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ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ ΣΠΟΥΔΩΝ

«ΜΟΡΙΑΚΗ & ΕΦΑΡΜΟΣΜΕΝΗ ΦΥΣΙΟΛΟΓΙΑ»

ΚΑΘΗΓΗΤΗΣ: ΜΙΧΑΗΛ ΚΟΥΤΣΙΛΙΕΡΗΣ

ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ

«Ο ΡΟΛΟΣ ΤΗΣ ΔΡΑΣΤΙΚΟΤΗΤΑΣ ΤΗΣ Lp-PLA2 ΦΩΣΦΟΛΙΠΑΣΗΣ ΣΤΗ ΛΕΜΦΩΜΑΓΕΝΕΣΗ ΤΟΥ ΣΥΝΔΡΟΜΟΥ SJOGREN»

ΑΔΡΙΑΝΟΣ ΝΕΖΟΣ

ΕΠΙΒΛΕΠΟΥΣΑ: ΚΛΕΙΩ Π. ΜΑΥΡΑΓΑΝΗ, ΑΝΑΠΛΗΡΩΤΡΙΑ ΚΑΘΗΓΗΤΡΙΑ

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«THE ROLE OF PHOSPHOLIPASE Lp-PLA2 ACTIVITY IN SJOGREN'S SYNDROME RELATED LYMPHOMAGENESIS»

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Πρόλογος

Η παρούσα διπλωματική εργασία πραγματοποιήθηκε στην Ιατρική σχολή του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών, στο εργαστήριο Φυσιολογίας. Θα ήθελα να ευχαριστήσω θερμά τον Καθηγητή κ. Μιχαήλ Κουτσιλιέρη για την δυνατότητα που μου έδωσε να συμμετάσχω στο Μεταπτυχιακό Πρόγραμμα, την Αναπληρώτρια Καθηγήτρια κα. Κλειώ Μαυραγάνη για την ευκαιρία που μου έδωσε να εργαστώ μαζί της στο πλαίσιο της διπλωματικής μου εργασίας, για την πολύτιμη καθοδήγηση της, τα σχόλια και τις επισημάνσεις της. Θα ήθελα να ευχαριστήσω, επίσης, την Αναπληρώτρια Καθηγήτρια κα. Ελένη Κοτσιφάκη για τη συνεργασία, τις πολύτιμες συμβουλές της και την βοήθεια της για τη μύηση μου στον κόσμο των φωσφολιπασών και της Lp-PLA2. Επίσης, ευχαριστώ τα μέλη του εργαστηρίου Φυσιολογίας της Ιατρικής σχολής και ιδιαιτέρως το Χαράλαμπο Σκαρλή, τη Χριστίνα Φλέσσα, τον Ηλία Τσινόκου, τον Κωνσταντίνο Μαρκάκη, τη Μαρίτα Κοροπούλη, τον Παναγιώτη Λεμπέση, τον Ευάγγελο Ζεβόλη και το Θανάση Μουστόγιαννη, καθώς και την παθολογοανατόμο κα Ασημίνα Παπανικολάου, Αιμοπαθολογοανατομικό Τμήμα, Νοσοκομείου «Ευαγγελισμός» για το ομαδικό πνεύμα εργασίας, την αλληλοκατανόηση και τις πολύτιμες συμβουλές τους καθ' όλη τη διάρκεια της εκπαιδευτικής διαδικασίας. Τέλος θα ήθελα να ευχαριστήσω τη σύζυγο μου Νάνσυ για την κατανόηση και τη στήριξη της, καθώς και τα παιδιά μου Κωνσταντίνο, Χριστίνα και Ευάγγελο.

Contents

ABSTRACT

Background: One of the major complications of primary Sjogren's syndrome (SS) is the development of B-cell non-Hodgkin's lymphoma (B-NHL). The contribution of tissue macrophages in the pathogenesis of B-NHL, based on previous histopathological studies, is significant. Extracellular lipoprotein-associated phospholipase A2 (Lp-PLA2) is a product of tissue macrophages which is found in the circulation associated with lipoproteins and has been involved in both cardiovascular and malignant diseases, including B-NHL lymphoma.

Objective: The purpose of the current study was to investigate the role of serum Lp-PLA2 activity as a potential biomarker for the development of B-NHL in the setting of primary SS.

Methods: Lp-PLA2 activity in serum was determined by measuring [^{3}H]PAF degradation products by liquid scintillation. The samples were obtained from 48 primary SS patients, 9 primary SS patients with lymphoma (SS-lymphoma) and 40 healthy controls. Additionally, an independent cohort of 25 primary SS patients, 17 primary SS-lymphoma and 10 healthy controls were tested using a commercially available ELISA kit. Lp-PLA2 mRNA and protein expression in minor salivary gland tissue samples derived from our cohort patients were also evaluated by Real-Time PCR and Western Blot, respectively.

Results: The activity of Lp-PLA2 showed statistically significant increase in patients with primary SS-lymphoma compared to primary SS patients without lymphoma [mean±SD (nmol/min/ml): 62.0±13.4 vs 47.7±14.7, p=0.003 and 19.7±4.7 vs 15.2±3.2, p=0.005, respectively], as well as healthy controls [mean±SD (nmol/min/ml): 62.0±13.4 vs 52.0±16.3, p=0.03 and 19.7±4.7 vs 15.7±3.1, p=0.06, respectively]. No statistically significant difference in Lp-PLA2 activity was observed between primary SS patients without lymphoma development and healthy controls. Lp-PLA2 mRNA and protein expression were found to be increased in SS patients with lymphoma, compared to SS without lymphoma and sicca controls ($p<0.05$).

Conclusions: Evaluation of extracellular Lp-PLA2 activity in primary SS patients may serve as a useful serological biomarker for B-NHL development in the context of primary SS.

ΠΕΡΙΛΗΨΗ

ΕΙΣΑΓΩΓΗ- ΣΚΟΠΟΣ: Μία από τις κυριότερες επιπλοκές του συνδρόμου Sjogren (σS) αποτελεί η ανάπτυξη Β-κυτταρικού non Hodgkin λεμφώματος (B-NHL), στην παθογένεια του οποίου η συμβολή των ιστικών μακροφάγων φαίνεται να είναι σημαντική βάσει προηγούμενων ιστοπαθολογικών μελετών. Ένα προϊόν των ιστικών μακροφάγων με εμπλοκή τόσο σε καρδιαγγειακά όσο και κακοήθη νοσήματα, συμπεριλαμβανομένων του B-NHL λεμφώματος, αποτελεί η εκκριτική φωσφολιπάση Lp-PLA2, η οποία ανευρίσκεται στην κυκλοφορία συνδεδεμένη με λιποπρωτεΐνες. Σκοπός της μελέτης είναι η διερεύνηση του ρόλου της Lp-PLA2 ως πιθανού βιοδείκτη για την ανάπτυξη B-NHL στα πλαίσια του σS.

ΥΛΙΚΟ: Η δραστικότητα της Lp-PLA2 μετρήθηκε αρχικά στον ορό 48 ασθενών με σS, 9 ασθενών με σS-λέμφωμα και 40 υγιών μαρτύρων με την μέθοδο των Blank et al. Στη συνέχεια, σε μια ανεξάρτητη κοορτή 25 ασθενών με σS, 17 ασθενών με σS-λέμφωμα και 10 υγιών μαρτύρων, η δραστικότητα της Lp-PLA2 προσδιορίστηκε με μια εμπορικώς διαθέσιμη ELISA. Διενεργήθηκε στατιστική ανάλυση με τα λογισμικά SPSS και GraphPad. Η mRNA και η πρωτεϊνική έκφραση της Lp-PLA2 προσδιορίστηκε, επίσης, σε επίπεδο ιστού βιοψίας ελασσόνων σιελογόνων αδένων σε διαθέσιμα δείγματα από τους συμμετέχοντες στη μελέτη (μάρτυρες, ασθενείς με σS και σS-λέμφωμα), με τις τεχνικές της ποσοτικής αλυσιδωτής αντίδρασης πολυμεράσης πραγματικού χρόνου και ανοσοαποτύπωσης κατά Western, αντιστοίχως.

ΑΠΟΤΕΛΕΣΜΑΤΑ: Η δραστικότητα της Lp-PLA2 ήταν στατιστικώς σημαντικά αυξημένη στους ασθενείς με σS-λέμφωμα σε σχέση με τους ασθενείς με σS χωρίς λέμφωμα ($p=0,003$ και $p=0,005$, αντιστοίχως), καθώς και με τους υγιείς μάρτυρες ($p=0,03$ και $p=0,06$, αντιστοίχως). Η mRNA και η πρωτεϊνική έκφραση της Lp-PLA2 στη βιοψία ελασσόνων σιελογόνων αδένων ασθενών με σS που εμφάνιζαν λέμφωμα ήταν σημαντικά αυξημένη σε σχέση με τους ασθενείς με σS χωρίς λέμφωμα και τους ξηροστομικούς μάρτυρες ($p<0.05$).

ΣΥΜΠΕΡΑΣΜΑΤΑ: Η εκτίμηση της Lp-PLA2 δραστικότητας σε ασθενείς με σS θα μπορούσε να είναι ένας χρήσιμος ορολογικός βιοδείκτης για την ανάπτυξη B-NHL στο πλαίσιο του σS.

1. INTRODUCTION

1.1 Sjogren's Syndrome

Sjogren's syndrome (SS) is a chronic autoimmune disorder which primarily affects women with a female:male ratio of about 9:1. The age of onset is 50 to 60 years and its prevalence has been estimated to range between 0.5% and 1%. The dominant characteristic of SS is the accumulation of periepithelial lymphocytic cell infiltrates in the exocrine glands, mainly labial salivary and lachrymal glands resulting in xerostomia and xerophtalmia (Figure 1). Approximately half of the SS patients experience systemic disease manifestations with mononuclear infiltrates or immunocomplex depositions in parenchymal organs such as kidney, lung, and liver [1-3]. Although SS is considered benign disease, nearly 5 to 10% of patients develop B-cell non-Hodgkin lymphoma resulting in increased mortality rates.

Figure 1. Sjogren's syndrome main characteristics: xerostomia and xerophtalmia (from internet website: http://www.rheumatologyspecialistcare.com.au/sjogren-syndrome-kogarah-sydney.html)

Current hypotheses suggest that immune response elements along with environmental and genetic factors are major contributors in SS pathogenesis. B-cell hyperactivity is considered a hallmark in disease pathogenesis; it characterized by hyperglobulinaemia and increased serum autoantibody levels including antinuclear antibodies, antibodies against ribonucleoproteinic complexes Ro/SSA and La/SSB, rheumatoid factor (RF) and cryoglobulins [4].

Among the autoimmune diseases, SS shows the highest incidence of lymphomas with an estimated relative risk of 4 to 40 fold higher than that of the general population and SS was found to confer the highest susceptibility for lymphoma risk among all autoimmune disorders, with a standardized incidence ratio of 18.9 [5-7] (Figure 2). This makes SS an

ideal model for the concomitant study of autoimmunity and malignant transformation [8].

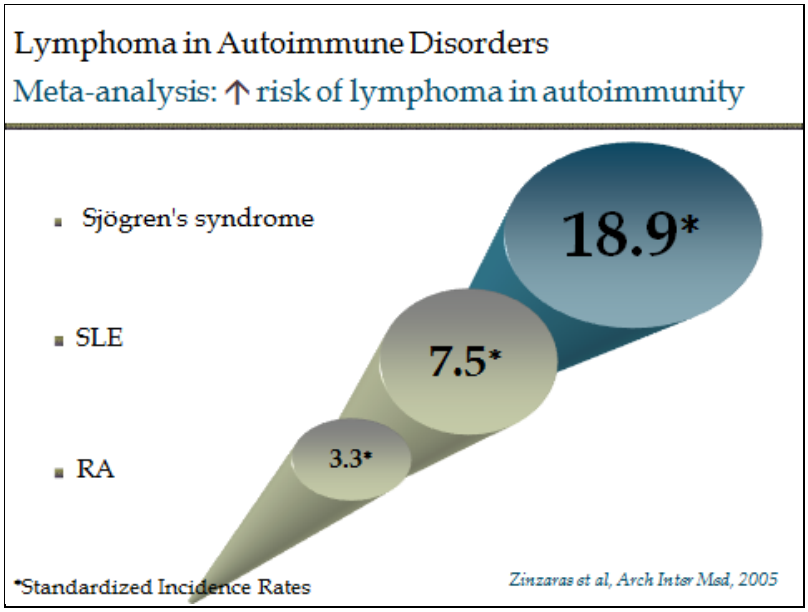

Figure 2. Risk of lymphoma in SS is the highest among systemic autoimmune diseases, in a meta-analysis by Zintzaras et al. [5]

1.1.1 SS pathogenesis

The main features of SS -characterizing an autoimmune disorder- include the presence of lymphocytic infiltrates composed by autoreactive T and B cells with associated tissue injury at the level of exocrine glands and circulating antibodies against several autoantigens such as the ribonucleoproteinic complexes Ro/SSA and La/SSB, the Fc receptor of immunoglobulins (RF) and cryoglobоulins[8].

The presence of lymphocytic cell infiltrates around the ducts and acini of exocrine -mainly salivary and lacrymal- glands is the major histopathological SS feature. Tissue biopsy from the easily accessible minor salivary gland (MSG) obtained from the lower lip is considered the gold standard for SS diagnosis [9]. According to the 2016 ACR-EULAR Classification Criteria for primary Sjögren's Syndrome MSG biopsy is considered compatible with SS diagnosis, when the average focus score is >1 [10]. According to Chisholm the focus score is calculated as the number of lymphocytic foci per 4 mm^2 surface in at least four informative lobules with a focus being defined as a cluster of at least 50 lymphocytes) [8]. These structural and functional changes result in exocrine gland tissue damage, impairing their secretory function considerably. The

presence of lymphocytic infiltrates in salivary gland tissue has long been suspected to be the main cause of secretory dysfunction observed in SS. However, clinical symptoms, such as xerostomia, seldom correlates with SS histological findings and data from animal models suggest that alternative pathways may lead to impaired glandular homeostasis. Such pathways include the induction of apoptosis of epithelial glands, alterations in aquaporin distribution or inhibition of neurotransmission by anti-muscarinic antibodies [11-14]. Other possible mechanisms for the SS-related exocrine gland dysfunction that is independent of immune system activation, has been also suggested, based on human and mouse models (including the overexpression of the bone morphogenetic protein-6 (BMP-6), impaired interaction of E-cadherin and components of the Hippo signalling pathway [15]).

Regarding the eye lacrimal gland, the activation of proinflammatory pathways with local production of cytokines and metalloproteinases results in damage of both corneal and bulbar conjunctival epithelium (keratoconjunctivitis sicca)[16]. Recent data from SS mouse model and SS patients show a downregulation of the PAX-6 gene, which is the master regulator of corneal lineage commitment, in association with ocular tissue damage that is dependent on the level of inflammation of the ocular surface [17].

Parenchymal organs such as the lung, kidney and liver can also be affected in SS patients leading to functional defects similar to MSG tissues. Peribronchial lymphocytic infiltrates have been observed in transbronchial tissue biopsies of SS patients who often experience dry cough and/or shortness of breath and demonstrate a mild small airway obstruction pattern [18]. Kidney tissue samples from SS patients reveal focal lymphocytic infiltrates surrounding the tubular epithelium which extend towards the interstitium. As a result, functional tubular impairment that is manifested mainly by hyposthenuria and renal tubular acidosis may occur [19]. These findings suggest that epithelium has a pivotal role in disease pathophysiology encouraging the term “autoimmune epithelitis” to describe the condition [20]. Figure 3 summarize typical SS patient clinical characteristics.

