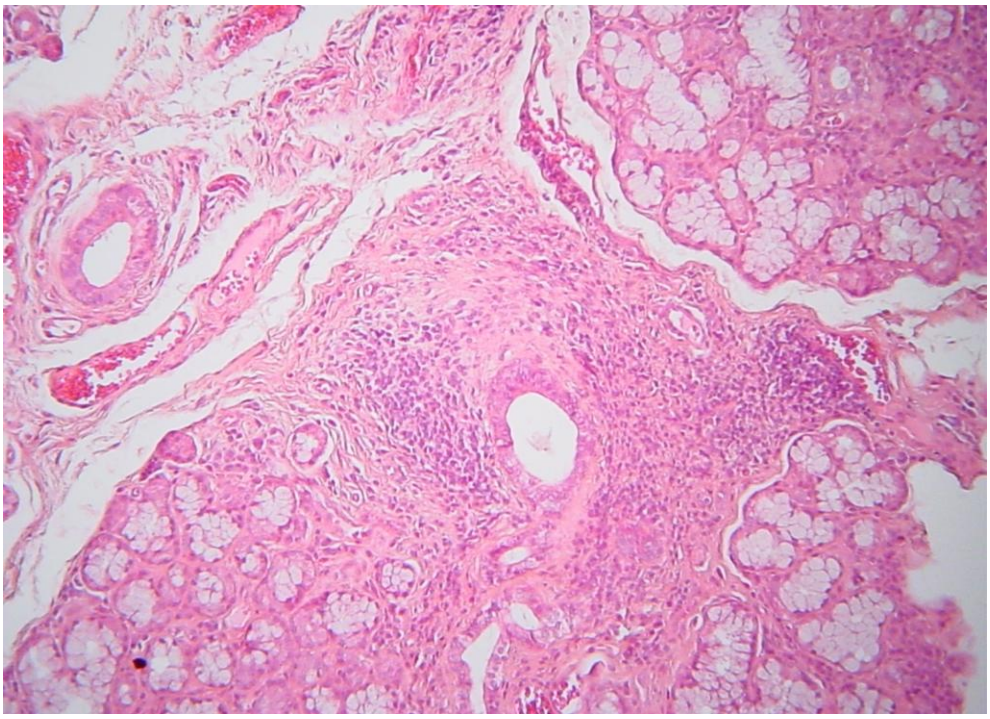


Fig. 9. Focal periductal lymphocytic infiltration in a Tarpley I SS patient (H&E,original magnification x100).

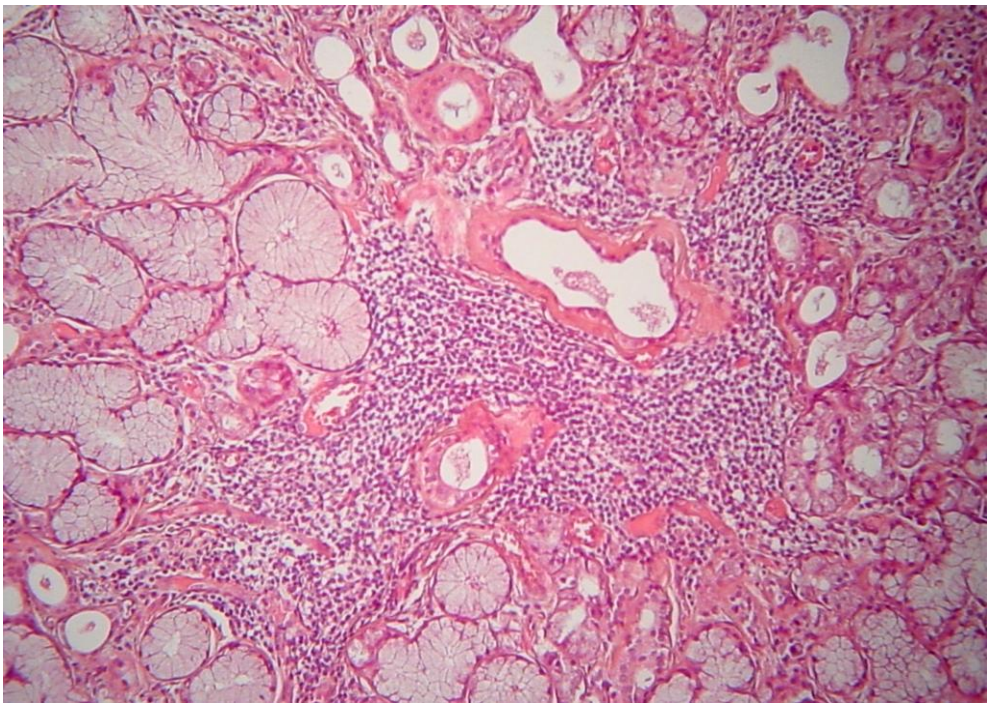


Fig. 10. Focal periductal lymphocytic infiltration with ductal dilation in a Tarpley II SS case (H&E, original magnification x100).

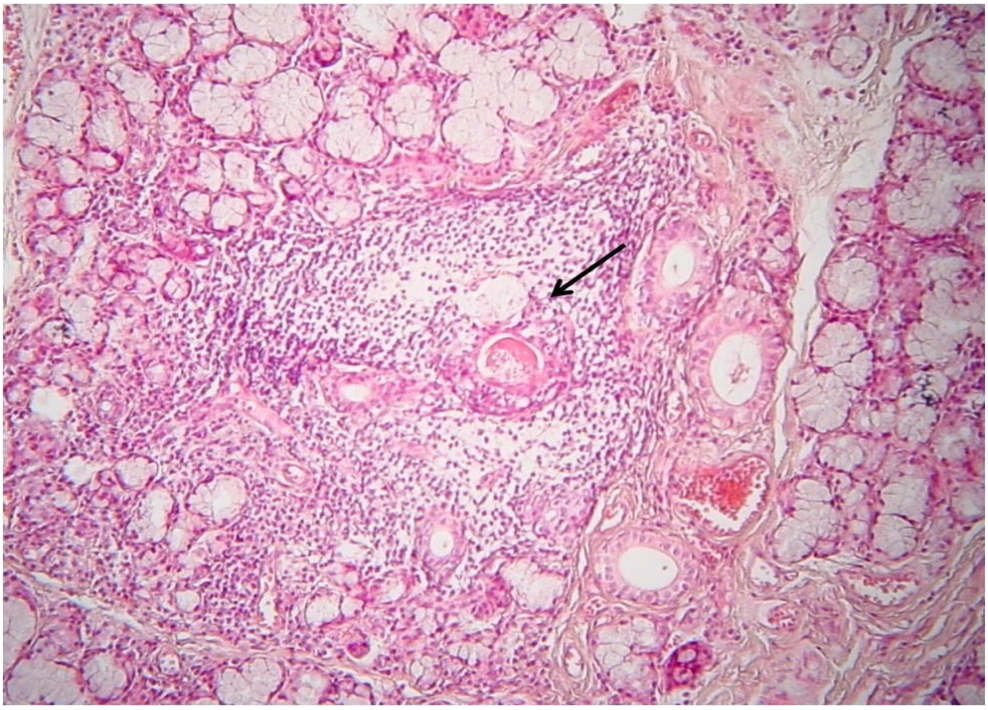


Fig. 11. Diffuse lymphocytic infiltration that disturbs normal glandular architecture in a Tarpley III SS case. Note the lymphocytic infiltration of the ductal epithelium (lymphoepithelial lesion, arrow). (H-E, original magnification x100).

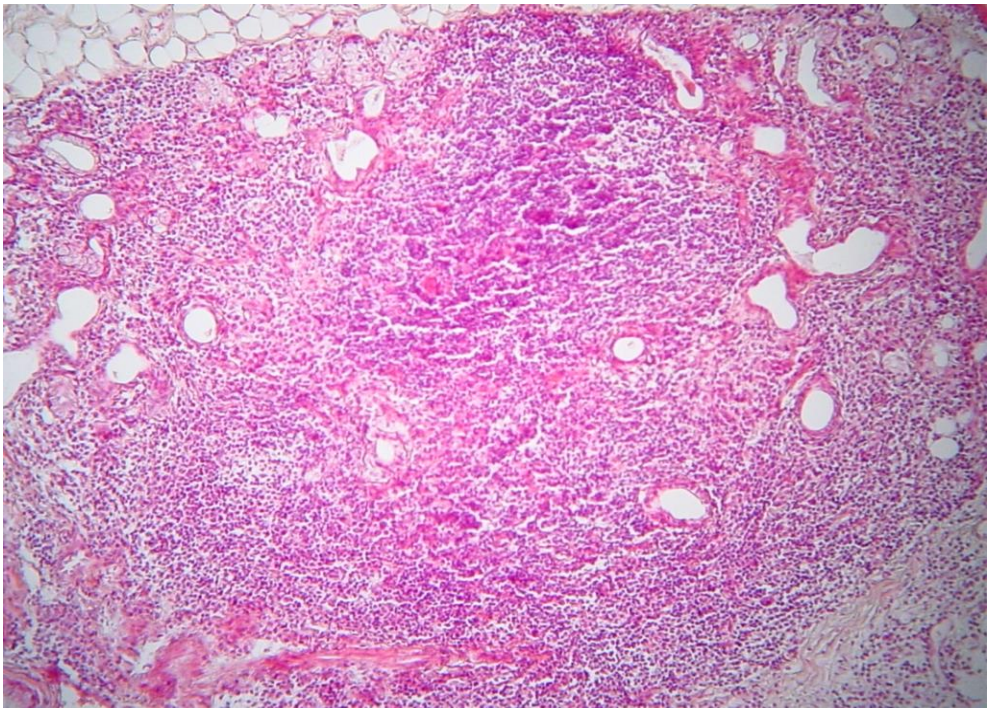


Fig. 12. Diffuse, extensive lymphocytic infiltration with lymphoid-like structure formation (arrow) and parenchymal destruction in a case of Tarpley IV SS (H&E, original magnification x100).

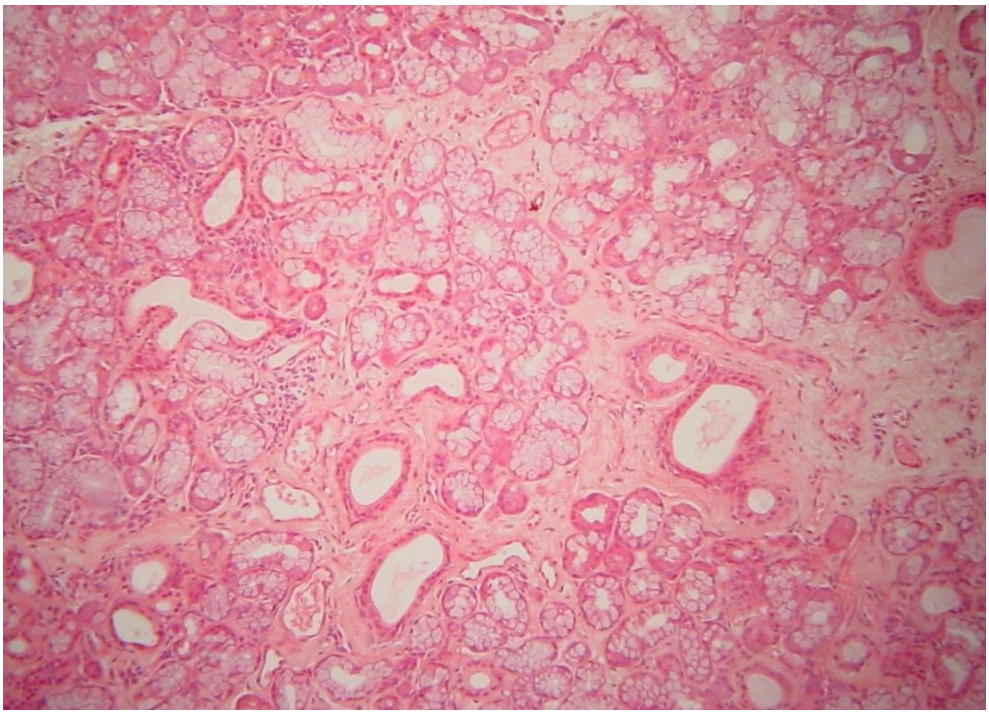


Fig.13. Dilated ducts, chronic inflammation and interstitial fibrosis in a case of CS (H&E, original magnification x100).

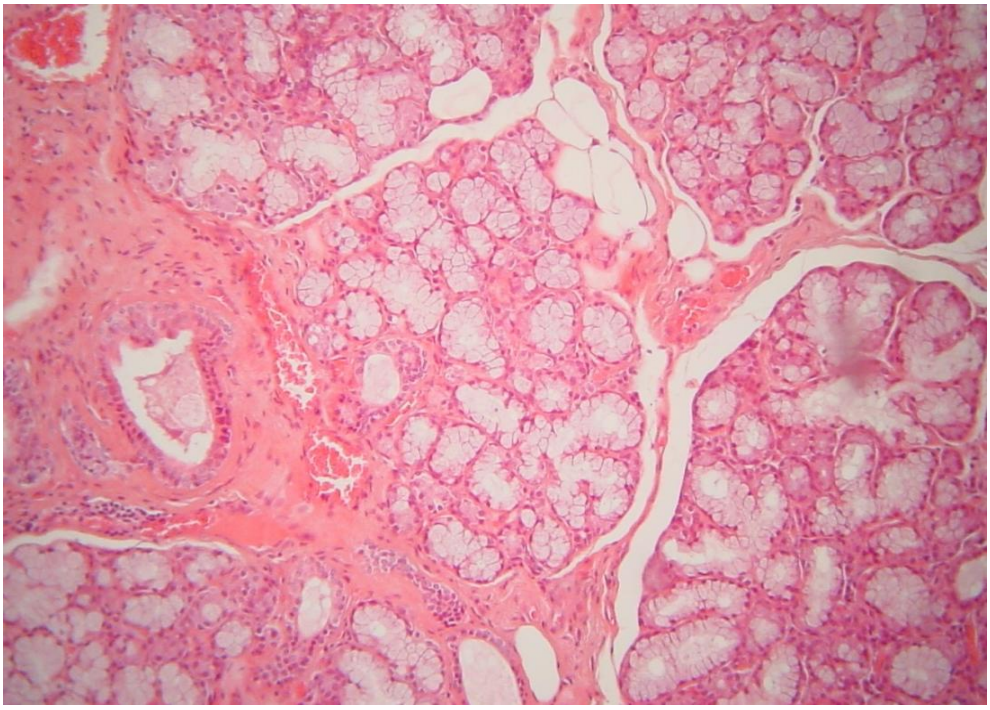


Fig. 14. Normal MSGs with mild focal chronic inflammation in a LLM case (H&E, original magnification x100).

6.6 Microscope slides preparation

All specimens in the study had been fixed in 10% neutral buffered formalin solution (4% formaldehyde) and embedded in paraffin blocks *(Diawax, Diapath, Martinengo, Italy),* as has been previously described.[440] The blocks of formalin-fixed paraffin-embedded tissues were clamped into a manual rotary microtome *(Leica RM2145)* for section cutting. Sections of 4μm were plated in a water bath (*Tissue Bath, P/S, NBIT Y2K, Chicago 47, USA*) at 62°C for 1 min and were then placed on positively-charged slides *(SuperFrost®Plus, Menzel-Glaser, D-38116 Braunschweig, 25*75*1.0 mm, Art No.J1800AMNZ),* to avoid tissue detachment during processing and staining. Finally, the slides were treated in a dry heat incubator *(Heratherm, Thermo Scientific, USA)* for 1 hour at 64°C to promote tissue adherence to the slide, and were afterwards stored in room temperature.

6.7 Immunohistochemistry

The formalin-fixed paraffin-embedded tissues were coded to ensure the double-blind study and 3 slides containing numbered serial sections were prepared for each one of the 30 blocks. The first slide was stained with a routine **H&E stain,** as has been previously described,[441] for histopathological evaluation and the other two were used for immunohistochemical staining with the **anti-L1NE-ORF2** and the **anti-APOBEC3B** antibodies (Table 19).

Table 19. Characteristics of antibodies used in present study.

Antibody	L1 ORF2	APOBEC3B
Antibody type	polyclonal	polyclonal
Host	rabbit	rabbit
Epitope	982-1281 amino acids	20-60 amino acids
Dilution	1:500	1:500
Epitope retrieval solution	EnVision™ FLEX Target Retrieval Solution	EnVision™ FLEX Target Retrieval Solution
Positive control	breast carcinoma	breast carcinoma
Source	Santa Cruz Biotechnology, Inc	Biorbyt
Catalog number	sc-67198	orb155694

6.7.1 Immunohistochemistry protocol

6.7.1.1 Deparaffinization and re-hydration

Prior to deparaffinization, tissue slides were placed in a dry heat incubator in 60°C for 15', in order to allow the drainage of melting paraffin. For deparaffinization, the slides were immersed twice into fresh xylene at room temperature, for 10' each time.

For re-hydration, the slides were transferred twice through 100% ethanol at room temperature, for 3' each time and were transferred twice through 95% ethanol at room temperature for 3'. Then, the slides were immersed into 40ml Tris-buffered saline solution containing Tween 20 *[EnVision™ FLEX Wash Buffer (20x concentrated), DAKO, K8007, Agilent Technologies, USA]* at room temperature for 10' and, finally, in distilled water for 10'.

6.7.1.2 Endogenous peroxidase blocking

Blocking of the endogenous peroxidase activity was achieved by incubating the slides in 3% H_2O_2 solution [5ml H_2O_2 diluted in100ml distilled water (*Hydrogen Peroxide Block 30%, Merck, Darmstadt, Germany)*] at room temperature for 10', followed by washing in Tris-buffered saline solution containing Tween 20 *[EnVision™ FLEX Wash Buffer (20x concentrated), DAKO, K8007, Agilent Technologies, USA]* at room temperature for another 10'.

6.7.1.3 Antigen retrieval

Slides were immersed into plastic pots containing 20ml Citrate buffer pH 6.1 *[EnVision™ FLEX Target Retrieval Solution (50x concentrated), DAKO, K8005, Agilent Technologies, USA]* and were placed in a microwave oven for 20', to induce heat-mediated antigen retrieval. Subsequently, the slides were cooled in room temperature for 20', followed by rinsing with Tris-buffered saline solution containing Tween 20 *[EnVision™ FLEX Wash Buffer (20x concentrated), DAKO, K8007, Agilent Technologies, USA]* at room temperature for 10'.

6.7.1.4 Immunohistochemical staining

For the immunohistochemical staining, each primary antibody was diluted in the specific antibody buffer *[EnVision™ FLEX Antibody Diluent, DAKO, K8006, Agilent Technologies, USA]* at room temperature for 1'. Then, the slides were incubated with the

primary antibody in appropriate dilution (anti-LINEORF2 1:500, anti-APOBEC3B 1:500) overnight at 4°C.

The next day the slides were rinsed with Tris-buffered saline solution containing Tween 20 *[EnVision™ FLEX Wash Buffer (20x concentrated), DAKO, K8007, Agilent Technologies, USA]* at room temperature for 10'. Then, they were incubated with the ready-to-use secondary buffered solution containing a stabilizing protein and an antimicrobial agent *[EnVision™ FLEX+ Rabbit (LINKER), DAKO, K8019, Agilent Technologies, USA]* at room temperature for 15'. The slides were rinsed again with Tris-buffered saline solution containing Tween 20 *[EnVision™ FLEX Wash Buffer (20x concentrated), DAKO, K8007, Agilent Technologies, USA]* at room temperature for 10'.

Slides were then incubated with *EnVision Flex+ [DAKO, K8002, Agilent Technologies, USA]* at room temperature for 25-30'. Once again, the slides were washed into Tris-buffered saline solution containing Tween 20 *[EnVision™ FLEX Wash Buffer (20x concentrated), DAKO, K8007, Agilent Technologies, USA]* at room temperature for 10'.

Slides were incubated with the appropriate DAB solution *[1-2 drops of EnVision™ FLEX DAB+ Chromogen (DM827) diluted into 100ml EnVision™ FLEX Substrate Buffer (DM823), DAKO, Agilent Technologies, USA]* at room temperature for 5'.

Slides were rinsed with Tris-buffered saline solution containing Tween 20 *[EnVision™ FLEX Wash Buffer (20x concentrated), DAKO, K8007, Agilent Technologies, USA]* at room temperature for 10' and with running faucet water for 10'. Finally, the slides were immersed at room temperature for 1' into Gill's hematoxylin (No. 2) solution, which is indicated for nuclear counterstain for chromogens such as DAB.

6.7.1.5 Dehydration and slide covering

Dehydration of slides was performed through 4 incubations into ethanol (70%, 80%, 95% and 100%) at room temperature for 1' in each ethanol concentration, followed by immersion into xylene at room temperature for 2' and slides' and placement of the cover slip using mounting solution (Table 20).

Table 20. Immunohistochemistry protocol.

Deparaffinization and re-hydration

- dry heat incubator, x15', 60°C
- fresh xylene, 2x10', room temperature (RT)
- ethanol 100%, 2x3', RT
- ethanol 95%, 2x3', RT
- Tris-buffered saline solution, x10', RT [EnVision™ FLEX Wash Buffer (20x concentrated), DAKO, K8007, Agilent Technologies, USA]
- distilled water, x10', RT

Endogenous peroxidase blocking

- H_2O_2 3% solution [5ml H2O2 diluted in 100ml distilled water] x10', RT
- Tris-buffered saline solution, x10', RT

Antigen retrieval

- 20ml Citrate buffer pH 6.1, microwave oven x20',
- cooling, x20', RT
- Tris-buffered saline solution, x10', RT

Immunohistochemical staining

- primary antibody, diluted in the specific antibody buffer [EnVision™ FLEX Antibody Diluent, DAKO, K8006, Agilent Technologies, USA] in appropriate dilution (anti-LINEORF2 1:500, anti-APOBEC3B 1:500), overnight, 4°C
- Tris-buffered saline solution, x10', RT
- ready-to-use secondary buffered solution [EnVision™ FLEX+ Rabbit (LINKER), DAKO, K8019, Agilent Technologies, USA], x15', RT
- Tris-buffered saline solution, x10', RT
- EnVision Flex+ [DAKO, K8002, Agilent Technologies, USA], x25-30', RT
- Tris-buffered saline solution, x10', RT
- DAB solution [1-2 drops of EnVision™ FLEX DAB+ Chromogen (DM827) into 100ml EnVision™ FLEX Substrate Buffer (DM823), DAKO, Agilent Technologies, USA], x5', RT
- Tris-buffered saline solution, x10', RT
- running faucet water, x10'
- Gill's hematoxylin (No. 2) solution, x1', RT

Dehydration and slide covering

- ethanol 70%, x1', RT
- ethanol 80%, x1', RT
- ethanol 95%, x1', RT
- ethanol 100%, x1', RT
- xylene, x2', RT
- cover slip using mounting solution

6.7.2 Evaluation of immunostaining

Immunostaining evaluation was performed by the two examiners independently (E-M.K. and E.P.). In cases with contradictory results, the slides were re-evaluated, until consensus was reached. **Positive control** for both antibodies consisted of an invasive lobular (40% classic variant, 60% pleomorphic variant) breast carcinoma (grade 3, stage $T_2N_0M_x$) from a 52-year old female patient. No staining was observed in a Tarpley II SS case in which the primary antibodies were omitted (**negative control**).

The staining positivity for both antibodies was determined according to the semi-quantified method proposed by Gwak et al[426] for the APOBEC3 immunostaining (Table 21). The staining intensity was graded as 0 (negative), 1 (mild), 2 (moderate) and 3 (strong). The staining extent was characterized according to the percentage of the positively stained cells: 0 (0-5%), 1 (6-19%), 2 (20-49%) and 3 (≥50%). Cells were evaluated as positive when they displayed staining in either the cytoplasm or the nucleus. The final immunostaining score (0-9) was calculated as the product of intensity and extent. According to the final score, cases were categorized as negative (score 0-3), mildly positive (+, score 4) and strongly positive (++, score 6 or 9).

Table 21. Immunohistochemistry evaluation method.

intensity	Extent	final score	classification
0 (no stain)	0 (0-5%)	0-3	negative
1 (mild)	1 (6-19%)	4	mildly positive
2 (moderate)	2 (20-49%)	6, 9	strongly positive
3 (strongly)	3 (≥50%)		

6.8 Computerized image analysis

Cases with a borderline intensity staining were also evaluated with Computerized Image Analysis (CIA), a fast and more objective method, in order to test the accuracy of our results.[442, 443] CIA required a semi-automated system with the following hardware features: Intel Pentium V, Digital Camera Sony (1600×1200), Olympus CX-310 microscope and the following Software: Windows XP/Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan. Representative tissue areas demonstrating even a slight expression of the immunohistochemical markers were characterized as low to high. A broad spectrum of 0-255 continuous staining intensity values (grey scale) was the basis for analyzing these different expression levels. Values increasing up to 255 were correlated to progressively reduced protein expression, whereas values decreasing to 0 were associated with a strong protein expression. Thus, value 0 and 255 corresponded to black and white color respectively.[444] Measurements were performed in 5 optical fields per case and at an original magnification ×400.

6.9 Statistical analysis

Statistical analysis was performed with the use of SPSS, V22.0 Software for Windows (SPSS Inc., Chicago). The differences of the immunohistochemical expression of L1 ORF2p and APOBEC3B between the 4 SS Tarpley subgroups as well as between the SS patients and sicca (CS) or/and non-sicca (LLM) controls were evaluated by Fisher's exact test. The relationship between the CIA and the visual evaluation results regarding intensity of both antibodies was evaluated with Pearson's correlation. The statistical significance level was set at $p<0.05$.

7 RESULTS

7.1 L1 ORF2p immunohistochemical expression

7.1.1 L1 ORF2p immunohistochemical expression in breast carcinoma

The immunohistochemical expression of L1 ORF2p was initially confirmed in an **invasive lobular breast carcinoma**. Positive immunostaining was observed only in the ***ductal*** and ***acinar neoplastic cells***, whereas the connective and adipose tissue as well as the endothelial cells did not express L1ORFp (fig.15A). Strong cytoplasmic L1ORFp expression was evident in the entire neoplastic ductal epithelium (fig.15B).

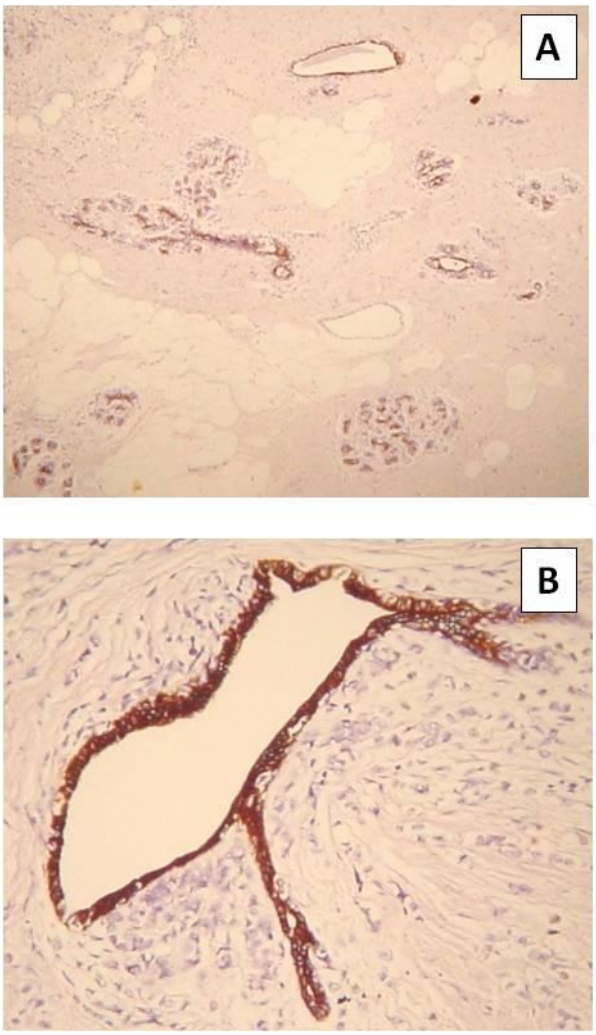
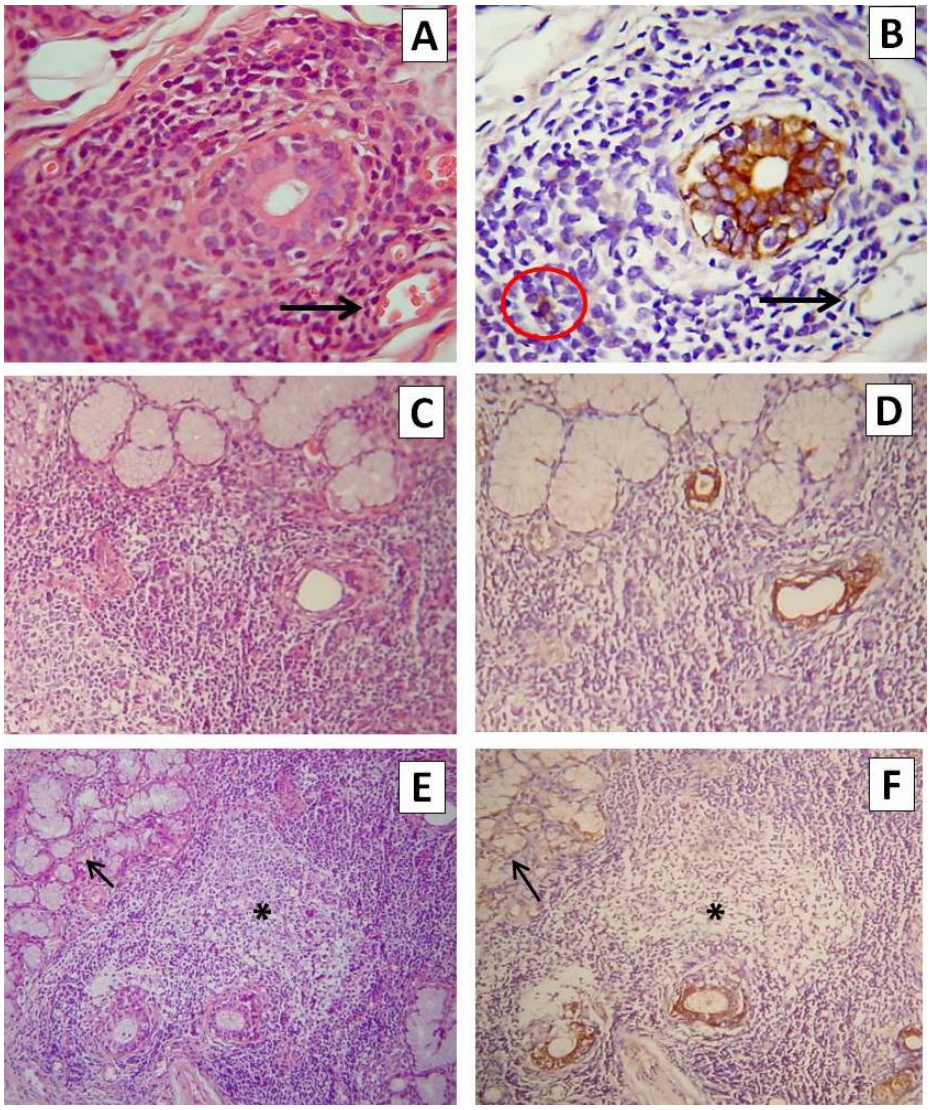


Fig. 15. (A, B) Strong cytoplasmic L1 ORF2p expression in the ductal and acinar cells of a breast carcinoma (positive control)[L1 ORF2p immunohistochemical stain, original magnification (A) x25, (B) x100].

