NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS

SCHOOL OF HEALTH SCIENCES

MEDICAL SCHOOL

DEPARTMENT OF CLINICAL AND LABORATORY MEDICINE

DEPARTMENT OF PATHOPHYSIOLOGY, Director: Professor Michael Voulgarelis

TITLE OF THE PhD THESIS

Καθορισμός κλινικών φαινοτύπων των ασθενών με πρωτοπαθές σύνδρομο Sjogren και αναζήτηση νέων βιοδεικτών της νόσου

LOUKAS CHATZIS MD

INTERNIST

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PhD thesis authored by the PhD candidate Loukas Chatzis at the National and Kapodistrian University of Athens entitled:

<< Determination of clinical phenotypes of patients with primary Sjogren's syndrome and uncovering new biomarkers for the disease>>

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Member of the three-member advisory committee: Prof. Tzioufas Athanasios (Supervisor)

Prof. Panayiotis Vlachoyiannopoulos

Associate Prof. Efstathia Kapsogeorgou

Members of the seven-member examination committee:

Prof. Voulgarelis Michail

Prof. Fotiadis Dimitrios

Prof. Mavragani Clio

Assistant Prof. Goules Andreas

Thesis submission: 23/11/2023

Grade: Excellent

ΙΠΠΟΚΡΑΤΕΙΟΣ ΟΡΚΟΣ

Ὄμνυμι Ἀπόλλωνα ἰητρὸν, καὶ Ἀσκληπιὸν, καὶ Ὑγείαν, καὶ Πανάκειαν, καὶ θεοὺς πάντας τε καὶ πάσας, ἵστορας ποιεύμενος, ἐπιτελέα ποιήσειν κατὰ δύναμιν καὶ κρίσιν ἐμὴν ὅρκον τόνδε καὶ ξυγγραφὴν τήνδε.

Ἡγήσασθαι μὲν τὸν διδάξαντά με τὴν τέχνην ταύτην ἴσα γενέτῃσιν ἐμοῖσι, καὶ βίου κοινώσασθαι, καὶ χρεῶν χρηίζοντι μετάδοσιν ποιήσασθαι, καὶ γένος τὸ ἐξ ωὐτέου ἀδελφοῖς ἴσον ἐπικρινέειν ἄῤῥεσι, καὶ διδάξειν τὴν τέχνην ταύτην, ἢν χρηίζωσι μανθάνειν, ἄνευ μισθοῦ καὶ ξυγγραφῆς, παραγγελίης τε καὶ ἀκροήσιος καὶ τῆς λοιπῆς ἁπάσης μαθήσιος μετάδοσιν ποιήσασθαι υἱοῖσί τε ἐμοῖσι, καὶ τοῖσι τοῦ ἐμὲ διδάξαντος, καὶ μαθηταῖσι συγγεγραμμένοισί τε καὶ ὡρκισμένοις νόμῳ ἰητρικῷ, ἄλλῳ δὲ οὐδενί.

Διαιτήμασί τε χρήσομαι ἐπ' ὠφελείῃ καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμὴν, ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἴρξειν.

Οὐ δώσω δὲ οὐδὲ φάρμακον οὐδενὶ αἰτηθεὶς θανάσιμον, οὐδὲ ὑφηγήσομαι ξυμβουλίην τοιήνδε. Ὁμοίως δὲ οὐδὲ γυναικὶ πεσσὸν φθόριον δώσω. Ἁγνῶς δὲ καὶ ὁσίως διατηρήσω βίον τὸν ἐμὸν καὶ τέχνην τὴν ἐμήν.

Οὐ τεμέω δὲ οὐδὲ μὴν λιθιῶντας, ἐκχωρήσω δὲ ἐργάτῃσιν ἀνδράσι πρήξιος τῆσδε.

Ἐς οἰκίας δὲ ὁκόσας ἂν ἐσίω, ἐσελεύσομαι ἐπ' ὠφελείῃ καμνόντων, ἐκτὸς ἐὼν πάσης ἀδικίης ἑκουσίης καὶ φθορίης, τῆς τε ἄλλης καὶ ἀφροδισίων ἔργων ἐπί τε γυναικείων σωμάτων καὶ ἀνδρῴων, ἐλευθέρων τε καὶ δούλων.

Ἃ δ' ἂν ἐν θεραπείῃ ἢ ἴδω, ἢ ἀκούσω, ἢ καὶ ἄνευ θεραπηίης κατὰ βίον ἀνθρώπων, ἃ μὴ χρή ποτε ἐκλαλέεσθαι ἔξω, σιγήσομαι, ἄῤῥητα ἡγεύμενος εἶναι τὰ τοιαῦτα.

Ὅρκον μὲν οὖν μοι τόνδε ἐπιτελέα ποιέοντι, καὶ μὴ ξυγχέοντι, εἴη ἐπαύρασθαι καὶ βίου καὶ τέχνης δοξαζομένῳ παρὰ πᾶσιν ἀνθρώποις ἐς τὸν αἰεὶ χρόνον. παραβαίνοντι δὲ καὶ ἐπιορκοῦντι, τἀναντία τουτέων.