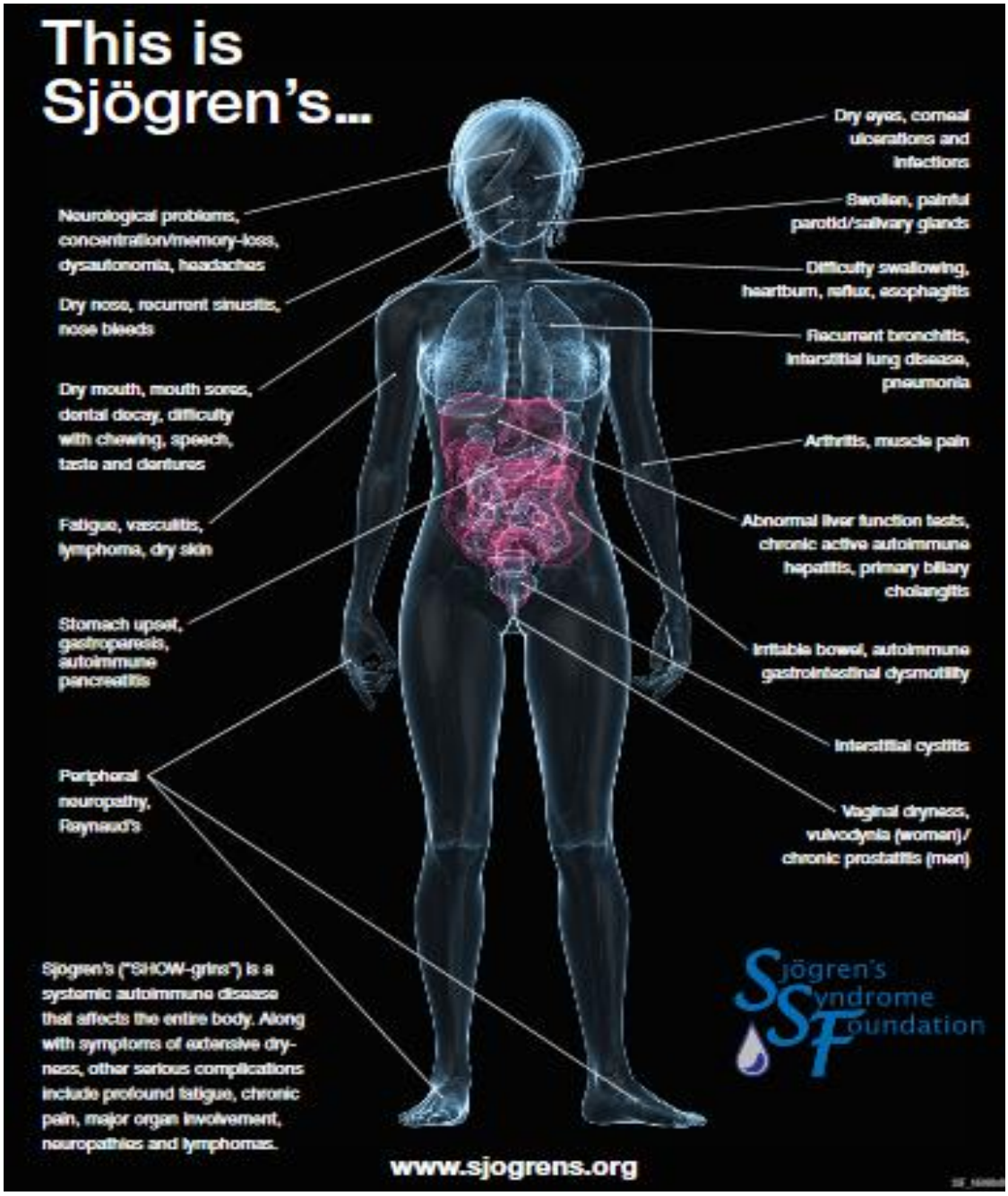

Figure 3. Typical clinical symptoms of SS patients (from the website of Sjogren's Syndrome Foundation, www.sjogrens.org).

MSG tissue biopsies have been proposed as reliable marker for SS diagnosis, due to the accessibility of the tissue and the absence of life threatening side effects. The histopathological picture of the MSG lesions reveals a robust immune response with several cellular populations within an inflammatory environment enriched in cytokines and chemokines [8].

1.1.2 Cellular populations and cytokine production

Data so far from SS patients as well as mouse SS models, show that the extent of cellular infiltrates in MSG tissues is highly variable (Figure 4). Based on the Tarpley biopsy score tissue infiltration can be classified as mild, intermediate and severe [21]. Moreover, the composition of cellular infiltrates in MSG tissues from SS patients is highly dependent on the level of lesion severity [22] .

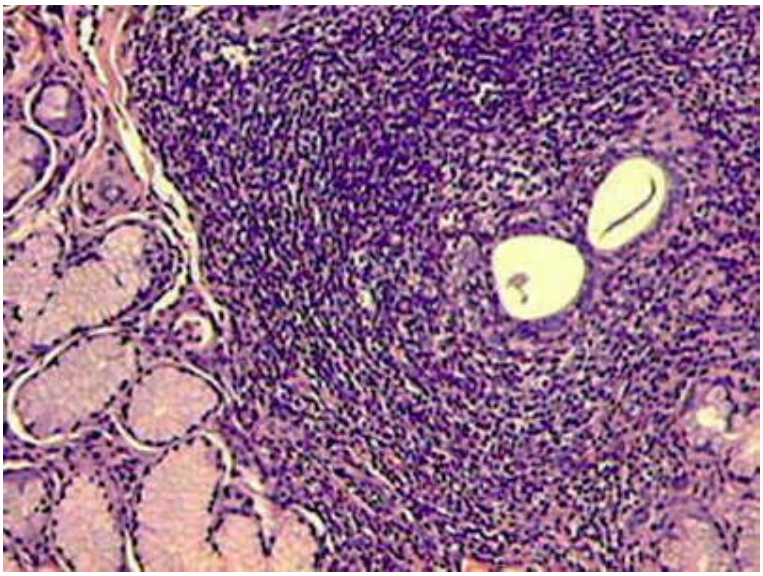

Figure 4. Typical lymphocytic infiltrates in minor salivary gland tissue biopsies of SS patients (Prof. HM Moutsopoulos).

T lymphocytes

T cells are considered as the major contributor of the immunopathological lesion observed in SS. The CD4+ T cells have been proposed as the predominant cell population in MSG infiltrates, however a recent study shows that cell composition of the mononuclear infiltrates in MSG is highly dependent on the severity of the histopathological lesion. Thus, while CD4+ T cells prevail in mild lesions, B cells are the dominant cellular type in advanced lesions (23).

Infiltrating lymphocytes in salivary tissues from SS patients produce (mainly) Th1 and Th2 cytokines. The disruption of the Th1/Th2 balance has long been proposed as a crucial event in the pathogenesis of SS and other autoimmune and inflammatory diseases. Of note, Th1 cytokines such interferon γ (IFNγ) are overexpressed in salivary

gland biopsies from SS patients at the mRNA and protein level and correlate with gland infiltration rates [23-26].

The putative role of the interleukin (IL)-33-ST2 axis in the disruption of Th1/Th2 balance in the pathophysiology of SS has been recently proposed. The levels of IL33 in serum and salivary gland were found to be increased in SS patients compared to controls suggesting that the secreted IL-33 could act synergistically with IL-12 and IL-23 to induce the secretion of IFNγ by natural killer (NK) and NKT cells, thus contributing to Th1 cytokine overproduction [27].

The Th17 cells constitute a subtype of the CD4+ T cells. They are cytokine-producing cells belonging to the interleukin 17 (IL-17) family and have been localized in abundance in the SS salivary gland tissue, especially in advanced lesions [28]. Serum IL-17 in SS was found to be increased and associated with disease duration and parotid gland swelling [29]. Also, Interleukin 22 (IL-22) produced by Th17 cells and a subset of NK cells (expressing the receptor NKp44), contribute to the inflammatory SS milieu [30].

The Th follicular (Tfh) cells constitute another CD4+ T cell subtype investigated for its role in SS. They are the major producers of the inteleukin-21 (IL-21) cytokine which is implicated in germinal center formation through the differentiation of B-cells into plasma-cells, and have been found in sera of SS patients in association with increased expression in the lymphocytic infiltrates of SS salivary gland tissues [31]. Staining techniques of salivary glands tissue biopsies have found that Tfh cells are present mainly in more organized structures with higher focus scores and in typical formation of germinal centers, and these patients will develop systemic features later in the course of the disease [32, 33]. Recent data shows that the CX3CL1 cytokine and its receptor CX3CR1, participates in the ectopic germinal center formation in salivary glands in SS patients [34]. Of note, a possible treatment approach for regulating the Tfh cells in SS patients is the use of the umbilical cord mesenchymal stem cells. One in vitro study reported the inhibition of the differentiation of circulating Tfh cells by umbilical cord mesenchymal stem cells which may help in controlling the germinal center formation and the progression of the disease [35].

Regulatory T lymphocytes (Tregs) -a Th population which suppress excessive activation of effector lymphocytes and maintain T- and B-cell tolerance to self antigens- have been

also studied in the setting of SS. While Tregs frequency was increased in tissues characterized by mild and moderate infiltrations, a diminished incidence has been observed in severe lesions suggesting that in mild and intermediate infiltrations Tregs prevent excessive Th17 expansion as opposed to advanced SS lesions where Tregs fail to control the immune mediated tissue damage [36].

B lymphocytes

B cells were found to be the major cell population of the advanced histopathological SS lesions [22]. They contain immunoglobulins with anti-Ro(SSA) and/or anti-La(SSB) reactivity [37]. Oligoclonal B lymphocytes and germinal center formation in salivary infiltrate, may represent a predisposing factor for lymphoma [38]. Comparative analysis by immunofluorescence between peripheral blood and parotid glands from primary SS patients has demonstrated reduced levels of CD27 expressing memory B cells in the periphery and recruitment of this cellular population in the inflamed salivary gland [39, 40]. In contrast, recent analyses by immunohistochemical analysis of B cell populations in salivary gland tissues show reduced number of memory CD27+ cells both in the periphery and salivary gland tissues. Moreover, CD27+ CD138+ plasma cells have been localized in the periphery of the B cell zone as opposed to the CD27+ CD138- subset, which reside within the B cell zone [41]. $CD21^{-/low}$ is a B cell subset (of memory) B cells that is increased in autoimmune diseases[42]. An unusual $CD21^{-/low}$ B cell population enriched in autoreactive clones has recently been found to be expanded in primary SS patients in comparison to healthy controls, particularly in those developing lymphoma. Given that CD21 potentiates B-cell receptor (BCR)-mediated signaling, these cells were anergic to BCR mediated signals but not to TLR stimulation, implying that exogenous or endogenous ligands might account for the break of peripheral B cell tolerance in this population [43].

Macrophages

Macrophages serve as APCs and thus are an important cellular component in SS histopathological lesions. Severe SS lesions show increased macrophage number characterized by heavy macrophage infiltration in tissue sections [22]. High Interleukin-18 (IL-18) -a cytokine produced mainly by macrophages but also other cell types- serum levels have been reported in patients with SS. Also, IL-18 producing macrophages have been found in association with cryoglobulinemia, low C4 levels and salivary gland

enlargement, which are recognized as adverse prognostic factors for lymphoma development [44]. Intrleukin-12 (IL-12) is a pleiotropic proinflammatory cytokine produced by dendritic cells and macrophages. A transgenic IL-12 mouse model has been recently shown to exhibit SS like features, while increased IL-12 levels have been measured in salivary gland tissues from SS patients indicating a favorable disease outcome with lower risk for lymphoma development [44]. IL-34, a recently identified pro-inflammatory cytokine that was originally ascribed as a cytokine that specifically promotes the growth and differentiation of CD14+ monocytes, has been found to be overexpressed in salivary glands of patients with SS and associated with expansion of a subset of monocytic lineage pro-inflammatory $CD14^{bright}CD16^{+}$ monocytes [45].

Dendritic cells

Dendritic cells (DC) have also been detected in labial salivary gland tissues of SS patients. Myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) are the two main DC types subtypes identified to date, based on their distinct morphological and functional characteristics. In mild SS lesions, peripheral depletion of blood mDCs is a common occurrence, possibly due to their accumulation into the salivary glands [46]. A similar pattern is observed with pDCs, a subset of dendritic cells resembling plasma cells, which are characterized by the presence of TLR-7 and 9 receptors and by their ability to produce large amounts of type I interferons (IFN) upon viral activation. While the number of pDCs in the peripheral blood was reduced in patients with primary SS compared to healthy controls, the opposite has been observed in salivary gland tissue; this may indicate that a local endogenous or exogenous trigger sensed by TLR receptors at the level of target organs attracts this specialized cell population [47-49]. Type I IFN inducible genes have been found to be upregulated in SS salivary gland tissues compared to controls, particularly which IFNα strongly correlates with expression of LINE-1 retroviral elements [50, 51]. In PB the overexpression of IFN-inducible genes resulting from peripheral type I IFN activity in association with disease activity was also detected; this result concurs with previous observations in lupus and other systemic autoimmune diseases. [52-56].

Epithelial cells

As already mentioned, lymphocytic infiltrations in exocrine and parenchymal organs surrounding epithelial structures designate the epithelium as a chief component of

disease pathogenesis. The capacity for intrinsic activation of salivary gland epithelial cells has been consistently supported by the presence of numerous immunoreactive molecules implicated in antigen-presentation (MHC-I and MHC-II), co-stimulation (CD40, B.7/CD80, PD-L1), cell adhesion (ICAM.1/CD54) and apoptosis (Fas, FasL). Activated epithelia have been also shown to produce pro-inflammatory cytokines (IL-1, -6, -8, TNFα and adiponectin), chemokines as well as the B-cell activating factor (BAFF) [57, 58]. The latter is a member of the tumour-necrosis factor (TNF) family essential for the development and survival of B lymphocytes and it has been found to be elevated in SS serum in association with antibodies against Ro/SSA and La/SSB antigens [59]. Recent findings suggest that the SS epithelial cells may serve as antigen-presenting cells, contributes to the creation of the inflammatory microenvironment, recruit B and T lymphocytes through lympho-attractant chemokines (CXCL13, CXCL21) and ensures B-cell lymphocyte survival and proliferation through BAFF production [58].

Negative feedback control mechanisms in inflammatory and immune response gene expression have been evaluated in SS. IκBA is an essential negative regulator of NFκB activation by controlling NFκB transcription. TNF-α induced protein 3 (TNFAIP3) serves as a negative feedback regulator of NFκB signaling in response to pro-inflammatory stimuli in different cell types. According to recent reports dysfunction in IκBA and TNFAIP3 account for the sustained activation of NFκB pathways in SS and that could explain the deregulated cytokines production observed in SS [60-62].

Apoptosis is considered a key event in disease pathogenesis given the presence of numerous pro-apoptotic molecules (Fas, Fas-ligand, Bax) in salivary gland epithelium [63, 64]. Constitutive expression of several anti-apoptotic proteins such as c-FLIP and Bcl-2 seem to act as compensatory tissue-repair mechanisms [63, 65, 66]. Recent data show B-cells induce salivary gland epithelial cell apoptosis through protein kinase C delta (PKCδ) activation [67]. As a consequence of increased apoptosis endogenous nucleic acids and autoantigens may be released triggering an inflammatory immune response at the level of the salivary gland tissue. Additionally, monocytes from SS patients show an impaired phagocytosis of apoptotic epithelial cells, contributing in the accumulation of apoptotic by-products [68]. Also, innate immunity contributes to the apoptosis of cultured salivary gland epithelial cells (SGECs) from SS patients through

the Toll-like receptor 3 which via the PI3K-Akt signalling pathway [69]. An alternative mechanism provided for the inappropriate auto-antigen presentation to the immune system includes the presence of exosomes in the cellular membrane which can be released from cultured salivary gland epithelial cells [70].

1.1.3. SS Etiopathogenesis

Despite efforts over the last several years to explain the etiopathogenesis of Sjogren's syndrome, a complete explanation remains elusive. According to the current thoughts, the interaction between genetic, environmental, hormonal and neuropsychological factors underlies the complex operating mechanisms leading to initiation and perpetuation of the aberrant immune responses SS (figure 5).