7.1.2 L1 ORF2p immunohistochemical expression in SS cases

7.1.2.1 Descriptive results

Eighteen out of 20 (90%) SS cases showed a positive L1 ORF2p immunoreaction. L1 ORF2p was expressed in all cases in the ***ductal epithelial cells*** (fig. 16B). L1ORF2 was not expressed in acinar cells (fig. 16D,F), in the lymphoid infiltrates (fig. 16B,D) or in the ***lymphoid-like structures*** (fig. 16F), while only few scattered inflammatory cells, possibly corresponding to MNCs, expressed cytoplasmic L1 ORF2p (fig. 16B, red cycle). L1 ORF2p was expressed in the ***striated muscle bundles*** (fig.16H) but was absent in the ***vascular endothelial cells*** (fig. 16B arrows, J) and the ***nerve bundles*** (fig.16J), as well as in ***areas of interstitial fibrosis*** (fig.16L) and ***lipoid degeneration*** (fig.16H).



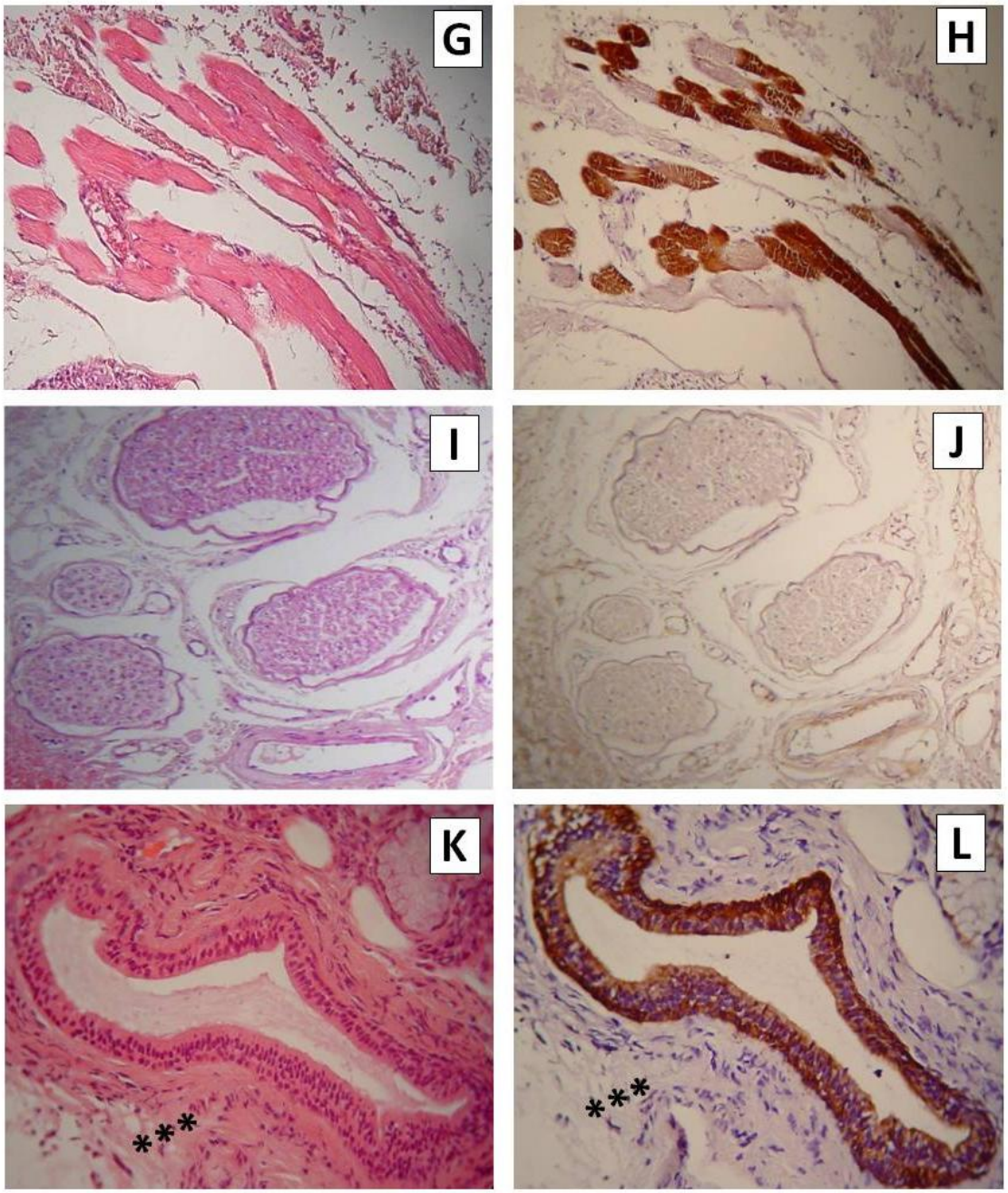


Fig. 16 L1 ORF2p positive expression in the ductal epithelium (B). Absence of staining was observed in the acinar cells (D, F),in the lymphoid infiltrates (B,D) or in the lymphoid-like structures (F), while only few scattered inflammatory cells, possibly corresponding to MNCs, expressed cytoplasmic L1 ORF2p (fig. 16B, red cycle). L1 ORF2p was expressed in the striated muscle bundles (H) while not in the vascular endothelial cells (B arrows, J) and the nerve bundles (J), as well as in areas of interstitial fibrosis (L) and lipoid degeneration (H) [(A),(C), (E), (G),(I),(K) H&E stain; (B),(D), (F), (H),(J),(L) L1 ORF2p immunohistochemical stain; original magnification (A), (B) x400, (C-F) x400, (G-L) X400].

The 20 SS cases were characterized as mildly positive/strongly positive or negative according to the immunoreaction in the ductal epithelium. L1ORF2 expression in the cytoplasm of the ductal cells showed often a slightly speckled-punctuate pattern and in some cases it was more prominent in the apical part. (fig. 17)**.** No difference in L1 ORF2p immunostaining was observed between intralobular and interlobular ducts.

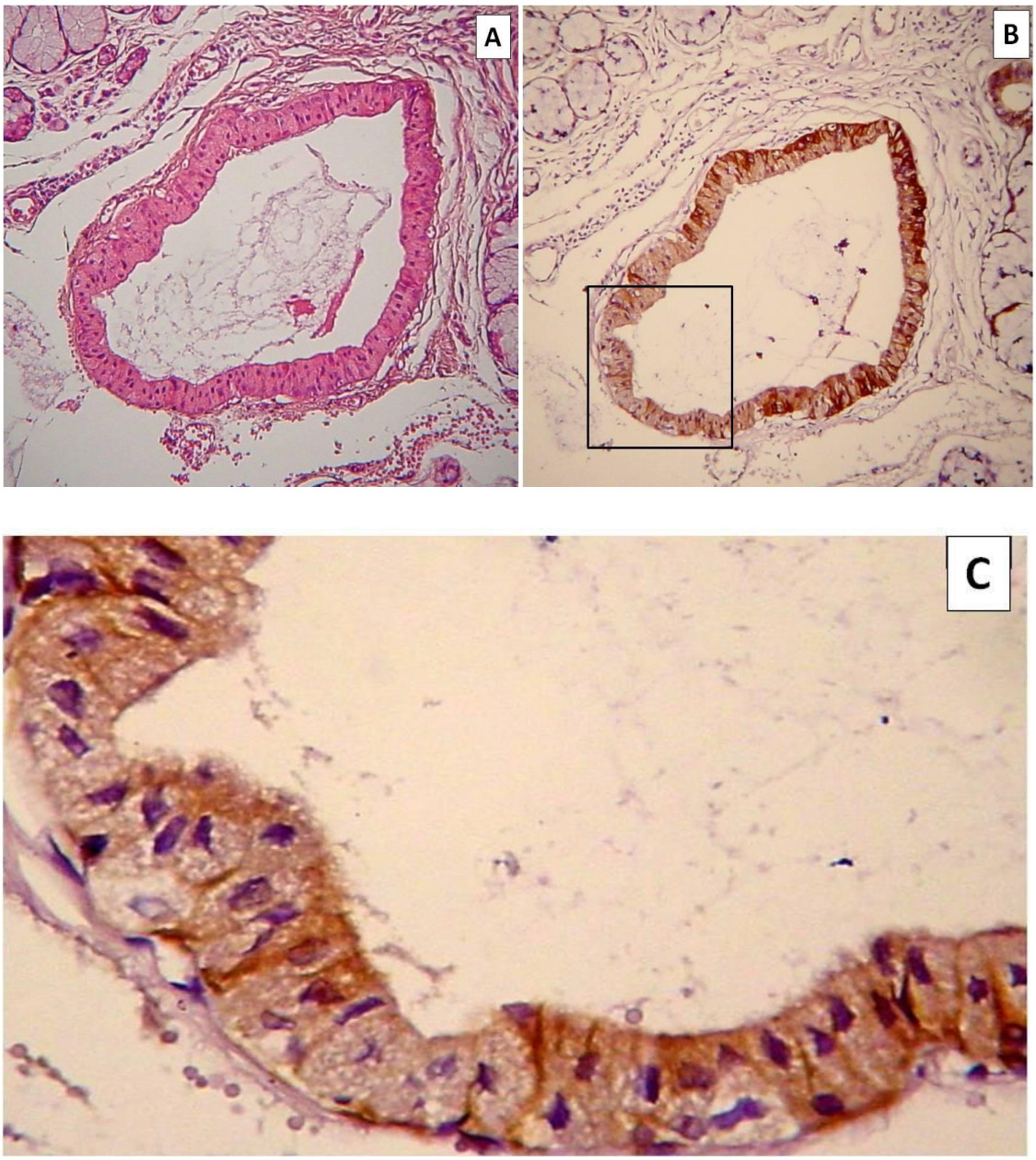


Fig. 17. L1 ORF2p cytoplasmic expression in the ductal epithelium (B, C). Note the fine speckled L1 ORF2p staining pattern (C) [(A) H&E stain; (B), (C) L1 ORF2p immunohistochemical stain; original magnification (A), (B) x100, (C) x400].

7.1.2.2 L1 ORF2p immunoexpression intensity

The L1 ORF2p intensity grading by the two examiners resulted initially in 16/18 (88,9%) cases with strong (grade 3) staining intensity. CIA was applied in 4 SS cases (2 Tarpley II, 1 Tarpley III and 1 Tarpley IV) with borderline results, in order to verify the L1 ORF2p staining intensity. The intensity outliers 100.25 and 155.96 corresponded to strong (grade 3) and mild (grade 1) intensity respectively, while intermediate values (129.44, 133.4) were considered indicative of a moderate intensity (grade 2).[444] The CIA intensity categorization was in agreement with the two examiners' grading in 3 out of 4 CIA-tested SS cases; in the 4th case (SS Tarpley II case), the CIA changed the intensity grade from 3 to 2. This case, though, showed L1 ORF2p expression in grade 3 extent, thus the intensity grade change did not influence the final classification of this case (final score 6, strongly positive case). Eventually, after incorporating the CIA results (fig. 18), **mild** (fig. 19A) **moderate** (fig. 19B) and **strong** (fig. 19C) L1 ORF2p intensity was observed in **1 (5%), 2(10%)** and **15(75%) SS cases** respectively. No staining was seen in 1 SS Tarpley II and 1 SS Tarpley IV case (Table 22).

Table 22. L1ORF2p staining intensity results in SS.

Study group	Intensity grade				
	0 (no stain)	1 (mild)	2 (moderate)	3 (strongly)	Total
SS TI	-	-	-	3	3
SS TII	1	-	2	4	7
SS TIII	-	-	-	7	7
SS TIV	1	1	-	1	3
SS total	2	1	2	15	20

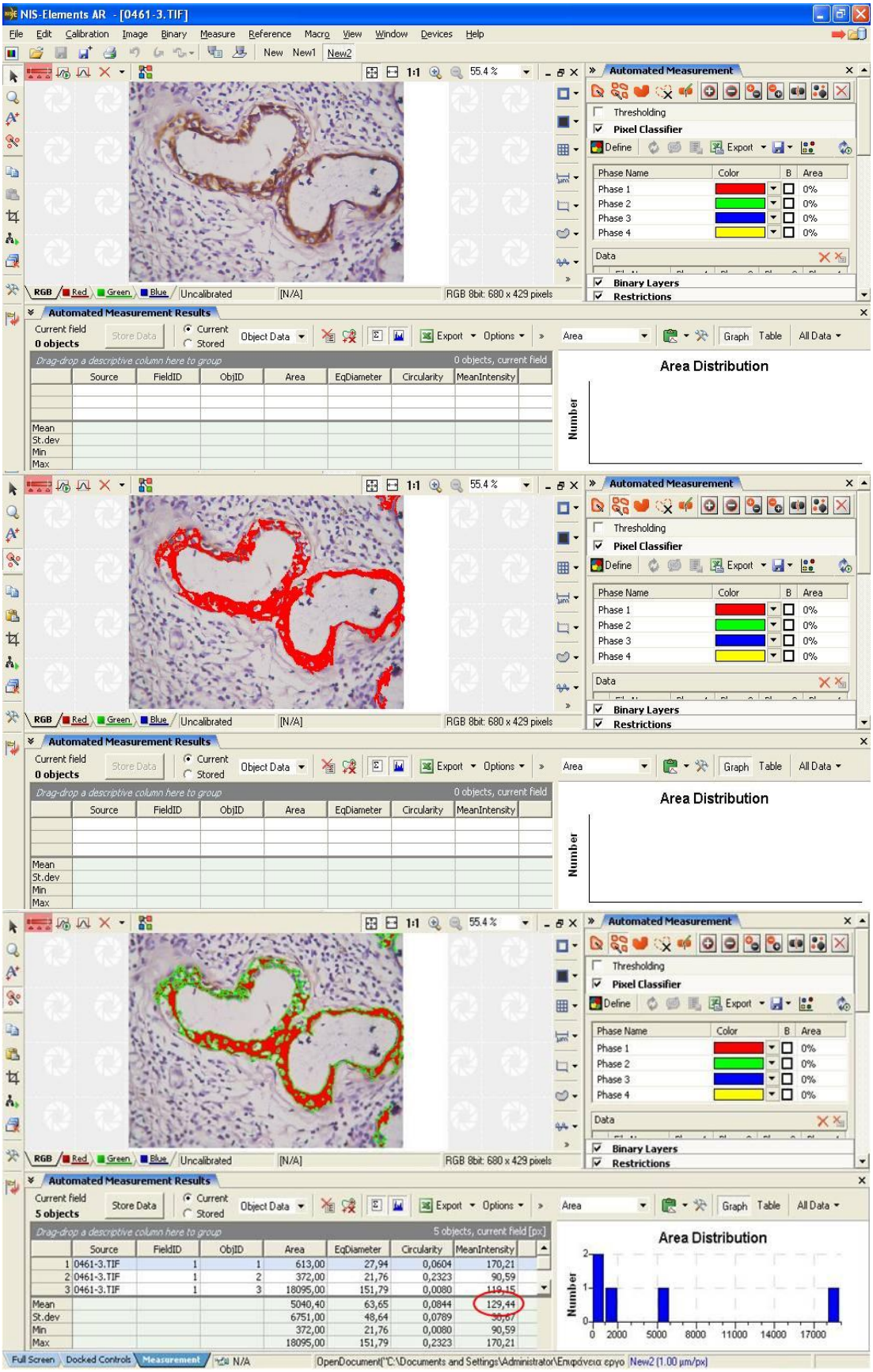


Fig. 18. CIA process for the L1 ORF2p intensity in a SS Tarpley II case (CIA value 129.44).

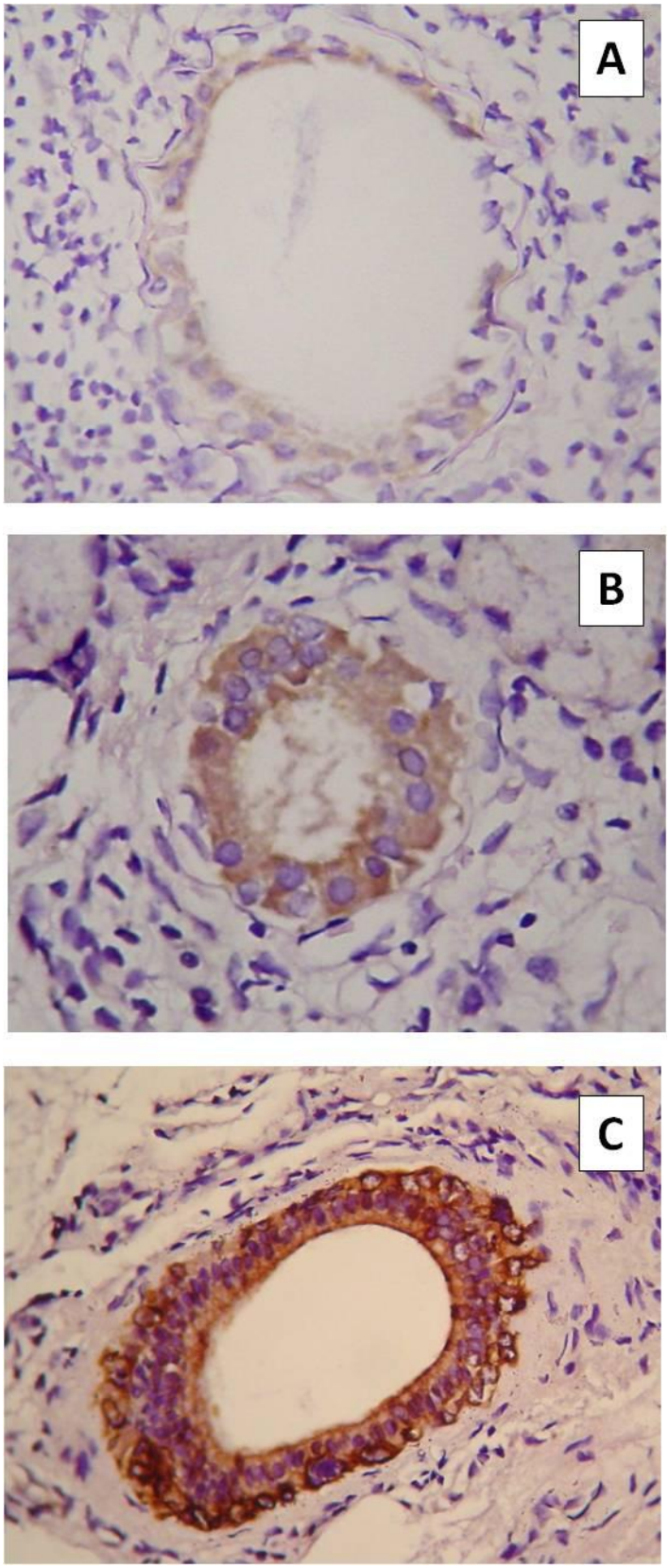


Fig. 19. L1 ORF2p staining of mild (A), moderate (B) and strong (C) intensity [L1 ORF2p immunohistochemical stain, original magnification (A), (B), (C) x400].

7.1.2.3 L1 ORF2p immunoexpression extent

The eighteen positive SS cases showed a L1 ORF2p staining extent of either **grade 2** (20-49% of the ductal cells) or **grade 3 (**≥ 50% of the ductal cells). L1 ORF2p expression was totally absent in 2 cases (extent **grade 0**) while no SS cases with L1 ORF2p expression in 6-19% of the ductal cells (extent **grade 1**) were found (Table 23). **Grade 2** extent was observed in 4/18 (22.2%) cases. In 3 out of these 4 cases [one of each Tarpley I, II and III] the final categorization was strongly positive, since they showed a strong (i.e. intensity grade 3) positive immunoreaction, leading to the final score of 6. In contrast, in one **grade 2** Tarpley II SS case (fig. 20**)** that showed moderate intensity (grade 2) of staining, the final score was 4. In 14/18 (77.8%) positive SS cases, positive immunostaining was observed in more than 50% of the ductal cells, corresponding to a maximum extent **grade** of **3** (fig. 21-24**)**.

Table 23. L1ORF2p staining extent results in SS.

Study group	Extent grade				
	0 (0-5%)	1 (6-19%)	2 (20-49%)	3 (≥50%)	Total
SS TI	-	-	1	2	3
SS TII	1	-	2	4	7
SS TIII	-	-	1	6	7
SS TIV	1	-	-	2	3
SS total	2	-	4	14	20

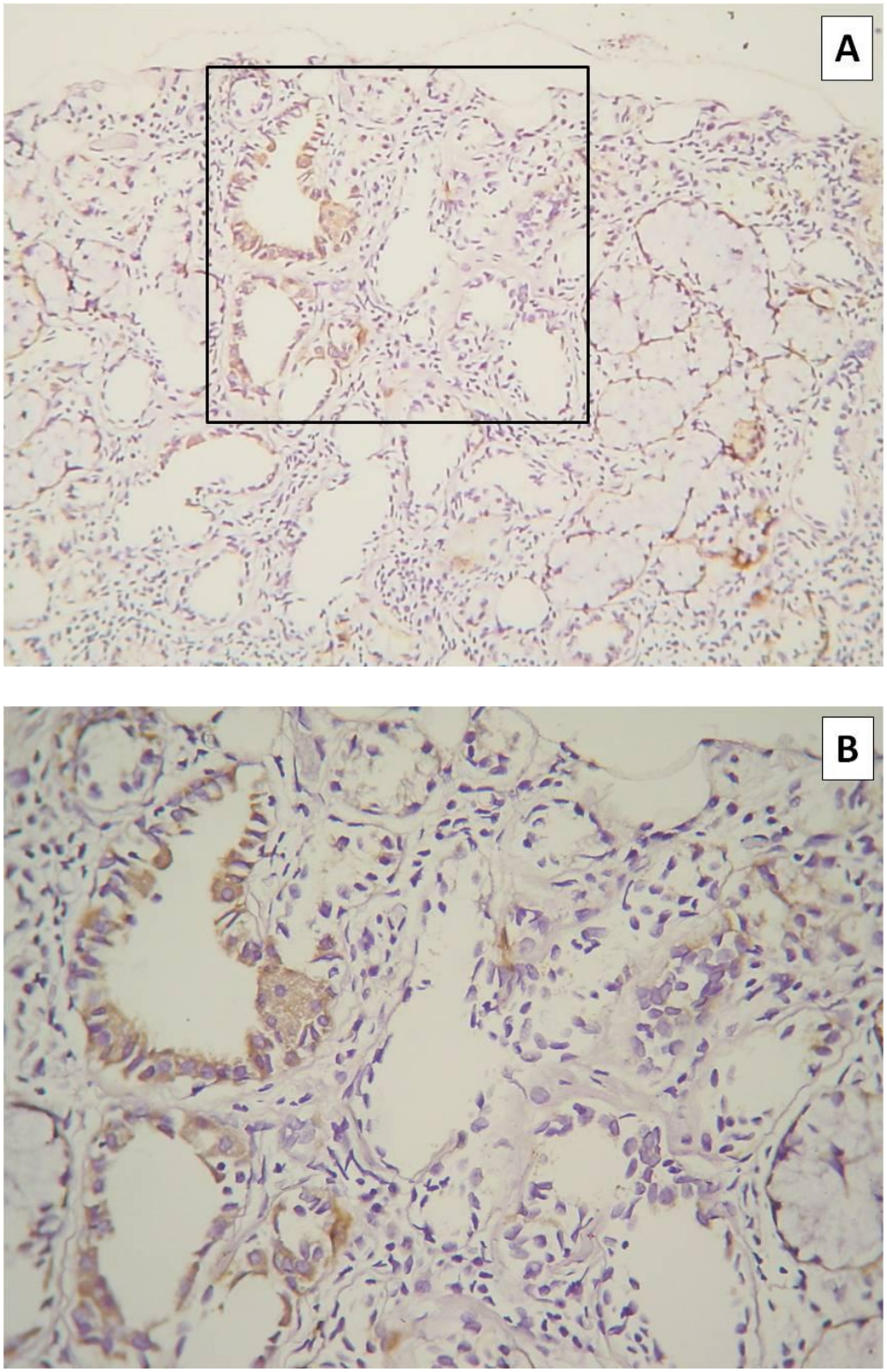


Fig. 20. L1 ORF2p expression of moderate intensity and extent grade 2 [L1 ORF2p immunohistochemical stain, original magnification (A) x100, (B) x400].

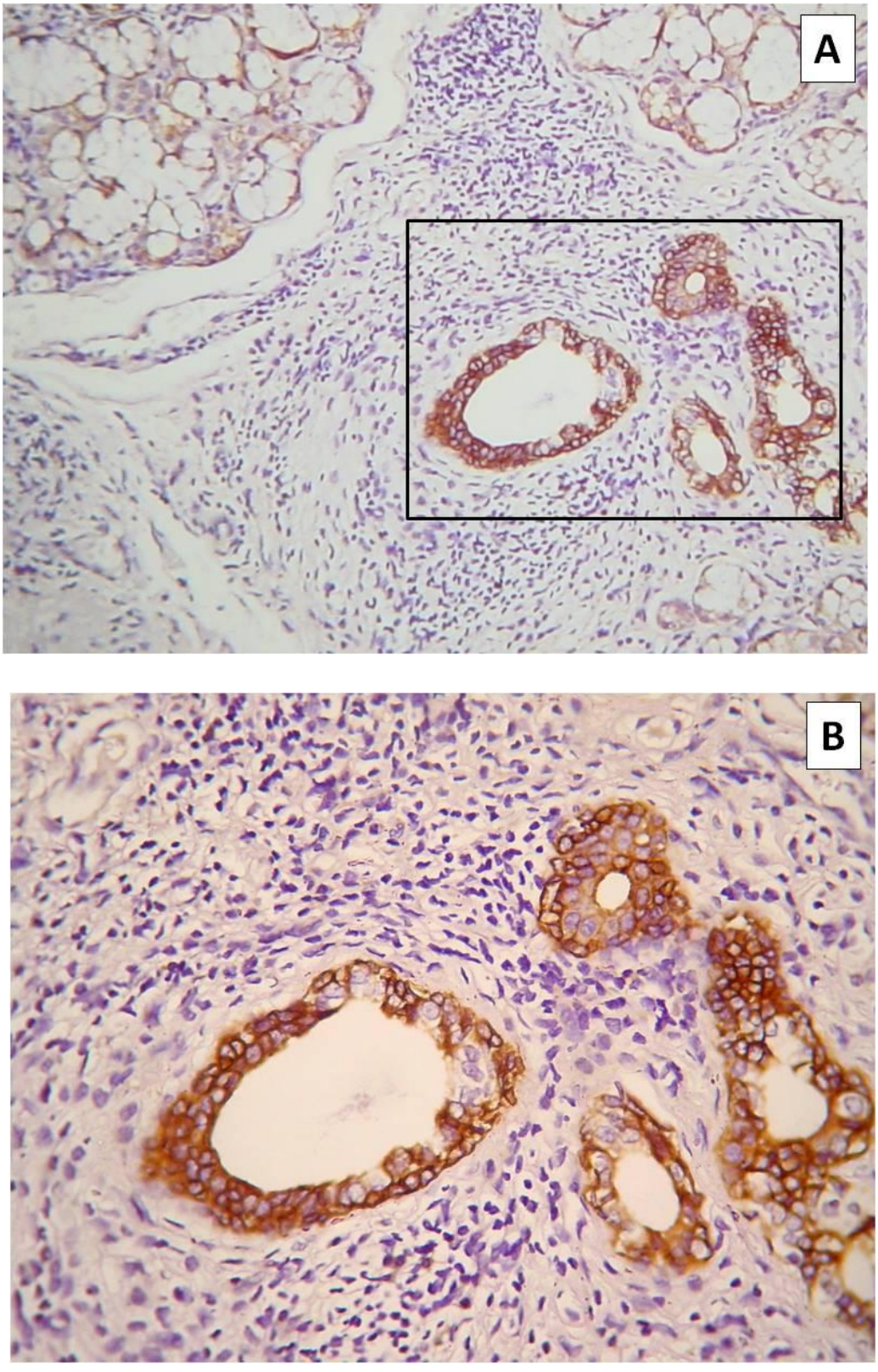


Fig. 21. Intense positive L1 ORF2p cytoplasmic expression of grade 3 extent in a SS Tarpley I patient. Note the negative periductal inflammatory infiltrate [L1 ORF2p immunohistochemical stain, original magnification (A) x100, (B) x400].