ΙΠΠΟΚΡΑΤΕΙΟΣ ΟΡΚΟΣ (Απόδοση στα νέα ελληνικά)

Ορκίζομαι στο θεό Απόλλωνα τον ιατρό και στο θεό Ασκληπιό και στην Υγεία και στην Πανάκεια και επικαλούμενος τη μαρτυρία όλων των θεών ότι θα εκτελέσω κατά τη δύναμη και την κρίση μου τον όρκο αυτόν και τη συμφωνία αυτή.

Να θεωρώ τον διδάσκαλό μου της ιατρικής τέχνης ίσο με τους γονείς μου και την κοινωνό του βίου μου. Και όταν χρειάζεται χρήματα να μοιράζομαι μαζί του τα δικά μου. Να θεωρώ την οικογένειά του αδέλφια μου και να τους διδάσκω αυτήν την τέχνη αν θέλουν να την μάθουν χωρίς δίδακτρα ή άλλη συμφωνία.

Να μεταδίδω τους κανόνες ηθικής, την προφορική διδασκαλία και όλες τις άλλες ιατρικές γνώσεις στους γιους μου, στους γιους του δασκάλου μου και στους εγγεγραμμένους μαθητές που πήραν τον ιατρικό όρκο, αλλά σε κανέναν άλλο.

Θα χρησιμοποιώ τη θεραπεία για να βοηθήσω τους ασθενείς κατά τη δύναμη και την κρίση μου, αλλά ποτέ για να βλάψω ή να αδικήσω. Ούτε θα δίνω θανατηφόρο φάρμακο σε κάποιον που θα μου το ζητήσει, ούτε θα του κάνω μια τέτοια υπόδειξη.

Παρομοίως, δεν θα εμπιστευτώ σε έγκυο μέσο που προκαλεί έκτρωση. Θα διατηρώ αγνή και άσπιλη και τη ζωή και την τέχνη μου. Δεν θα χρησιμοποιώ νυστέρι ούτε σε αυτούς που πάσχουν από λιθίαση, αλλά θα παραχωρώ την εργασία αυτή στους ειδικούς της τέχνης.

Σε όσα σπίτια πηγαίνω, θα μπαίνω για να βοηθήσω τους ασθενείς και θα απέχω από οποιαδήποτε εσκεμμένη βλάβη και φθορά, και ιδίως από γενετήσιες πράξεις με άνδρες και γυναίκες, ελεύθερους και δούλους. Και όσα τυχόν βλέπω ή ακούω κατά τη διάρκεια της θεραπείας ή και πέρα από τις επαγγελματικές μου ασχολίες στην καθημερινή μου ζωή, αυτά που δεν πρέπει να μαθευτούν παραέξω δεν θα τα κοινοποιώ, θεωρώντας τα θέματα αυτά μυστικά.

Αν τηρώ τον όρκο αυτό και δεν τον παραβώ, ας χαίρω πάντοτε υπολήψεως ανάμεσα στους ανθρώπους για τη ζωή και για την τέχνη μου. Αν όμως τον παραβώ και επιορκήσω, ας πάθω τα αντίθετα.

HIPPOCRATIC OATH (Translation by W.H.S. Jones)

I swear by Apollo Healer, by Asclepius, by Hygieia, by Panacea, and by all the gods and goddesses, making them my witnesses, that I will carry out, according to my ability and judgment, this oath and this indenture.

To hold my teacher in this art equal to my own parents; to make him partner in my livelihood; when he is in need of money to share mine with him; to consider his family as my own brothers, and to teach them this art, if they want to learn it, without fee or indenture; to impart precept, oral instruction, and all other instruction to my own sons, the sons of my teacher, and to indentured pupils who have taken the Healer's oath, but to nobody else.

I will use those dietary regimens which will benefit my patients according to my greatest ability and judgment, and I will do no harm or injustice to them. Neither will I administer a poison to anybody when asked to do so, nor will I suggest such a course. Similarly, I will not give to a woman a pessary to cause abortion. But I will keep pure and holy both my life and my art. I will not use the knife, not even, verily, on sufferers from stone, but I will give place to such as are craftsmen therein.

Into whatsoever houses I enter, I will enter to help the sick, and I will abstain from all intentional wrong-doing and harm, especially from abusing the bodies of man or woman, bond or free. And whatsoever I shall see or hear in the course of my profession, as well as outside my profession in my intercourse with men, if it be what should not be published abroad, I will never divulge, holding such things to be holy secrets.

Now if I carry out this oath, and break it not, may I gain for ever reputation among all men for my life and for my art; but if I break it and forswear myself, may the opposite befall me.

CURRICULUM VITAE

PERSONAL INFORMATION

NAME …….. : LOUKAS CHATZIS

DATE OF BIRTH …… : 07 MARCH 1989

E-MAIL…….. : lukechatzis@gmail.com

EDUCATION

10/2019 – 10/2023
PhD candidate, National & Kapodistrian University of Athens, School of Medicine, PhD thesis title: << Determination of clinical phenotypes of patients with primary Sjogren's syndrome and uncovering new biomarkers for the disease>>

09/2019 – 05/2023
National & Kapodistrian University of Athens, Faculty of Medicine
Masters in **"Infectious diseases"**. *Graduated with a 9.63/10* ***"Excellent"*** *degree.*

03/07/2020
Internal medicine specialty title acquisition

09/2006 – 08/2012
National & Kapodistrian University of Athens, Faculty of Medicine
Bachelor in "Medicine". Graduated with an 8,1/10 "Very Good" degree.