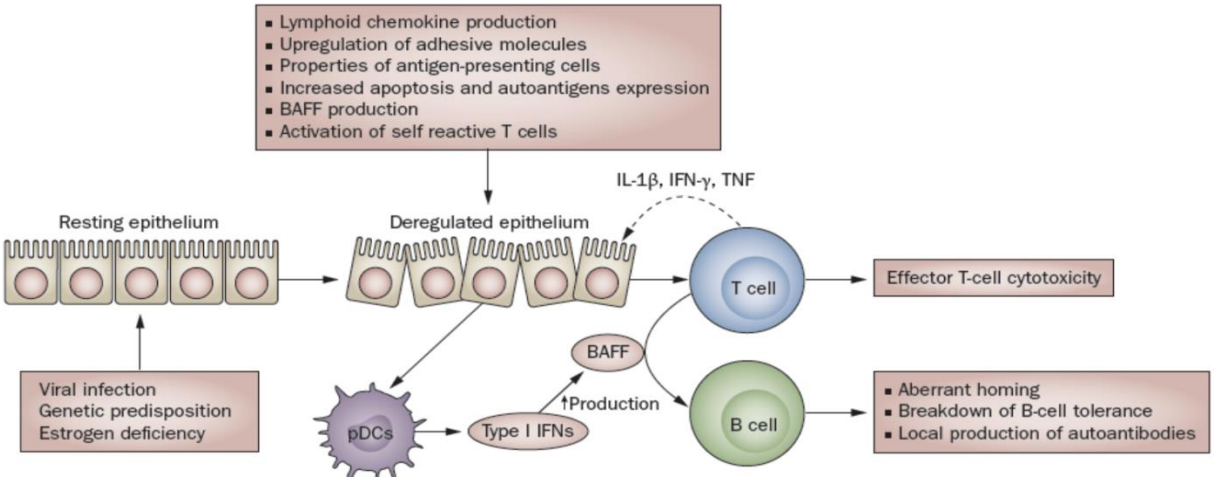

Figure 5. Cellular and molecular pathways implicated in the pathogenesis of Sjögren's syndrome [71].

Genetic Factors

The increased incidence of SS and other autoimmune diseases among monozygotic twins along with the familial aggregation observed strongly suggests genetic factors contribute to significantly to disease pathogenesis. A large number of immunogenetic studies consistently reveal increased prevalence of certain MHC II alleles, often in association with specific autoantibody responses. Also, single nucleotide polymorphisms (SNPs) outside the HLA locus, are implicated in IFN pathways such as IFN regulating factor-5 (IRF5) and signal transducer and activator of transcription 4 (STAT4) [72-79]. Polymorphisms of genes implicated in B-cell differentiation and activation such as

BAFF gene [80] and lymphotoxin α /lymphotoxin B/ TNF locus- have been also found to increase disease risk [81]. Two recent large-scale association studies of SS in European-American and Han Chinese populations respectively, confirmed the strong association of the MHC alleles as well as IRF-5, STAT4, TNFAIP3 and suggest genes of both innate and adaptive immunity are involved in SS [82, 83]. Another study in Scandinavian and UK participants showed that polymorphisms in TNIP1, an inhibitor of the NF-κB pathway, are associated with antibody-positive primary SS [84]. A protective polymorphism for SS development has been reported in the NCR3/NKp30 gene which codes for the natural killer (NK)-specific activating receptor which regulates the cross talk between NK and dendritic cells and type II IFN secretion. The promoter SNP rs11575837 (G>A) is associated with reduced gene transcription and function as well as protection in pSS [85]. Germinal centre-like structures (GC) are found in the minor salivary glands of high risk for lymphoma development SS patients. A recent genetic study supports the hypothesis that patients with or without GC have distinct disease phenotypes. SNPs in the CCL11 (eotaxin) gene were associated with GC-like structures while possible associations for GC-like structures showed other SNPs in B cell activation related genes as well as in the NF-κB pathway genes [86]. A functional deletion of 6.7Kb of the leucocyte immunoglobulin-like receptor A3 (LILRA3) has been recently discovered to occur in patients with SS as well as in other autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, lupus and Non Hodgkin B cell lymphomas [87-90]. In SS patients the presence of this functional deletion correlates with leucopenia as well as positive anti-Ro/SSA and anti-La/SSB antibodies [87].

Epigenetics

Over the last few years a lot of interest has been focussed on intricate involvement of epigenetic modifications and the development of autoimmune disorders. Disturbances of several microRNAs regulating the expression of genes linked to innate immune responses such as mir146a have been reported in SS patients [91, 92] [92, 93]. Deep sequencing of small RNAs from SS patients has revealed previously unidentified microRNAs with significant disease specificity [94].

According to Shi et al, miR-146a is overexpressed while miR-155 is underexpressed in the peripheral mononuclear blood cells of SS patients and the expression levels of both correlated with the patients' clinical features [95].

Imbalances of methylation mechanisms have been previously implicated in the pathogenesis of autoimmunity [96-98]. In SS, a coordinated overexpression of methylating enzymes implicated in de novo and maintenance methylation has been observed at the level of SS salivary gland possibly as a compensatory response aimed at controlling against inappropriate overexpression of endogenous LINE-1 retroelements [50].

Genome-wide methylation analyses in SS patients identified distinct CpGs in naive CD4+ T cells compared to healthy controls. Genes involved in SS pathogenesis were found to be hypomethylated in SS patients included LTA (encoding lymphotoxin α) and interferon signature pathway genes (STAT1, IFI44L). Oppositely, the transcription factor gene RUNX1 was hypermethylated in SS patients suggesting a possible connection to lymphoma predisposition [99].

The FOXP3 promoter, the major regulator of the T regulatory cell (Tregs) development, was found hypermethylated in CD4+ T cells from SS patients compared to healthy controls, while mRNA and protein levels of the FOXP3 gene were decreased in CD4+ T cells in SS patients suggesting the downregulation of the FOXP3 expression influences Treg development in the setting of SS [100].

Global DNA methylation was tested in salivary gland epithelial cells (SGEC), peripheral T cells and B cells from SS patients. In SGEC from SS patients global DNA methylation was reduced with no difference observed between T and B cells. This reduction was associated with a decrease in DNA methyl transferase (DNMT) 1 and an increase in Gadd45-alpha expression. However, this SGEC demethylation may be attributed in part to the infiltrating B cells through an alteration of the PKC delta/ERK/DNMT1 pathway [101].

Environmental Factors

Viruses

Taking into account the data so far regarding the characteristic activation of epithelial cells and the presence of type I IFN signature in the setting of SS, viruses have been long been proposed as putative triggers for disease initiation. Numerous viruses have

been proposed for SS etiopathology; including cytomegalovirus, Epstein-Barr virus, human herpes virus type-6, HTLV-1, retroviruses, HCV virus and enteroviruses with inconclusive results [102-104]. Epstein-Barr virus-encoded small RNA (EBER) derived from EBV infected cells in conjunction with the SS related autoantigen La/SSB leads to type-I IFN production through TLR3 activation [105]. A recent study reported that active EBV infection is selectively associated with ectopic lymphoid structures in the salivary glands of patients with SS and appears to contribute to local growth and differentiation of disease-specific autoreactive B cells [106]. Endogenous retroviral elements (LINE 1) have been also proposed as potential primary triggers in generation of type I IFN responses [49, 51].

Stress

Neurohormonal alterations have been implicated as potential contributors in personality traits and psychopathology features of patients with primary SS. Increased number of stressful life events prior to disease onset in association with defective coping strategies have been proposed as potential SS triggers in a genetically susceptible individual [107].

Hormonal factors

The predominance of female gender in combination with disease onset around menopause age strongly suggests of hormonal contribution in SS. In accordance to human epidemiological data, a healthy mouse model developed SS-like autoimmune exocrinopathy after ovariectomy as a result of increased apoptosis of salivary epithelium; increased expression of the retinoblastoma-associated protein 48 (RbAp48) which is a major determinant of estrogen deficiency-dependent apoptosis in the exocrine glands has been implicated [108, 109]. The enzyme aromatase catalyzes the production of estrogens from androgens. An aromatase deficient mouse model developed severe - SS mimicking autoimmune lymphocytic exocrinopathy [110]. Given the immunomodulatory actions of estrogens in normal salivary gland epithelial cells, the previously reported estrogen unresponsiveness of salivary gland epithelial cells derived from SS salivary gland tissues could be related to the intrinsic epithelial activation observed in SS [111, 112].

SS patients are also characterized by reduced salivary gland tissue levels of androgens and particularly the active form of testosterone, dehydrotestosterone (DHT). This is

either due to low systemic DHEA levels-the main source of DHT in females-or to defective intra-acrine DHT production. DHT has been previously shown to serve as an apoptosis inhibitor of salivary epithelial cells. As a result, androgen deficiency leads to unopposed induction of estrogen deficiency-dependent apoptosis in menopausal SS patients leading to excessive autoimmune reactions [113-115].

1.1.4 The role of Interferons in SS pathogenesis

During last two decades, several reports have revealed the role of type I interferon (IFN) system in the pathogenesis of systemic and organ-specific disorders including SS [47, 55, 116]. Type I IFNs (IFN α/β) are proteins that normally provide protection from viral infections through the activation of hundreds of genes implicated in antiviral response, the so called "IFN signature" [49]. Microarray analysis of minor salivary glands (MSGs), peripheral mononuclear cells, whole blood and peripheral blood (PB) CD14+ monocytes revealed a prominent type I IFN signature in patients with SS, often in association with autoantibodies against Ro/SSA and La/SSB antigens [53, 116, 117]. Additionally, elevated plasma type I IFN activity has been also demonstrated in plasma from SS patients compared to healthy individuals. As previously reported, pDCs are the professional type I IFN producing cells which are reduced in the periphery and preferentially recruited by MSGs derived from SS patients [47, 52, 54, 118]**.**

Although type I IFNs were proposed as predominant contributors in the pathogenesis of SS, recent data suggest a prevailing role of type II IFNs in disease pathogenesis [117, 119, 120]. Involvement of the type II IFN -IFNγ- in SS has been previously well demonstrated in both humans and animal models [47, 119, 121-124]. IFNγ is predominantly produced by T and NK cells and to a lesser extent by dendritic cells, macrophages and B cells [125]**.** Following ligation of the IFNγ receptor, induction of interferon II signature genes occurs, promoting antimicrobial protection (host defense), apoptosis, inflammation and tissue damage [126, 127].

Whether type I or II IFNs predominate in SS pathogenesis remains controversial. Possible reasons study discrepancies could account on the disease heterogeneity extending from mild to severe/life-threatening disease, [128] together with the significant overlap between the genes induced by types I and II IFNs [119] or the type of sample (PB or tissue) used in different studies [25, 47, 53, 117, 119]. In our laboratory we investigated the contribution of both type I and II IFN signatures in the generation of

SS clinical phenotypes, including lymphoma development. We found that type I IFN signature in PB of SS patients predominate over the type II signature in SS MSG biopsies. Moreover we observed a concomitant presence of low IFNα and high IFNγ transcript levels in MSG tissues, which was strongly associated with lymphomagenesis in the setting of SS. Taking together we propose that the IFNγ/α mRNA ratio in MSG diagnostic biopsies could serve as a putative novel tissue biomarker in the diagnosis of salivary extranodal SS-related lymphoma [129].

1.1.5. SS as a model of lymphoproliferation

Lymphoproliferation in autoimmunity has been a subject of investigation for years [130-133]. In patients with SS, this association was first reported by Talal and Bunim in the early sixties [134], confirmed by other studies and a meta-analysis published in 2005 which found that SS has the higher risk to lymphoma development among autoimmune diseases [5, 135]. Lymphoma is the leading cause of mortality in primary SS, with a 2 to 8-fold increased risk compared to the control population [1, 131, 136]. The most common type of NHL in the context of SS is extra nodal marginal zone (MZ) mucosa-associated lymphoid tissue (MALT) lymphoma which affects approximately 65% of the whole SS population with lymphoma [6]. Other marginal and non-marginal zone lymphomas have been also described including splenic and nodal MZ, as well as lympho-plasmacytoid and diffuse large B-cell (DLBC) lymphomas [7, 8] (figure 6). SS-related MALT lymphomas affect mainly parotid and sub-mandibular salivary glands (SGs) which are the major disease target organs, and follow an indolent course in the majority of cases; however naso-pharynx, pulmonary and gastrointestinal involvement needs to be excluded [6]. Though the traditional view considers lymphoma development as the end result of a long-lasting inflammatory process in the SS salivary glands, a growing body of evidence supports an adverse profile present early in disease course. Although the sequential events between B-cell hyperactivity and malignant transformation remain elusive, a significant body of evidence reveals several clinical, serological, histopathological, and immunological parameters associated with adverse outcome and increased mortality rates in approximately 5-10% of SS population [1, 137, 138] (figure 6).

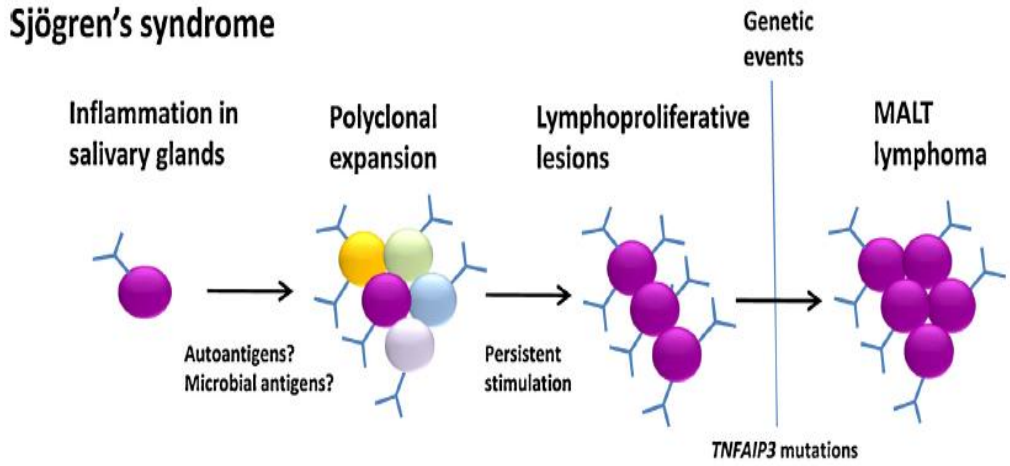

Figure 6. Proposed model for SS related lymphoproliferation [139].

Clinical and laboratory characteristics such as peripheral neuropathy, palpable purpura, low complement C4 levels, and cryoglobulinemia, along with the presence of germinal center formation and current research findings such as increased incidence of IL-18 expressing macrophages and low IL-12 levels in salivary gland biopsies, have been recognized as the key adverse predictors for lymphoma development [140]. In a recent meta-analysis lymphadenopathy and parotid enlargement have been added to adverse predictors for lymphoma development in the setting of SS [141]. Another study evaluating lymphoma-associated biomarkers of well-characterized patients with SS, demonstrated that among SS patients with salivary gland swelling, only those with positive biomarkers, such as low complement C4, cryoglobulinaemia, leukopenia and anti-La antibodies exhibited an increased risk of lymphoma evolution [142]. Furthermore, CD4+ T lymphocytopenia is associated with B-NHL development in patients with SS, and shows significantly positive correlation with other risk factors (parotid swelling, vasculitis, rheumatoid factors, low complement, cryoglobulinemia) [143].

Soluble and genetic mediators in lymphoma development in SS

A growing body of data supports the distinct prognostic nature of SS, already present at time of SS diagnosis [1]. Moreover, it has been also recognized that patients with earlier SS onset display more aggressive clinical phenotypes [144]. Taken together, these data suggest that genetic factors are important contributors in the pathogenesis of SS related lymphoma [145].