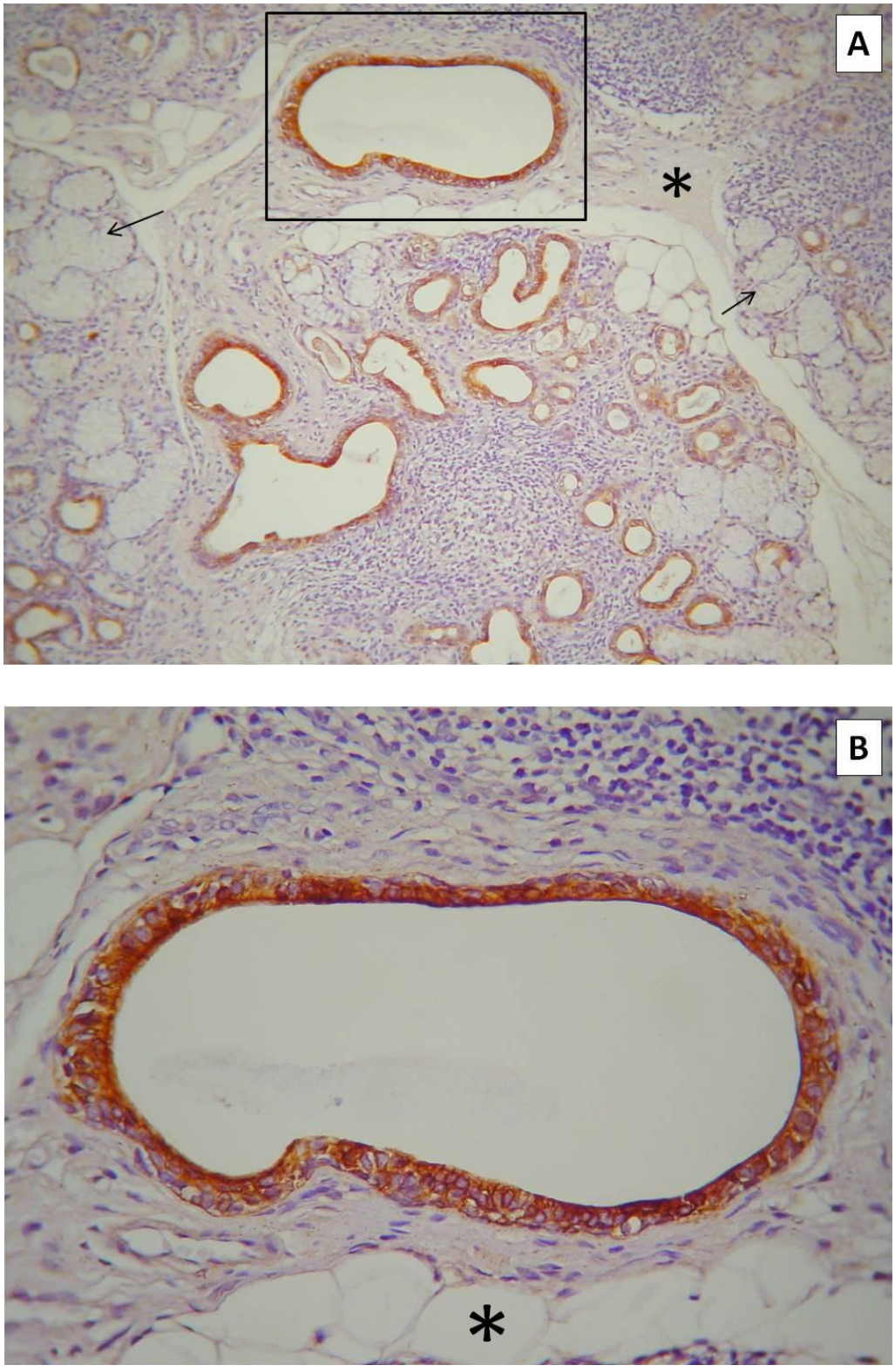
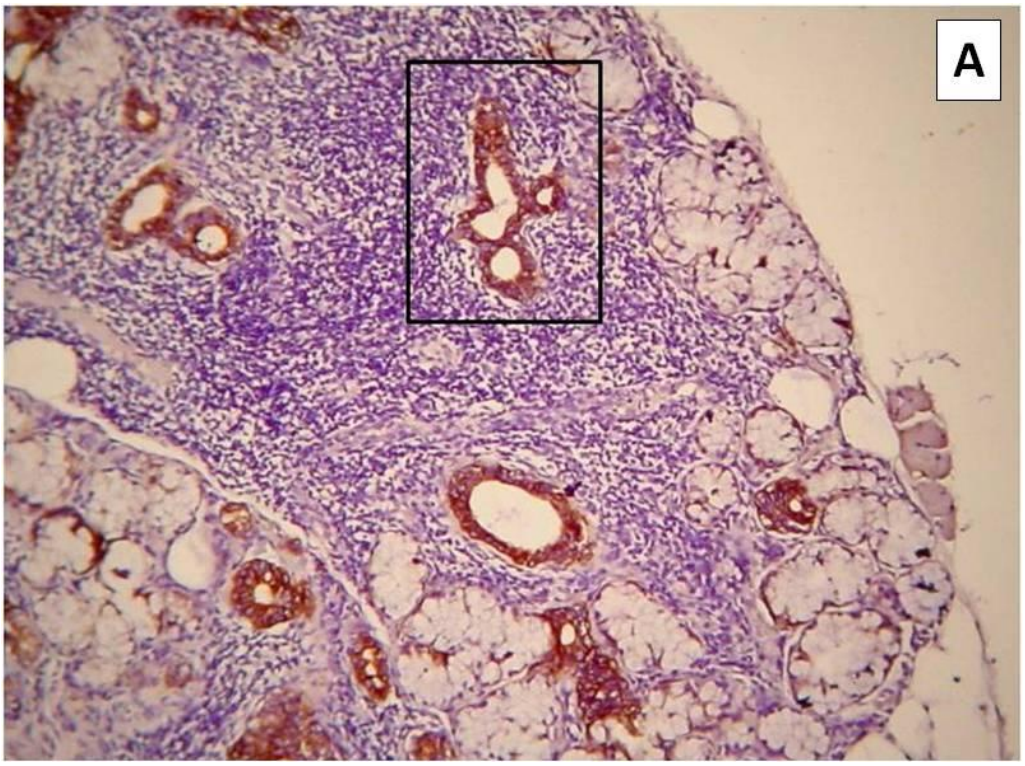


*Fig. 22. Intense positive L1 ORF2p cytoplasmic expression in the ductal epithelium of a Tarpley II SS patient (grade 3 extent). Note the absence of L1 ORF2p expression in the acinar cells (A, arrows) and the areas of intestinal fibrosis (A, *) and lipoid degeneration (B, *) [L1 ORF2p immunohistochemical stain, original magnification (A) x100, (B) x400].*



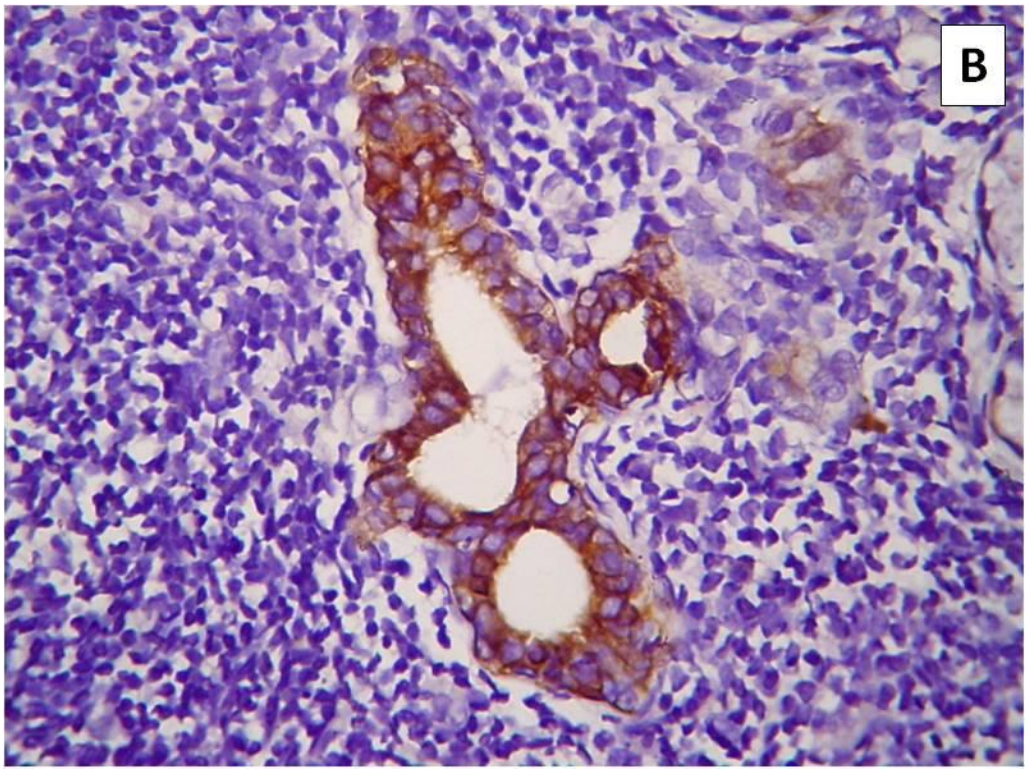


Fig. 23. Intense L1 ORF2p cytoplasmic expression in the ductal epithelial cells of a Tarpley III SS patient (extent grade 3). Note the absence of L1 ORF2p immunoreaction in the periductal lymphocytic infiltrate [L1 ORF2p immunohistochemical stain, original magnification (A) x100, (B) x400].

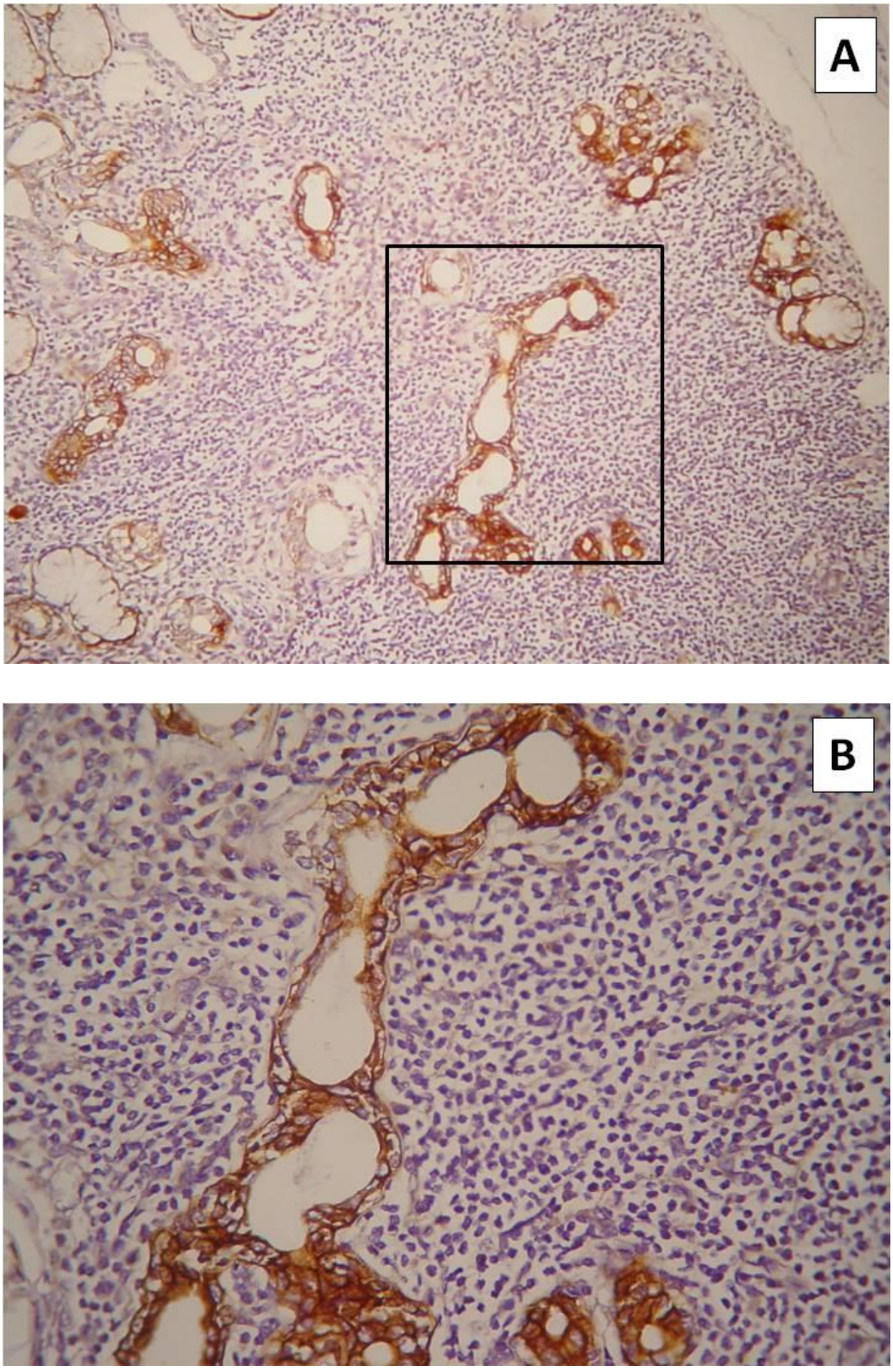


Fig.24. The only L1 ORF2p positive SS Tarpley IV case (intensity and extent both grade 3) [L1 ORF2p immunohistochemical stain, original magnification (A) x100, (B) x400].

7.1.2.4 L1 ORF2p final score and classification

The product of intensity and extent resulted in a final score of five different values, i.e. 0, 3, 4, 6 and 9 (Table 24). L1 ORF2p was totally absent in 1 Tarpley II and 1 Tarpley IV SS patient (**final score 0**), while another one Tarpley IV case showed mild *(intensity grade 1)* L1 ORF2p reactivity across the whole tissue section *(extent grade 3)*, leading to a **final score** of **3**. These 3/20 (15%) SS cases were **classified** as L1 ORF2p **negative**. In order to rule out the possibility that the negative findings in these cases were the result of an unspecified technical fault, the immunohistochemical test was repeated, giving once again the same results. Sixteen out of seventeen (94.1%) L1 ORF2p **positive** SS cases showed a **final score** of **6** (4 cases) or **9** (12 cases) and were **classified** as **strongly positive**. Only 1 SS Tarpley II case displayed **a final score** of **4,** corresponding to the **classification** of **mildly positive**. All Tarpley I and III cases and 5 out of 7 SS Tarpley III cases showed strong positivity (**final score** of **6** or **9**, diagram 2). Tarpley IV was the only subgroup in which most cases (2/3) had a final score of 0 or 3. This difference, though, in final score was not statistically significant among the Tarpley subgroups ($p>0.05$). Finally, **17/20 (85%) L1 ORF2p positive SS cases** were observed. No statistically significant difference was observed between the four Tarpley subgroups regarding L1 ORF2p final classification (diagram 3, $p>0.05$).

Table 24. L1ORF2p final score and cases classification in SS.

Study group	Final score			
	0-3	4	6 or 9	Total
	negative	mildly positive	stronglyly positive	
SS TI	-	-	3	3
SS TII	1	1	5	7
SS TIII	-	-	7	7
SS TIV	2	-	1	3
SS total	3	1	16	20

17/20 (85%) positive SS cases

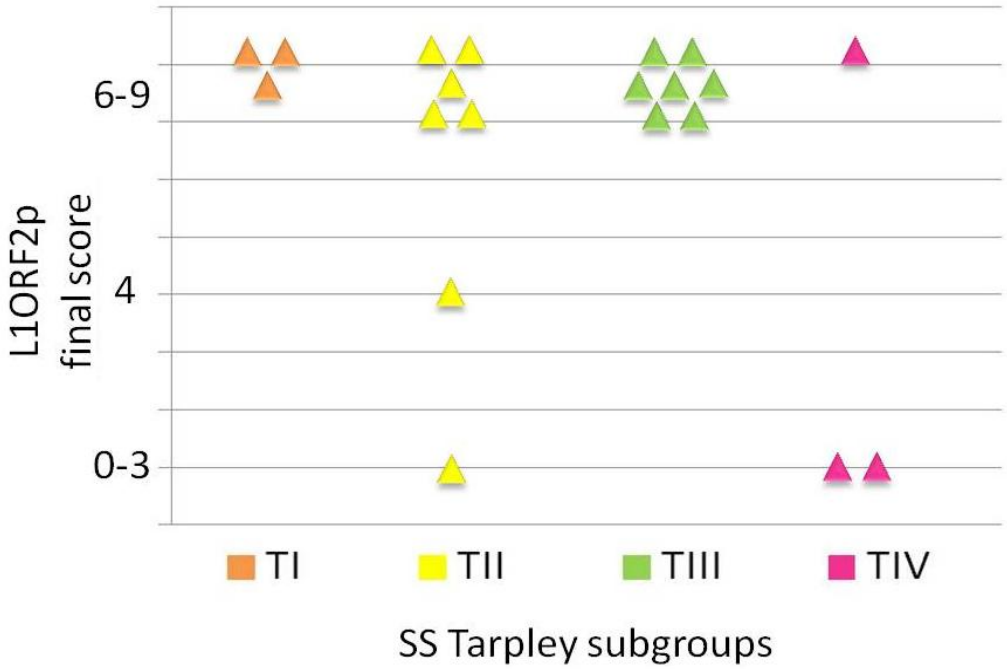


Diagram 2. L1 ORF2p final score in the SS Tarpley subgroups. Strong positivity (final score 6 or 9) was observed in all Tarpley I and III cases, 5/7 Tarpley II cases, but only in 1/3 Tarpley IV cases (p>0.05).

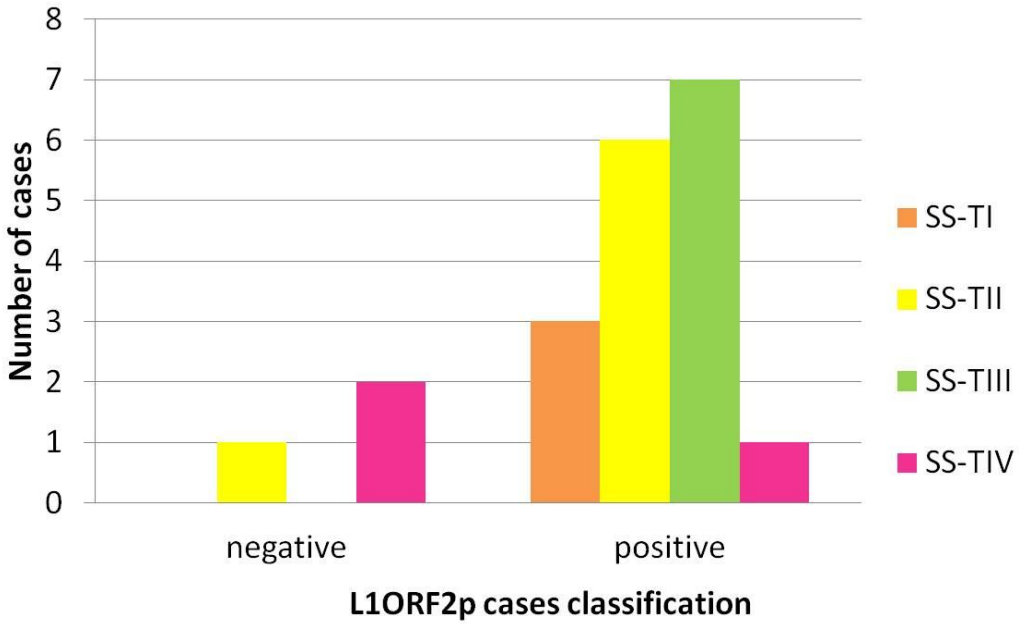


Diagram 3. L1 ORF2p expression in SS cases. The L1 ORF2p final classification (positive or negative) did not differ significantly between the SS Tarpley subgroups (p>0.05).

7.1.3 L1 ORF2p immunohistochemical expression in controls

Similarly to the SS cases, L1 ORF2p expression in controls was observed consistently in the cytoplasm of the ***ductal epithelial cells***, regardless of their interlobular or intralobular location. No difference in L1 ORF2p immunostaining between the ducts in the normal glands of LLM cases and the inflamed ones in CS (fig. 25) and LLM (fig. 26) cases was found. The L1 ORF2p immunoreaction was absent in the ***acinar cells*** and the ***chronic inflammatory cells***, as well as in the ***vascular endothelial cells*** and the ***nerve bundles*** in all CS and LLM cases. In contrast, positive L1 ORF2p immunostaining was observed in the ***muscle bundles***.

7.1.3.1 Sicca controls (CS)

All CS cases showed strong (**intensity grade 3**, Table C1) L1 ORF2p expression in more than 50% of the ductal cells (**extent grade 3**, Table C2). The **final score** in 5/5 CS cases was **9** and they were classified as **strongly positive** (Table C3).

7.1.3.2 Non-sicca controls (LLM)

Four out of five LLM cases displayed strong (**intensity grade 3**, Table 25) L1 ORF2p expression in more than 50% of the ductal cells (**extent grade 3**, Table 26), resulting in the **final score** of **9** and their classification as **strongly positive** (Table 27). One LLM case expressed L1 ORF2p in a moderate (**grade 2)** intensity (Table 25) and extent (Table 27) pattern [**final score 4**, classification as **mildly positive** (Table 26)]. As the intensity grade in this LLM case was considered as borderline, it was further evaluated with the CIA, which resulted in a CIA value of 130.03. This value was intermediate of the intensity outliers 100.25 (grade 3) and 155.96 (grade 1), thus the **grade 2** intensity in this case was confirmed.

No statistically significant difference of L1 ORF2p immunoexpression, regarding intensity, extent and final classification between sicca and non-sicca controls was found ($p > 0.05$).

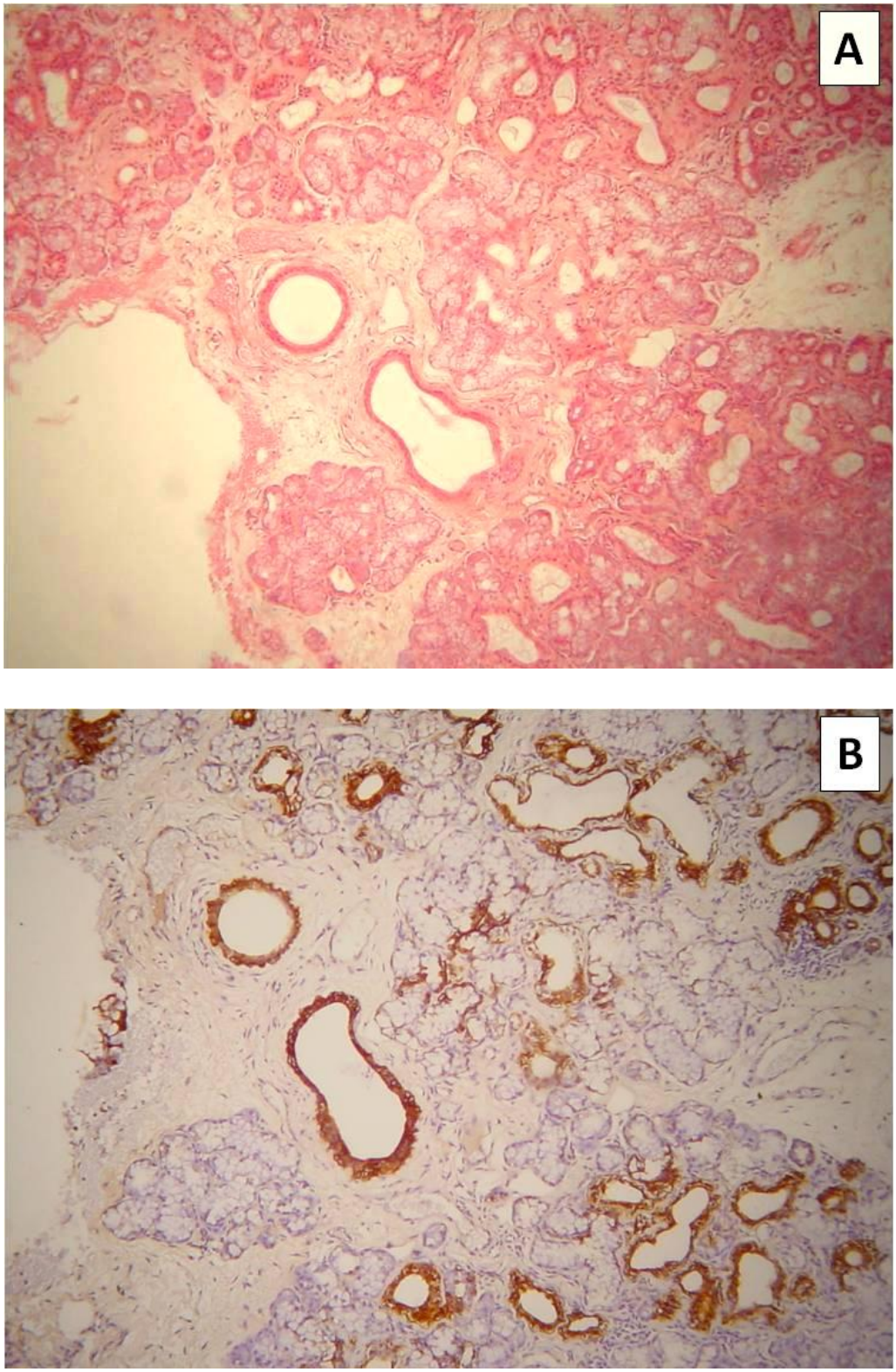


Fig. 25. Strong cytoplasmic L1 ORF2p expression (of extent grade 3) in a CS case [A, H&E stain; B, LIORF2p immunohistochemical staining; original magnification (A), (B) x100]

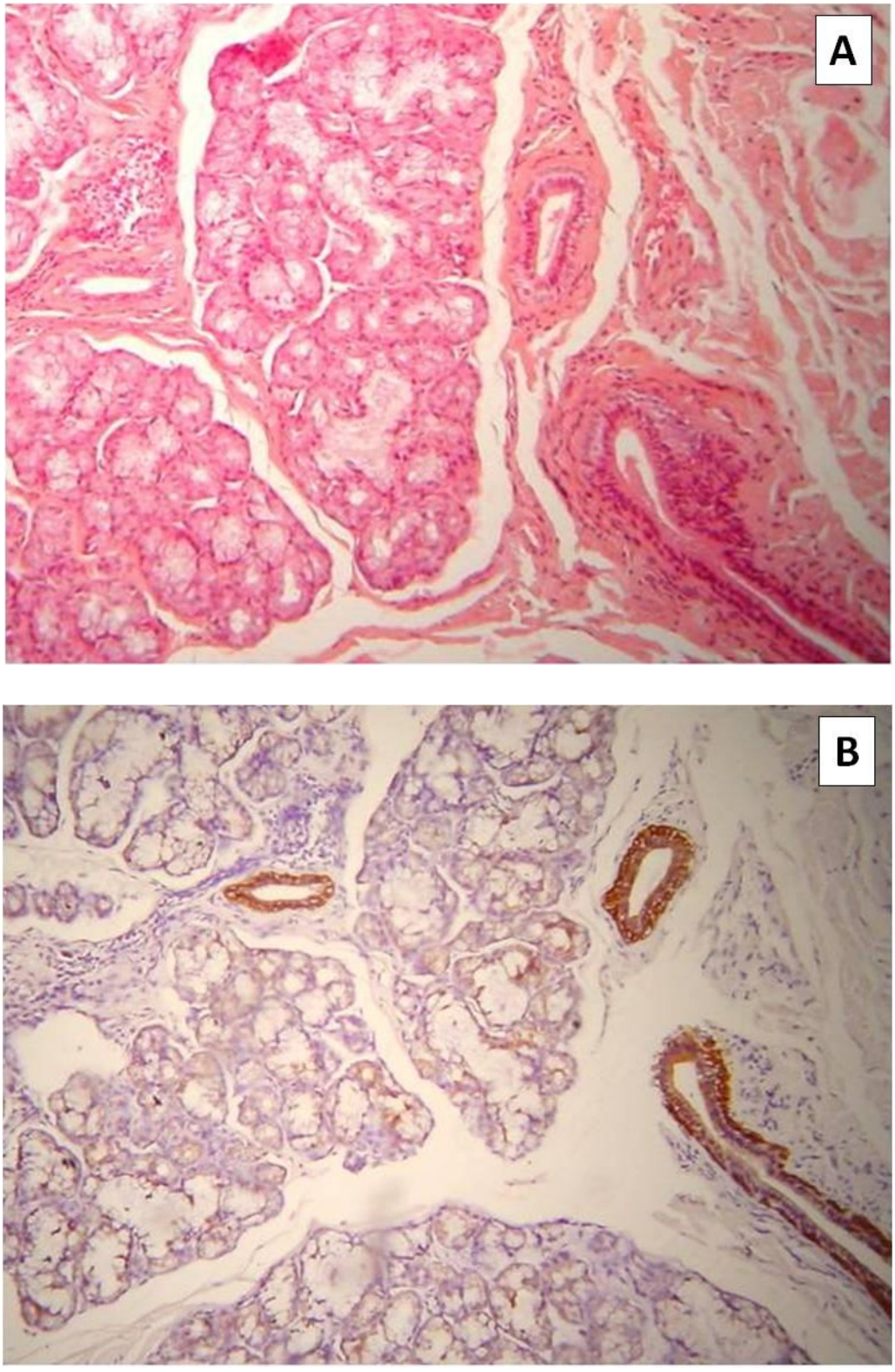


Fig. 26. Strong L1 ORF2p expression in the ductal cells of normal glands in a LLM case (extent grade 3) [A, H&E stain; B, LIORF2p immunohistochemical staining; original magnification (A), (B) x100].

Table 25. L1ORF2p staining intensity results in controls.					
Study group	Intensity grade				
	0 (no stain)	1 (mild)	2 (moderate)	3 (strongly)	Total
CS	-	-	-	5	5
LLM	-	-	1	4	5
Total controls	-	-	1	9	10

Table 26. L1ORF2p staining extent results in controls.					
Study group	Extent grade				
	0 (0-5%)	1 (6-19%)	2 (20-49%)	3 (≥50%)	Total
CS	-	-	-	5	5
LLM	-	-	1	4	5
Total controls	-	-	1	9	10

Table 27. L1ORF2p final score and cases classification in controls.				
Study group	Final score			
	0-3	4	6 or 9	Total
	negative	mildly positive	stronglyly positive	
CS	-	-	5	5
LLM	-	1	4	5
Total controls	-	1	9	10

5/5 CS and 5/5 LLM positive cases

7.1.4 L1 ORF2p immunohistochemical expression in SS cases VS controls

The **L1 ORF2p positive immunostaining in the ductal epithelium of MSGs** was the main common finding among the SS patients and the controls. The negative L1 ORF2p expression in the acinar cells, the inflammatory cells, the vascular endothelial cells and the nerve bundles, as well as the positive immunoreaction in muscle bundles in SS cases were also in common with the L1 ORF2p staining pattern in sicca (CS) and non-sicca (LLM) controls.

7.1.4.1 L1 ORF2p immunoexpression intensity

The L1 ORF2p immunoexpression intensity grade varied from 0 to 3 among the SS cases, while no stain (0) was found in 2 SS cases. In contrast, 5/5 CS cases and 4/5 LLM cases showed an L1 ORF2p intensity of grade 3, except for 1 LLM case with grade 2 intensity. **The difference in the incidence of intensity, though, was not statistically significant between the SS, CS and LLM groups (p>0.05).** Diagram 4 illustrates the intensity grading in SS patients and CS and LLMs controls. The bars in diagram 4 are filled with portions of the L1 ORF2p immunohistochemical figures from our sample.

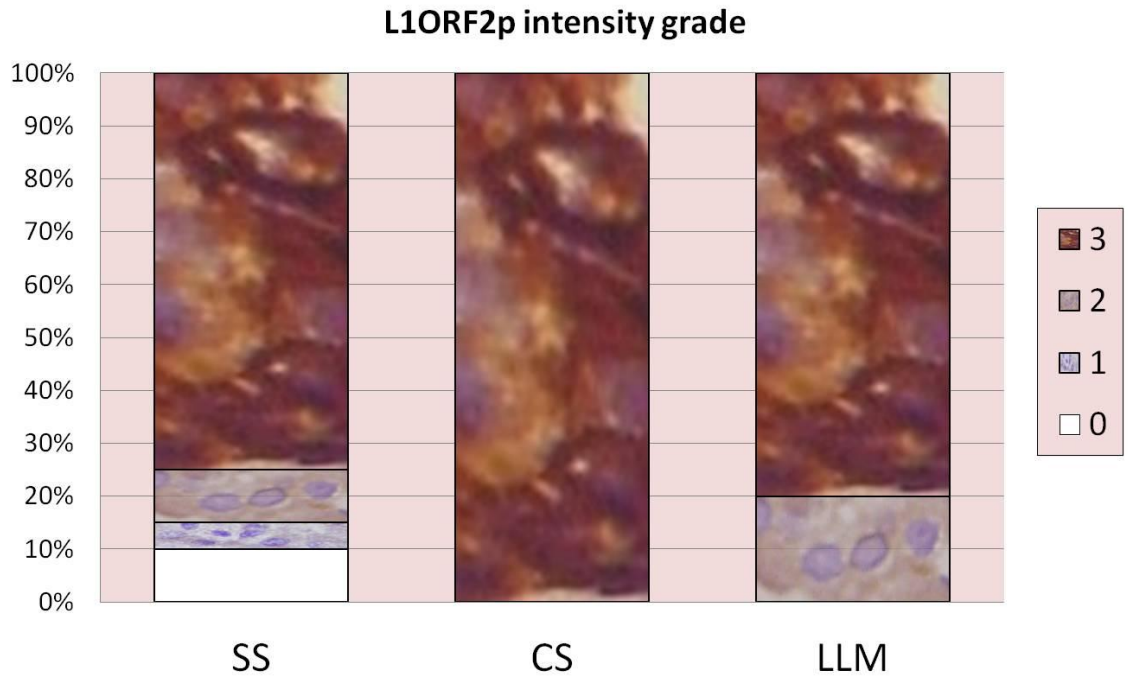


Diagram 4. L1 ORF2p intensity grades in SS, CS and LLM cases. The incidence of the L1 ORF2p intensity grades was not significantly different between the SS patients and the controls (p>0.05).