B-cell activation and germinal center formation are key pathogenetic events in both SS and NHL and molecules implicated in these processes are candidate biomarkers for SS related lymphoproliferation [146-148] These biomarkers include serum BAFF levels [129, 146, 149, 150], other B cell growth factors including FMS-like tyrosine kinase 3 ligand (Flt-3L) and chemokines involved in organization of ectopic lymphoid follicles such as CXC chemokine ligand 13 (CXCL13) that have been shown to be elevated in serum derived from SS patients complicated by lymphoma [151-153]. Increased frequency of the minor T allele of the rs9514828 *BAFF* variant was detected in the high risk for lymphoma development group, in contrast the minor A allele of the rs12583006 which was more prevalent in the low risk group. Haplotypes in the 5′ regulatory region of *BAFF* gene may also discriminate the high risk from low risk SS patients for lymphoma development. SS patients with lymphoma display lower frequencies of the TACC and TTCT haplotypes compared to low risk SS and HC respectively αlong together with a higher frequency of the TTTC haplotype compared to the low risk SS [154]. Interestingly, an increased prevalence reaching 70% of SS patients carrying the His159Tyr mutation of the *BAFF* receptor gene–was detected, particularly in those patients complicated by MALT lymphoma and whose disease onset occurred at a younger age [146], results supports the activation of the alternate NF-κB pathway, as evidenced by increased NFκB2 expression levels in B cells derived from SS patients bearing the His159Tyr mutation as a contributing factor for MALT lymphoma development [146].

Uncontrolled inflammatory responses have been linked to lymphoma development particularly of MALT type [155-158]. Extensive lymphocytic infiltration [159] along with increased percentage of IL-18 producing macrophages [44] and heightened transcript levels of both IFNγ [129] and inflammasome molecules [160] in minor salivary gland (MSG) tissues have been related to lymphoma development in the context of SS . In line with these findings, serum levels of IL-18 and apoptosis-associated speck-like protein (ASC) were found to be increased in high risk SS patients and SS-lymphoma subsets [160]. In view of the implication of the A20 (A20 is also known as TNFAIP3) protein in controlling the NFκB pathway, the participation of TNFAIP3 in the negative feedback regulation of NFκB signaling has been established. Several studies explored the contribution of a functional Tumor necrosis factor alpha-induced protein 3 (*TNFAIP3*) variant which encodes the A20 gene in both inflammatory and

malignant disorders [161-163]. In patients with SS of French [164, 165], UK [165] and Greek origin [166], the prevalence of the coding rs2230926 *TNFAIP3* variant, has been previously found to be increased in SS-lymphoma [165, 166], increasing the risk by approximately 2.5-fold. In the Greek SS cohort the presence of the variant was associated with higher serum IgM and LDH levels, higher transcripts of the anti-apoptotic Bcl-XL molecule in peripheral blood and lower leucocyte and neutrophil counts [166]. Of interest, approximately one-fifth of SS-lymphoma cases with younger age at disease onset (≤ 40 years) carried the rs2230926 variant, supporting the concept of increased mutational load in SS patients with lymphoma presenting earlier in life. Similarly, the wild type variant of the immunoreceptor *LILRA3* (Leukocyte immunoglobulin-like receptor A3) which has been related/connected in chronic inflammatory disorders [88, 167] was detected in all primary SS patients ≤40 years of age afflicted by lymphoma in comparison to 81.8% in primary SS-non lymphoma patients and 83.2% of HC. As expected, LILRA3 protein serum levels were increased in this SS subset [168]. The major histocompatibility complex P5 (*HCP5*) is implicated in lymphoma development in SS in an Italian cohort, in which the rs3099844 variant of this gene increases lymphoma risk by approximately seven fold [169].

The malignant transformation of cells is often driven by defects in the immunosurveilant mechanism of inflammatory response [170]. In salivary gland tissues derived from SS patients with lymphoma, IFNα transcripts were found to be downregulated, implying a similar mechanism may occur in SS related malignant transformation [129]. In order to explore potential genetic contributors for the dampened type IFNα responses observed in salivary glands from SS-lymphoma patients, we tested SS patients of Greek and Italian origin with or without by lymphoma [171] for genetic variants of the three-prime repair exonuclease 1 (*TREX1*) gene, which increases susceptibility in lupus patients with neuropsychiatric manifestations [172] and is involved in type I IFN pathways [173]. We did not detect any difference in the rs3135941 and rs3135945 variants between the groups, however the frequency of minor genotype of the rs11797A allele was found to be remarkably reduced in SS-lymphoma patients of non-MALT type. Since the presence of the rs11797 AA genotype was found to be related with increased type I IFN inducible genes in MSG tissues, we postulate that genetically diminished type I IFN responses could offer an alternative explanation for SS related lymphomagenesis [171].

Epigenetic alterations have been associated with NHL pathogenesis in general [174], and in SS in particular with defective expression of miR200b-5p [175] and DNA methylating enzymes [50] in MSG tissues derived from SS patients with lymphoma. Variations of the *MTHFR* gene and particularly the rs1801133 (c.677C > T) TT genotype show increased frequencies among primary SS patients complicated by non-MALT lymphoma in association with decreased methylation levels [176]. Oppositely, reduced prevalence of the rs11801131 (c.1298A > C) C allele was observed in the primary SS non-MALT patient group compared to controls and to patients without increasing the possibility of double-strand breaks which serve as markers of DNA damage. These findings suggest that a defective DNA methylation patterns and subsequent silencing of oncogenes together with genomic instability provide additional operating mechanisms in SS related lymphomagenesis [177] (figure 7).

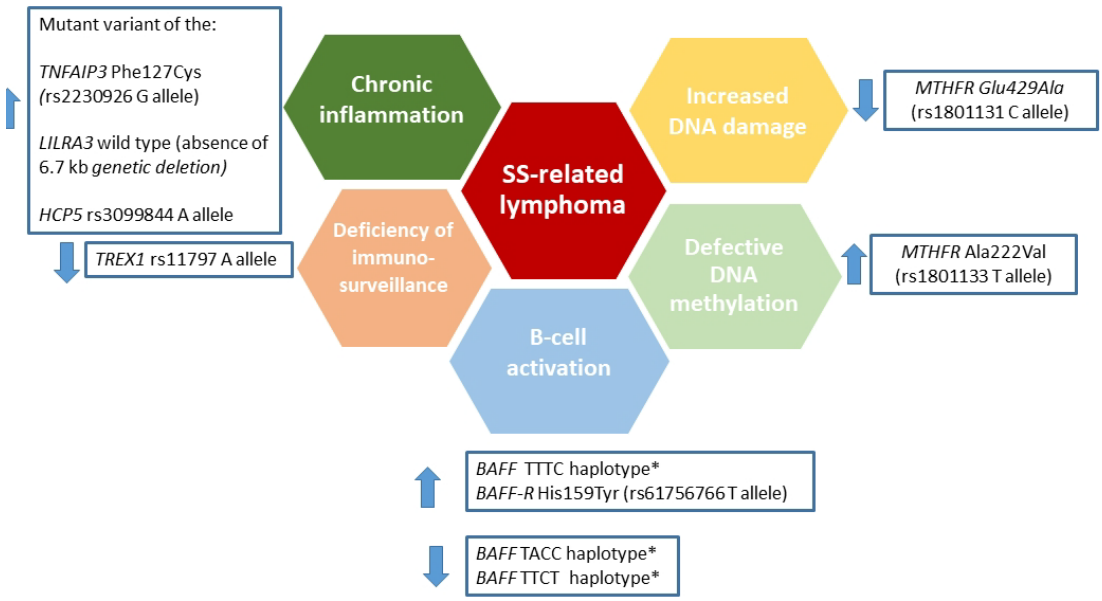

Figure 7. Lymphomagenesis in the setting of Sjogren's syndrome-Genetic contributors and potential pathogenetic mechanisms involved. SS: Sjogren's syndrome; TNFAIP3: tumor necrosis factor-alpha induced protein 3; LILRA3: leukocyte immunoglobulin-like receptor subfamily A member 3; HCP5: major histocompatibility complex P5 gene; TREX-1: three prime repair exonuclease 1; BAFF: B cell activating factor; BAFF-R: B cell activating factor receptor; MTHFR: methylene tetrahydrofolate reductase, *contain alleles for rs1224141, rs12583006, rs9514828 and rs9514827 respectively [145].

1.2 Phospholipase Lp-PLA2

The enzyme Lipoprotein associated phospholipase A 2 (Lp-PLA2), formerly called platelet-activating factor acetylhydrolase (PAF-AH) is an enzyme belonging to the PLA 2 superfamily and functions as a catalyst in PAF hydrolysis. It is present in plasma partly bound to LDL (approximately 70–85%), while the rest is bound to HDL [178, 179]. In humans Lp-PLA2 is coded by the PLA2G7 gene which translates to a protein with a molecular mass of 45-kDa consisting by 441 amino acids (Figure 8).

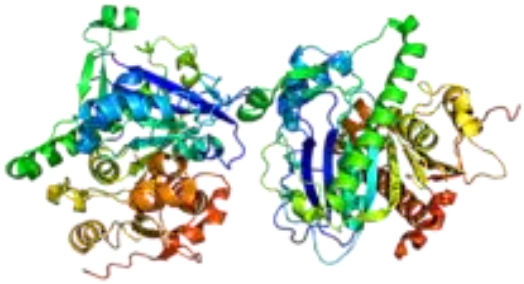

Figure 8. Crystal structure of human plasma platelet activating factor acetylhydrolase (Protein Data Bank, https://www.rcsb.org/structure/3D59)

Its distribution varies, depending on the extent of its glycosylation [180]. Lp-PLA2 preferably hydrolyses phospholipids with short acyl chains of up to 6 carbon atoms in length at the sn-2 position [179] (Figure 9).

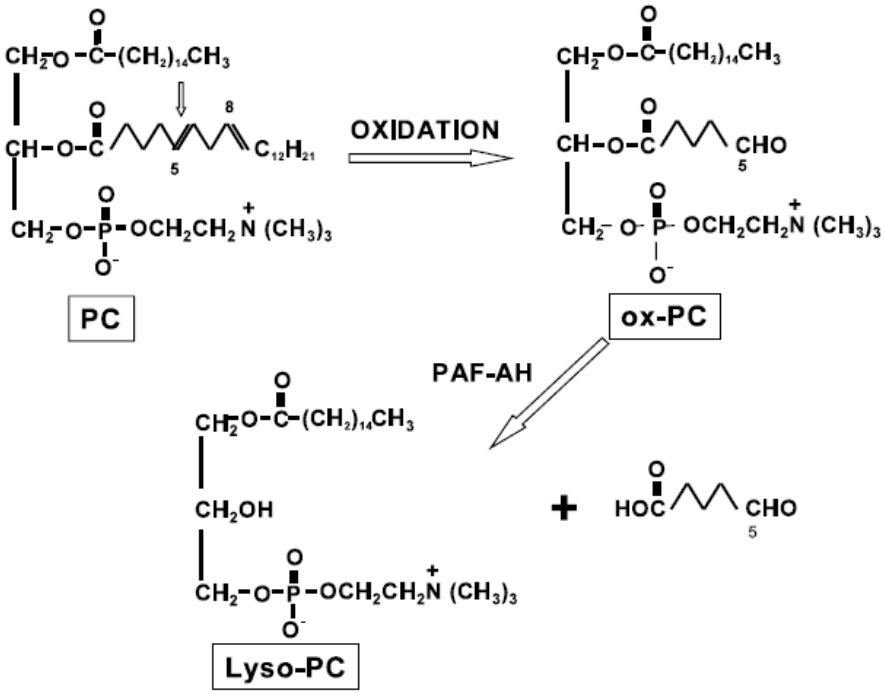

Figure 9. Hydrolysis of oxidized phospholipids by Lp-PLA2 [179].

Lp-PLA2 is present in atherosclerotic lesions where it is produced by macrophages [181, 182]. Oxidation of LDL is considered a prerequisite event in the atherogenic process. It is mediated by endothelial cells, smooth muscle cells, and macrophages in the arterial intima and oxidized LDL (oxLDL) have been localized in abundance in atherosclerotic lesions [182]. Oxidative modification of LDL includes: peroxidation of the lipids, conversion of oxPC to lysophosphatidylcholine (lysoPC) (up to 50%), an increase in density, an increase in net negative charge resulting in increased electrophoretic mobility, breakdown of apolipoprotein B (ApoB) into smaller peptides, and binding of oxidized lipids on ApoB [183-185]. Oxidation of LDL has various atherogenic effects such as its increased accumulation in macrophages and smooth muscle cells leading to foam cell formation, increased production of adhesion molecules, induction of monocytes/macrophages and smooth muscle cell proliferation [182, 186]. During LDL oxidation a significant amount of oxPC is degraded to lysoPC. [187]. The fragmentation of polyunsaturated sn-2 fatty acyl groups in LDL phosphatidylcholine produces molecules with short acyl chains at the sn-2 position, such as POVPC, that can serve as substrates to Lp-PLA2 which is gradually inactivated during the oxidation process [179]. In recent years, several epidemiological studies indicate that the level of circulating Lp-PLA2 is an

independent predictor of coronary artery disease [188, 189]. It has not yet elucidated whether Lp-PLA2 is just an indicator of vascular inflammation characterized by macrophage accumulation and increased Lp-PLA2 production, or it is a causative factor of vascular inflammation and plaque formation [179, 190].

Recent findings show that secreted (extracellular) and intracellular phospholipases A2 (PLA2) have diverse functions in tumorigenesis and imply that Lp-PLA2 is implicated in the process [191]. Lp-PLA2 expression is aberrantly regulated in a variety of cancers [192] (Figure 10), and this effect appears to be most prominent among members of the PLA2 superfamily.

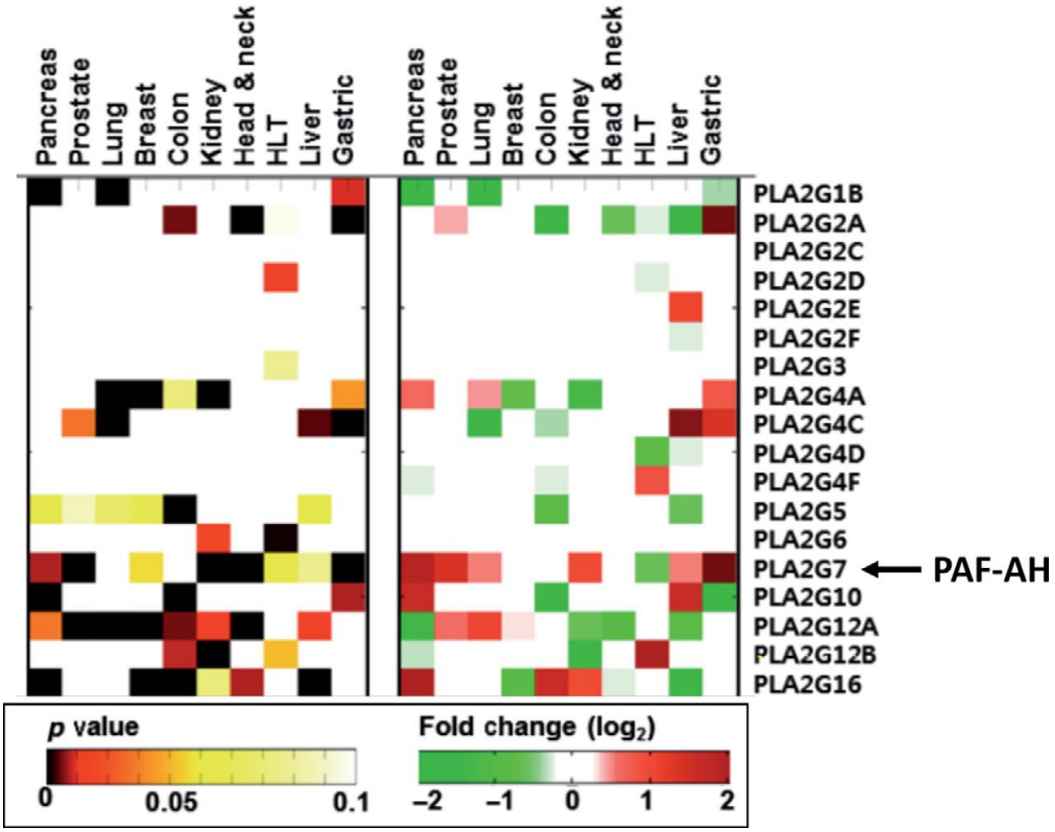

Figure 10. Expression profiles of phospholipases A2 in cancer. Comparison of gene expression profiles of the genes encoding 18 human PLA2 between normal and cancer samples [192].

The mechanisms whereby Lp-PLA2 is thought to regulate signaling events have been traditionally linked with either decreases in biologically active phospholipids such as PAF and oxidatively fragmented phospholipids (OxPL), or increases in oxidized and/or short/medium chain fatty acids and lyso-phosphatidylcholine (lyso-PC). It is not always

emphasized, however, that these products can potentially be further metabolized in a tissue-specific manner and generate “secondary” metabolites that can have signaling roles, act as biosynthetic precursors, and/or have direct functions in metabolism, as proposed for other PLA2 enzymes [193]. Lp-PLA2 appears to have diverse roles and has diametrically opposite functions that either enhance or suppress tumorigenesis. The diversity of phospholipid substrates hydrolized by Lp-PLA2 combined with the biological activities of the primary and secondary lipid products it provides some rationale for its involvement in cancer.

A number of secreted PLA2 proteins elicit signaling events in catalytic activity-independent fashions and by binding to specific receptors [193]. According to Stafforini et al, this mechanism has not been reported to be involved in Lp-PLA2-mediated signaling, this possibility need to be investigated to further elucidate the role of Lp-PLA2 in tumorigenesis and to develop approaches to regulate the function and expression of this enzyme for the benefit of patients suffering from malignant diseases[191].

Lp-PLA2 can potentially have both pro- and antitumorigenic effects that may be related, in part due to the fact that its substrates, PAF and OxPL, also can have diverse functions in tumors and tumor microenvironments. For example, PAF, one of the key substrates for the enzyme, is a unique growth regulator with diverse functions [194]. Earlier studies established that PAF can have dual effects depending on its point of action in the cell cycle [194]. These include stimulation of the mitogenic response in G0-arrested cells and inhibition of the G1 to S transition. In certain conditions, challenge with PAF and/or OxPL substrates in the absence of further metabolism can lead to apoptosis [195] and cell cycle arrest [196].

In contrast, a number of studies reported increased cellular proliferation [197] and suppression of DNA repair [198] following treatment with PAF. In addition, diverse responses also have been reported when Lp-PLA2 substrates elicit downstream signals through participation of the receptor for PAF (PAFR) [199]. A second issue to consider is that the products of the reaction, lyso-PC and oxidized fatty acids, can have important biological functions. Moreover, further metabolism of Lp-PLA2 products can give rise to pro-tumorigenic signaling molecules. For example, metabolism of lyso-PAF/lyso-PC by lyso-PLD/autotaxin may contribute to oncogenesis via lyso-PA, a well-recognized mediator of tumorigenic signaling. Lyso-PA-mediated engagement of LPA receptors

whose expression is frequently deregulated in cancer cells and tissues [200] activates members of the RHO family of small GTPases and drives cell migration and invasion [201]. In models of ovarian cancer, lyso-PA recently was shown to induce loss of junctional β-catenin, stimulate clustering of β1 integrins, and enhance the conformationally active population of surface β1 integrins [202]. Further, lyso-PA treatment initiated nuclear translocation of β-catenin and transcriptional activation of Wnt/β- catenin target genes resulting in gain of mesenchymal marker expression.

Serum lyso-PA is an established indicator of tumor initiation and progression in breast cancer [203], ovarian cancer [204], and multiple myeloma [205]. Thus, Lp-PLA2 has potential effects on tumorigenesis through its ability to alter the levels of several key lipid signaling molecules. To understand how Lp-PLA2 impacts various types of cancer, often in opposite manners, requires consideration of the state of activation of closely related metabolic pathways, as they may play defining roles on the net effect of Lp-PLA2 in individual malignancies [191].

1.2.1 Cell Proliferation and Apoptosis

Cell culture studies have reported both pro- and antitumorigenic roles for PAF, based on its ability or its metabolically inert analog carbamoyl-PAF (cPAF) to affect cellular growth. In transformed and non-transformed mast cells, cPAF was shown to induce a significant decline in cell proliferation in a dose- and time-dependent manner, by reducing DNA synthesis and arresting cells at G2-M and G0-G1, mimicking chemotherapy treatment [196]. Moreover, Lp-PLA2 expressed by mast cells appeared to decrease these effects as the use of PAF instead of metabolically inactive cPAF resulted in much less pronounced effects. Similarly, PAF and cPAF decreased the growth of a variety of colon cancer cells [206] and this effect was partially mediated by interaction with its receptor PAFR. Data from cell cultures of intestinal cells [207], colon cancer cells [206], keratinocytes [196] and mast cells [196] following exposure to PAF show that the cell fate is apoptosis, through activation of caspase 3 and increased PARP-1 cleavage. Similarly, Chen and coworkers reported that oxidatively truncated phospholipids specifically hydrolyzed by Lp-PLA2 induce apoptosis in PAFR-independent manner, and that overexpression of Lp-PLA2 suppressed the ability of these

lipids to induce apoptosis [208]. These pro-tumorigenic effects of Lp-PLA2 could occur only under certain conditions.

In contrast to these findings, several studies have presented evidence supporting the role of the PAF-induced stimulation of cellular growth [197, 209], and that PAF rapidly regulates genes involved in proliferation, (anti)-apoptosis, and migration, suggesting that PAF acts as an activator of proliferation [210]. Moreover, studies in breast and ovarian cancer cells show that PAF stimulates cellular growth and that these effects can be inhibited by pharmacologic blockade of PAFR [197, 209]. Though the results are controversial they could be explained by the differential experimental conditions used in these studies (e.g., exposure time, concentration and quality of exogenous PAF used, metabolism by endogenous Lp-PLA2) and further studies are needed for clarification of the role of PAF and Lp-PLA2 in tumorigenesis. It has been proposed that PAF could play dual functions: one as a promoter of apoptosis where it is required to maintain homeostasis, and the other as a contributor to tumorigenesis where it acts as a classic mediator of inflammation and inhibitor of DNA repair [196]. The balance between these opposing forces could reveal the net effect of PAF and Lp-PLA2 on tumorigenesis.

Tumor invasion and metastasis are hallmarks of malignant cancer cells that involve coordinated communication among stromal cells in the tumor microenvironment and individual tumor cells [192]. Metastasis to distant sites- rather than the primary tumor- is the main cause of deaths [211]. The key event in metastatic disease is the autonomous ability of tumor cells to mobilize, migrate, and, through participation of lymph and blood vessels, invade and proliferate in distant tissues and organs [212, 213]. Phospholipases and especially Lp-PLA2 has been found to contribute to metastatic events by affecting intra- and intercellular signaling events that affect motility, adhesion, and invasion. However, in vitro and in vivo studies have been found that increassed Lp-PLA2 expression reduce both spontaneous and induced motility and adhesion through a mechanism that involves αvβ3 integrin upregulation [214, 215]. Another important aspect is the ability of tumor to invade distant organs and tissues through proteolytic digestion of the extracellular matrix (ECM) and basement membrane by matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent endopeptidases that degrade ECM components, for example MMP-2 and MMP-9 degrade type IV collagen -a major constituent of the basement membrane- creating a microenvironment that promote tumor cell migration [216]. Interestingly, Lp-PLA2 has been shown to inhibit both MMP-2 and MMP-9 expression in the mouse model brain [217] and, on the other

hand, PAF has been reported to induce expression of these enzymes in vivo [218, 219]. Studies in experimental pulmonary metastasis of B16F10 murine melanomas support these findings, by a PAF mediating expression of MMP-9 in the lung [220, 221].

An additional characteristic of metastatic tumor cells is the ability to adhere to endothelial cells before extravasation into distant tissues. PAF has been shown to enhance the adhesiveness of melanoma and colon carcinoma cells to endothelia [222]. Moreover, nonspecific inhibition of Lp-PLA2 by cigarette smoke increased the adherence of breast cancer cells to the lung endothelium [211], while exogenous administration of recombinant Lp-PLA2 decreased endothelial invasiveness in mouse models [223]. Taking all data together, Lp-PLA2 seems to control multiple key steps involved in the metastatic potential of tumor cells to distant tissues and organs. Additional work in experimental models of metastatic disease is necessary in order to clarify the exact role of Lp-PLA2.

1.2.2 Angiogenesis

Neovascularization is a key feature of malignant tumors and involves communication between cancer cells and surrounding endothelial cells. The major inducer of tumor angiogenesis is vascular endothelial growth factor (VEGF) which binds to the VEGF receptor (VEGFR) tyrosine kinases VEGFR1 and VEGFR2 to mediate its signal [192]. Current data support that PAF induce VEGF production [224] and blocking PAFR decreases VEGF expression in models of tumorigenesis [225, 226]. Moreover, based on the ability of Lp-PLA2 to decrease PAF levels, the forced expression of Lp-PLA2 reverted the proangiogenic phenotype of tumor cells and tumor-derived endothelial cells [214], as well as expression of Lp-PLA2 in cancer cells significantly reduced vascularization and growth in vivo, increasing the survival of animals with Lp-PLA2-expressing tumors [215]. However, administration of recombinant Lp-PLA2 was shown to increase VEGF mRNA and protein levels in a model of transient cerebral ischemia [217]. A possible explanation for the conflicting results may arise from other studies showing that signaling events elicited by PAF via PAFR can lead to diverse, often opposite, effects [194]. Another mechanism that Lp-PLA2 can potentially increase VEGF levels is through the products it generates. Lyso-PC and lyso-PA stimulate secretion of angiogenic factors including VEGF [227-229] and FGF2 [229] by

endothelial, stromal, and cancer cells. Thus, Lp-PLA2 appears to have both pro- and antiangiogenic effects that are likely to affect tumorigenesis and other diseases in opposite manners, probably due to differences in signaling events elicited by substrates, primary products, and metabolites of these molecules.

1.2.3 Enzymatic Activity-Independent Effects

According to Stafforini et , a possibility that has not been explored in depth but should be considered in the future is that Lp-PLA2 may have catalytic activity-independent functions in tumorigenesis. Bioinformatic studies aimed at elucidating novel protein–protein interactions have revealed a number of potential partners for Lp-PLA2 that can be assessed through a variety of online databases. Further studies are needed to clarify whether Lp-PLA2 plays a role in tumorigenesis by regulating the expression and/or function of partner proteins and receptors, in addition to its catalytic activity-dependent functions [191].

1.2.4 Lp-PLA2 in Colon, prostate, breast cancer and melanoma

The role of Lp-PLA2 has been investigated in colon cancer, both in in vitro and in vivo studies using transgenic mice lacking the Lp-PLA2 expression [230]. Absence of Lp-PLA2 robustly decreased intestinal polyposis and colon tumor formation in Apc(Min/+) mice [206]. The studies suggested that the observed effects were due to an anti-tumorigenic role of PAF characterized by aberrant function of the tumor suppressor gene adenomatous polyposis coli (Apc). In colonic epithelial cells, exposure to a PAF analog led to dephosphorylation of Akt at serine-473 and induction of the intrinsic pathway of apoptosis [206]. These data are consistent with increased apoptosis due to substrate accumulation in the absence of Lp-PLA2. However, these effects could be related to decreased generation of lipid metabolites such as lyso-PA, or other catalytic activity-independent effects. Further studies are needed to determine whether cell-specific deletion-related effects of Lp-PLA2 are due to changes in tumor cells, in the tumor-associated microenvironment, or both. Of note, a publication on the genetics of oncogene cooperation in colon cancer reported that altered expression of several genes was important for malignancy to proceed [231] The gene encoding Lp-PLA2 (Lp-PLA2,

also known as PLA2G7) was identified as a “cooperation response gene” whose expression was synergistically increased 10.7-fold by loss-of-function of p53 and Ras activation in mouse and human colon cancer cells. Importantly, silencing the expression of Lp-PLA2 led to a 42% decrease in the size of tumors developing in vivo. Current data support that Lp-PLA2 is an important contributor to colon tumorigenesis and targeting expression and/or enzymatic activity of Lp-PLA2 should be considered as therapeutic potential.

Interesting data have been found regarding the role of Lp-PLA2 in prostate cancer. Nearly half of human prostate cancers have a gene rearrangement formed by fusion of 50 regulatory elements of an androgen-regulated gene to the coding region of a member of the E twenty-six (ETS) gene family of transcription factors [232, 233]. The most common ETS rearrangement is the fusion between the androgen-regulated transmembrane protease serine 2 (TMPRSS2) gene and the ETS-related gene ERG, an oncogenic transcription factor. ERG rearrangement is an early event in prostate cancer, however, the impact of ETS rearrangements in the initiation and progression of prostate cancer is unclear [234]. Some studies suggest an association with more aggressive tumors and poor prognoses [233], while others report no such association and still others report favorable outcomes [234]. Gene expression analyses consistently shows that Lp-PLA2 expression strongly correlates with that of ERG [235, 236] (Figure 11), due to the ability of ERG to transcriptionally regulate Lp-PLA2 [237], in accordance with silencing ERG expression study that shows decreased Lp-PLA2 mRNA levels [238]. Interestingly, a meta-analysis including studies that compare gene expression profiles in benign versus prostate cancer tissues and ERG rearrangement -positive versus -negative cases also identified Lp-PLA2 as one of the top-ranking ERG+ regulated genes [234].

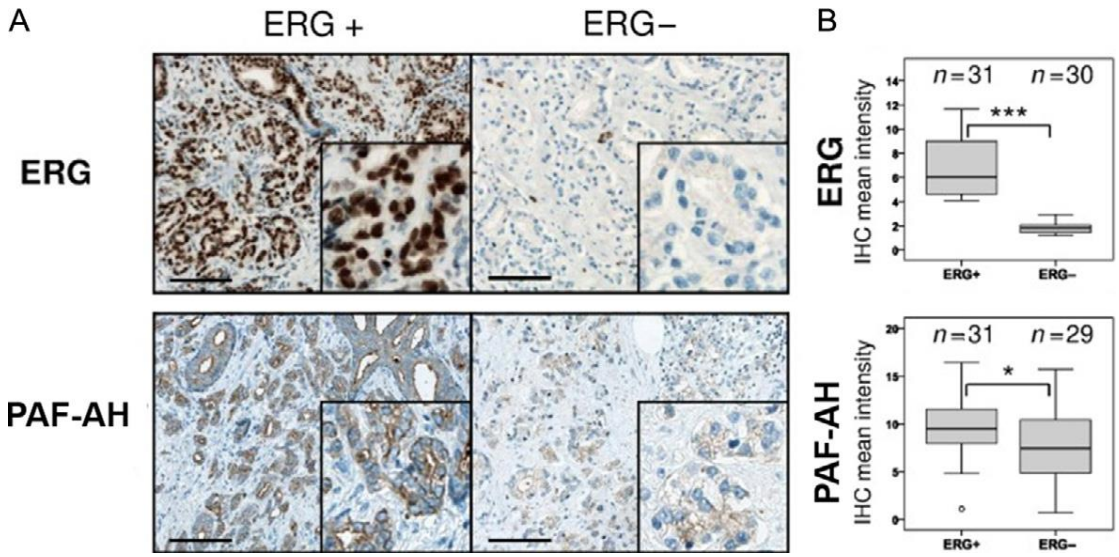

Figure 11. Lp-PLA2 is upregulated in ERG positive prostate cancer tumor tissues by immunohistochemistry [234].

Another study investigated whether Lp-PLA2 overexpression contributes to prostate tumorigenesis [238] and concluded that inhibiting Lp-PLA2 activity or expression may provide an anti-proliferative, pro-apoptotic, and anti-migratorial therapeutic approach to treat prostate tumors.

Lp-PLA2 could be a key molecule in the pathogenesis of prostate cancer through other mechanisms, including its relationship with the previously overrepresented in prostate cancer tissues choline-containing compounds (ChoCC, such as choline, phosphocholine, and glycerophosphocholine) [239]. The genes encoding Lp-PLA2 and choline kinase alpha were identified as the two genes whose expression most closely accompanied the increase in ChoCC observed in prostate cancer.