7.1.4.2 L1 ORF2p immunoexpression extent

The L1 ORF2p immunoexpression extent grade in the SS group was 0 (in 2/20 cases), 2 (in 4/20 cases) or 3 (in 14/20 cases). In contrast, all CS and 4/5 LLM cases showed staining of grade extent 3 (diagram 5). **The grade of extent, though, did not differ significantly between the SS, CS and LLM cases (p>0.05).**

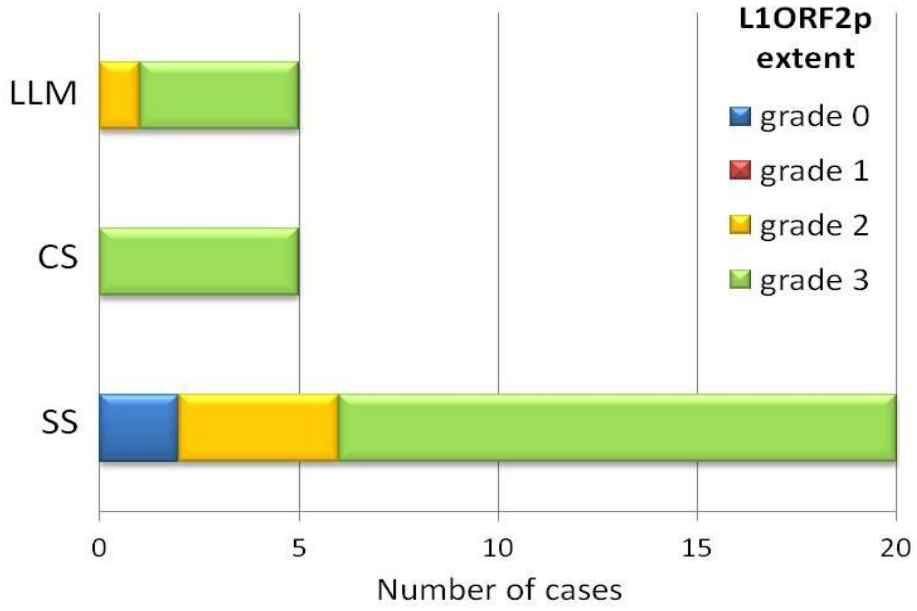


Diagram 5. L1 ORF2p immunoexpression extent in SS, CS and LLM cases.

7.1.4.3 L1 ORF2p final score and classification

The final L1 ORF2p immunostaining results are presented in detail in Tables 28 and 29. Cases that had been also evaluated with CIA are marked with "*" or "**". CIA changed the intensity grade from 3 to 2 in case "**". Only 2 positive cases (1 SS Tarpley II case and the 1 LLM case) had a final score of 4 and were initially classified as mildly positive. In order to compare the L1 ORF2p immunoreaction between the SS patients and the control groups, these two cases were grouped together with the strongly positive SS and LLM cases respectively for the statistical analysis. This new classification ended up in **17/20 (85%) SS cases, 5/5 (100%) CS cases and 5/5 (100%) LLM cases with positive L1 ORF2p expression**. No statistically significant difference in the L1 ORF2p final score and classification was observed between the SS patients in total and the CS (p>0.05) or LLM (p>0.05) group separately or between the SS patients and the two control groups together (p>0.05). However, the separate analysis of the four

Tarpley categories resulted in a significantly different final score (***p= 0.038***, diagram 6) and classification (***p= 0.038***, diagram 7) between the **SS Tarpley IV** group and all of the controls.

Table 27. L1ORF2p immunostaining results in SS cases.					
case	**study group**	**extent**	**intensity**	**final score**	**classification**
1	SS TI-1	3	3	9	strongly positive
2	SS TI-2	3	3	9	strongly positive
3	SS TI-3	2	3	6	strongly positive
4**	SS TII-1	3	2	6	strongly positive
5*	SS TII-2	2	2	4	mildly positive
6	SS TII-3	3	3	9	strongly positive
7	SS TII-4	2	3	6	strongly positive
8	SS TII-5	3	3	9	strongly positive
9	SS TII-6	3	3	9	strongly positive
10	SS TII-7	0	0	0	negative
11	SS TIII-1	2	3	6	strongly positive
12	SS TIII-2	3	3	9	strongly positive
13*	SS TIII-3	3	3	9	strongly positive
14	SS TIII-4	3	3	9	strongly positive
15	SS TIII-5	3	3	9	strongly positive
16	SS TIII-6	3	3	9	strongly positive
17	SS TIII-7	3	3	9	strongly positive
18	SS TIV-1	3	3	9	strongly positive
19*	SS TIV-2	3	1	3	negative
20	SS TIV-3	0	0	0	negative

Table 29. L1ORF2p immunostaining results in controls.					
case	**study group**	**extent**	**intensity**	**final score**	**classification**
1	CS-1	3	3	9	strongly positive
2	CS-2	3	3	9	strongly positive
3	CS-3	3	3	9	strongly positive
4	CS-4	3	3	9	strongly positive
5	CS-5	3	3	9	strongly positive
6*	LLM-1	2	2	4	mildly positive
7	LLM-2	3	3	9	strongly positive
8	LLM-3	3	3	9	strongly positive
9	LLM-4	3	3	9	strongly positive
10	LLM-5	3	3	9	strongly positive

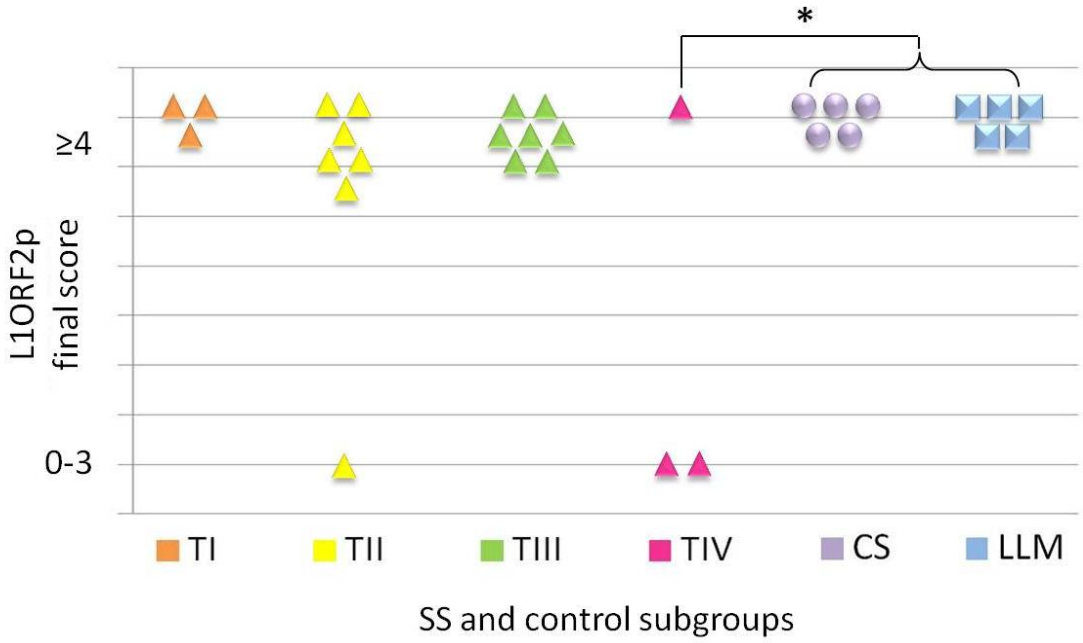


Diagram 6. Seventeen out of twenty SS cases and all of the controls had a final score ≥ 4. The final score was statistically different between the SS Tarpley IV cases and the controls in total ***(p=0.038)***.

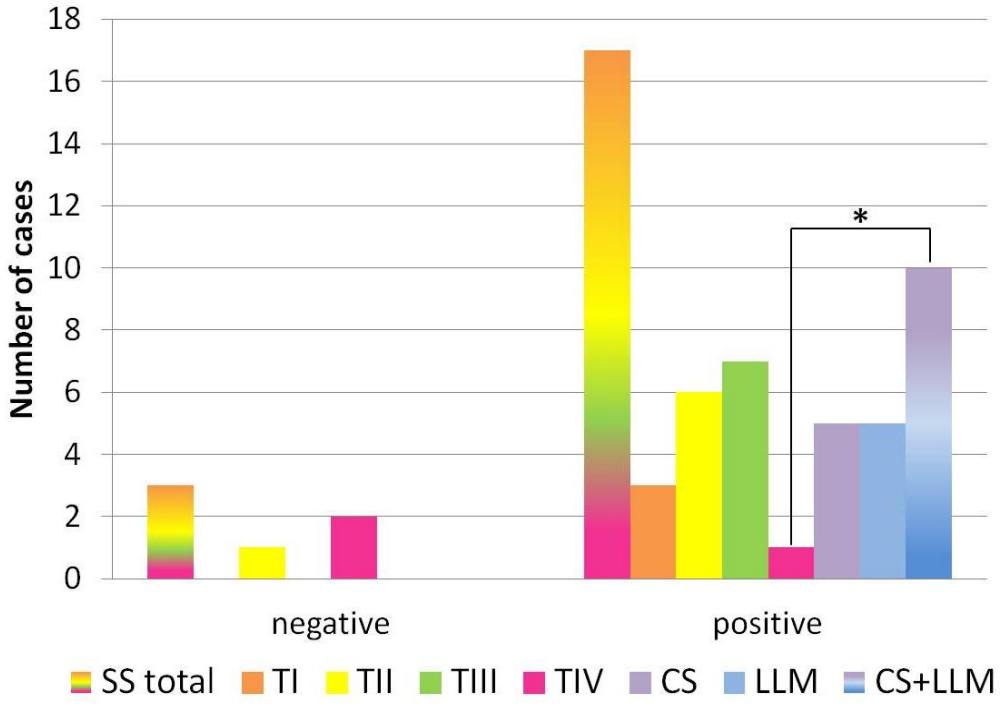


Diagram 7. The number of L1 ORF2p positive cases was significantly lower in the SS Tarpley IV group compared with the two control groups in total ***(p=0.038)***.

7.2 APOBEC3B immunohistochemical expression

7.2.1 APOBEC3B immunohistochemical expression in breast carcinoma

As in L1 ORF2p, the immunohistochemical expression of APOBEC3B was first evaluated in the invasive lobular breast carcinoma control case. APOBEC3B was found in the cytoplasm of the neoplastic ductal (fig.27A) and acinar (fig.27B) cells, while it was absent in the tumor stroma.

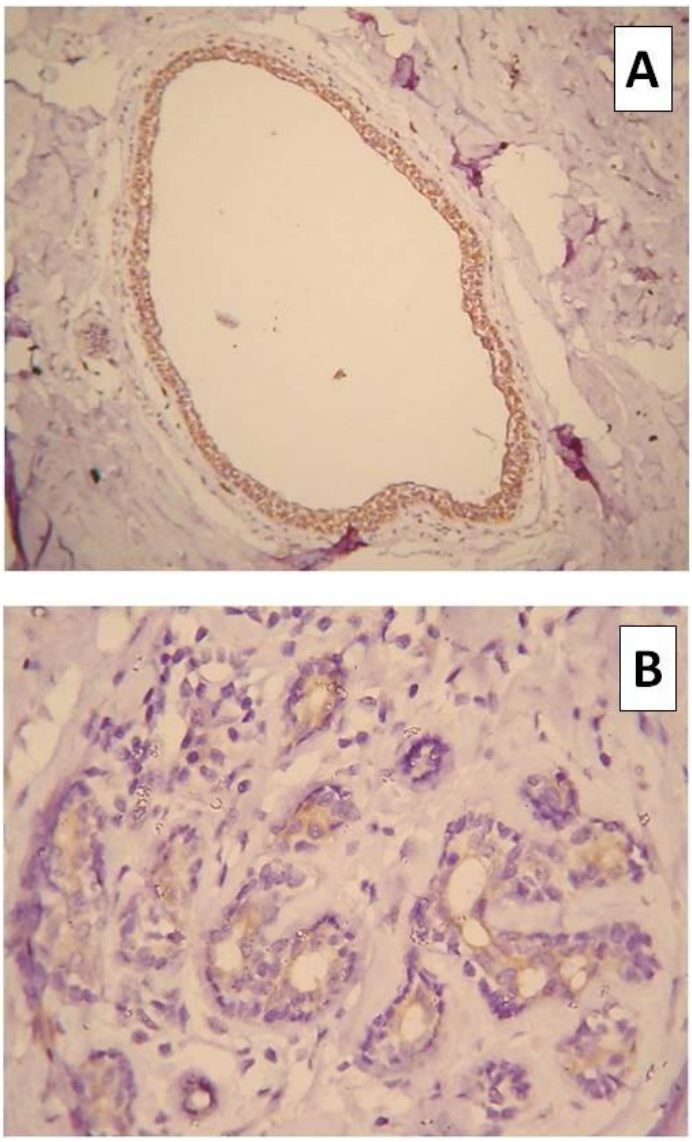
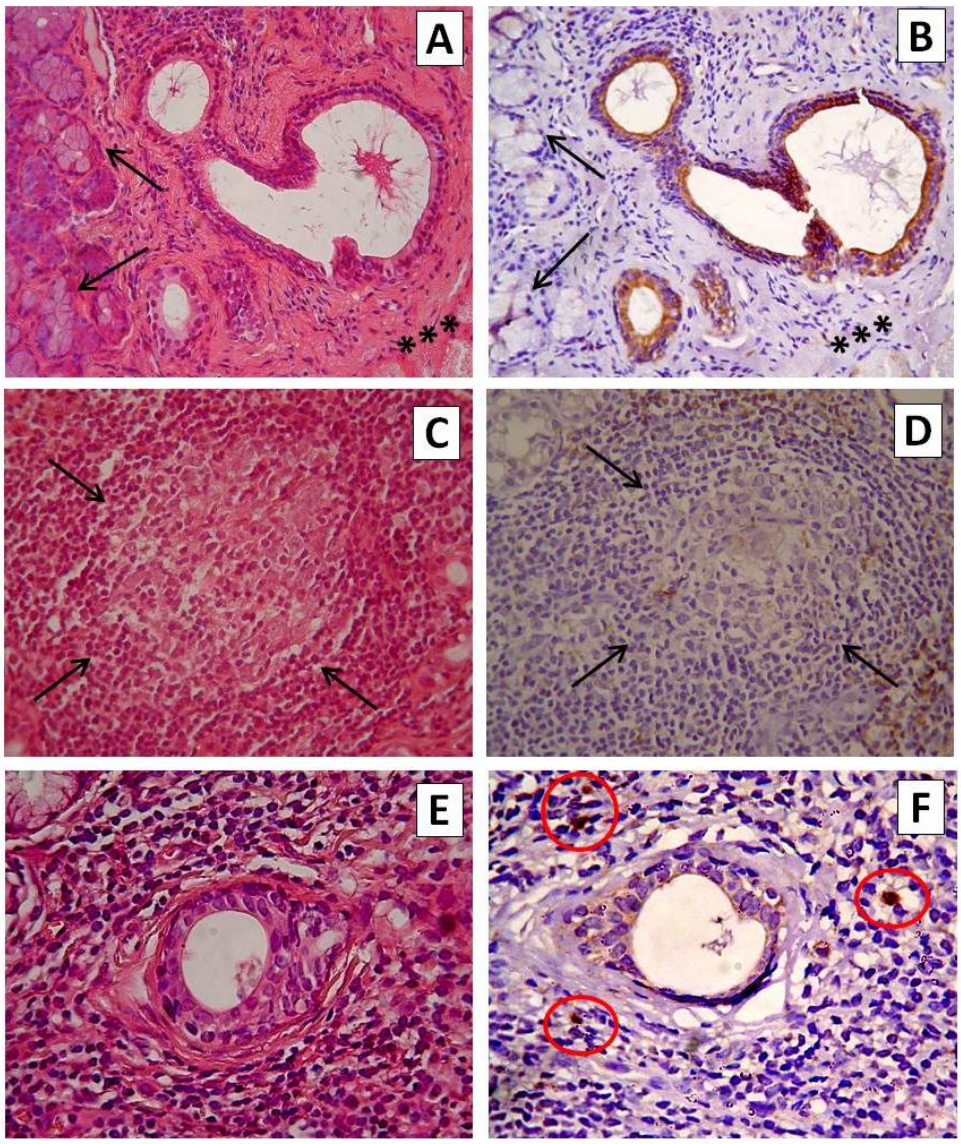


Fig. 27. Cytoplasmic APOBEC3B expression in the neoplastic ductal (A) and acinar cells (B) of breast carcinoma (positive control) and negative immunoreaction in the connective tissue [APOBEC3B immunohistochemical stain, original magnification (A) x100, (B) x400].

7.2.2 APOBEC3B immunohistochemical expression in SS cases

7.2.2.1 Descriptive results

All SS cases expressed APOBEC3B. APOBEC3B immunoreaction was consistently observed in the ***ductal epithelial cells*** (Fig.28B) in positive SS cases. APOBEC3B was not expressed in the ***acinar cells*** (Fig.1B, D), in the areas of ***lymphocytic infiltration***(Fig.28F) as well as in the ***lymphoid-like structures*** (Fig.28D), while only few scattered inflammatory cells, possibly corresponding to MNCs, expressed cytoplasmic APOBEC3B (Fig.28F). APOBEC3B expression was also absent in the ***vascular endothelial cells*** (Fig.28H) and the ***muscle bundles*** (Fig.28J), as well as in ***areas*** of ***interstitial fibrosis*** (Fig.28B) and ***lipoid degeneration*** (Fig.28L). Positive APOBEC3B immunoreaction was noticed in the ***nerve bundles*** (Fig.28H).



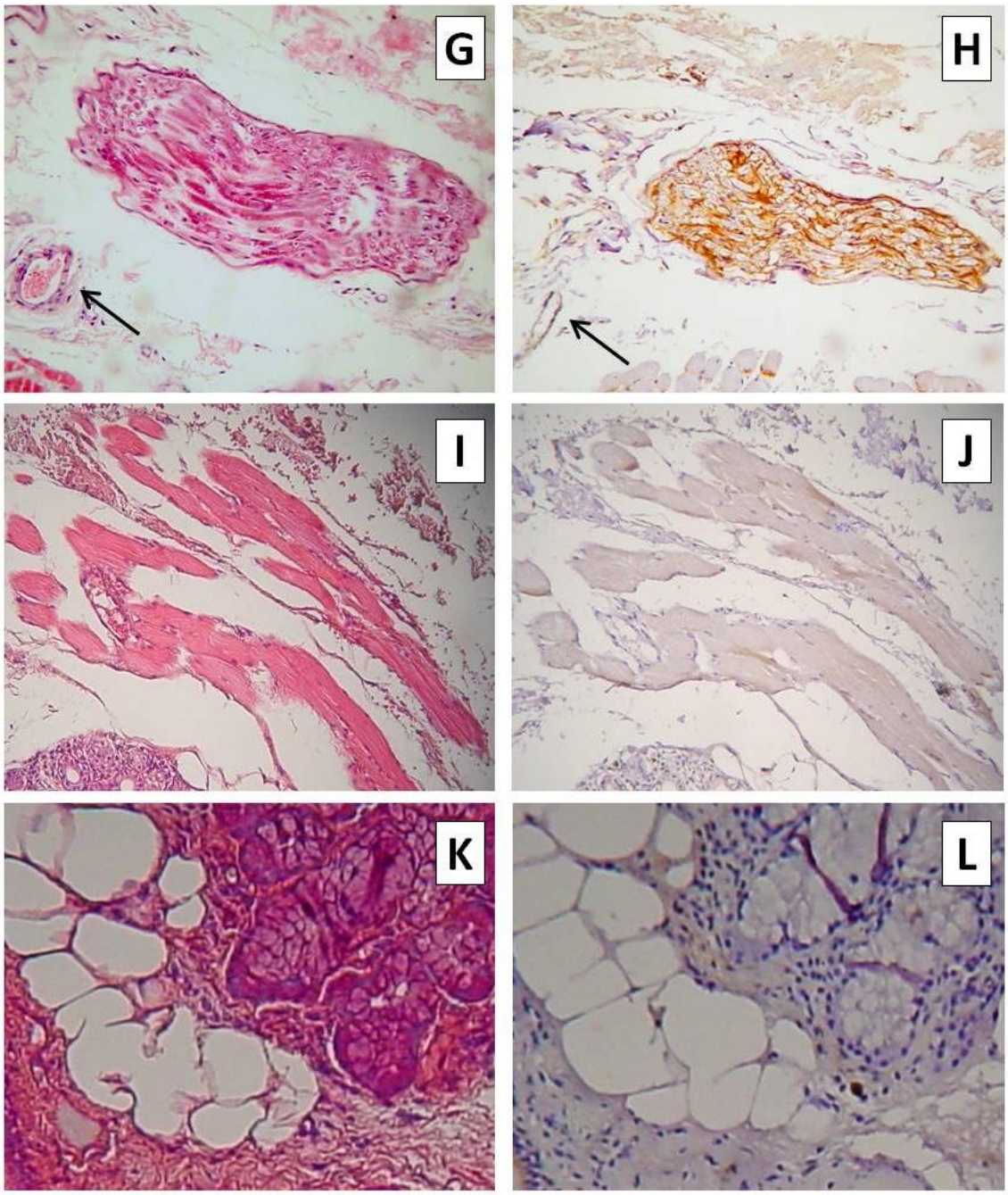


*Fig. 28. APOBEC3B positive expression in the ductal epithelium (B). Absence of staining was observed in the acinar cells (B arrows, D), the lymphocytic infiltration (F), the lymphoid-like structures (D white arrow), the vascular cells (H arrow), the muscle bundles (J) and the areas of interstitial fibrosis (B ***) or lipoid degeneration (L). Positive APOBEC3 immunoreaction in the nerve bundles (H) and scattered inflammatory cells (F, red cycles) [(A), (C), (E), (G), (I), (K) H&E stain; (B), (D), (F), (H), (J), (L) APOBEC3B immunohistochemical stain; original magnification (A), (B), (E), (F), (G), (H), (I), (J) x400, (C), (D), (K), (L) x100].*

The 20 SS cases were classified as mildly positive/strongly positive or negative according to the immunoreaction in the ductal epithelium. All positive SS cases expressed APOBEC3B in the **cytoplasm** of the ductal epithelial cells, often with a **fine speckled pattern** (Fig. 29A-C). In most cases the staining was found mostly in the **apical part** of the ductal cell cytoplasm. No difference of the APOBEC3B immunoreaction was noticed between the intralobular and interlobular ducts.

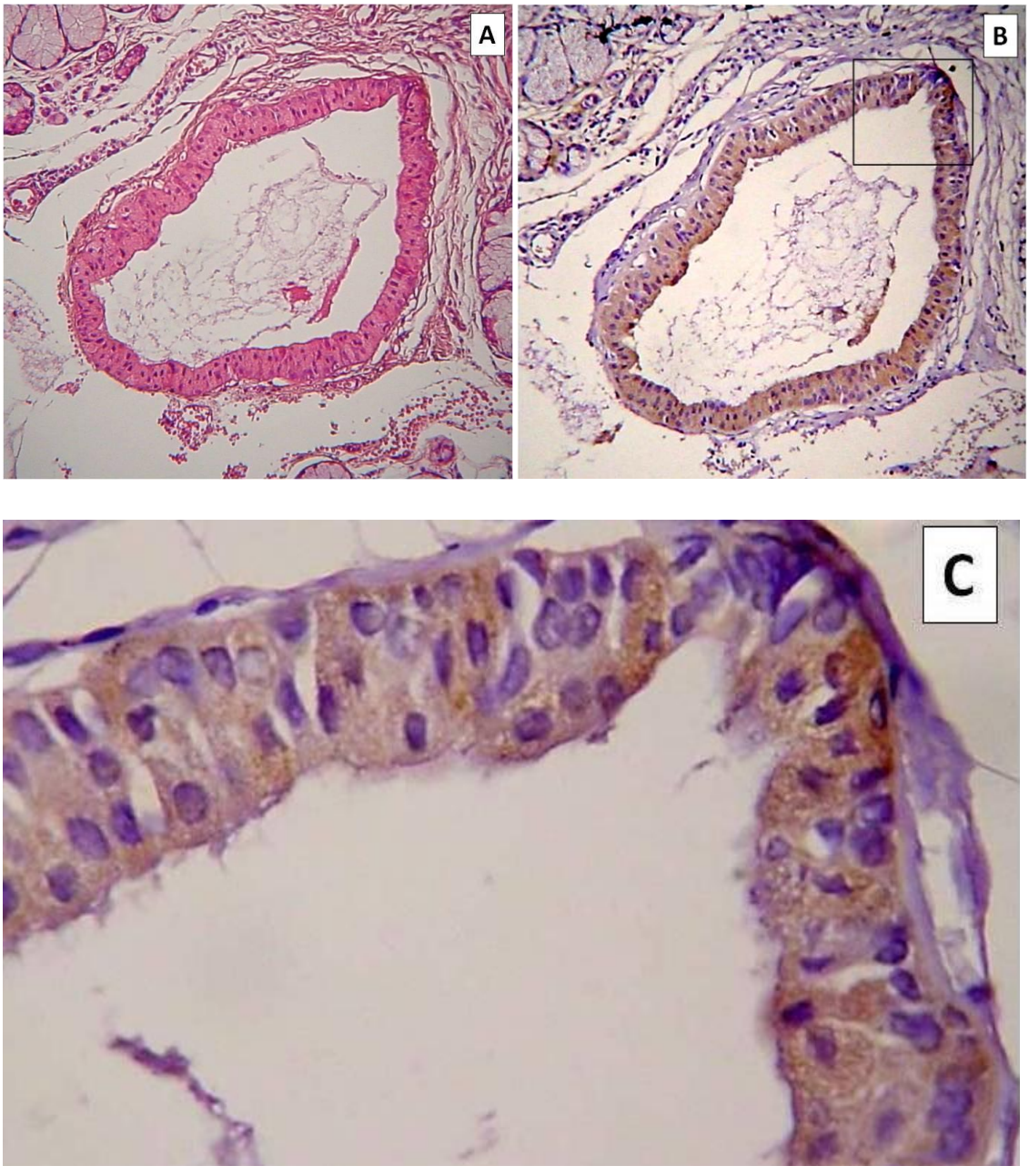


Fig. 29. APOBEC3B cytoplasmic expression in the ductal epithelial cells. Note the fine speckled APOBEC3B staining pattern (C) [(A) H&E stain; (B), (C) L1 ORF2p immunohistochemical stain; original magnification (A), (B) x100, (C) x400].

7.2.2.2 APOBEC3B immunoexpression intensity

The APOBEC3B intensity grading by the two examiners resulted initially in 4/20, 13/20 and 3/20 cases with mild (grade 1), moderate (grade 2) and strong (grade 3) intensity respectively. The CIA was applied in 8 SS cases (2 from each SS Tarpley subgroup) with borderline results, in order to verify the APOBEC3B staining intensity. The intensity outliers 119.96 and 205.85 determined the strong (grade 3) and mild (grade 1) intensity, respectively.[444] The values 190.32, 195.25, which were close to 205.85, were regarded as mild intensity. The intermediate values (163.58, 168.27, 170.84 and 172.3) were interpreted as moderate intensity (grade 2). The CIA intensity classification was in agreement with the two examiners' grading in 5 out of 8 CIA-evaluated cases. In 1 SS Tarpley I case the CIA decreased the intensity grade from 2 to 1, resulting in its classification as negative, despite the immunopositivity in extent grade 3 (final score 3, classification as negative). CIA also decreased the intensity grade from 3 to 2 in 2 SS Tarpley IV cases. In the latter 2 cases, though, the APOBEC3B was expressed across the whole section (extent grade 3); thus, the intensity modification did not influence the final classification (final score 6, classification as strongly positive). According to the CIA results (fig.30), **mild** (fig. 31A) **moderate** (fig. 31B) and **strong** (fig. 31C) APOBEC3B intensity was observed in **5(25%), 14(70%)** and **1(5%) SS cases** respectively (Table 30). **The APOBEC3B intensity was significantly lower in the SS Tarpley I compared with the Tarpley II cases *(p=0.008)***.

Table 30. APOBEC3B staining intensity results in SS.