In melanoma patients the level of Lp-PLA2 mRNA in blood cells has been proposed as a potential biomarker to predict clinical outcome and survival when combined with expression of the genes cathepsin D (acts as a protease that may facilitate cell growth at distant sites), TXNRD1 (plays a key role in redox homoeostasis), and IRAK3 (functions as a negative regulator of Toll-like receptor signaling, expressed in monocytes and macrophages) [240]. In this study, including melanoma patients treated with the immunotherapeutic drug tremelimumab, an IgG2 antibody that targets CTLA-4, increased Lp-PLA2 expression predicted increased survival, suggesting that decreasing the levels of phospholipid substrates may limit key responses that contribute to melanoma. Lp-PLA2 expression combined with that of cathepsin D, TXNRD1, and

IRAK3 seems to predict survival of melanoma patients independently of tremelimumab therapy.

From the biomarker perspective, genetic variation of the Lp-PLA2 gene, serological expression, or mNRA expression may provide information when assessed not independently, but in combination with other genes. Lp-PLA2 levels in tissue or blood may provide valuable information about progression free survival and/or overall survival in malignancies. Lp-PLA2 is included is a group of genes associated with progression-free survival of glioblastoma patients [241]. In breast cancer patients, increased levels of Lp-PLA2 were associated with a decrease in HR (0.11) and Lp-PLA2 transcripts were identified as members of a group of 65 genes expressed in the microenvironment surrounding breast tumors, with increased expression being associated with mammary cancer progression [242]. Lp-PLA2 was also proposed as a potential serological marker of hepatitis C virus-associated hepatocellular carcinoma (HCC) [243]. Finally, Lp-PLA2 transcript levels may serve as a marker for non-Hodgkin's lymphoma [244], especially in DLBCL where it was found to be highly overexpressed compared with normal peripheral blood B cells or lymph node and multiple myeloma [245].

The role of Lp-PLA2 in tumorigenesis remains to be elucidated. Data so far highlight the role for this enzyme as a modulator of certain types of cancer and not as a driver. In a group of malignancies, such as those that develop in the prostate and large intestine, Lp-PLA2 appears to actively contribute to the disease process. In these cases, inhibiting the activity may provide a novel approach to complement existing therapies that target other pathways or cellular processes [191].

The usefulness of inhibiting Lp-PLA2 for cancer therapeutic purposes in general has a number of limitations such as putative problems with cardiovascular system. On the other hand, data so far suggest that Lp-PLA2 may have beneficial effects in melanoma, multiple myeloma, and glioblastoma. Further studies will clarify the exact protective of deleterious in each malignancy and each tumor cell-type.

1.2.6 Lp-PLA2 in inflammatory and autoimmune diseases

Numerous studies have measured plasma Lp-PLA2 activity in a variety of patient populations. Modest increases in the activity of the enzyme have been observed in a wide range of pathologies, particularly those having an inflammatory component [246,

247], including pregnancy-induced and essential hypertension, vascular disease, ischemic stroke, diabetes mellitus, glomerulonephritis, rheumatoid and non-rheumatoid arthritis, and chronic cholestasis [248]. In most cases, the activity is increased during the acute phase, which is consistent with expression being controlled by inflammatory stimuli.

Lower than normal Lp-PLA2 activity has been associated with certain disease. They include asthma [249, 250], systemic lupus erythematosus [251], juvenile rheumatoid arthritis [252], multiple organ failure [253], acute myocardial infarction [254], sepsis [255], and Crohn's disease [256]. However, it's not clear whether the decreased activity observed in these disorders is a consequence of the pathology or it might have been a pre-existing condition that contributed to the disease. There is evidence supporting the former, as found in Crohn's patients with high disease activity, who had significantly lower plasma Lp-PLA2 than healthy controls [256]. Four months following surgery, when the patients were in remission, Lp-PLA2 plasma levels were normalized. In situ inactivation of Lp-PLA2 can occur through oxidation [257, 258]. Thus, any disorder having an oxidative or a free radical-generating component is likely to exhibit decreased acetylhydrolase activity (i.e., acquired deficiency). Since plasma LDL level is a strong determinant of circulating Lp-PLA2, in patients with systemic lupus erythematosus, for example, the decreased Lp-PLA2 activity correlated with reduced LDL levels [251]. These cases demonstrate that Lp-PLA2 activity can be either up- or down-regulated in various disease states.

2. AIM OF OUR STUDY

Currently there is a need for novel biomarkers of the early diagnosis of SS lymphoproliferative disease. Thus, the purpose of the current study was to investigate the role of serum Lp-PLA2 activity, as well as mRNA and protein expression as a potential biomarker for the development of B-NHL in the setting of primary SS.

3. PATIENTS AND METHODS

2.1. Patients

In the present case-control study serum samples were obtained from two individual SS cohorts: (i) 48 SS patients, 9 SS patients complicated by lymphoma (SS-L) and 40 apparently healthy individuals served as a healthy control (HC) group and (ii) 25 SS patients, 17 SS-L patients και 10 HC. Blood samples were allowed to clot for 30 minutes at 25^{o}C. Blood was centrifuged at 2000 x g for 15 minutes at 4^{o}C. The serum was transferred to new 1.7ml tubes and stored at -80^{o}C. Labial minor salivary gland (MSG) biopsy tissues were also taken as a routine part of the diagnostic evaluation of SS from 16 SS patients, 12 SS-L patients and 6 patients complained for sicca symptoms without diagnosis for SS served as sicca controls for RNA and/or protein extraction. Twelve formalin-fixed paraffin-embedded MSG tissue sections (5 μm) derived from 4 SS patients, 4 SS-L patients and 4 patients complained for sicca symptoms without diagnosis for SS served as sicca controls were also included. The mean age ± standard deviation (SD) and the female percentage in our study participants groups were: 57.3±12.5 years and 92.0% females in SS, 59.3±11.0 years and 92.0% females in SS-L and 54.8±10.2 years and 96.0% females in HC. All patients were followed in the Outpatient Clinic of the Department of Pathophysiology, School of Medicine, University of Athens. All patients fulfilled the 2002 American/European criteria for the classification of primary SS [259] and the 2016 ACR/EULAR SS classification criteria [10]. The study was approved by Laiko General Hospital of Athens Ethics Committee (ΔΣ12/20-4-16). All subjects gave informed consent in accordance with the Declaration of Helsinki. Lymphoma diagnosis was based on World Health Organization classification criteria. In the current study, all SS-lymphoma patients were further classified as SS-MALT patients.

Clinical, serological and histopathological characteristics were also recorded after thorough chart review. These included the presence of arthralgias, arthritis, subjective and objective measures of oral and ocular dryness, salivary gland enlargement, Raynaud's phenomenon, lung involvement (documented by pulmonary-function tests and X-ray and/or computed-tomography scans), interstitial nephritis or glomerulonephritis (documented by biopsy), liver involvement (documented by liver biopsy showing changes compatible with primary biliary cirrhosis or autoimmune

cholangitis), including palpable purpura, vasculitis, peripheral neuropathy (verified by nerve-conduction studies), lymphoma development (histologically diagnosed), anti-Ro/SSA and/or anti-La/SSB autoantibodies, rheumatoid factor (RF), complement C3- and C4-levels, cryoglobulinemia, hypergammaglobulinemia (total gammaglobulins>2 g/L), leucopenia (white-blood-cell count<4000/mm3) and lymphopenia (number of lymphocytes<1000/mm3). C4 hypocomplementemia was defined as complement levels <20mg/dl and rheumatoid factor positivity as rheumatoid factor levels more than 20IU/ml. No evidence of monoclonality was detected in MSG SS-non lymphoma tissues included in the study. In the first SS cohort data for the presence of plaque formation, measurements for intima media thickness (IMT), as a marker of subclinical atherosclerosis, as well as traditional risk factors for atherosclerosis was also obtained The presence of subclinical atherosclerosis was defined by the presence of plaque and/or arterial wall thickening (defined as IMT score >0.90 mm in carotid and femoral arteries) as determined by ultrasound (iU22, Philips, Royal Philips Electronics of the Netherlands). Both the carotid (common carotid, bifurcation, and internal carotid) and the femoral (common femoral and superficial femoral) arteries were evaluated in each individual. The mean carotid artery IMT was defined as the average of 36 IMT readings (common, bifurcation, and internal carotid arteries, right and left side, far and near wall, with three sampling points per segment) and the mean femoral artery IMT was the average of twenty-four IMT readings (common and superficial femoral arteries, right and left side, far and near wall, with three sampling points per segment), as previously described. Plaque formation was defined as a focal protrusion of more than 50% of the surrounding wall. The radiologist in charge of the ultrasound scanning was unaware of the clinical diagnosis of the subjects under evaluation [260].

2.2 Methods

2.2.1 Measurement of extracellular Lp-PLA2 activity

Extracellular Lp-PLA2 activity was measured in human serum samples of our first SS cohort stored at -80^{o}C for <6 months. The analysis measured [^{3}H]PAF degradation products {[^{3}H] CH_3COOH} in liquid scintillation counter. Briefly, each sample (serum, lipoprotein solution, lipoprotein eluate fractions, or purified Lp-PLA2 preparations) was

incubated with 10μM [^{3}H]PAF in PBS, pH 7.4, for 10 min at 37°C. The lipids were extracted at the end of incubation by the method of Folch et al. [261], and the amount of radioactivity recovered in the aqueous phase was determined by liquid scintillation.

In the second independent SS cohort, we performed the assay protocol for extracellular Lp-PLA2 with a commercially available PAF Acetylhydrolase Assay Kit (Cayman Chemical), according to manufacturer's instructions. All measurements were performed in duplicate. Cayman's PAF Acetylhydrolase Assay Kit provides an accurate and convenient method for measurement of Lp-PLA2 activity. The assay uses 2-thio PAF which serves as a substrate for all Lp-PLA2s. Upon hydrolysis of the acetyl thioester bond at the sn-2 position by Lp-PLA2, free thiols are detected using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent). Briefly, we evaluate two no-Enzyme Control Wells – by adding 10 µl DTNB Assay Reagent and 15 µl Assay Buffer to at least two wells and these wells correct for any non-enzymatic hydrolysis of the substrate-, two Positive Control Wells (Human Recombinant Lp-PLA2) -by adding 10 µl DTNB Assay Reagent, 10 µl Lp-PLA2, and 5 µl Assay Buffer- and the Sample Wells -by adding 10 µl DTNB Assay Reagent, 10 µl sample, and 5 µl Assay Buffer (two wells for each sample). In order to obtain reproducible results, the amount of Lp-PLA2 that was added at each well should cause an absorbance increase between 0.01 and 0.1/min. Then we cover with the plate cover and incubate for 30 minutes at room temperature to allow any free thiols in the sample to react with DTNB. We initiate the reactions by adding 200 µl substrate solution to all wells. Then we carefully shake the 96-well plate for 30 seconds to mix and read the absorbance once every minute at 412 nm using a plate reader (Versamax, Molecular Devices, USA) to obtain at least five time points. At each time point, we determined the average absorbance of the No-Enzyme Control wells and subtracted these values from all sample values for each respective time point. Then we determined the change in corrected absorbance (ΔA412) per minute by plotting the average values as a function of time to obtain the slope (rate) of the linear portion of the curve. We selected two points on the linear portion of the curve and determined the change in absorbance during that time using the following equation: ΔA412/min. =A412 (Time 2) - A412 (Time 1)Time 2 (min.) - Time 1 (min.)4. We used the following formula to calculate the Lp-PLA2 activity. The reaction rate at 412 nm can be determined using

the DTNB extinction coefficient of 10.66 mM. One unit of enzyme hydrolyzes one μmol of 2-thio PAF per minute at 25°C.

Lp-PLA2 Activity (μmol/min/ml) = ΔA412/min.10.66 mM-1x0.225 ml0.01 ml x Sample dilution.

2.2.2. MSG tissue RNA and protein extraction

Total RNA was extracted from MSG tissue biopsies (stored at -80^{o}C) by TRIZOL Reagent (Thermo Scientific, USA) according to manufacturer's instructions. Briefly, we add 1 mL of TRIzol™ Reagent (Thermo Fisher, USA) per 50–100 mg of MSG tissue and homogenize using a homogenizer. We incubate for 5 minutes to permit complete dissociation of the nucleoproteins complex and add 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent used for lysis, then securely cap the tube and incubate for 2–3 minutes. We centrifuged the sample for 15 minutes at 12,000 × g at 4°C. The mixture separated into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase. We transferred the aqueous phase containing the RNA to a new tube. Then we added 0.5 mL of isopropanol to the aqueous phase, per 1 mL of TRIzol™ Reagent used for lysis. We incubated for 10 minutes and centrifuged for 10 minutes at 12,000 × g at 4°C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube and we discarded the supernatant with a micropipettor. Then we resuspended the pellet in 1 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis. We vortexed the sample briefly, then centrifuged for 5 minutes at 7500 × g at 4°C, discarded the supernatant with a micropipettor and air dry the RNA pellet for 5–10 minutes. We resuspended the pellet in 20–50 μL of RNase-free water by pipetting up and down and incubated in a water bath or heat block set at 55–60°C for 10–15 minute. The quantity and quality of RNA samples were spectrophotometrically tested (Biospec Nano, Japan). A commercial preparation of total RNA from normal salivary glands pooled from 24 male/female Caucasians (ages 15-60; cause of death: sudden death) was used as a source of healthy RNA (Clontech Laboratories, Inc). Protein extraction was performed by lysis of the tissue in (RIPA buffer 50 mM Tris HCl, 150 mM NaCl; Sigma) supplemented with a protease inhibitor cocktail and phosphatase inhibitor cocktail (Cell Signaling Technology),. Total protein was extracted from MSG tissues by radioimmunoprecipitation assay extraction buffer (RIPA buffer) supplemented with protease and phosphatase cocktail inhibitor and 1 mM PMSF (all reagents purchased

from New England Biolabs, Canada). Briefly, 0.25-0.5ml of RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl,1 mM Na_2EDTA,1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4 and 1 µg/ml leupeptin] was added per 0.25-0.50mg of MSG tissue and homogenization for 1 min was performed. Then samples were stored on ice for 30 minutes, brief sonication of the tissue lysate was also performed followed by centrifugation of tissue extracts for 10 minutes at 14,000 x g at 4^{o}C. We removed the supernatant and stored at -80^{o}C upon use. Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA) was used for determination of protein concentration. We pipetted 25µL of each BSA standard or unknown (diluted 1:100) sample in triplicate into a microplate well (standard samples working range = 20-2000µg/mL). We added 200µL of the working reagent [we prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B)] to each well and mixed the plate thoroughly on a plate shaker for 30 seconds. Then we covered plate and incubated at 37°C for 30 minutes, cool plate to room temperature and measured the absorbance at 562nm on a plate reader (Versamax, Molecular Devices, USA). We prepared a standard curve by plotting the average Blank-corrected 562nm measurement for each BSA standard versus its concentration in µg/mL and used the standard curve to determine the protein concentration of each unknown sample.

2.2.6 cDNA synthesis and Real-Time PCR

One µg of total RNA obtained from MSG samples was reverse-transcribed using Superscript III reverse transcriptase system from Invitrogen (Thermo Fisher, USA). Briefly, we added the following components to a nuclease-free microcentrifuge tube: 1µl (500 ng) of oligo$(dT)_{18}$, 1µg total RNA, 1µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH) and nuclease free water (Qiagen, Germany) up to 13 µl. We heated the mixture to 65°C for 5 minutes and incubated on ice for at least 5 minutes and collected the contents of the tube by brief centrifugation Then we added: 4µl 5X First-Strand Buffer, 1µl 0.1 M DTT, 1µl RNaseOUT™ Recombinant RNase Inhibitor (Thermo Fisher, USA) and 1µl of SuperScript™ III RT (200 units/µl). We mixed by pipetting gently up and down. Then we incubated at 50°C for 60 minutes and inactivated the reaction by heating at 70°C for 15 minutes. Complementary DNA

samples were diluted 1:10 with nuclease free water (Qiagen, Germany) immediately after synthesis and stored at -20°C.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to quantify specific cDNAs using the Bio-Rad IQ5 thermocycler and the Kapa Biosystems SYBR Green (Kapa Biosystems, South Africa). Specific primers for each gene were designed using the Beacon Designer software trying to amplify only cDNA (exon-intron spanning), as follows: PLA2G7 forward primer: CTGCTATTGGCATTGACCTGGC and reverse primer AGGTAGAGCCAAGACTTGTCCC as well as GAPDH as a normalization gene, forward primer CATCACTGCCACCCAGAAGA and reverse primer TCCACCACCCTGTTGCTGTA. Briefly, the reaction was carried out in a total volume of 20 μL per reaction. Reaction mixture included 2 μL of template cDNA, 0.4 μM of each primer, 10μl 2× KAPA SYBR Green Mix (Kapa Biosystems, South Africa), and sterile water. Amplification protocol started with an initial denaturation step 95°C for 4 min followed by 40 cycles at 95°C for 5s (denaturation) and 63°C (annealing and extension) for 30s. To assess product specificity, amplicons were checked by melting curve analysis. Melting curves were generated from 65°C to 95°C with increments of 0.5°C/cycle for 15s at each cycle and all inconsistent results were discarded. Then, the threshold values were recorded for each sample in the logarithmic portion of the amplification curve. All reactions were performed in duplicate. MSG healthy RNA pool was the reference sample for MSG tissues. Each sample's threshold cycle values for the target and house-keeping genes were subtracted from the corresponding values of the reference sample. Finally, the target gene values were divided by the housekeeping gene values for each sample, and the result was the relative expression value for each unknown sample [129].

2.2.5 Western Blot analyses

An equal amount of total protein lysates (25μg) was heated at 95^{o}C for 5 minutes, electrophoresed on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels (29:1 acrylamide. Applichem, Germany) under denaturing conditions for 2 hours, and transferred overnight at 4^{o}C onto PVDF membranes (Bio-Rad, USA). The blots were blocked with TBST (20 mmoles/liter Tris HCl, pH 7.6, 137 mmoles/liter NaCl, and

0.1% Tween 20) containing 5% nonfat dried milk at room temperature for 90 minutes. The membranes were probed overnight at 4^{o}C with primary antibody against Lp-PLA2 (PLA2G7) Rabbit Polyclonal Antibody (Origene, USA) at 1:1.000 dilution in TBST containing 5% milk and another day GAPDH as control (monoclonal anti-mouse antibody, Santa Cruz Biotechnology). The blots were washed 3x 5 minutes, followed by incubation with a secondary goat antibody raised against rabbit IgG or secondary goat antibody raised against mouse IgG conjugated to horseradish peroxidase (1:2,000 dilution) (both purchased from Santa Cruz Biotechnology). The bands were visualized by exposure of the blots to x-ray film after incubation with freshly made enhanced chemiluminescent substrate for 2 minutes (GAPDH) or 20 minutes (PLA2G7) (SuperSignal; Pierce/ Thermo Scientific, USA). Densitometry analysis was performed using ImageJ image processing software (http://rsb.info.nih.gov/ij). As a positive control for the Lp-PLA2 protein expression was used a total protein lysate from human placenta tissue sample available in our laboratory (high RNA expression based on Human Protein Atlas, https://www.proteinatlas.org/ENSG00000146070-PLA2G7/tissue).

2.2.6 Immunohistochemistry

Immunohistochemical detection of Lp-PLA2 (PLA2G7) was performed by a standard immunoperoxidase technique using the SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling, USA) in 12 formalin-fixed paraffin-embedded MSG tissue sections (5 μm) derived from 4 sicca, 4 SS patients and 4 SS-L patients. Briefly, paraffin sections were rehydrated in successive baths of xylene, 100%, 96%, 80%, 70% ethanol, and distilled water. The sections were washed with PBS (phosphate buffered saline) 3 times. Antigen retrieval was performed by microwaving for 10 minutes in 0.01 M Citrate buffer (pH 6.0). Incubation for 10 minutes at room temperature with Power block Universal Blocking Reagent (BioGenex, USA) and 10 minutes with 3% H_2O_2 (BioGenex, USA) were performed to block non-specific antibody binding and endogenous peroxidase activity, respectively. Incubation of serial sections with Lp-PLA2 (PLA2G7) Rabbit Polyclonal Antibody (Origene, USA) at 1:25 dilution and concentration-matched isotype control antibody (PharMingen, San Diego, CA) was performed for 30 minutes at room temperature. Polymer–horseradish peroxidise (HRP) Reagent (Cell Signaling, USA) was applied for 30 minutes at room temperature and

after a washing step the substrate diaminobenzidine (DAB) solution (Cell Signalling, USA) was performed for 5-10 minutes. Biopsy sections were counterstained with haematoxylin for 2 minutes (Mayers Haematoxylin solution, Sigma Aldrich Inc, USA), dehydrated in successive baths of water, 70%, 80%, 96%, 100% ethanol, and xylene and coverslip mounted with 2 drops of Aqueous Mounting Media (Chembiotin, Canada). Negative control staining was performed by replacing primary antibody with PBS. Positive immunoreactivity appears as brown color. As a positive control was used a human tonsil section sample, available in our laboratory according to Lp-PLA2 (PLA2G7) Rabbit Polyclonal Antibody (Origene, USA) datasheet.

2.2.7 Statistics

Statistical analysis was performed by SPSS v.21 package and Graph Pad PRISM. Two-group comparisons of continuous data were assessed using t-tests, or the Mann-Whitney test, when the data did not have a normal distribution. Difference was considered statistically significant if $p<0.05$.

3. Results

3.1 Clinical and serological characteristics of study participants

Tables 1 and 2 depict SS patients complicated or not by lymphoma from the first and the second cohort, respectively, did not differ in the clinicopathological and laboratory characteristics described.

Table 1. Clinicopathological and laboratory characteristics of the first SS cohort.

	pSS non lymphoma (n=48)	pSS-lymphoma (n=9)	p-value
Age (mean±S.D.)	58.3±13.1	63.4±11.6	ns
Female sex (%)	44 (91.7)	9 (100.0)	ns
Age of onset (mean±SD)	49.8±14.5	52.2±10.7	ns
Disease duration (mean±SD)	8.5±5.9	11.2±6.3	ns
Dry mouth (%)	46(95.8)	9(100.0)	ns
Dry eyes (%)	45(93.8)	9(100.0)	ns
Abnormal Schirmer's test (%)	33/44(75.0)	8(88.9)	ns
Rose Bengal Stain (≥4/9) (%)	14/34(41.2)	1/5(20.0)	ns
Focus score (number of foci/4mm^2) (mean±SD)	1.9±0.9	3.2±2.9	ns
Tarpley score (mean±SD)	1.8±1.1	2.3±1.6	ns
Antinuclear Antibodies ≥1/320 (%)	43(89.6)	9(100.0)	ns
Antibodies against Ro/SSA (%)	34(70.8)	9(100.0)	ns
Antibodies against La/SSB (%)	22(45.8)	5(55.6)	ns
Salivary gland enlargement (%)	6/40(15.0)	6 (66.7)	ns
Rheumatoid Factor positivity (>20 IU/ml)(%)	18/47(38.3)	8(88.9)	ns
Arthralgias/Myalgias (%)	20/42(47.6)	6(66.7)	ns
Arthritis (%)	7(14.6)	1/8(12.5)	ns
Raynaud's Phenomenon (%)	6/42(14.3)	5(55.9)	ns
Palpable Purpura (%)	7(14.6)	2(22.2)	ns
Leucopenia (<3000 white blood cells) (%)	1(2.1)	0 (0.0)	ns
C-reactive protein positivity (>5mg/l) (%)	5(10.4)	1(11.1)	ns
Erythrocyte sedimentation rate (mm/h) (mean±SD)	30.8±22.3	31.0±19.0	ns
Low Complement 4 (<20mg/dl) (%)	23(47.9)	7(77.8)	ns
Hypergammaglobulinemia (>18%)	23/45(51.5)	4(44.4)	ns
Lactate Dehydrogenase levels (IU/L) (mean±SD)	205.2±58.0	200.4±57.0	ns

Table 2. Clinicopathological and laboratory characteristics of the second SS cohort.

	pSS non lymphoma (n=25)	pSS-lymphoma (n=17)	p-value
Age (mean±S.D.)	58.6±11.1	58.9±10.6	ns
Female sex (%)	25(100.0)	15(88.3)	ns
Age of onset (mean±SD)	48.4±14.4	48.7±11.2	ns
Disease duration (mean±SD)	10.2±8.4	10.2±8.1	ns
Dry mouth (%)	24(96.0)	17(100.0)	ns
Dry eyes (%)	23(92.0)	16(94.1)	ns
Abnormal Schirmer's test (%)	13/18 (72.2)	12/14 (85.7)	ns
Rose Bengal Stain (≥4/9) (%)	9/16 (56.3)	2/7 (28.6)	ns
Focus score (number of foci/4mm^2) (mean±SD)	1.4±0.7	5.2±3.0	ns
Tarpley score (mean±SD)	1.6±0.8	3.3±0.5	ns
Antinuclear Antibodies ≥1/320 (%)	21(84.0)	17(100.0)	ns
Antibodies against Ro/SSA (%)	17(68.0)	16(94.1)	ns
Antibodies against La/SSB (%)	8/24(33.3)	11(64.7)	ns
Salivary gland enlargement (%)	4(16.0)	10(58.8)	ns
Rheumatoid Factor positivity (>20 IU/ml)(%)	9/24(37.5)	15/16(93.8)	ns
Arthralgias/Myalgias (%)	17(68.0)	7(41.2)	ns
Arthritis (%)	4(16.0)	3(17.6)	ns
Raynaud's Phenomenon (%)	8(32.0)	4(23.6)	ns
Palpable Purpura (%)	5(20.0)	7(41.2)	ns
Leucopenia (<3000 white blood cells) (%)	0(0.0)	0(0.0)	ns
C-reactive protein positivity (>5mg/l) (%)	2(8.0)	4(23.5)	ns
Erythrocyte sedimentation rate (mm/h) (mean±SD)	24.0±20.0	35.6±32.9	ns
Low Complement 4 (<20mg/dl) (%)	11(44.0)	13(76.5)	ns
Hypergammaglobulinemia (>18%)	10/24(41.7)	11(64.7)	ns
Lactate Dehydrogenase levels (IU/L) (mean±SD)	236.2±76.3	255.9±98.8	ns

3.2 Lp-PLA2 activity in SS cohorts

Figure 12 and 13 show that the activity of Lp-PLA2 is significantly increased in patients with primary SS who developed lymphoma compared to primary SS patients without lymphoma [mean±SD (nmol/min/ml): 62.0±13.4 vs 47.7±14.7, p=0.003 and 19.7±4.7 vs 15.2±3.2, p=0.005, respectively], as well as healthy controls [mean±SD (nmol/min/ml): 62.0±13.4 vs 52.0±16.3, p=0.03 and 19.7±4.7 vs 15.7±3.1, p=0.06, respectively]. No

statistical significant differences in Lp-PLA2 activity were detected between primary SS patients without lymphoma development and healthy controls.

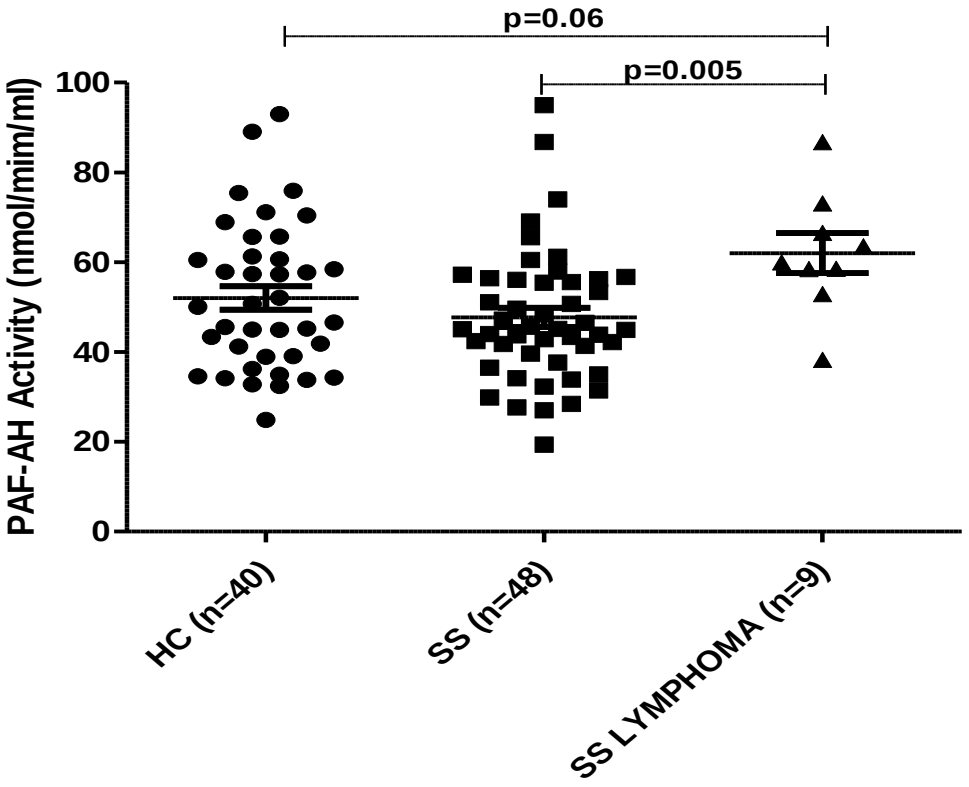

Figure 12. Lp-PLA2 activity in the first SS cohort. HC: healthy control, SS: Sjogren's Syndrome, SS LYMPHOMA: Sjogren's Syndrome complicated by lymphoma.

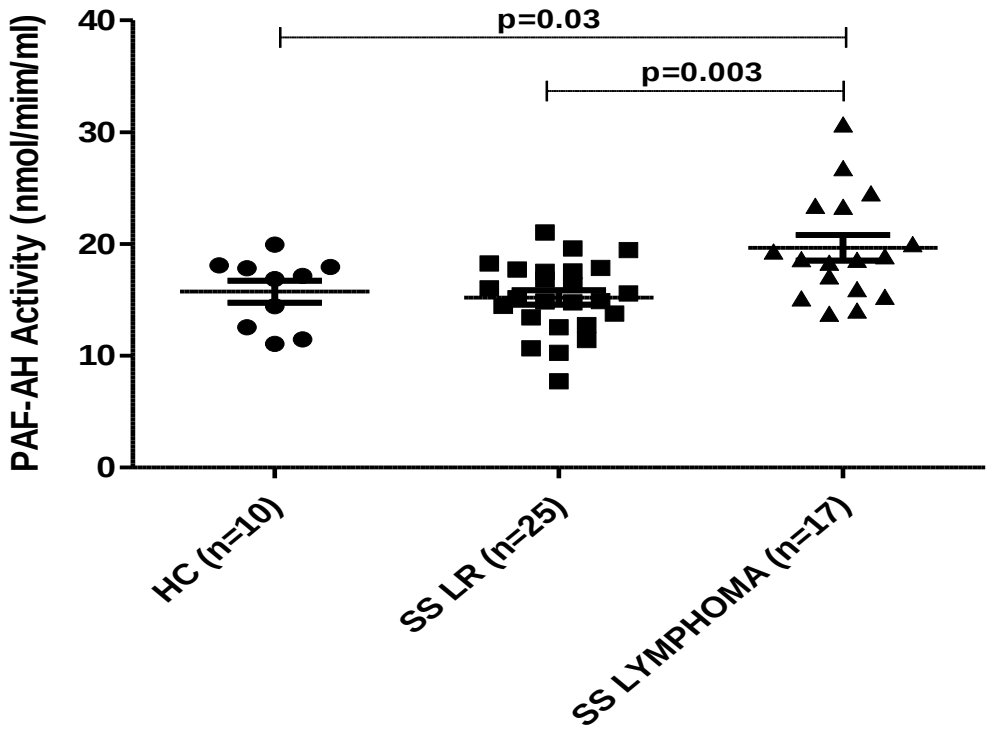

Figure 13. Lp-PLA2 activity in the second SS cohort. HC: healthy control, SS: Sjogren's Syndrome, SS LYMPHOMA: Sjogren's Syndrome complicated by lymphoma.