Study group	Intensity grade				
	0 (no stain)	1 (mild)	2 (moderate)	3 (strongly)	Total
SS TI	-	3	-	-	3
SS TII	-	-	6	1	7
SS TIII	-	2	5	-	7
SS TIV	-	-	3	-	3
SS total	-	5	14	1	20

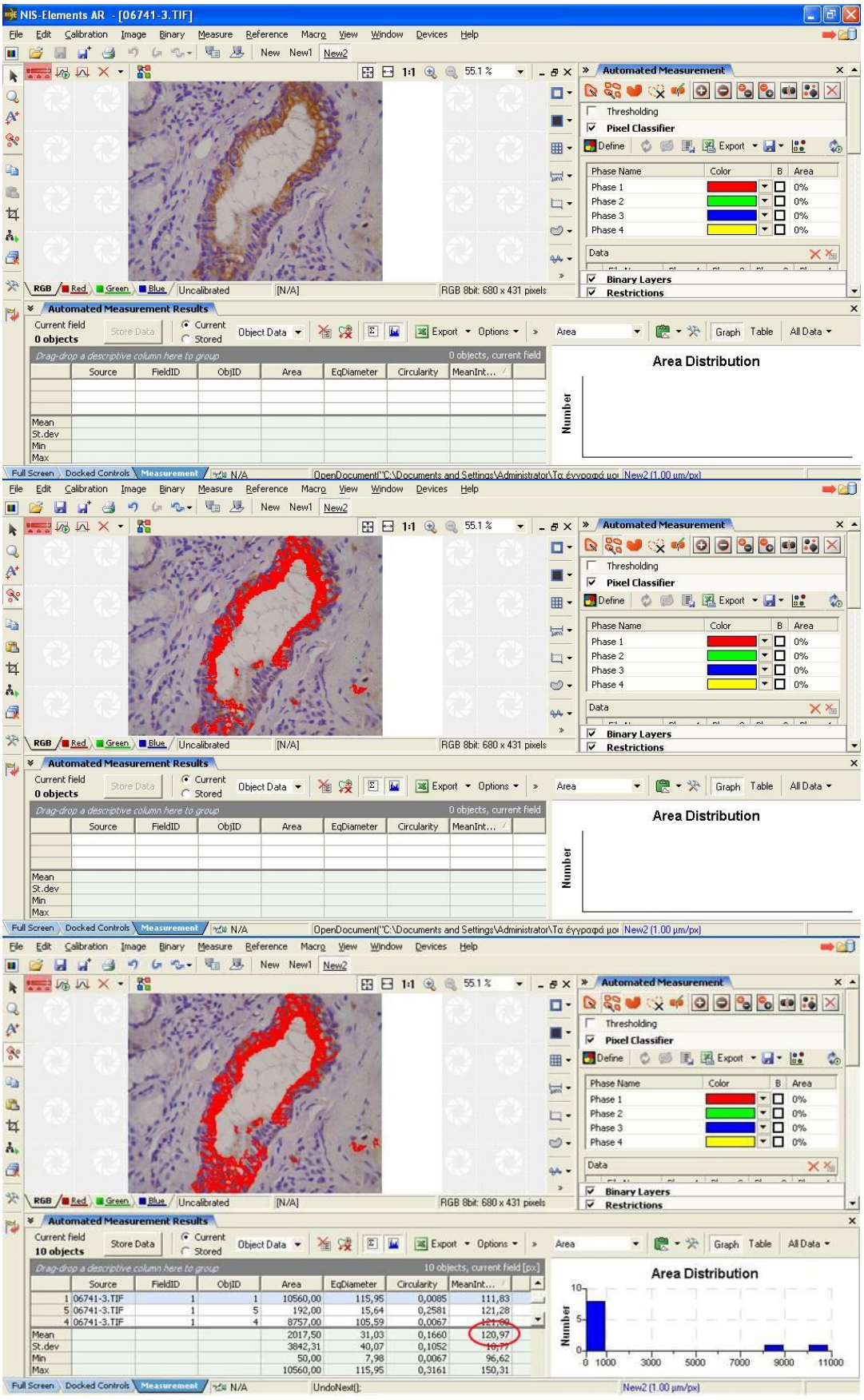


Fig. 30. CIA process for the APOBEC3B intensity in a SS Tarpley II case (CIA value 120.97).

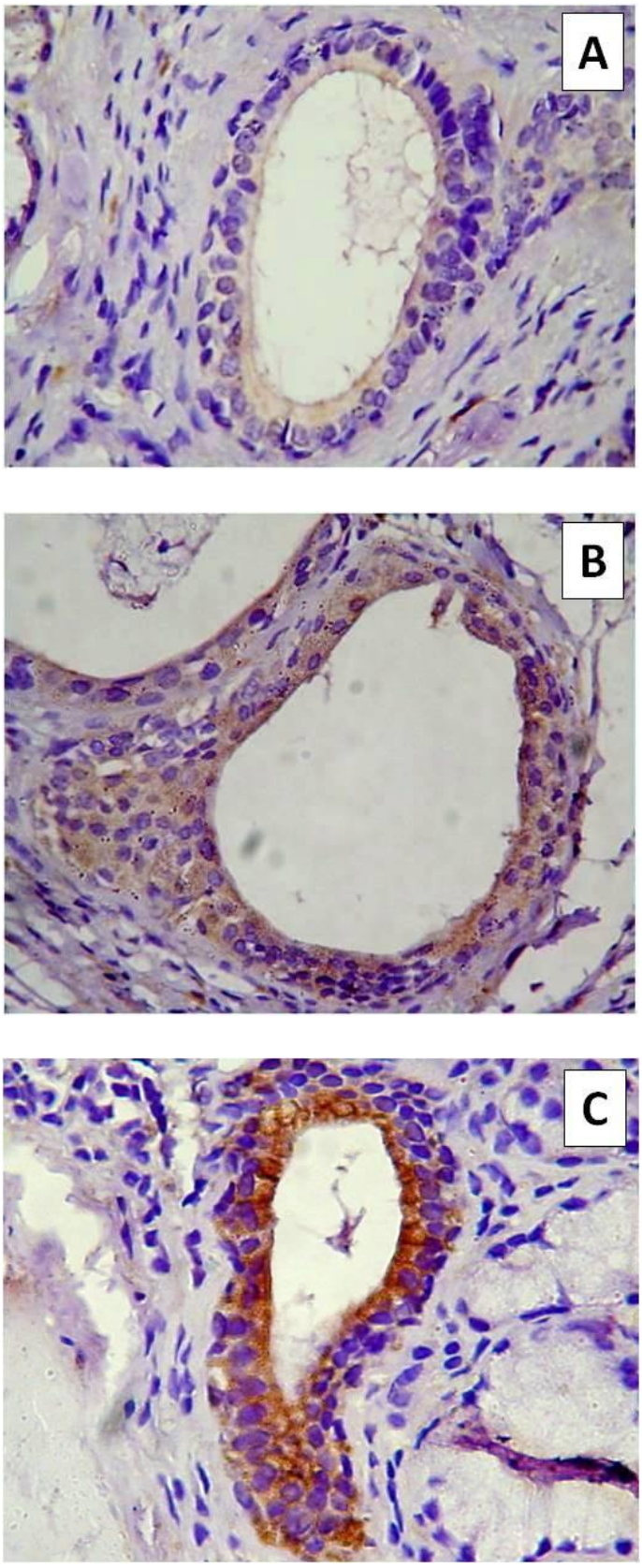


Fig. 31. APOBEC3B staining of mild (A), moderate (B) and strong (C) intensity [APOBEC3B immunohistochemical stain, original magnification (A), (B), (C) x400].

7.2.2.3 APOBEC3B immunoexpression extent

The APOBEC3B immunoreaction was found in more than 50% of ductal epithelial cells **(extent grade 3) in all SS cases of each Tarpley subgroup** (Table 31, fig. 32-35).

Table 31. APOBEC3B staining extent results in SS.

Study group	Extent grade				
	0 (0-5%)	1 (6-19%)	2 (20-49%)	3 (≥50%)	Total
SS TI	-	-	-	3	3
SS TII	-	-	-	7	7
SS TIII	-	-	-	7	7
SS TIV	-	-	-	3	3
SS total	-	-	-	20	20

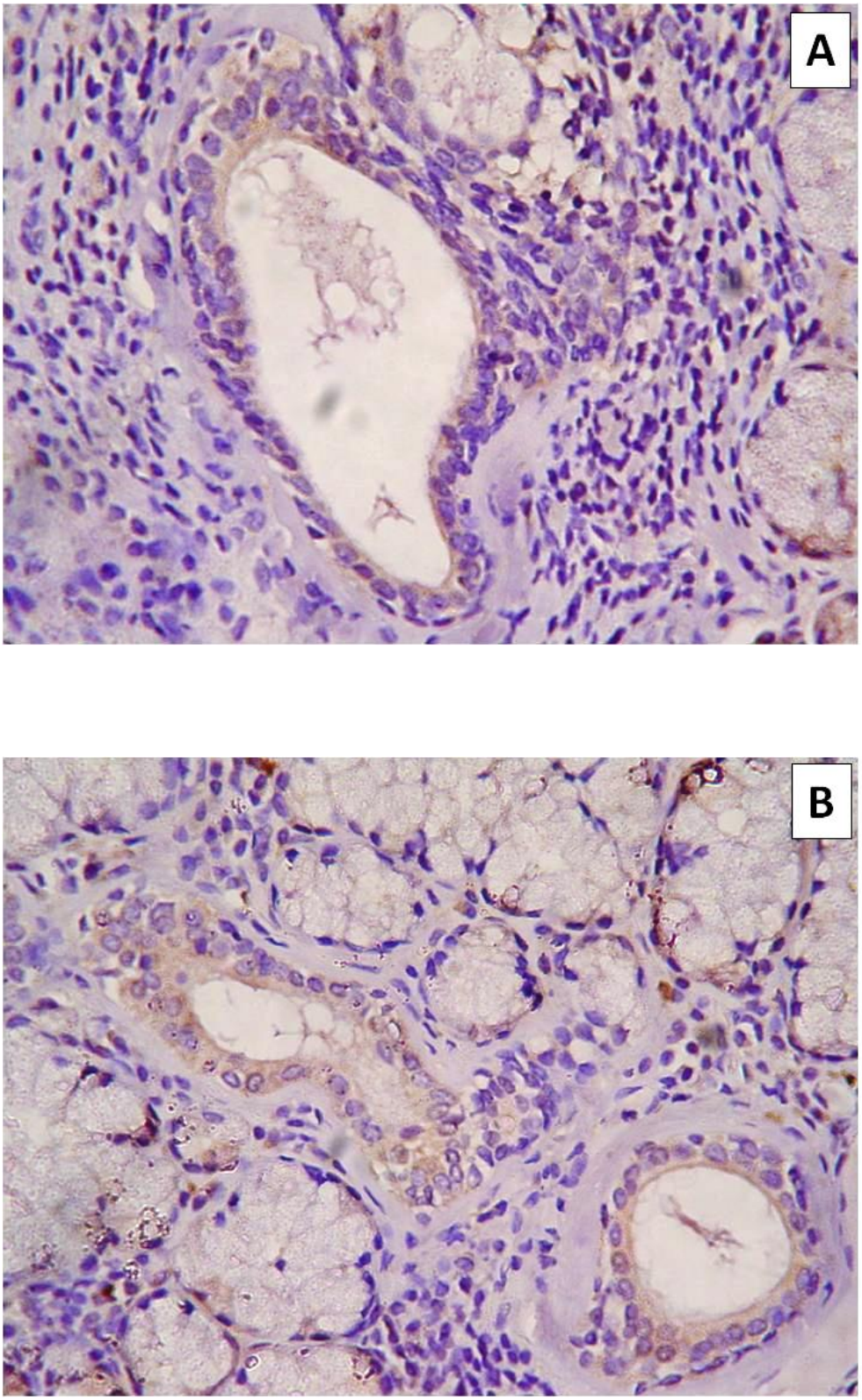


Fig. 32. Mild APOBE3B expression in the ductal epithelium with (A) and without (B) periductal lymphocytic infiltration in a SS Tarpley I patient, [APOBEC3B immunohistochemical stain, original magnification (A), (B) x400].

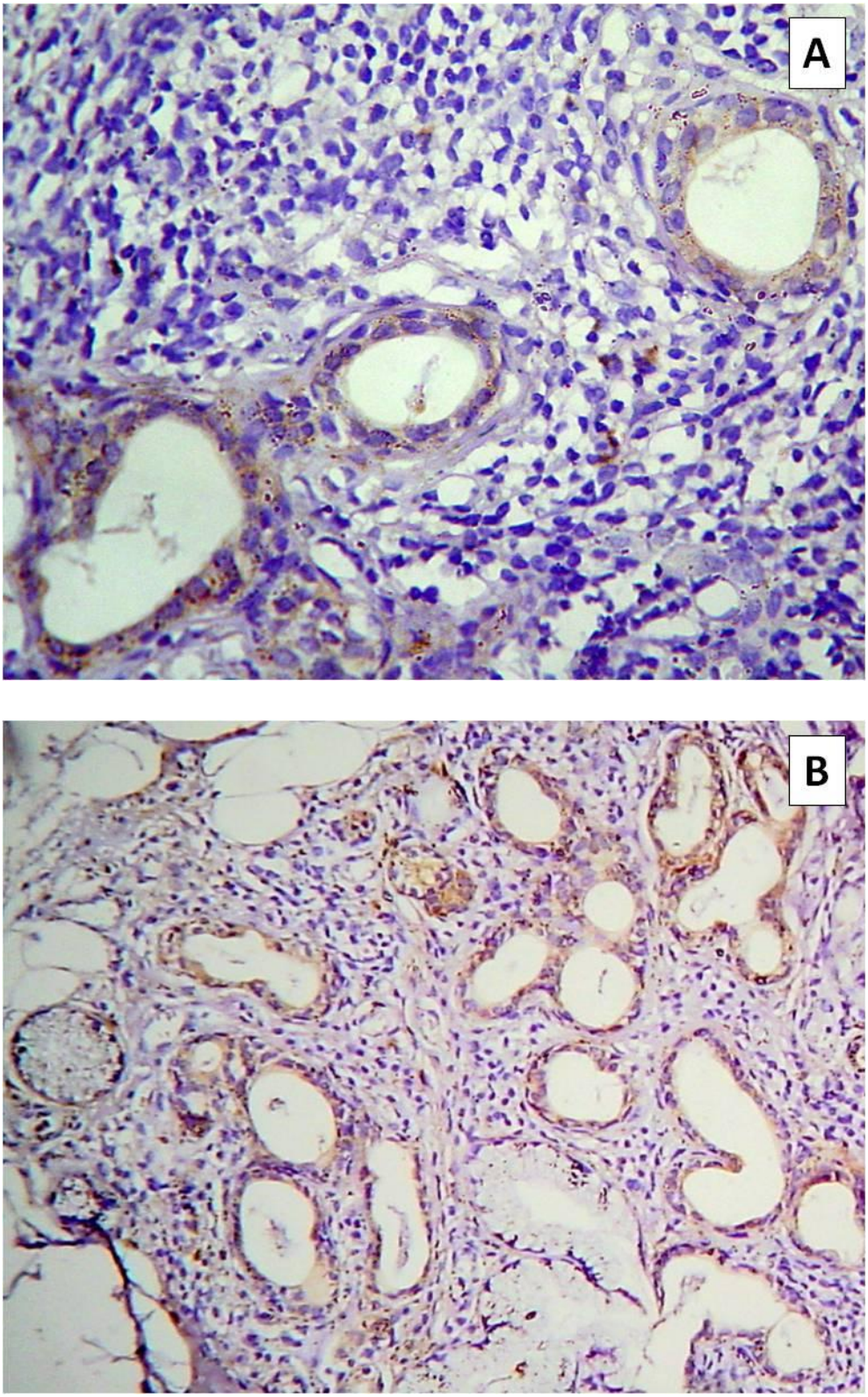


Fig. 33. Moderate APOBE3B expression in the ductal epithelium with (A) and without (B) periductal lymphocytic infiltration in a SS Tarpley II case. Note a few scattered APOBEC3B positive inflammatory cells in (A) [APOBEC3B immunohistochemical stain, original magnification (A) x400, (B) x100].

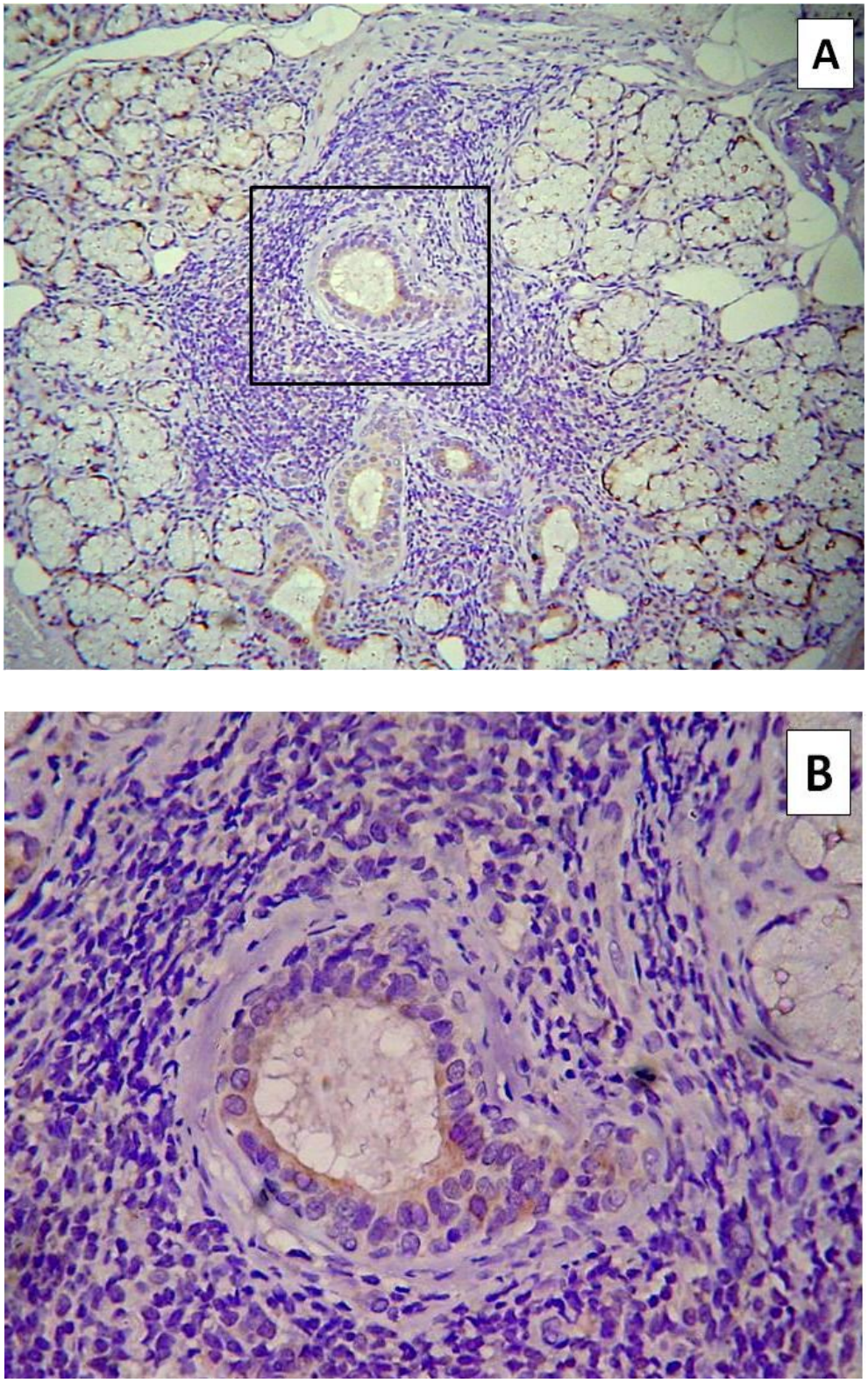


Fig. 34. Moderate APOBE3B expression in the ductal epithelium of a SS Tarpley III case (extent grade 3). Note the negative APOBEC3B expression in the acinar cells (A) and the diffuse lymphocytic infiltrate (B) [APOBEC3B immunohistochemical stain, original magnification (A) x100, (B) x400].

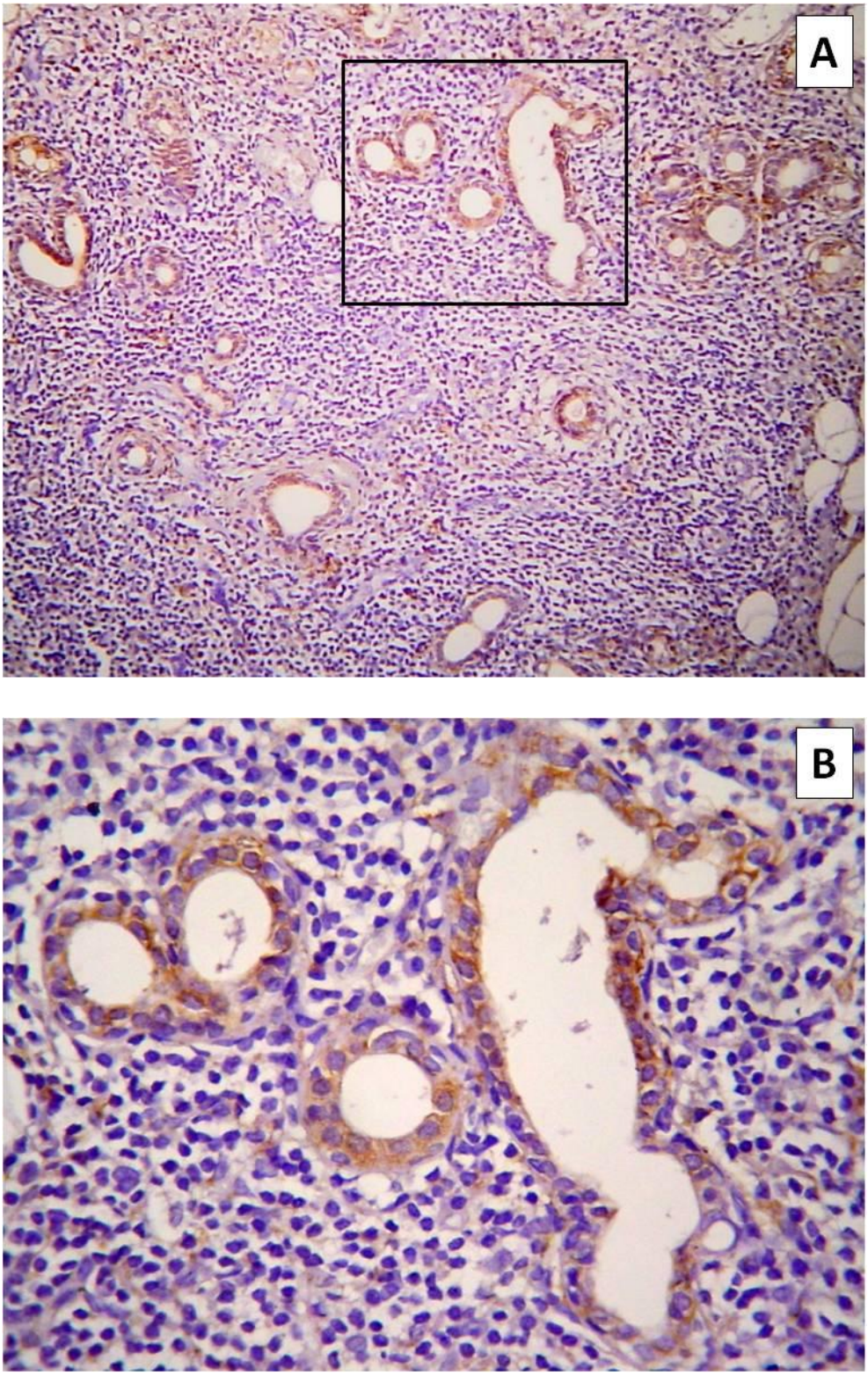


Fig. 35. Positive moderate APOBEC3B expression in the ductal epithelium of a SS Tarpley IV patient. Note the APOBEC3B negative diffuse lymphocytic infiltration [APOBEC3B immunohistochemical stain, original magnification (A) x100, (B) x400].

7.2.2.4 APOBEC3B final score and classification

The product of intensity and extent resulted in a final score of three different values, i.e. 3, 6 and 9 (Table 32). No SS case showed total absence of APOBEC3B immunostaining, but, based on the **final score 3** (as a product of grade 1 intensity and grade 3 extent), **5 (25%) SS cases** were classified as **negative**. All SS Tarpley I cases were among the finally sorted as negative cases. **Fourteen** out of twenty **(70%) SS cases** had a **final score** of **6**, while only **1 SS case (5%)** had a **final score** of **9**. These **15** SS cases were classified as **strongly positive**. No mildly positive case was observed**. A statistically significant difference was found between the SS Tarpley I and II subgroups regarding the APOBEC3B final score** (diagram 8, ***p=0.008***) **and classification** (diagram 9, ***p=0.008***).

Table 32. APOBEC3B final score and cases classification in SS.

Study group	Final score			
	0-3	4	6 or 9	Total
	negative	mildly positive	strongly positive	
SS TI	3	-	-	3
SS TII	-	-	7	7
SS TIII	2	-	5	7
SS TIV	-	-	3	3
SS total	5	-	15	20

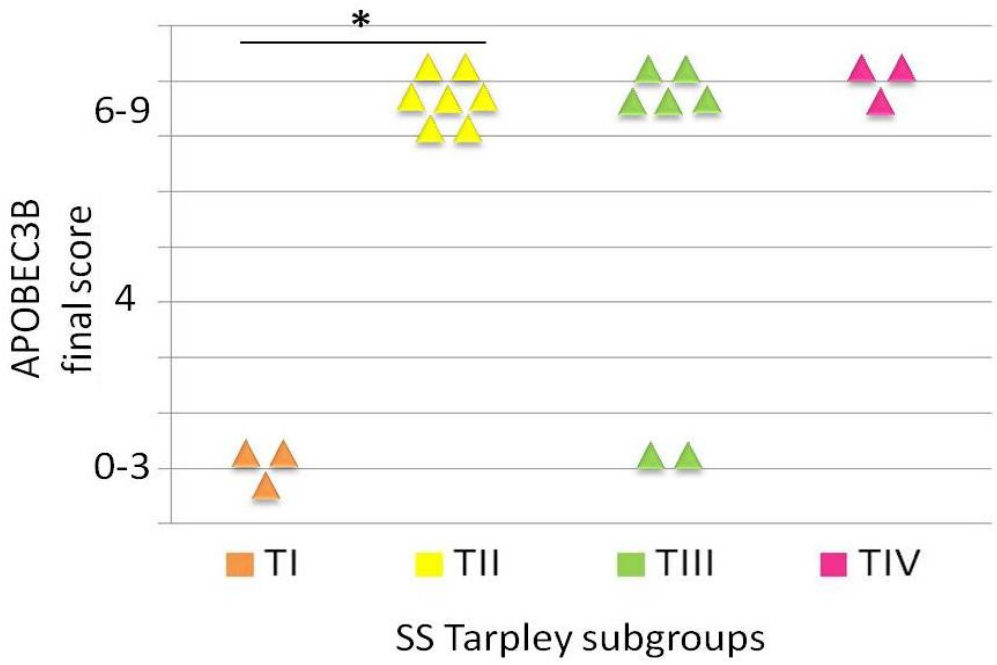


Diagram 8. A final score of 6 or 9 was found in all Tarpley II and IV cases, in 5/7 Tarpley II cases, but in no Tarpley I case. A statistically significant difference in APOBEC3B final score was found between the SS Tarpley I and II subgroups ***(p=0.008)****.*

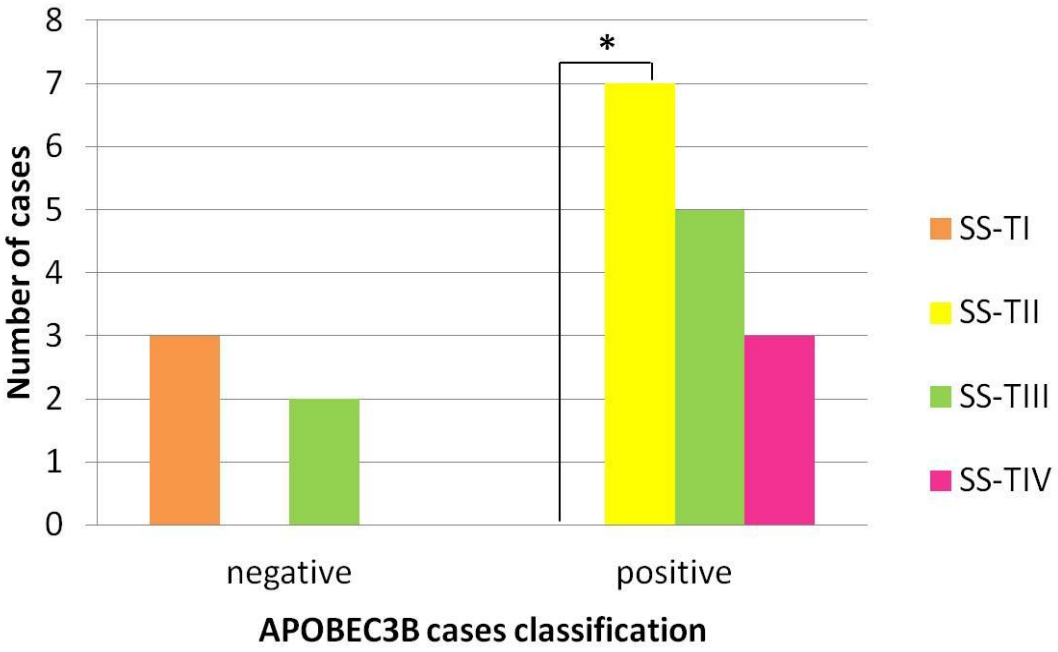


Diagram 9. Fifteen out of twenty SS cases were APOBEC3B positive. APOBEC3B positive expression was observed in all Tarpley II SS cases but in no Tarpley I SS case ***(p=0.008)****.*

7.2.3 APOBEC3B immunohistochemical expression in controls

The APOBEC3B immunostaining in the control groups was found consistently in the cytoplasm of the ***ductal epithelial cells***, regardless of their interlobular or intralobular location, similarly to the SS cases. (fig. 36)No difference was observed in the APOBEC3B expression between ducts in the normal glands and the inflamed ones in 3/5 LLM. In 2/5 LLM cases, the APOBEC3B intensity was greater in the ductal epithelium of the inflamed glands compared to the normal ones. Since the non-sicca control group was selected in order to study the immunohistochemical expression of the two antibodies in the normal glands, the intensity in these 2 LLM cases was finally determined according to the staining of the normal glands (fig. 37). The APOBEC3B immunoreaction was absent in the ***acinar cells*** and the ***chronic inflammatory cells***, as well as in the ***vascular endothelial cells*** and the ***muscle bundles*** in all CS and LLM cases. In contrast, positive APOBEC3B immunostaining was observed in the ***nerve bundles***.

7.2.3.1 Sicca controls (CS)

All CS cases showed strong (**intensity grade 3**, Table 33) APOBEC3B immunoreaction in more than 50% of the ductal cells (**extent grade 3**, Table 34). One CS case was initially characterized by the two examiners as of moderate (grade 2) but borderline intensity, thus it was further evaluated with the CIA. As the CIA value (131.75) was close to the outlier (119.96) corresponding to the strong intensity, this case was finally characterized to be of intensity grade 3. The **final score** in **5/5 CS cases** was **9** and they were classified as **strongly positive** (Table 35).

7.2.3.2 Non-sicca controls (LLM)

Three out of five **(60%) LLM cases** displayed moderate (**intensity grade 2**, Table 33) APOBEC3B immunostaining in more than 50% of the ductal cells (**extent grade 3**, Table 34), resulting in the **final score** of **6** and their classification as **strongly positive** (Table 35). Two LLM cases expressed APOBEC3B in a mild (**grade 1)** intensity (Table 33) and despite the grade 3 extent of staining (Table 34) they had a **final score 3** and were classified as **negative** (Table 35).

The APOBEC3B intensity was significantly lower in the LLM than CS cases ***(p= 0.008)***.