3.3 PLA2G7 mRNA expression in MSG tissues

As shown in figure 14, mRNA transcripts of the PLA2G7 gene were increased statistically significant in SS patients complicated by lymphoma compared to both SS patients without the presence of lymphoma and sicca controls (4.7±4.4 vs 1.3±2.1, p=0.002 and 4.7±4.4vs 0.4±0.3, p=0.003, respectively). No significant difference was observed between SS patients and sicca controls (p=0.48).

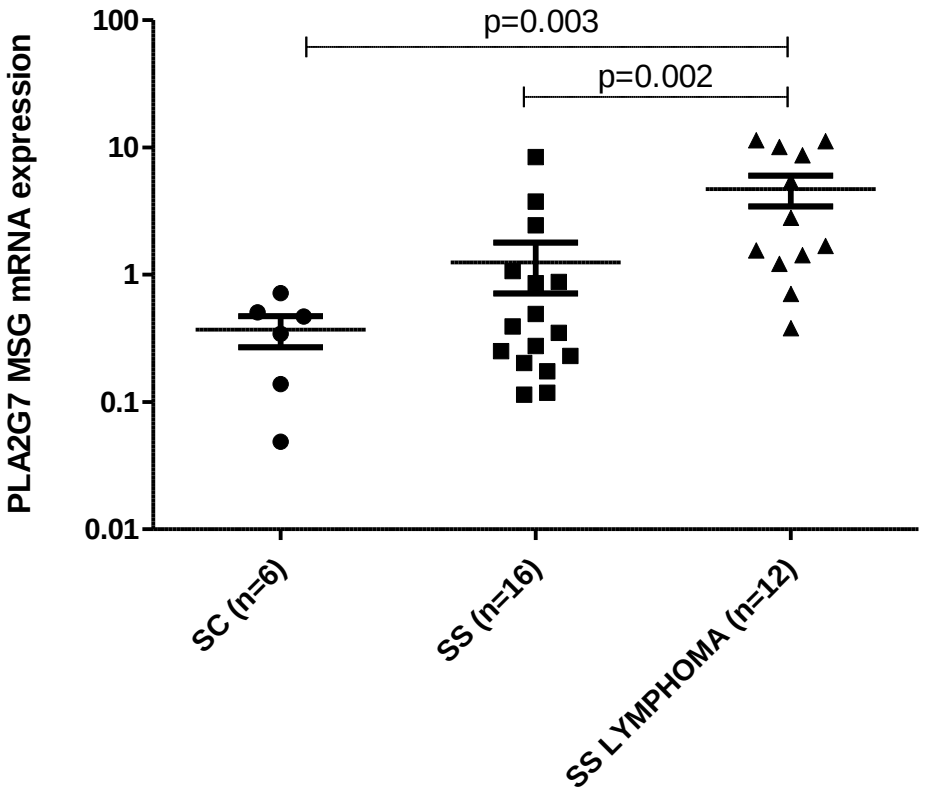

Figure 14. PLA2G7 mRNA expression in MSG tissues. SC: non autoimmune controls with sicca complaints, SS: Sjogren's Syndrome, SS LYMPHOMA: Sjogren's Syndrome complicated by lymphoma.

3.3 Lp-PLA2 protein expression in MSG tissues by Western Blot

As shown in Figure 15 and 16, increased Lp-PLA2 protein expression was found in MSG total protein extracts derived from SS patients complicated by lymphoma compared to both SS patients without the presence of lymphoma and sicca controls as tested by Western Blot (1.93±0.33 vs 1.12±0.37, p=0.048 and 1.93±0.33 vs 0.39±0.65, p=0.03, respectively). A marginal increased Lp-PLA2 MSG protein expression was observed between SS patients and sicca controls (p=0.08). As depicted in figure 4, two samples derived from MSG tissues of SS patients complicated by lymphoma did not express the reference protein GAPDH (though Lp-PLA2 protein was expressed), probably due to methodological pitfalls and were excluded from the final analysis.

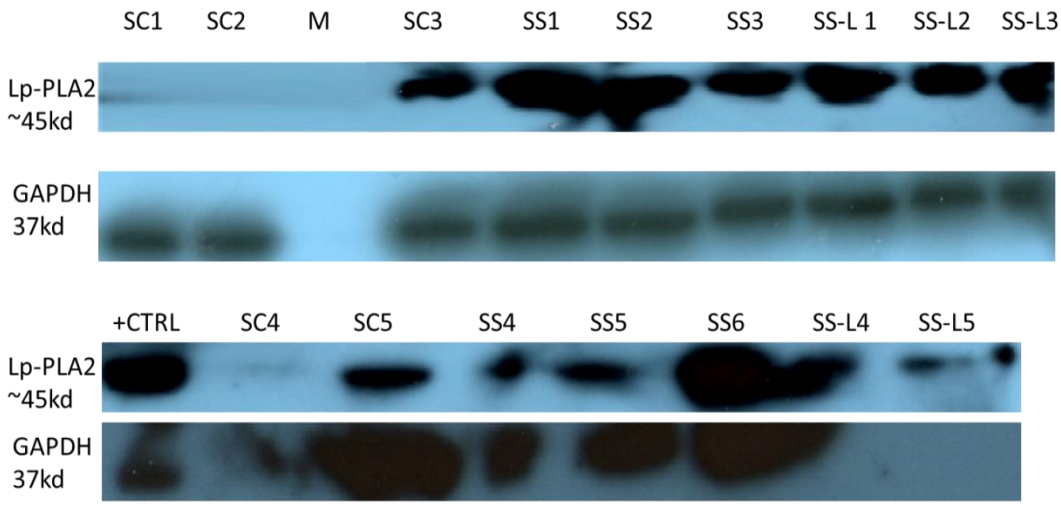

Figure 15. LpPLA-2 and GAPDH MSG protein expression tested by Western Blot. SC: non autoimmune controls with sicca complaints, SS: Sjogren's Syndrome, SS LYMPHOMA: Sjogren's Syndrome complicated by lymphoma.

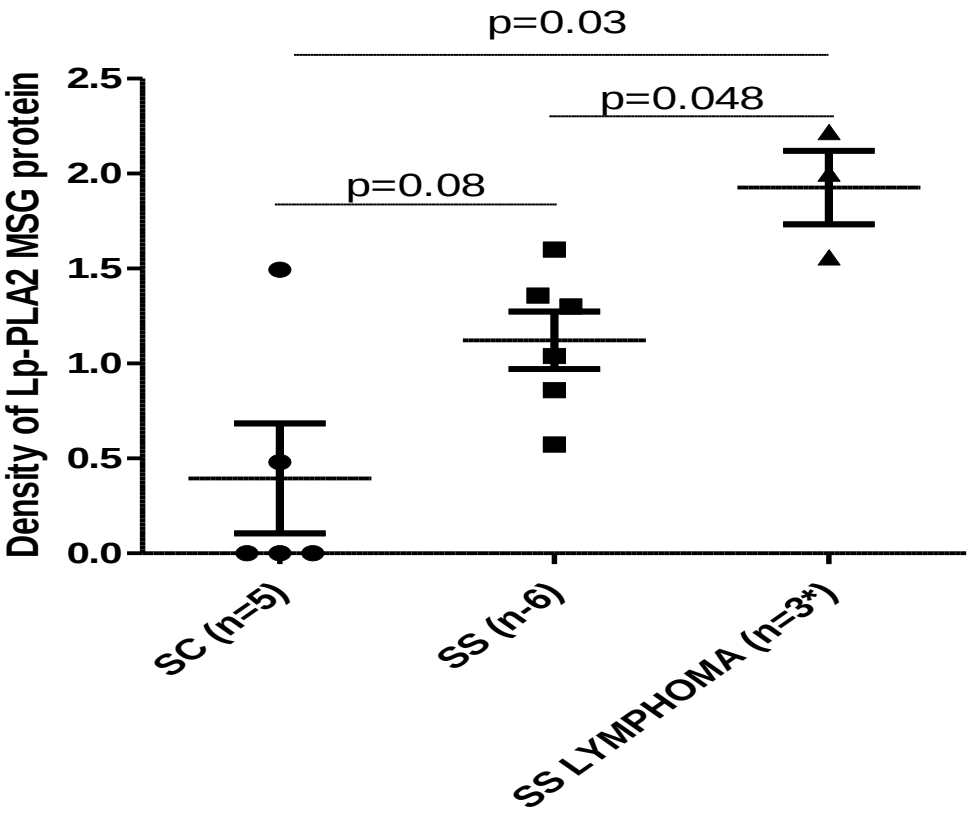

Figure 16. LpPLA-2 MSG protein density tested by Western Blot. SC: non autoimmune controls with sicca complaints, SS: Sjogren's Syndrome, SS LYMPHOMA: Sjogren's Syndrome complicated by lymphoma.

3.4 Lp-PLA2 protein expression in MSG tissues by immunohistochemistry

Increased Lp-PLA2 protein expression was found in MSG tissue sections derived from SS patients complicated by lymphoma compared to both SS patients without the presence of lymphoma and sicca controls as tested by immunohistochemistry. The expression was found mainly within lymphocytic infiltrates including tissue macrophages (the major Lp-PLA2 producing cell type) and lower expression was reported in salivary gland epithelial cells (data not shown). Statistical analysis remains to be done.

3.5 Lp-PLA2 activity in the first SS cohort according the presence of plaque and IMT

Since the role of Lp-A2 activity in atherosclerosis is well established in the literature, we checked if our results were influenced by the presence of atherosclerosis in our groups. In our first cohort we had available data regarding the presence of plaque formation and the presence of the intima media thickness>0.9 (IMT>0.9), a marker of subclinical atherosclerosis. No significant differences were observed between SS patients (with or without the presence of lymphoma). It seems that the presence or absence of atherosclerosis did not influence our results (Figure 17).

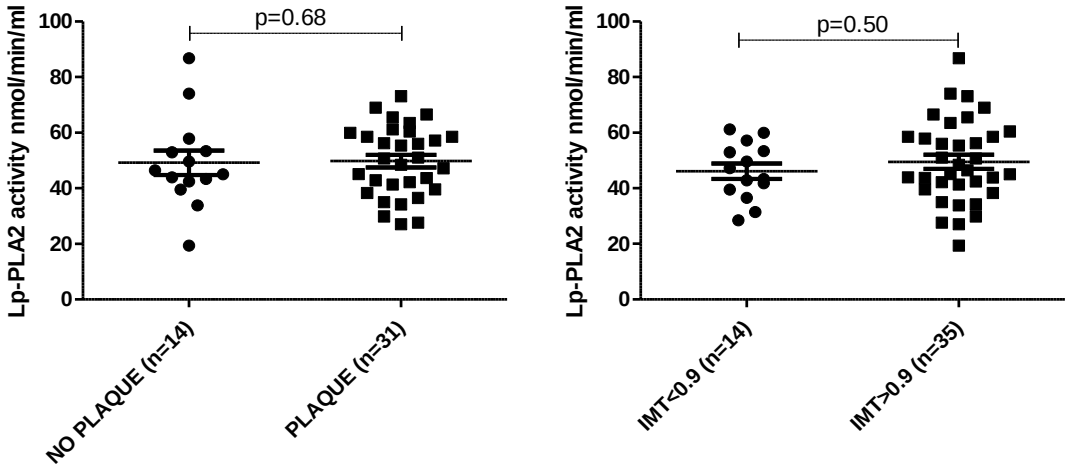

Figure 17. Lp-PLA2 activity in Sjogrne's Syndrome (SS) patients with and without the presence of atherosclerotic plaque formation and intima media thickness>0.9. No

difference was found between SS patients without plaque and with the presence of plaque [mean±SD (nmol/min/ml): 49.2±16.4 vs 49.8±16.7, p=0.68] and between patients with IMT<0.9 and patients with IMT>0.9 [mean±SD (nmol/min/ml): 46.1±10.2 vs 49.5±15.0, p=0.50].

4. DISCUSSION

The role of Lp-PLA2 activity in atherosclerosis is an undoubtable event [189]. However, recent data support that Lp-PLA2 activity, as well as mRNA and/or protein expression contribute in the tumorigenesis of multiple malignancies including prostate, breast, melanoma, multiple myeloma and lymphoma [191]. In our study, we investigated the potential impact of Lp-PLA2 in SS related lymphomagenesis. Our results show firstly that serum Lp-PLA2 activity is significantly increased in SS patients complicated with lymphoma compared to both SS non lymphoma patients and healthy controls by two different methods in two independent cohorts. Moreover, based on the available data of the presence of atherosclerosis in our first SS cohort, no difference was found regarding the presence/absence of atherosclerosis and the levels of Lp-PLA2 activity. Secondly, mRNA and protein expression of Lp-PLA2 molecule was significantly increased in MSG tissue biopsy samples derived from SS patients with lymphoma compared to both SS and sicca controls. Finally, this expression seems to be attributed in the lymphocytic infiltrates (including tissue macrophages) compartment of SS tissue biopsies. As far as we know, this is the first time in the literature that Lp-PLA2 molecule has been evaluated in SS and particularly in the SS related lymphomagenesis.

Our results are supported by two different methods: one is considered as the gold standard technique with the measurement of [^{3}H]PAF degradation products of human serum samples by liquid scintillation; the other, which is quicker ans simpler in its application uses ELISA the serum Lp-PLA2 activity, in two independent SS cohorts. Moreover, the finding that the mean expression seems to decrease (no statistically significant) between healthy controls and SS without the presence of lymphoma patients, are in accordance with previous findings in another autoimmune disorder, SLE [251]. Generally in a disorder having an oxidative or free radical-generating component is likely to exhibit decreased acetylhydrolase activity as is found in lupus. This decreased

Lp-PLA2 activity has been found to be correlated with reduced LDL levels in lupus patients [251]. On the other hand, the absence of statistical significant difference between the SS non lymphoma group and the HC by two methods imply an involvement of increased Lp-PLA2 activity in lymphoproliferative complications of SS.

Furthermore, our findings of mRNA and protein expression in MSG tissue biopsies strengthen our conclusions, which show that Lp-PLA2 expression is higher only between SS patients with lymphoma and controls as well as SS without lymphoma patients. Our immunohistochemistry results validate those reported in the literature showing the main expressing cells in SS patients to be infiltrates of monocytes (and tissue macrophages) and lymphocytes. Also they show that SS MSG tissues are characterized by lymphocyte infiltration and especially in SS patients complicated by lymphoma (higher number of tissue macrophages and B monoclonal cells), while sicca patients lack the presence of these infiltrates. Our findings suggest a probable implication of increased Lp-PLA2 activity and expression in pathophysiological processes underlying immunocomplexes mediated manifestations and lymphoma development among SS patients.

Our data show a strong correlation between Lp-PLA2 and monocyte (differentiating into tissue macrophages) and lymphocyte levels, however, a larger sample size of SS patients is required to confirm and buttress our findings. The role of LpPLA2 should also be evaluated in SS mouse models and in transgenic models with salivary gland tissue specific overexpression of the Lp-PLA2 molecule. These studies should examine the role of Lp-PLA2 activity as a risk factor for the development of lymphoma in SS.

In conclusion, the clarification of the molecular patterns leading to Lp-PLA2 elevated activity and mRNA/protein expression in samples derived from SS patients complicated by lymphoma may provide a novel reliable biomarker for lymphoproliferation as well as shed light on new targets for therapeutic interventions and enable personalized medicine in SS.

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