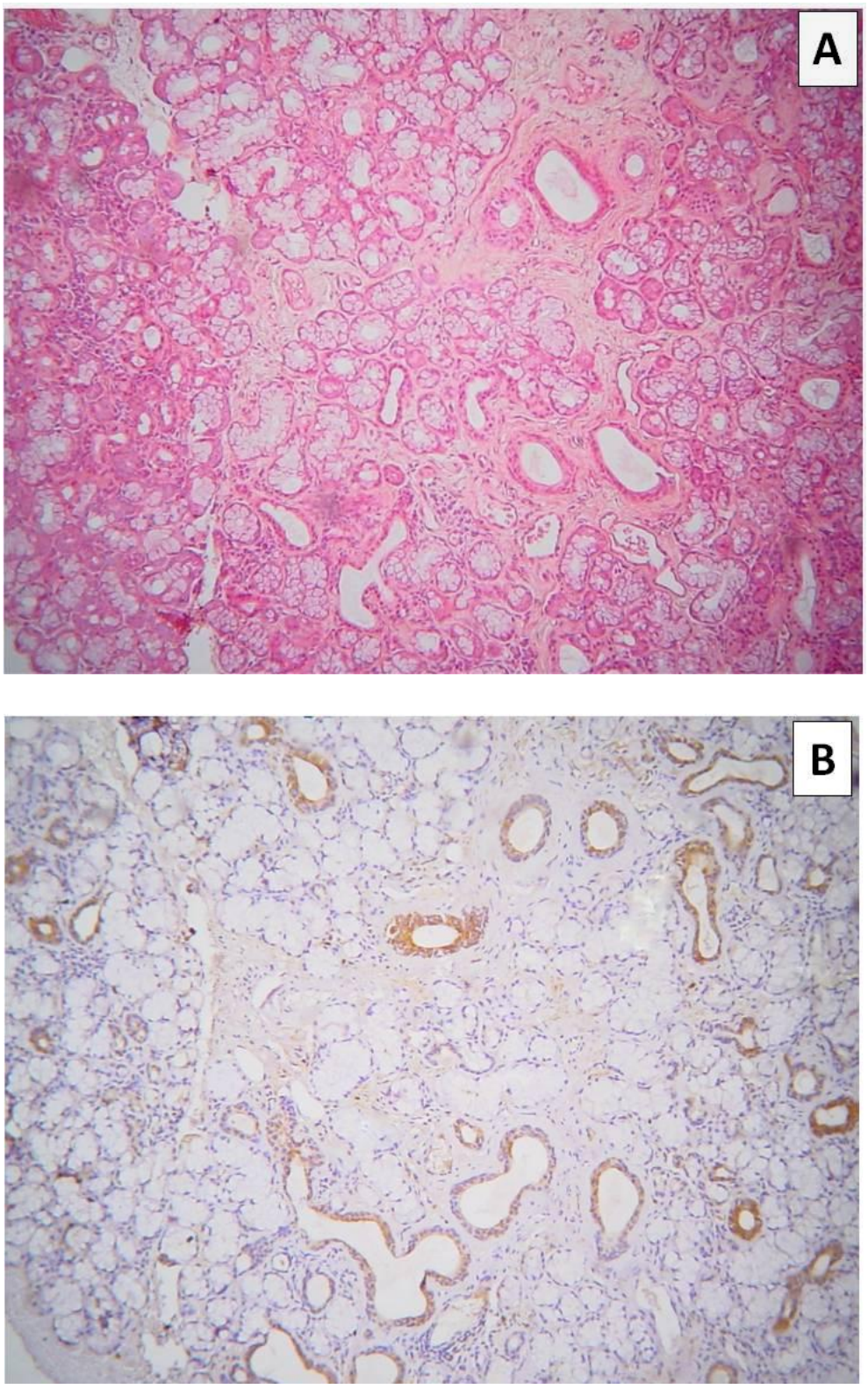
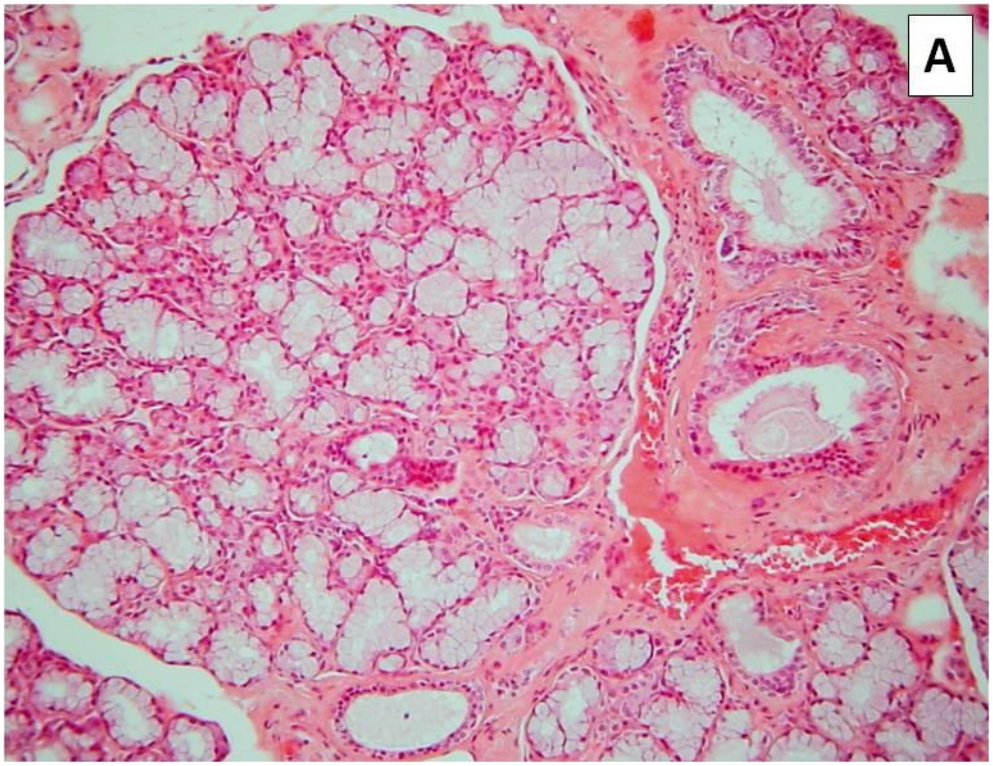


Fig. 36. Strong APOBEC3B expression (extent grade 3) in the ductal cells of a CS case. Note the absence of staining in the acinar cells and the APOBEC3B negative chronic inflammatory cells [A, H&E σταιν; B, APOBEC3B immunohistochemical stain; original magnification (A), (B) x100].



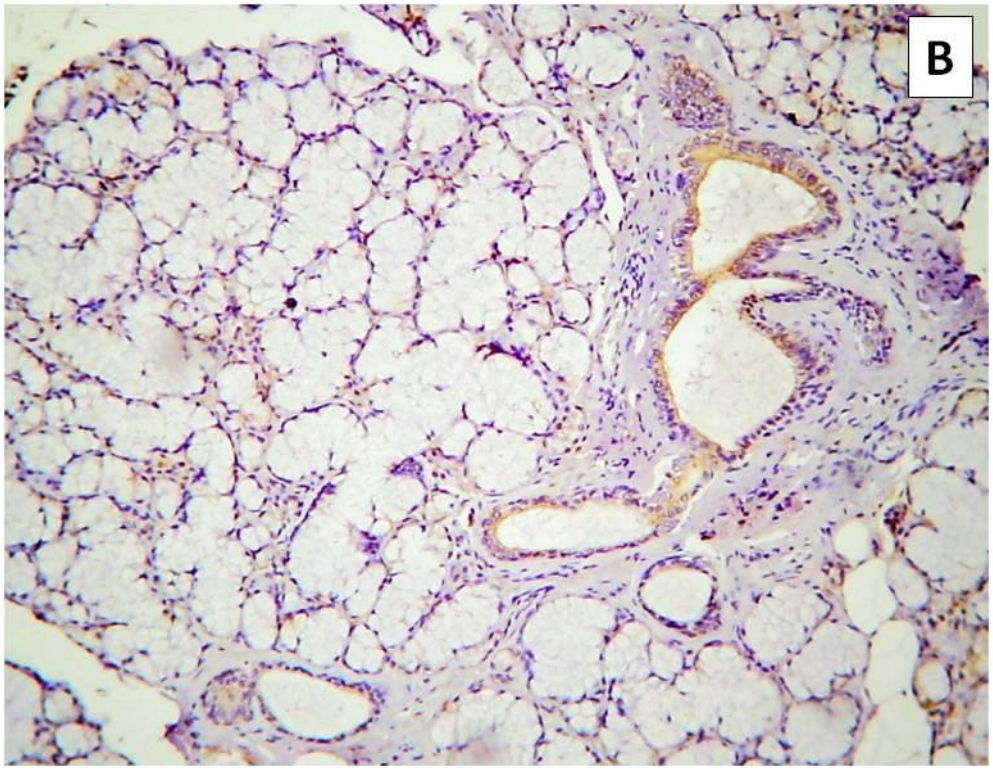


Fig. 37. Moderate APOBEC3B expression in the ductal epithelium in a LLM case [A, H&E stain; B, APOBEC3B immunohistochemical stain; original magnification (A), (B) x100]..

Table 33. APOBEC3B staining intensity results in controls.					
Study group	Intensity grade				
	0 (no stain)	1 (mild)	2 (moderate)	3 (strongly)	Total
CS	-	-	-	5	5
LLM	-	2	3	-	5
Total controls	-	2	3	5	10

Table 34. APOBEC3B staining extent results in controls.					
Study group	Extent grade				
	0 (0-5%)	1 (6-19%)	2 (20-49%)	3 (≥50%)	Total
CS	-	-	-	5	5
LLM	-	-	-	5	5
Total controls	-	-		10	10

Table 35. APOBEC3B final score and cases classification in controls.				
Study group	Final score			
	0-3	4	6 or 9	Total
	negative	mildly positive	strongly positive	
CS	-	-	5	5
LLM	2	-	3	5
Total controls	2	-	8	10

7.2.4 APOBEC3B immunohistochemical expression in SS cases VS controls

Similar to the L1 ORF2p immunostaining, the APOBEC3B expression in the ductal epithelium of MSGs was the main common finding among the SS patients and the controls. The negative APOBEC3B immunoreaction in the acinar cells, the inflammatory cells, the vascular endothelial cells and the muscle bundles, as well as the positive expression in the nerve bundles in SS cases were also in common with the APOBEC3B immunostaining pattern in sicca (CS) and non-sicca (LLM) controls.

7.2.4.1 APOBEC3B immunoexpression intensity

The APOBEC3B immunoexpression intensity grade varied from 1 to 3 among the SS cases. All CS cases showed a uniform strong (intensity grade 3) expression, while the intensity was characterized as mild or moderate in 2 and 3 LLM cases respectively. The difference in intensity grade was statistically significant between the SS cases in total and the CS cases ***(p≈0)***, as well as between the CS and LLM group ***(p=0.008)***. Diagram 10 illustrates the intensity grades in SS patients and CS and LLM controls. The bars in diagram 10 are filled with portions of the APOBEC3B immunohistochemical figures from our sample.

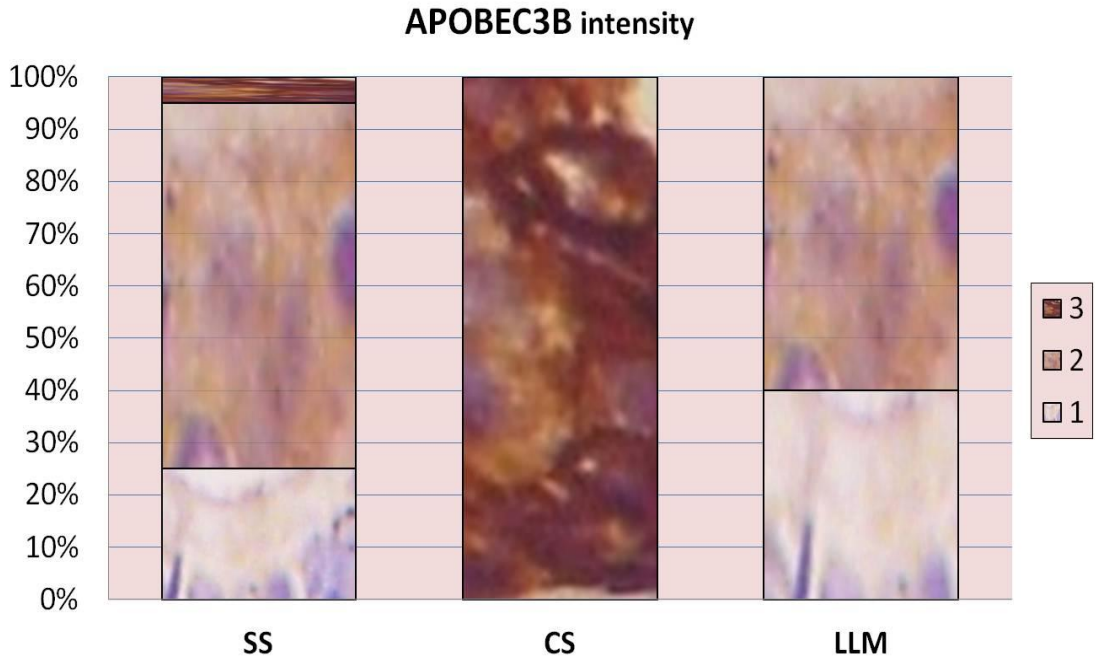


*Diagram 10. APOBEC3B intensity grades in SS, CS and LLM cases. APOBEC3B intensity grade was significantly different between the SS and CS **(p≈0)**, as well as between the CS and LLM group **(p=0.008)**.*

7.2.4.2 APOBEC3B immunoexpression extent

The APOBEC3B immunoexpression extent grade was the same (grade 3) in all SS, CS and LLM cases.

7.2.4.3 APOBEC3B final score and classification

The final APOBEC3B immunoexpression results are shown in detail in Tables 36 and 37. Cases in which the intensity was confirmed with CIA are marked with "*" or "**". CIA changed the intensity grade in cases marked with "**". In all SS cases with a CIA-induced intensity change, a decrease of one degree in the intensity grade was produced. In contrast, CIA increased the intensity grade in 1 CS case. The APOBEC3B staining extent was of grade 3 in all cases, thus the final score and classification as positive or negative was only intensity-dependent. **Positive APOBEC3B expression**, resulting from a **final score** of **6 or 9**, was observed in **15/20 (75%) SS**, **5/5 (100% CS** and **3/5 (60%) LLM cases**. No statistically significant difference in the APOBEC3B immunopositivity was observed between the SS patients in total and CS ($p>0.05$) or LLMs ($p>0.05$) or the two control groups together ($p>0.05$). When the four Tarpley subgroups were included in the analysis separately, though, the APOBEC3B final score (diagram 11) and classification (diagram 12) **in the SS Tarpley I subgroup was found significantly lower from Tarpley II cases *(p= 0.008)*, CS *(p= 0.018)* and from the total of controls *(p= 0.035)*.** The other three Tarpley subgroups did not differ significantly from each other or from each control group or from the total of controls.

Table 36. APOBEC3B immunostaining results in SS cases.					
case	study group	extent	intensity	final score	classification
1*	SS TI-1	3	1	3	negative
2	SS TI-2	3	1	3	negative
3**	SS TI-3	3	1	3	negative
4*	SS TII-1	3	2	6	strongly positive
5	SS TII-2	3	2	6	strongly positive
6*	SS TII-3	3	3	9	strongly positive
7	SS TII-4	3	2	6	strongly positive
8	SS TII-5	3	2	6	strongly positive
9	SS TII-6	3	2	6	strongly positive
10	SS TII-7	3	2	6	strongly positive
11	SS TIII-1	3	1	3	negative
12*	SS TIII-2	3	1	3	negative
13*	SS TIII-3	3	2	6	strongly positive
14	SS TIII-4	3	2	6	strongly positive
15	SS TIII-5	3	2	6	strongly positive
16	SS TIII-6	3	2	6	strongly positive
17	SS TIII-7	3	2	6	strongly positive
18**	SS TIV-1	3	2	6	strongly positive
19**	SS TIV-2	3	2	6	strongly positive
20	SS TIV-3	3	2	6	strongly positive

Table 37. APOBEC3B immunostaining results in controls.					
case	study group	extent	intensity	final score	classification
1	CS-#1	3	3	9	strongly positive
2	CS-#2	3	3	9	strongly positive
3	CS-#3	3	3	9	strongly positive
4	CS-#4	3	3	9	strongly positive
5**	CS-#5	3	3	9	strongly positive
6	LLM-#1	3	2	6	strongly positive
7	LLM-#2	3	2	6	strongly positive
8	LLM-#3	3	1	3	negative
9	LLM-#4	3	1	3	negative
10	LLM-#5	3	2	6	strongly positive

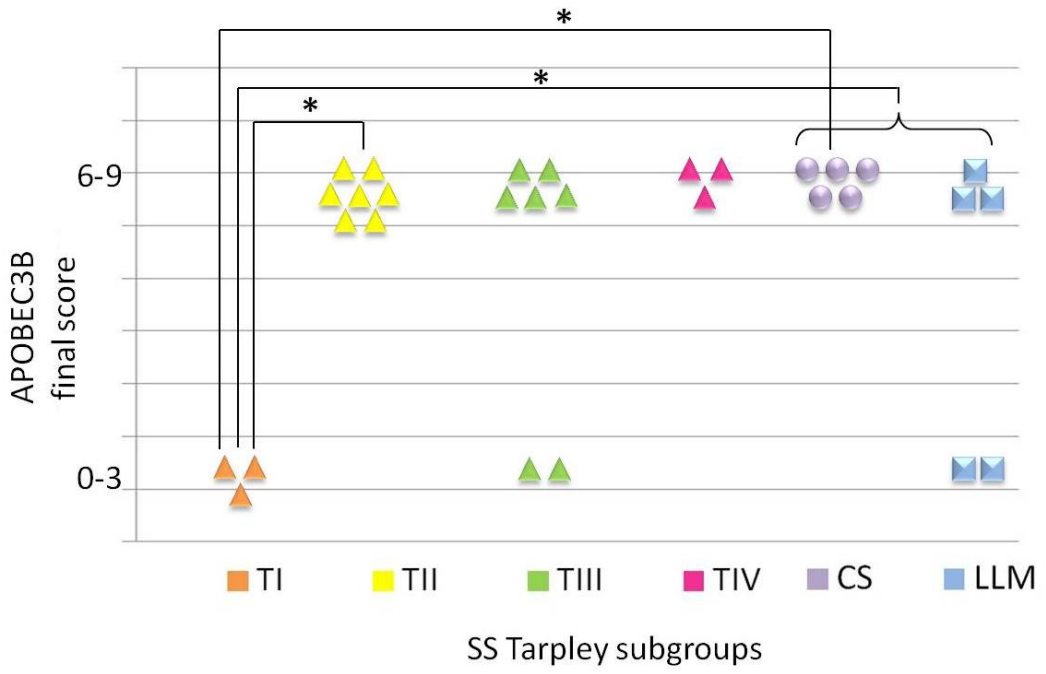


Diagram 11. Fifteen out of twenty SS cases, all CS cases and 3/5 LLM controls had a final score of 6 or 9. The difference in the final score was statistically significant between the SS Tarpley I cases and the SS Tarpley II cases ***(p=0.008)****, the CS group* ***(p=0.018)*** *and the total of controls* ***(p=0.035)****.*

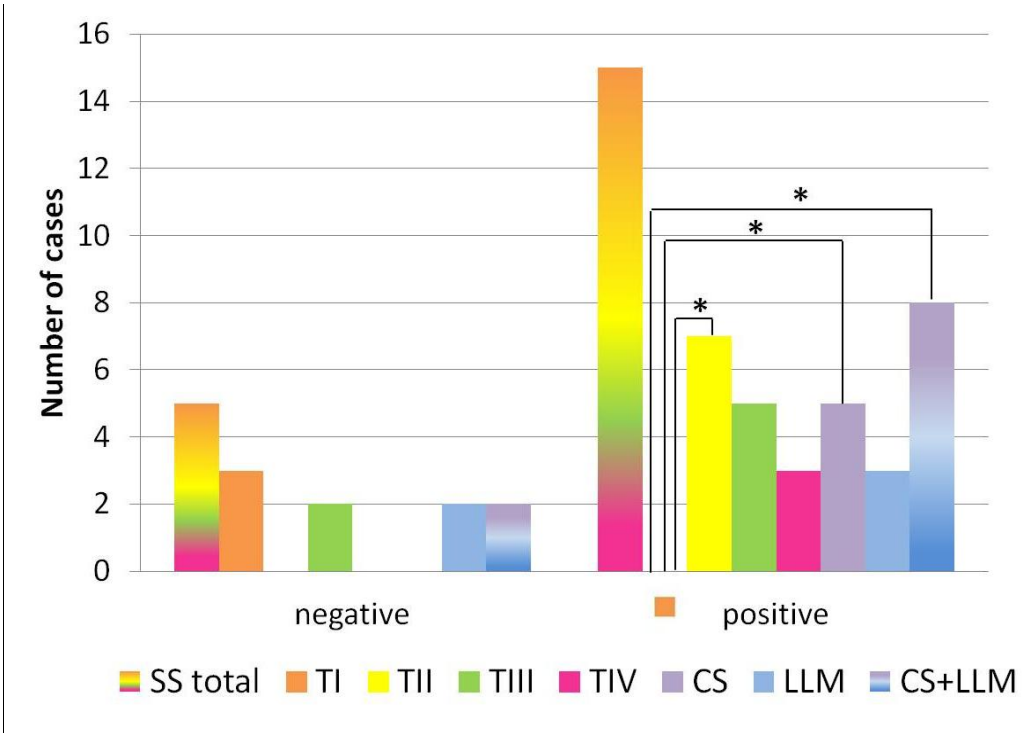


Diagram 12. The number of APOBEC3B positive cases was significantly lower in the SS Tarpley I group compared with the SS Tarpley II group ***(p=0.008)****, the CS group* ***(p=0.018)*** *and the total of controls* ***(p=0.035)****.*

7.3 L1 ORF2p VS APOBEC3B immunohistochemical expression

Positive immunoreaction of both L1 ORF2p and APOBEC3B was consistently observed in the cytoplasm of the ***ductal epithelial cells*** in SS patients of every Tarpley subgroup, regardless of the presence of a lymphocytic infiltrate (fig. 38, 39), as well as in the sicca (CS, fig. 40) and non-sicca controls (LLM, fig. 40). The staining of both antibodies showed often a fine speckled pattern, and was found to be more intense in the apical part of the cytoplasm. The ***muscle bundles*** expressed the L1 ORF2p, but not the APOBEC3B (fig. 41), while a positive APOBEC3B immunoreaction was observed in the ***nerve bundles***, in contrast to a negative L1 ORF2p one (fig. 41).

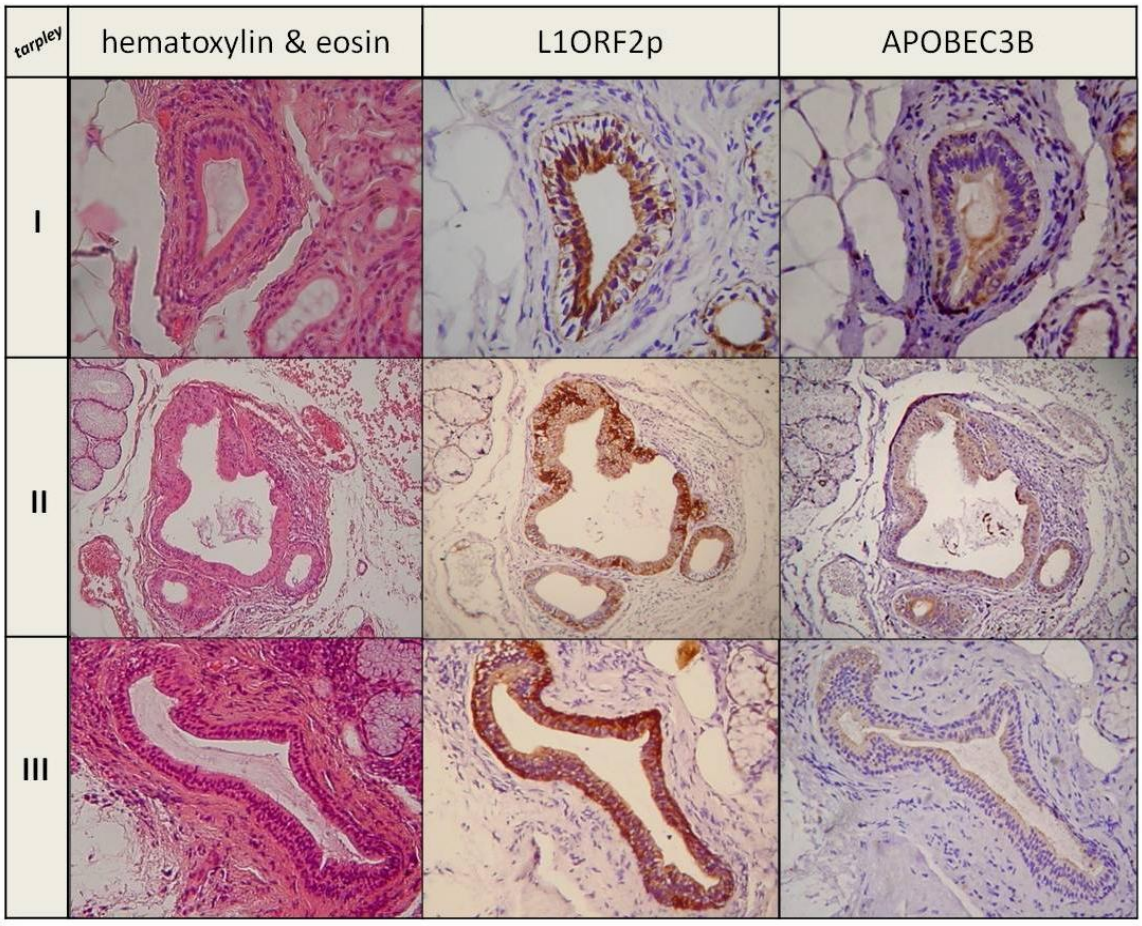


Fig.38. Cytoplasmic L1 ORF2p and APOBEC3B immunoexpression in ducts without periductal lymphocytic infiltrate in SS Tarpley I, II and III cases (original magnification x400).

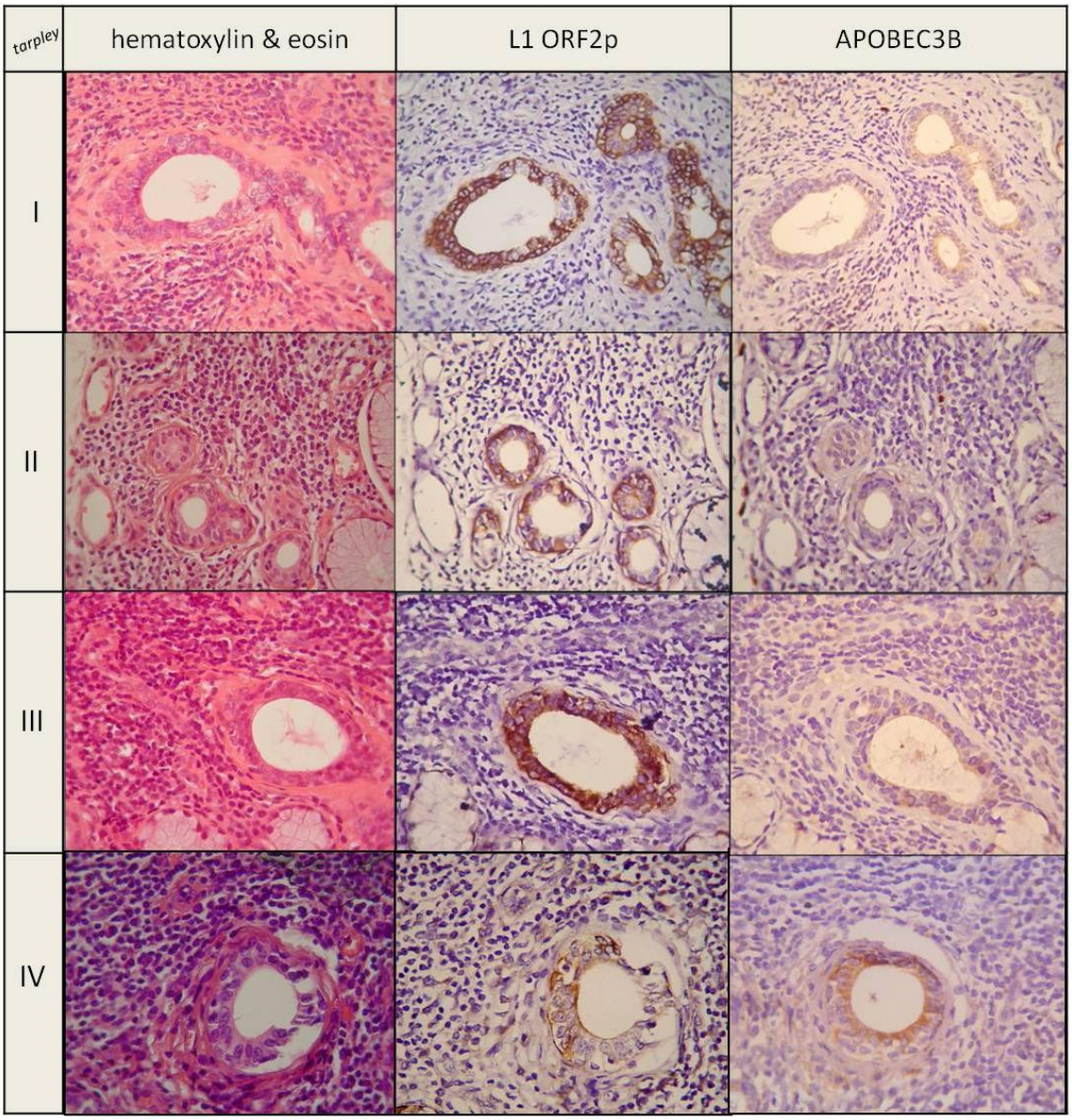


Fig.39. Cytoplasmic L1 ORF2p and APOBEC3B immunoexpression in ducts with prominent periductal lymphocytic infiltrate in SS Tarpley I, II, III and IV cases (original magnification x400).

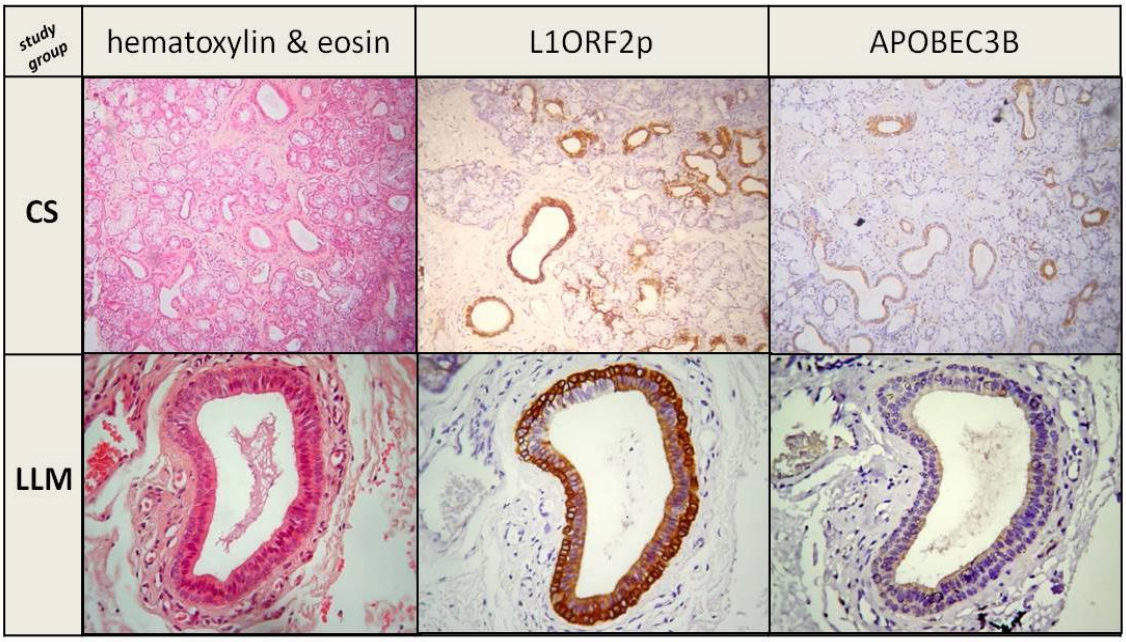


Fig. 40. Cytoplasmic L1 ORF2p and APOBEC3B immunoexpression in the ductal epithelium of CS and LLM control groups (original magnification CSx100, LLMx400).

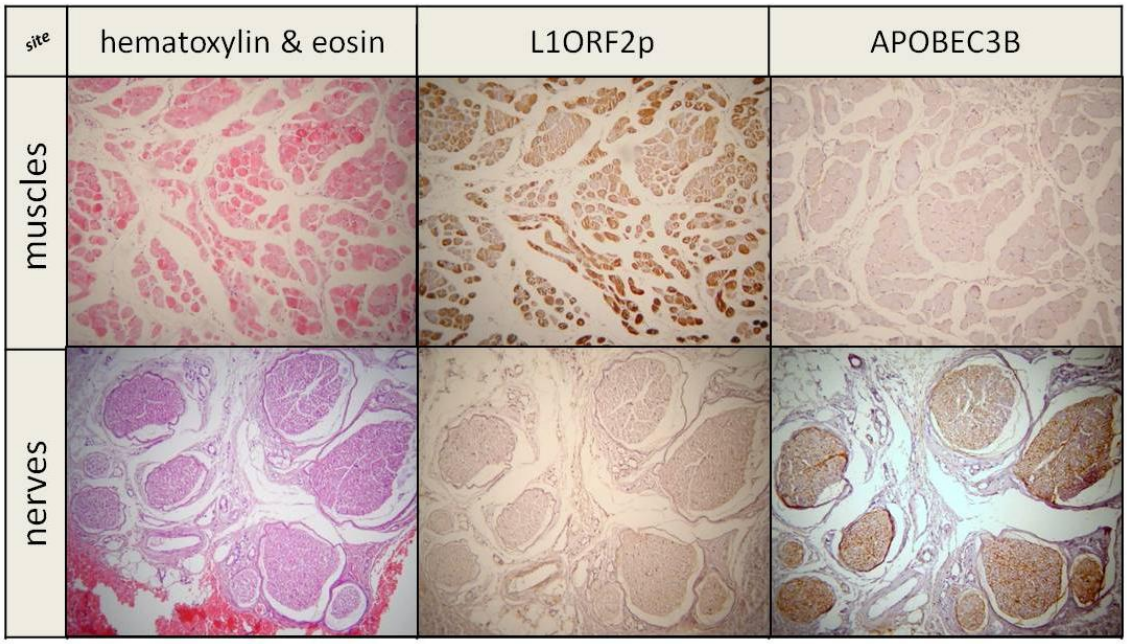


Fig 41. L1 ORF2p and APOBEC3B immunostaining in the muscle and nerve bundles of SS patients (original magnification x400).

7.3.1.1 L1 ORF2p VS APOBEC3B immunohistochemical intensity

The CIA was applied to evaluate the intensity grading results in 5 and 9 borderline cases of L1 ORF2p and APOBEC3B immunostaining respectively. **CIA decreased the intensity by 1 grade in 1/5 L1 ORF2p tested cases and in 3/9 APOBEC3B tested cases, while it increased the intensity by 1 grade in 1/9 APOBEC3B case.** Among these cases, two were considered of borderline intensity for both L1 ORF2p and APOBEC3B. In the first case, CIA decreased the L1 ORF2p but not the APOBEC3B intensity grade; in the second case, CIA decreased the APOBEC3B intensity grade, but did not change the L1 ORF2p intensity grade. In 4/5 (80%) L1 ORF2p and in 5/9 (55.6%) APOBEC3B of cases that were evaluated with CIA, CIA results were in agreement with the visual evaluation by the two researchers. The final score, though, was estimated as the product of extent and intensity and the final scores of 6 and 9 were grouped together. Thus, **the CIA results changed the final score and** consequently **the classification in only 1/9 APOBEC3B tested cases but in none L1 ORF2p case**. Fig. 42 represents the correspondence of CIA values to the intensity grades of the L1 ORF2p and APOBEC3B evaluated cases.

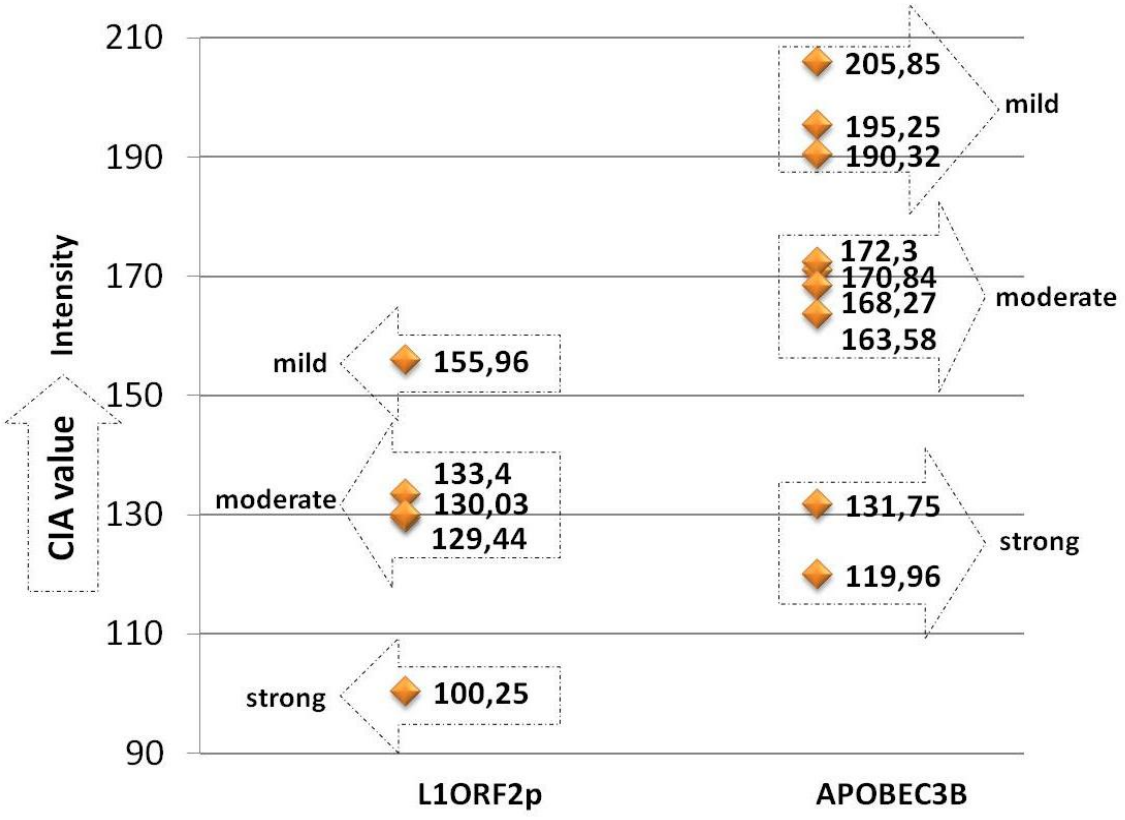


Fig 42. CIA values correspondence to intensity grades of both antibodies.

In 12/20 SS cases and 4/5 LLM cases the intensity grade of L1 ORF2p was greater than the APOBEC3B. Among these 12 SS cases, were all the Tarpley I and III cases, as well as 1 Tarpley II and 1 Tarpley IV case. In contrast to Tarpley I and III cases, 2 out of 3 Tarpley IV cases showed **a lower intensity of L1 ORF2p compared with APOBEC3B**. In all CS cases, as well as in 4/7 Tarpley II SS cases the intensity grade was the same for both antibodies (diagram 13).

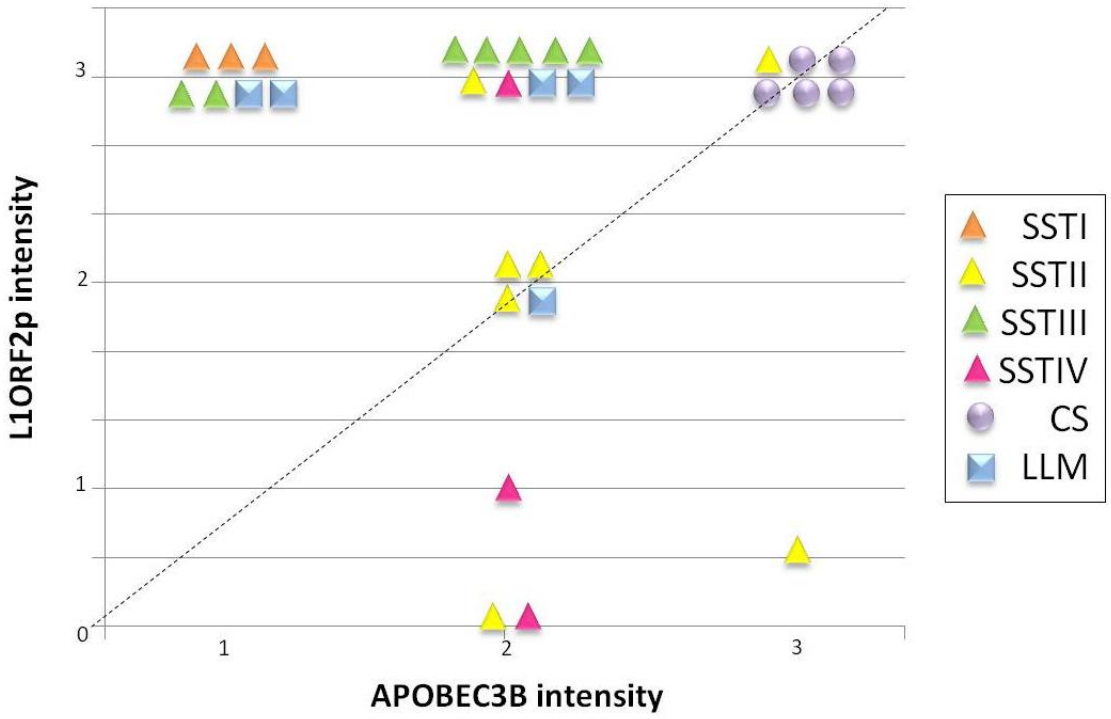


Diagram 13. L1 ORF2p and APOBEC3B intensity grades for the 30 study cases.

7.3.1.2 L1 ORF2p VS APOBEC3B immunohistochemical extent

The extent of staining was similar for both antibodies. The L1 ORF2p immunostaining extent varied between 2 and 3 in the SS group, except for the two cases with no staining, as well as in the LLM group, while 5/5 CS cases displayed L1 ORF2p immunoreaction in an extent grade of 3. **A uniform APOBEC3B expression of extent grade 3 was observed in 20/20 SS cases and all sicca and non-sicca controls**.

7.3.1.3 L1 ORF2p VS APOBEC3B immunohistochemical final score and classification

As it can be easily noticed in diagram 14, most of the SS and LLM cases, as well as all the CS cases were L1 ORF2p or/and APOBEC3B positive. In particular, 17/20 and 15/20 SS cases were L1 ORF2p and APOBEC3B positive respectively, while 5/5 and 3/5 cases were L1 ORF2p and APOBEC3B positive respectively.

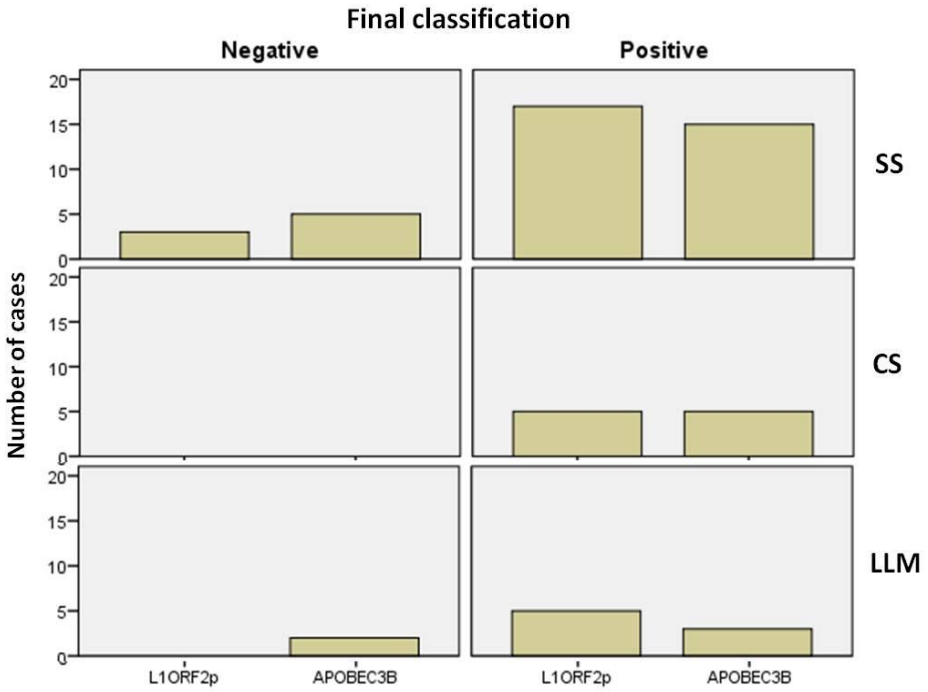


Diagram 14. Final classification categories for both antibodies in the three study groups.

When the concurrent expression of both antibodies was evaluated in each of the three study groups, **12/20 (60%) SS**, **5/5 (100%) CS** and **3/5 (60%) LLM** cases were found **double positive**. This difference, though, was not statistically significant (p>0.05). Next, the L1 ORF2p and APOBEC3B concurrent expression was evaluated in each SS and control case separately, resulting in **0/3(0%) Tarpley I, 6/7 (85.7%) Tarpley II, 5/7 (71.4%) Tarpley III and 1/3 (33.3%) Tarpley IV double positive cases** (diagram 15). The L1 ORF2p and APOBEC3B double positive expression was found to differ significantly between SS **Tarpley I** and **II subgroup *(p=0.03)***, as well as between **Tarpley I** and **CS cases *(p=0.018)***. It is also worth mentioning that the 3/20 L1 ORF2p negative SS cases (2/3 SS Tarpley IV cases and 1 Tarpley I case) showed strong positivity (final score of 6) for APOBEC3B. Moreover, a strongly positive L1

ORF2p expression (final score of 6 or 9) was observed in all APOBEC3B negative cases (3 SS Tarpley I, 2 SS Tarpley III and 2 LLM cases) (diagram 16).

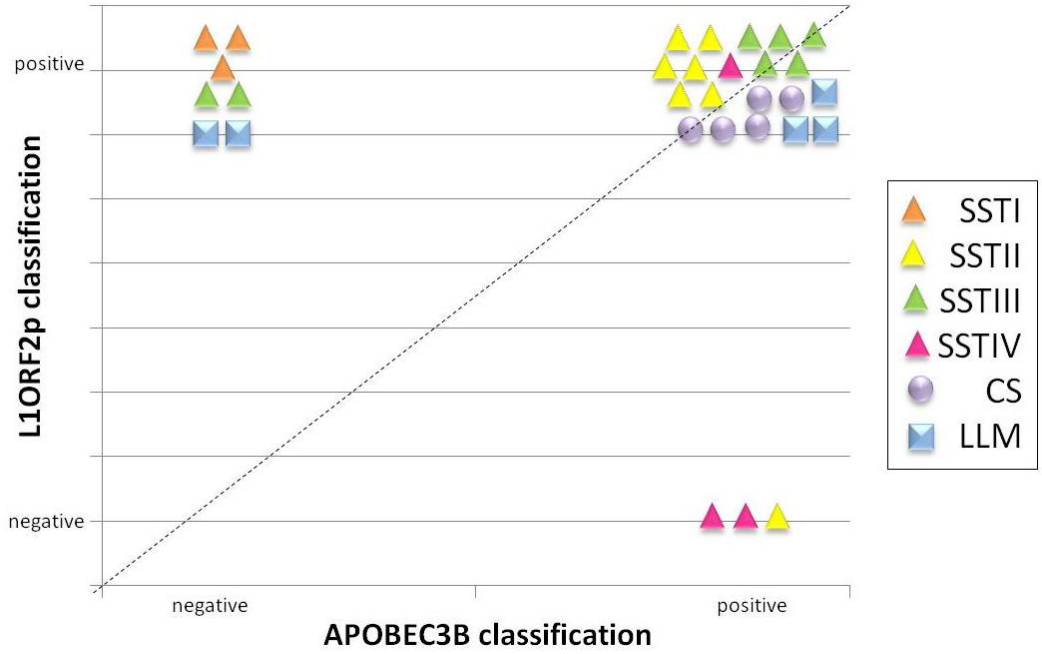


Diagram 15. L1 ORF2p and APOBEC3B double classification in each SS, CS and LLM case.

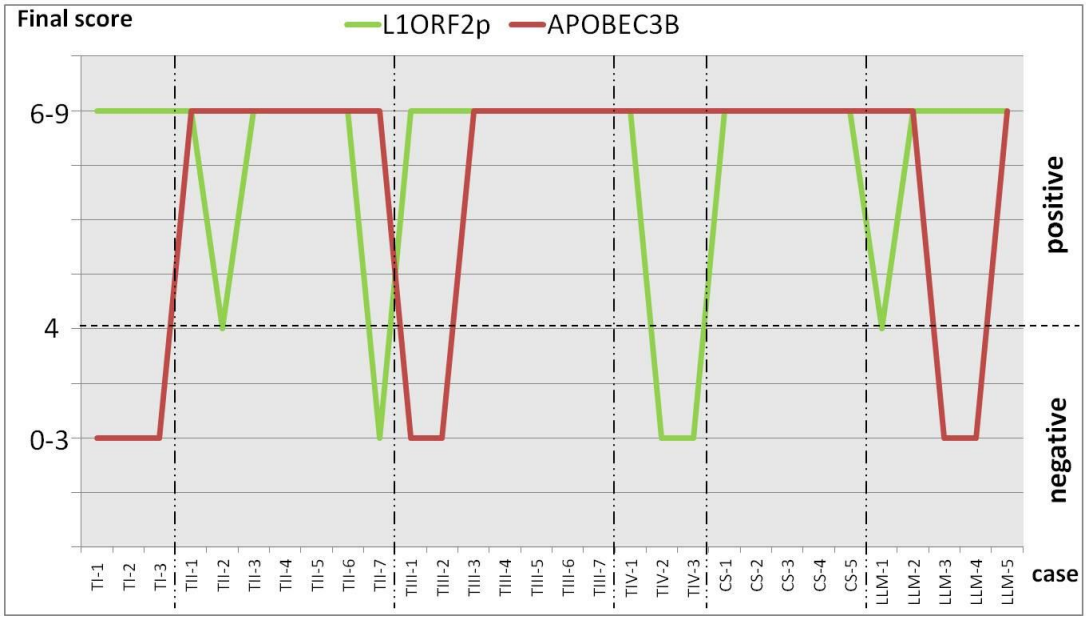


Diagram 16. L1 ORF2p and APOBEC3B final score and classification in each SS, CS and LLM cases.

8 DISCUSSION

In the present study, L1 ORF2p and APOBEC3B proteins were immunohistochemically detected for the first time in the ductal epithelial cells of MSGs of SS patients and non-autoimmune controls. The cytoplasmic immunoreactivity of both proteins tested was in accordance with their expression in the breast carcinoma specimen that was selected as positive control,[227, 426] as well as with the results of previous immunohistochemical studies.[198, 222, 223, 227, 421, 427-429]

Study method

L1 ORF2p and APOBEC3B have been mostly evaluated with molecular methods while studies regarding the immunohistochemical profile of the L1 ORF2p and APOBEC3B proteins are scarce in the literature. The high percentage of L1 adenosine content (up to 40%), which may cause a defective transcription elongation, as well as the unconventional translation mechanism of L1 RNA that results in low levels of L1 ORF2p (~one ORF2p molecule per L1 mRNA) may have possibly hindered the L1 ORF2p detection.[209] On the other hand, the high degree of homology between the APOBEC3 proteins has caused difficulty in raising an antibody specific for each member of the group.[408] Gwak et al[426] raised an antibody against the APOBEC3B N-terminal amino acid residues 80-127. This amino acid sequence, though, is 100%, 70%, 50% and 29% homologous with APOBEC3F, APOBEC3D, APOBEC3G and APOBEC4.[365] Thus, the results of their study may well have corresponded to an APOBEC protein other than APOBEC3B.[426] The anti-APOBEC3B antibody used in the current study, targets the N-terminal amino acid residues 20-60 of the APOBEC3B protein, similarly to the antibody used by Onguru et al,[421] and this sequence is specific for it.[365] Since the immunohistochemical expression of L1 ORF2p and APOBEC3B proteins has been confirmed in neoplastic breast tissue,[227, 426] a breast carcinoma tissue sample was used as a positive control.

The aim of this study was to comparatively evaluate the immunohistochemical expression of L1 ORF2p and APOBEC3B proteins in the MSGs of SS patients and non autoimmune controls. The immunostaining results for both antibodies were evaluated according to the grading system proposed by Gwak et al.[426] This system characterizes as positive only cases with at least a moderate intensity of a minimum extent grade 2. In our opinion, this strict criterion is useful in the evaluation of new, not widely tested

antibodies, as it increases the sensitivity of the evaluation and reduces the possibility of false positive results due to overestimation. Additionally, the use of the same grading system for both antibodies facilitated the comparative evaluation of the L1 ORF2p and APOBEC3B results.

Although immunohistochemistry is a well validated method that provides information not only for the presence but also for the distribution of the antigen,[442] the results are usually evaluated by semiquantitive methods which poses a question of accuracy.[442] To overcome this limitation and in order to provide a more accurate evaluation on the staining intensity, a Computerized Image Analysis (CIA) was performed in cases scored as borderline between two intensity grades by the two researchers. Automated immunohistochemical measurements via CIA are related to target antigen concentration and are more accurate in ranges of staining that appear weak to the human eyes.[442] Moreover, the digital values of CIA that correspond to intensity grades can resolve intra- and inter-observer disagreement about the staining intensity because CIA does not classify the results into groups, but provides a numerical value for each case evaluated that increases the sensitivity of the immunohistochemical analysis.[445, 446] In our study, the intensity grade of the L1 ORF2p and APOBEC3B immunostaining was evaluated in 5 and 9 cases respectively. More APOBEC3B than L1 ORF2p cases were chosen for CIA evaluation, as APOBEC3B staining was predominantly of moderate intensity (18/30, 60%), in contrast to the clearly strong L1 ORF2p intensity (24/30, 80%). Moreover, as APOBEC3B was expressed in all cases in an extent of grade 3, even cases with a moderate (grade 2) intensity were finally classified as strongly positive (final score 6). This finding points out that the APOBEC3B final classification was only intensity-dependent, a finding that emphasizes the need for a more accurate scoring of intensity. CIA changed the intensity grade in only 1/5 L1 ORF2p and in 4/9 APOBEC3B cases but the CIA results were moderately to significantly correlated with the two researchers' visual findings *(r=0.85 for L1 ORF2p, r=0.64 for APOBEC3B).* A high degree of agreement has also been reported in studies comparing the CIA results regarding the intensity of immunoexpression with manual visual semiquantitative evaluation.[443, 446]

L1 ORF2p expression

In the present study, we identified cytoplasmic L1 ORF2p expression in the ductal cells of MSGs of SS patients and controls. L1 elements have been associated with the development of autoimmune diseases, including SS, as they are inducers of the type I IFN pathway.[157, 326, 341, 342, 348] Considering the role of L1 ORF2p in SS, three mechanisms are proposed (fig. 43). According to one hypothesis, L1 ORF2p is the translational product of an active L1 element that may promote the immune response in SGECs via Toll-like Receptor (TLR)-dependent or independent mechanisms.[58] Apoptotic cell death, a well-described antigen-presentation mechanism of the SGECs, may expose L1 elements to the immune system.[48] The demethylated L1 CpG-enriched DNA could bind to TLR9, while L1 mRNA could bind either to TLR7 or other cytosolic receptors, including RIG-I like receptors, of pDCs and activate them to produce type I IFN.[58, 157, 447] Moreover, SGECs are also capable to produce type I IFNs,[58] while TLR7 and TLR9 have been found in the ductal epithelium of parotid glands in SS patients.[62] Thus, L1 element may act as an intrinsic trigger factor of type I IFNs in an autocrine manner. Alternatively, apoptotic death of SGECs' or exosomes' formation may expose L1 RNA or its encoded ORF1p and ORF2p to the immune system and trigger the generation of autoantibodies.[157] As a result, DNA (L1 and endogenous nucleic acids) and autoantibodies-containing immune complexes may be recognized by TLR9 found in the endosomal compartment of pDCs, via the FcγRIIα receptor, and stimulate type I IFN production.[58] Finally, L1 ORF2p has a key role in the successful L1 retrotransposition,[173] and L1 retrotransposition-competent elements integrating into new genomic sites may affect mRNA splicing or exons resulting in the production of a protein that is acknowledged as foreign by the immune system.[157]

The cytoplasmic L1 ORF2p expression observed in the current study is in line with most previous immunohistochemical/immunocytochemical analyses.[198, 222, 223, 227] Cytoplasmic expression has been associated with the L1 ORF2p large size that complicates the protein nuclear import via passive diffusion while the entrance of L1 ORF2p to the nucleus, which is required for retrotransposition, is facilitated by the truncation of a C-terminal fragment.[154] Moreover, differences in the sensitivity or cross reactivity of the various antibodies used in previous studies may be responsible for the different results regarding the cellular protein localization.[209]

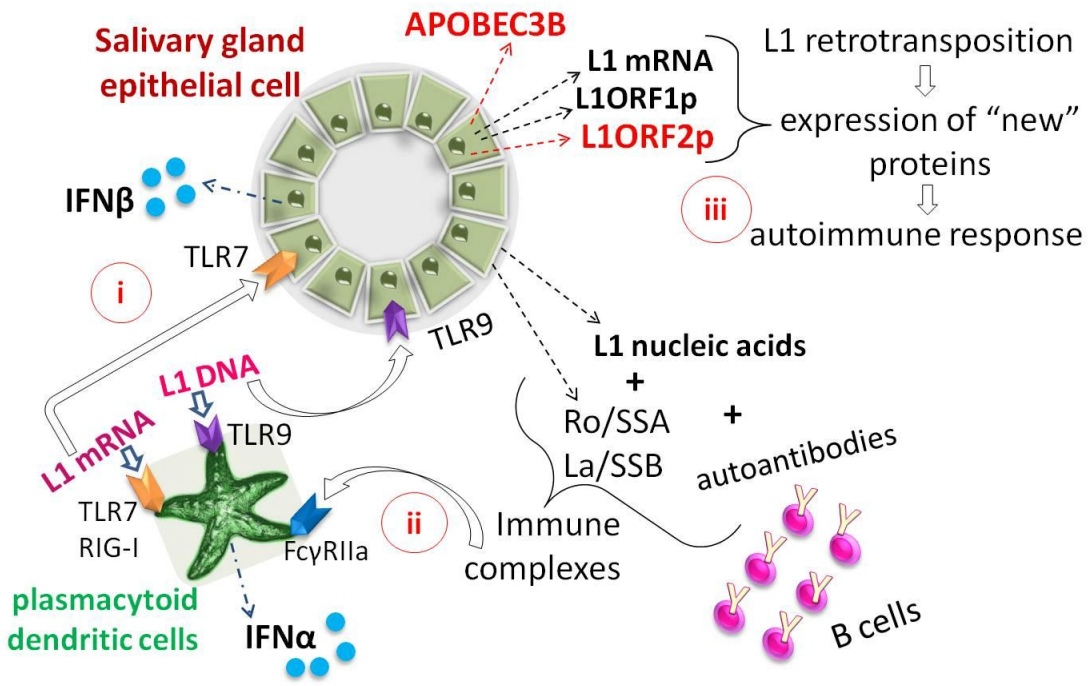


Fig. 43. L1 may trigger the autoimmune response in SS via different mechanisms: i) via TLR or other cytosolic receptors found in pDCs or SGECs, ii) via L1-containing immune complexes or iii) via retrotransposition and expression of new proteins that will be recognized as foreign by the immune system.

Martin et al[448] reported the co-expression of full-length L1 RNA and L1 ORF1p in mouse embryonal carcinoma cell lines, where L1 ORF1p was not associated with any known cytoplasmic organelles and its punctuate pattern was attributed to its detection within RNPs. Additionally, the cytoplasmic expression of L1 ORF2p has been related with a potential localization in cytoplasmic granules, such as stress granules or P-bodies, where L1 ORF1p has been found to co-localize with the Ro 60/TROVEC2 protein.[234] The localization in cytoplasmic granules may be responsible for the punctuate pattern of L1 ORF2p staining observed in the present study. This finding has not been reported in previous L1 ORF2p immunohistochemical studies while interestingly, a punctuate immunostaining has been mentioned for L1 ORF1p in the literature.[198, 240] Taken that the Ro60/TROVEC2 autoantigen binds endogenous retroelements, including L1,[449] the theory of L1 localization in cytoplasmic granules may gain more interest in the future, since stress granules have been recognized very recently as potential autoantibody targets in another autoimmune disorder, i.e. the systemic sclerosis.[450]

We found positive L1 ORF2p expression in 17/20 SS cases and in all CS and LLM cases. Our findings are in accordance with the studies by Mavragani et al, [251, 340] who found full-length L1 mRNA expression in pSS patients, sicca and non-sicca controls via real-time PCR. Furthermore, Sirivanichsuntorn et al[250] studied the L1 methylation profile in MEC using 14 cases of normal SGs as controls. Since L1 expression is expected in hypomethylated tissues, their finding that L1 elements are hypomethylated in normal SGs, although to a lesser extent compared to neoplastic SGs, is in line with our results about L1 ORF2p expression in the normal MSGs of SS and non SS patients. No significant difference in the L1 ORF2p positive immunoreaction was observed between SS patients in total and controls in the present study. Mavragani et al[251] reported increased full-length L1 mRNA levels in the MSGs of low-risk for lymphoma development pSS patients and sicca controls with autoimmune disease manifestations in comparison to non-autoimmune sicca controls and healthy controls. Although the results of our study seem to be in contradiction with the above findings, it is known that post-transcriptional events may influence the amount of mRNA that will eventually translate into the final product, and in our case into L1 proteins, and in particular L1 ORF2p.

When the four Tarpley subcategories of the present study were analyzed separately, a significantly lower L1 ORF2p expression was found in the SS Tarpley IV subgroup compared to the total of CS and LLM cases (*p= 0.038).* Since the high biopsy focus score is considered as an adverse prognostic factor for lymphoma development in SS patients,[9] this result is in line with the aforementioned study by Mavragani et al,[251] who found significantly lower L1 mRNA levels in the type I SS patients, i.e. those in high risk for lymphoma, compared to type II ones. These findings may be indicative of various genomic deviations among SS patients that predispose them to a different susceptibility for lymphoma development.

APOBEC3B expression

Since L1 retroelements are found in both autoimmune and non-autoimmune patients, it's possible that their uncontrolled expression triggering autoimmunity is the result of a deregulated epigenetic mechanism. APOBEC3 proteins are among the inhibitory factors of the endogenous L1 retroelements.[219, 374, 392] A coordinated expression of the APOBEC3A and APOBEC3B mRNA with L1 mRNA overexpression

has been reported in the MSGs of SS patients, indicating that the APOBEC3 enzymes may act as a host-defensive mechanism against the L1-deleterious effect in SS.[356]

In line with data supporting that APOBEC3 enzymes are plausible to be found were L1 elements are present,[392] a cytoplasmic APOBEC3B expression was observed in the ductal cells of MSGs of SS patients in the present study, similarly to L1 ORF2p expression. Our finding coincides with the cytoplasmic expression found in most previous APOBEC3B immunohistochemical studies,[421, 427-429] except for the study by Kosumi et al,[430] who regarded only nuclear APOBEC3B expression as positive. APOBEC3B has been reported to shuttle between the cytoplasm and nucleus, containing two distinct nuclear export and import signals,[374] but its L1 inhibitory properties are independent of its subcellular localization.[219]

APOBEC3B protein was expressed in all SS patients, although, according to the grading system applied, 3/3 SS Tarpley I cases with mild APOBEC3B intensity were finally classified as APOBEC3B negative. Despite the low number of patients in each study subgroup, separately analysis of the four Tarpley subcategories resulted in a significantly lower APOBEC3B expression in SS Tarpley I cases compared to the CS controls *(p=0.018)*. This difference suggests that the APOBEC3B inhibitory mechanism is probably attenuated at the initial stage of SS. Although APOBEC3B enzymes are not the only restricting factor of L1 retroelements,[206] the weak immunostaining of APOBEC3B in SS Tarpley I patients may allow for the inadequately controlled L1 elements to trigger autoimmunity. If the difference in the APOBEC3B positivity between SS Tarpley I and CS cases could be confirmed in a larger group of patients, this difference could be of help in clarifying borderline histopathologically SS cases. The initial exclusion of 5 borderline SS cases from our SS study group, although it reduced the number of available Tarpley I SS cases, it probably helped to highlight this difference in APOBEC3B expression. A significantly higher APOBEC3B immunopositivity was also observed in the SS Tarpley II cases compared to Tarpley I cases. This finding may indicate a compensatory increase in APOBEC3B levels in line with the ongoing inflammation, in order to achieve a more efficient inhibition of the L1-induced autoimmune response in SS.

APOBEC3B protein was expressed in the ductal epithelium of the MSGs in all CS cases but only in 3 out of 5 normal MSGs in LLM cases. A more intense

APOBEC3B immunostaining was observed in the inflamed MSGs compared to normal glands in LLM cases, although only the staining in the normal glands was taken into account for the final APOBEC3B classification of these cases. The APOBEC3B and other APOBEC3 enzyme levels have been found up-regulated in some tissues, such as the liver, in case of viral infection, as a result of the chronic pro-inflammatory cytokines overexpression.[414] Based on these observations, the stronger APOBEC3B expression in the inflamed MSGs compared to normal glands detected in the present study could be attributed to a local overexpression of pro-inflammatory factors. It should be mentioned, though, that no difference was found in the APOBEC3B immunoreaction between the inflamed and non-inflamed ducts in SS cases.

L1 ORF2p and APOBEC3B expression

The high percentage of double positive expression of L1 ORF2p and APOBEC3B proteins in the present study illustrates the coordinated expression of the "intrinsic enemies", i.e. L1 elements, and its inhibitory machine, i.e. APOBEC3 enzymes. Although the 3 L1 ORF2p negative cases showed a strong APOBEC3B expression, the absence of L1 ORF2p expression cannot be attributed only to the APOBEC3B effect, as several other host mechanisms are involved in L1 restriction.[206] It is more probable, though, that the low to absent APOBEC3B expression in 5 SS cases may have contributed to the strong L1 ORF2p expression in these cases.

We have also observed a L1 ORF2p positive expression in the skeletal muscle bundles, in which APOBEC3B was negative. Conversely, APOBEC3B was expressed in the nerve bundles that were L1 ORF2p negative. These findings could not be easily compared with the relevant information in the pertinent literature. L1 expression in the skeletal muscle has been reported previously in the mRNA level.[214] L1 mRNA has also been detected in fetal and adult brain[212, 214, 216] and neurons[216] but no data exist regarding the L1 proteins immunohistochemical expression in the nerves. Similarly, APOBEC3B mRNA has been reported in human brain and skeletal muscles,[372] but data concerning the APOBEC3B protein immunohistochemical expression in nerves and muscles are missing.

Moreover, APOBEC3B and L1 ORF2p proteins were mostly absent in the inflammatory infiltrates of MSGs in the present study. Although a low to absent APOBEC3B mRNA expression has been found in freshly isolated monocytes and

lymphocytes,[372] no data regarding APOBEC3B protein expression in inflammatory cells are available for comparison with our observations (see Table 15). L1 ORF2p expression in inflammatory cells has also not been described in the literature (see Table 11). In addition, blood vessels in the current study were L1 ORF2p and APOBEC3B negative. Ergun et al,[222] investigated the association between the L1 ORF1p and ORF2p expression and the stage of blood vessels differentiation and maturation. In their study, the endothelial cells of the mature blood vessels showed immunostaining were double positive for L1 ORF1p and ORF2p, while small and immature capillaries were double negative.[222]

Undoubtedly, the small number of cases that were evaluated and the semiquantitative evaluation method used restricts the range of conclusions that could be drawn. Moreover, due to the retrospective nature of our study, data regarding the immunological profile of cases included were largely absent, thus, their correlation with the immunohistochemical findings was not feasible.

Despite these limitations, the current study is the first report of the expression of L1 ORF2p and APOBEC3B proteins in the MSGs of SS patients and healthy controls. Moreover, our study provides evidence for the cellular localization of these molecules within the ductal epithelium, which conforms to the potential role of L1 retroelements as an intrinsic antigen of the SGECs that could activate the immune response in SS patients. Pharmaceutical inhibitors against the L1 ORF2-reverse transcriptase are considered a promising future treatment choice in autoimmunity.[341, 342] Since APOBEC3 enzymes are not the only L1 inhibitory factors, future studies using a larger sample of SS patients should focus on the correlation between APOBEC3B as well as other APOBEC3s, such as APOBEC3A and APOBEC3G, with additional L1 restriction factors, including the DNTs and the TREX exonuclease, and investigate their collective effect on the inhibition of L1-mediated autoimmune response.

9 CONCLUSIONS

1. L1 ORF2p and APOBEC3B are expressed in the MSGs of SS patients and non autoimmune controls. Thus, these two proteins represent endogenous elements of the salivary glands.
2. L1 ORF2p may trigger the autoimmune response in SS via different mechanisms; thus the pharmaceutical inhibition of the L1 ORF2-reverse transcriptase is considered a promising future treatment choice in autoimmunity.
3. The L1 ORF2p and APOBEC3B co-expression in the ductal epithelium of SS patients possibly suggests that APOBEC3 acts as an inhibitory mechanism, against the inappropriate endogenous expression of L1 elements.
4. The lower APOBEC3B expression in Tarpley I SS patients possibly indicates that the epigenetic regulation of L1s by APOBEC3 deaminases may be impaired, resulting in an inadequate control of L1 elements and activation of the type I IFN pathway in SS.

10 ABSTRACT

IMMUNOHISTOCHEMICAL STUDY OF THE EXPRESSION OF LINE-1 (ORF2p) AND APOBEC3B PROTEINS IN THE LABIAL MINOR SALIVARY GLANDS OFSJÖGREN'S SYNDROME PATIENTS

Study Objective: Sjögren's syndrome (SS) is a chronic autoimmune disease that involves approximately 0.5-1% of the general population, showing a clear predilection for middle-aged female patients. The histopathological lesion in SS is characterized by a focal mononuclear cell infiltration of the exocrine glands, in proximity to the glandular epithelium (autoimmune epithelitis), that results in the degradation of glandular parenchyma. A multifactorial aetiopathogenesis of SS has been proposed, in which an interplay between immunological, genetic, epigenetic and environmental factors is eminent. Among the several pathogenetic mechanisms that have been proposed, the activation of the interferon (IFN) pathway is considered a hallmark event. The Long Interspersed Nuclear Elements-1 (L1s) are potential trigger factors that stimulate plasmacytoid dendritic cells, activating the type I IFN pathway in SS. L1s are mobile elements accounting for nearly 20% of the human genome. L1 elements contain two non-overlapping open reading frames (ORF1 and ORF2), which encode for L1 ORF1p and ORF2p proteins. The human L1 expression is regulated by various transcriptional and posttranscriptional mechanisms, including the inhibitory effect of the Apolipoprotein B mRNA-Editing enzyme, catalytic polypeptide-like 3C (APOBEC3) deaminases. APOBEC3 family constitutes of 7 proteins: APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F, APOBEC3G and APOBEC3H. A coordinate expression of APOBEC3B and L1 mRNA has been reported in the minor salivary glands (MSGs) of SS patients.

The aim of the present study was to comparatively evaluate the immunohistochemical expression of L1 ORF2p and APOBEC3B proteins in the labial minor salivary glands (MSGs) of SS patients.

Materials and Methods: The material of the present study derived from the Histopathological Archives of the Department of Oral Medicine and Pathology, Faculty of Dentistry, National and Kapodistrian University of Athens. L1 ORF2p and APOBEC3B expression was assessed by a routine immunohistochemical method in the MSG tissues from 20 SS patients, classified in four subcategories according to

Tarpley's histological criteria. The MSGs derived from 5 cases diagnosed as "Chronic Sialadenitis (CS), not compatible with SS" and the normal MSG tissue included in 5 excisional biopsies of lower lip extravasation cysts (lower lip mucoceles, LLM) were used as sicca and non-sicca controls respectively. An invasive lobular breast carcinoma was used as the positive control of both L1 ORF2p and APOBEC3B. The immunopositivity for both antibodies was determined according to a semi-quantified method as the product of extent and intensity of staining. Cases with a borderline intensity staining were also evaluated with a Computerized Image Analysis (CIA). The relationships between the L1 ORF2p and APOBEC3B expressions in SS patients and sicca- (CS) or non-sicca (LLM) controls were evaluated by Fisher's exact test. Pearson correlation coefficient test was also used to compare the correlation of the intensity value between the CIA results and the visual evaluation results for both antibodies. The statistical significance level was set at $p<0.05$.

Results: A strong cytoplasmic expression of both antibodies was observed in the breast carcinoma sample. Almost 90% of SS cases and all of the CS and LLMs controls expressed L1 ORF2 protein. L1 ORF2p was expressed in all cases in the cytoplasm of the ductal epithelial cells, often with a speckled pattern. In 15/20 (75%) SS cases a strong L1 ORF2p intensity was observed, while in 14/18 (77.8%) of the L1 ORF2 positive SS cases, immunostaining was seen in more than 50% of the ductal cells. According to the final score based on the product of intensity and extent of expression, 17/20 (85%) SS cases were found to be L1 ORF2 positive. The L1 ORF2p final positive or negative classification did not differ significantly between the SS Tarpley subgroups or between the SS cases (in total), the CS and the LLM cases. The separate analysis of the four SS Tarpley subgroups resulted in a statistically significant difference between the SS Tarpley IV cases and the controls in total *($p= 0.038$).*

All SS, CS and LLM cases expressed APOBEC3B protein in the cytoplasm of the ductal epithelial cells, often with a speckled pattern. A moderate APOBEC3B intensity was found in 14/20 (70%) SS cases, while the intensity grade was significantly lower in the SS Tarpley I compared to the Tarpley II cases *($p=0.008$).* In all SS cases, APOBEC3B expression was seen in more than 50% of the ductal cells. According to the final score, based on intensity and extent, 15/20 (75%) of SS cases were found to be APOBEC3B positive. A statistically significant difference was found between the SS Tarpley I and II subgroups regarding the APOBEC3B final classification as positive or

negative *(p=0.008)*. A statistically significant difference in APOBEC3B intensity grade was observed between the SS cases in total and the CS cases *(p≈0)*, as well as between the CS and LLM group *(p=0.008)*. Moreover, the APOBEC3B final score was significantly lower in the SS Tarpley I cases in comparison with the SS Tarpley II cases *(p=0.008)*, the CS group *(p=0.018)* and the total of controls *(p=0.035)*.

No difference in L1 ORF2p and APOBEC3B immunostaining was observed between intralobular and interlobular ducts. In 4/5 (80%) L1 ORF2p and in 5/9 (55.6%) APOBEC3B of cases that were evaluated with CIA, CIA results were in agreement with the visual evaluation by the two researchers. A moderate to strong correlation was observed between the CIA and the visual evaluation results (*r=0.85* for L1 ORF2p, *r=0.64* for APOBEC3B). A double positive L1 ORF2p and APOBEC3B expression was seen in 12/20 (60%) SS, 5/5 (100%) CS and 3/5 (60%) LLM cases *(p>0.05)*. In 3/20 (15%) L1ORF2p negative SS cases a strong APOBEC3B positivity was observed, while 5/20 (25%) APOBEC3B negative SS cases showed a strong L1ORF2p expression. The difference in L1ORF2p and APOBEC3B double positivity was statistically significant between SS Tarpley I and II subgroup *(p=0.03)*, as well as between the SS Tarpley I and the CS cases *(p=0.018)*.

Conclusions: The L1 ORF2p and APOBEC3B co-expression in the ductal epithelium of SS patients possibly indicates the role of APOBEC3 as an inhibitory mechanism, against the inappropriate endogenous expression of L1 elements. The lower levels of APOBEC3B in SS Tarpley I patients in the present study may denote that the impaired epigenetic regulation of L1s by APOBEC3 deaminases may contribute to the activation of type I IFN pathway, as an initiating co-factor in SS pathogenesis. The elevated APOBEC3B positivity that was observed in the SS Tarpley II cases compared to the Tarpley I, may also reflect a compensatory increase in APOBEC3B levels in line with the ongoing inflammation, in order to achieve a more efficient inhibition of the L1-induced autoimmune response in SS. Since APOBEC3B is not the only L1 inhibitory factor, the epigenetic regulation of L1 elements in SS needs further evaluation.

11 ΠΕΡΙΛΗΨΗ

ΑΝΟΣΟΪΣΤΟΧΗΜΙΚΗ ΔΙΕΡΕΥΝΗΣΗ ΤΗΣ ΕΚΦΡΑΣΗΣ ΤΩΝ ΜΟΡΙΩΝ LINE-1(ORF2) ΚΑΙ APOBEC3B ΣΕ ΕΛΑΣΣΟΝΕΣ ΣΙΑΛΟΓΟΝΟΥΣ ΑΔΕΝΕΣ ΚΑΤΩ ΧΕΙΛΟΥΣ ΑΣΘΕΝΩΝ ΜΕ ΣΥΝΔΡΟΜΟ SJÖGREN

Αντικείμενο μελέτης: Το σύνδρομο Sjögren (Sjögren's syndrome, SS) είναι μία χρόνια αυτοάνοση νόσος που προσβάλλει περίπου το 0,5-1% του γενικού πληθυσμού, με σαφή προτίμηση στις γυναίκες μέσης ηλικίας. Η ιστοπαθολογική εικόνα του SS χαρακτηρίζεται από την εστιακή διήθηση των εξωκρινών αδένων από μονοκύτταρα φλεγμονώδη κύτταρα σε εγγύτητα με το αδενικό επιθήλιο («αυτοάνοση επιθηλίτιδα»), η οποία καταλήγει στην καταστροφή του αδενικού παρεγχύματος. Η αιτιοπαθογένεια του SS θεωρείται πολυπαραγοντική, με το προτεινόμενο μοντέλο να βασίζεται στην αλληλεπίδραση μεταξύ ανοσολογικών, γενετικών, επιγενετικών και περιβαλλοντικών παραγόντων. Μεταξύ των διαφόρων παθογενετικών μηχανισμών, καθοριστική θεωρείται η ενεργοποίηση της οδού της ιντερφερόνης (IFN). Ένα πιθανό διεγέρτη της οδού της IFN τύπου Ι πιστεύεται ότι αποτελούν τα ρετρομεταθετά στοιχεία, που συμπεριφέρονται ως ενδογενή ιογενή ερεθίσματα. Στην κατηγορία των ρετρομεταθετών στοιχείων ανήκουν τα μακρά διάσπαρτα πυρηνικά στοιχεία-1 (long interspersed nuclear elements, LINEs-1, L1s). Τα L1s περιέχουν δύο μη-επικαλυπτόμενα ανοικτά πλαίσια ανάγνωσης (open reading frames, ORF1 και ORF2), που κωδικοποιούν τις πρωτεΐνες ORF1p και ORF2p. Η έκφραση των L1 μεταθετών στοιχείων ρυθμίζεται από ποικίλους μεταγραφικούς και μεταμεταγραφικούς μηχανισμούς, συμπεριλαμβανομένων των ενζύμων Apolipoprotein B mRNA-Editing enzyme, catalytic polypeptide-like 3C (APOBEC3). Η APOBEC3 οικογένεια αποτελείται από 7 μέλη: APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F, APOBEC3G και APOBEC3H. Η έκφραση των μορίων APOBEC3B και L1 σε επίπεδο mRNA έχει αναφερθεί στους ελάσσονες σιαλογόνους αδένες (εΣΑ) ασθενών με SS.

Ο σκοπός της παρούσας μελέτης είναι η ανοσοϊστοχημική διερεύνηση της έκφρασης των πρωτεϊνών L1 ORF2p και APOBEC3B σε εΣΑ κάτω χείλους ασθενών με SS.

Υλικό και Μέθοδος: Το υλικό της μελέτης συλλέχθηκε αναδρομικά από το αρχείο του Ιστοπαθολογικού Εργαστηρίου της Στοματολογίας της Οδοντιατρικής Σχολής του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών. Η έκφραση των L1 ORF2p και APOBEC3 μορίων ελέγχθηκε με τη συνήθη ανοσοϊστοχημική τεχνική σε 20

μονιμοποιημένα σε παραφίνη ιστοτεμάχια προερχόμενα από μερική βιοψία εΣΑ κάτω χείλους με διάγνωση εστιακής λεμφοκυτταρικής σιαλαδενίτιδας συμβατής με SS τα οποία ταξινομομήθηκαν σε 4 υποομάδες με βάση την ταξινόμηση των Tarpley και συν., καθώς και σε 5 ιστοτεμάχια προερχόμενα από μερική βιοψία εΣΑ κάτω χείλους με διάγνωση χρόνιας μη ειδικής σιαλαδενίτιδας μη συμβατής με SS (ΧΣ) και σε 5 ιστοτεμάχια εΣΑ κάτω χείλους, προερχόμενα από ολική βιοψία βλεννωδών κύστεων (ΒΛ). Ως θετικός μάρτυρας για την έκφραση και των δύο αντισωμάτων χρησιμοποιήθηκε ένα ιστοτεμάχιο διηθητικού λοβιακού καρκινώματος μαστού. Η θετική έκφραση και για τα δύο αντισώματα ελέχθηκε ως το γινόμενο της τιμής της έκτασης και της τιμής της έντασης της ανοσοϊστοχημικής χρώσης. Οι περιπτώσεις στις οποίες η ένταση μπορούσε να ενταχθεί σε κάποια κατηγορία έντασης οριακά ελέγχθηκαν με τη μέθοδο της αυτοποιημένης ανάλυσης εικόνας μέσω ηλεκτρονικού υπολογιστή (Computerized Image Analysis,CIA). Το Fisher's exact test εφαρμόστηκε για τον στατιστικό έλεγχο της έκφρασης του κάθε αντισώματος μεταξύ των SS υποομάδων καθώς και μεταξύ των SS, ΧΣ και ΒΛ περιπτώσεων, ενώ η δοκιμή συσχέτισης κατά Pearson έλεγξε την συσχέτιση των αποτελεσμάτων για την ένταση της ανοσοϊστοχημικής χρώσης μεταξύ της CIA και των δύο ερευνητών. Ως επίπεδο στατιστικής σημαντικότητας ορίστηκε το $p<0,05$.

Αποτελέσματα: Έντονη κυτταροπλασματική έκφραση και για τα δύο αντισώματα παρατηρήθηκε στο θετικό μάρτυρα καρκίνου μαστού. Σχεδόν το 90% των SS περιπτώσεων και το σύνολο των ΧΣ και ΒΛ εξέφραζε την L1 ORF2 πρωτεΐνη. Η L1 ORF2p εκφραζόταν σε όλες τις περιπτώσεις στο κυτταρόπλασμα των κυττάρων των πόρων, συχνά με ένα στικτό πρότυπο χρώσης. Στις 15/20 (75%) SS περιπτώσεις παρατηρήθηκε ισχυρή ένταση της L1 ORF2p, ενώ στις 14/18 (77.8%) SS περιπτώσεις η L1 ORF2p εκφραζόταν σε περισσότερο από 50% των κυττάρων των πόρων. Με βάση το τελικό σκορ που προέκυψε ως το γινόμενο της τιμής της έντασης και της έκτασης, 17/20 (85%) SS περιπτώσεις ήταν L1 ORF2p θετικές. Η τελική κατηγοριοποίηση σε θετικές και αρνητικές L1 ORF2p περιπτώσεις δε διέφερε σε στατιστικά σημαντικό (ΣΣ) βαθμό μεταξύ του συνόλου των SS περιπτώσεων και των ομάδων της ΧΣ και της ΒΛ. Με βάση, όμως, την ξεχωριστή ανάλυση των 4 SS Tarpley υποομάδων, ΣΣ διαφορά στην έκφραση της L1 ORF2p παρατηρήθηκε μεταξύ της SS Tarpley IV υποομάδας και του συνόλου των μαρτύρων *($p= 0,038$).*

Όλες οι περιπτώσεις SS, ΧΣ και ΒΛ εξέφραζαν την APOBEC3B πρωτεΐνη στο κυτταρόπλασμα των κυττάρων των πόρων, συχνά με ένα στικτό πρότυπο χρώσης. Μέτριας έντασης έκφραση της APOBEC3B παρατηρήθηκε σε 14/20 (70%) SS περιπτώσεις, ενώ η ένταση της APOBEC3B ήταν χαμηλότερη σε ΣΣ βαθμό στην SS Tarpley I υποομάδα συγκριτικά με την Tarpley II *(p=0,008)*. Σε όλες τις SS περιπτώσεις η APOBEC3B εκφραζόταν σε >50% των κυττάρων των πόρων. Με βάση το τελικό σκορ, 15/20 (75%) SS περιπτώσεις ήταν APOBEC3B θετικές. Στην τελική κατηγοριοποίηση σε APOBEC3B θετικές ή αρνητικές περιπτώσεις παρατηρήθηκε ΣΣ διαφορά μεταξύ των υποομάδων SS Tarpley I and II *(p=0,008)*. Επίσης, ΣΣ ήταν η διαφορά στην ένταση της APOBEC3 μεταξύ του συνόλου των SS και των ΧΣ περιπτώσεων *(p≈0)*, καθώς και μεταξύ των ομάδων ΧΣ και ΒΛ (p=0,008). Το τελικό σκορ της APOBEC3B ήταν μικρότερο σε ΣΣ βαθμό στις SS Tarpley I περιπτώσεις σε σχέση με τις Tarpley II περιπτώσεις *(p=0,008)* καθώς και με την ομάδα της ΧΣ *(p=0,018)* και με το σύνολο των μαρτύρων *(p=0,035)*.

Η έκφραση των L1 ORF2p και APOBEC3B δε διέφερε μεταξύ των ενδολόβιων και μεσολοβίδιων πόρων. Σε 4 από τις 5 (80%) L1 ORF2p και 5 από τις 9 (55.6%) APOBEC3B περιπτώσεις στις οποίες η ένταση ελέγχθηκε με τη CIA, τα αποτελέσματα της CIA συμφώνησαν με αυτά των δύο ερευνητών. Μεταξύ των αποτελεσμάτων της CIA και των δύο ερευνητών παρατηρήθηκε μέτρια έως έντονη συσχέτιση, (*r=0.85* για την L1 ORF2p, *r=0.64* για την APOBEC3B). Ταυτόχρονα θετική έκφραση για την L1 ORF2p και την APOBEC3B διαπιστώθηκε σε 12/20 (60%) SS, 5/5 (100%) ΧΣ και 3/5 (60%) ΒΛ περιπτώσεις *(p>0.05)*. Οι 3/20 (15%) L1ORF2p αρνητικές SS περιπτώσεις είχαν έντονη APOBEC3B έκφραση, ενώ οι 5/20 (25%) APOBEC3B αρνητικές SS περιπτώσεις εξέφραζαν την L1ORF2p σε έντονο βαθμό. Η διαφορά στην ταυτόχρονα θετική έκφραση των L1 ORF2p και APOBEC3B ήταν ΣΣ μεταξύ των υποομάδων SS Tarpley I και II *(p=0,03)*, καθώς και μεταξύ των περιπτώσεων SS Tarpley I και ΧΣ *(p=0,018)*.

Συμπεράσματα: Η συν-έκφραση των L1 ORF2p και APOBEC3B πρωτεϊνών στο επιθήλιο των πόρων των ασθενών με SS πιθανά υποδεικνύει τη συμμετοχή των APOBEC3 ενζύμων ως ένα ανασταλτικό μηχανισμό εναντίον της υπέρμετρης έκφρασης των L1 μεταθετών στοιχείων. Η χαμηλότερη έκφραση της APOBEC3B που παρατηρήθηκε στην παρούσα μελέτη στην SS Tarpley I υποομάδα ίσως υποδηλώνει έμμεσα πως ο ανεπαρκής έλεγχος των L1 στοιχείων από τις APOBEC3 απαμινάσες

μπορεί να συμβάλλει στην ενεργοποίηση της οδού της IFN τύπου I, ως ένας συμπαράγοντας στα αρχικά στάδια του SS. Αντίστοιχα, τα αυξημένα επίπεδα του APOBEC3B μορίου στις SS Tarpley II σε σχέση με τις Tarpley I περιπτώσεις πιθανά αναδεικνύει μία αντισταθμιστική αύξηση των APOBEC3B επιπέδων παράλληλα με τη φλεγμονώδη διήθηση των εΣΑ, με στόχο τον πιο αποτελεσματικό έλεγχο της μεσολαβούμενης από τα L1 αυτοάνοσης απόκρισης στο SS. Δεδομένου ότι το APOBEC3B ένζυμο είναι ένας μόνο από τους ανασταλτκούς παράγοντες των L1s, μελλοντικές έρευνες είναι αναγκαίες για τη μελέτη της επιγενετικής ρύθμισης των L1 στοιχείων στο SS.

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13 ABBREVIATIONS

A= adenine

apoB= apoliapolipoprotein B

APOBEC3= apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like3

ACA= Anti-centromere antibodies

ACR=American College of Rheumatology

AECG= American-European Consensus Group

AID= Activation-induced cytidine deaminase

AIDS= Acquired Immunodeficiency Syndrome

AMA=Anti-mitochondrial antibodies

APC= antigen-presenting cell

BAFF= B cell activating factor

bcl= B cell lymphoma

C=carboxyl

CC= coiled coill

cDNA= complementary DNA

CIA= Computerized Image Analysis

CpG= cytosine-guanine

CSR= class switch recombination

CTD= C-terminal domain

DC= dendritic cell

DLBC= diffuse large B-cell

DNMT= DNA methyltransferases

DN=Double negative

DSB= double-stranded DNA break

ds= double-stranded

EBV= Epstein–Barr virus

ECC= embryonal carcinoma cell

EN= endonuclease

ESC= embryonic stem cell

ESSDAI= EULAR Sjögren's Syndrome Disease Activity Index

FDC= follicular dendritic cell

FLS= Focal Lymphocytic Sialadenitis

Foxp3= forkhead box p3

GCT= germ cell tumor

HCV= Hepatitis C virus

HEV= high endothelial venule

HIV= human immunodeficiency virus

HLA= human leukocyte antigen

HRV=Human Retrovirus

HTLV-1= Human T lymphotropic Virus Type 1

H&E=hematoxylin-eosin

IAP=intracisternal A-type retroviral particle

ICAM= intercellular adhesion molecule-

IFN= Interferon

IL= interleukin

iPSC= induced pluripotent stem cell

IRGs/ISGs= IFN-responsive/stimulated genes

ISG= IFN signature

La/SSB= La/SS antigen B

LINE= long interspersed nuclear element

LTR= long terminal repeat

L1= long interspersed nuclear element-1

MALT= Mucosa-associated lymphoid tissue

mDC= Myeloid DC

MHC= major histocompatibility complex

miRNAs= microRNAs

MNC= mononuclear cell

MSGs= minor salivary glands

MEC= mucoepidermoid carcinoma

MSC= mesenchymal stem cell

NAHR= nonallelic homologous recombination

N= amino

NES= nuclear export signal

NHL= non-Hodgkin's lymphoma

NK=Natural Killer

NLS= nuclear localization signal

OH= hydroxyl residue

ORF= open reading frame

OSS= ocular staining score

PAMP= pathogen associated molecular pattern

PBMC= peripheral blood mononuclear cell

pDC= plasmacytoid DC

PIWI= P-element induced wimpy testis

PRR=Pattern recognition receptor

RA= rheumatoid arthritis

RF=Rheumatoid factor

Ro/SSA= Ro/SS antigen A

RNP= ribonucleoprotein particle

RRM= RNA recognition motif

RT-PCR=reverse transcriptase-polymerase chain reaction

RT= reverse transcriptase

SAMHD1= SAM domain and HD domain-containing protein 1

SGECs= salivary glands epithelial cells

SICCA= Sjögren's International Collaborative Clinical Alliance

SINE=short interspersed nuclear element

SIV=simian immunodeficiency virus

SLE= systemic lupus erythematosus

SS=Sjögren's syndrome

ss= single stranded

TE= Transposable elements

T_{FH}= follicular Th cell

TGF= transforming growth factor

Th= T helper

TLR=Toll-like receptor

TNF= tumor necrosis factor

TPRT= target-site primed reverse transcription

Treg= T regulatory cell

TREX1=Three Prime Repair Exonuclease 1

TRIM21= tripartite motif-containing protein 21

TROVE2=TROVE domain family, member 2

TSD= target site duplication

T= Thymine

UTR= untranslated region

U= Uracil

VCAM= vascular cell adhesion molecule

vif= viral infectivity factor