09/2003 – 09/2006
First public school of Cholargos
Pre-tertiary education. ***Graduated with an 19,256/20,000 "Excellent" degree.***

CURRENT POSITION

09/2021 - present: **Laiko University Hospital, Department of Pathophysiology**
- Academic instructor at the Department of Pathophysiology of the University of Athens
- Supervising internist at Laiko general Hospital

WORK EXPERIENCE

01/09/2021- current

Academic instructor at the Department of Pathophysiology of the University of Athens

09/03/2020- 31/08/2021

Working as a fellow in internal medicine at Laiko General University Hospital, after internal medicine title acquisition

09/07/2015- 09/03/2020

Internal medicine resident at Laiko General University Hospital

06/09/2013 - 31/01/2015

Obligatory rural service in the medical center of Distomo, Boeotia - Transferred from 12/2013 to the dialysis unit of Livadeia hospital.

-Crucial role in the duties of the nephrology clinic (diagnostic, therapeutic, educational, research), the dialysis unit (puncture and assessment of arteriovenous fistulas and grafts, insertion of central venous catheters (femoral – jugular – subclavian), managing dialysis emergencies and the outpatient unit.

-Shift fulfillment in the emergency department of the cardiology and the internal medicine units of Livadeia hospital *(Director: Maria Gennadiou Spantidou).*

01/02/2013 - 31/05/2013

Participation in the 4-month postgraduate program directed by "Hygeia" General Hospital for junior physicians.

-Attendance of the daily duties of the Internal medicine and the Intensive Care Unit.

-Specialized training in the most up-to-date imaging and radiotherapy methods.

-Close collaboration with the director of the Rheumatology clinic *(Director: Professor Kitas George)*

11/2012 – 12/2012

Voluntary participation at the emergency ward and the rheumatology outpatient clinic of the Pathophysiology department of Laiko University Hospital *(Director: Professor Tzioufas Athanasios).*

01/09/2012 – 31/09/2012

Attending physician at the rheumatology department of Leiden University Medical Center (LUMC), taking part in the medical and research workshops, the medical visit of the inpatients and the daily outpatient rheumatology clinic *(Director: Prof. Huizinga Tom).*

10/08/2012 – 16/08/2012

Participation in the voluntary program of "Helping the Greek Islands" taking part in all the daily procedures and night shifts of the Peripheral hospital of Kos Island.

SCHOLARSHIPS AND AWARDS

- Awarded with a 3-year financial aid grant for medical students by the "Dimitrios Mavrokordatos" foundation after assessment and examinations during the fourth year of medical school.

- Scholarship for the participation in the Postgraduate program held by "ΥΓΕΙΑ" hospital for junior doctors after examinations and interview (duration: 4 months).

- Scholarship from the Greek Society of Hypertension to take part in the European Society of Hypertension Summer School From 7th September to 13th September 2018, in Les Diablerets, Switzerland

- Bursary from the European League Against Rheumatism (EULAR) to attend the 2019 EULAR Congress held in Madrid, Spain

- Bursary from the European League Against Rheumatism (EULAR) to attend the 2020 EULAR Congress held online

- Scholarship from the university of Brest (UBO) [Bourse de mobilité / Biologie Santé)] for a 4-month mobility at the B Lymphocytes and Autoimmunity lab (UMR1227) to conduct a project including Imaging Mass Cytometry in the context of my PhD

- 2nd award for the best abstract presented entitled "Deep spatial profiling of Sjogren Syndrome patients by imaging mass cytometry" in the fourteen symposium of ΕΠΕΜΥ held in Rodos, 29 September to 02 October 2022

- 1st award for the best abstract presentation entitled "Deep spatial profiling of Sjogren Syndrome patients by imaging mass cytometry" in the 28th Hellenic rheumatology symposium held in Athens, 8-11 December 2022

CONTINUING MEDICAL EDUCATION - RESEARCH – PRESENTATIONS

- Participation in the educational program **"Basic surgical skills"** held by the research and educational department of ELPEN in cooperation with "Hygeia" General Hospital.

(29-30/04/2013)

- Successful completion of the post-graduate program **"Methodology of writing scientific papers"** organized by the university of Thessaly.

(02-31/05/2014)

- Certification in **Newborn Life Support (NLS)** after successfully completing the corresponding course organized by the European Resuscitation Council (ERC).

(31/05/2014)

- Certification in **Advanced Life Support (ALS)** and training in resuscitation techniques for adults after the completion of the corresponding course organized by the European Resuscitation Council (ERC).

(01-02/11/2014)

PUBLICATIONS

1. Autoimmune epithelitis beyond the exocrine glands: an unusual case of anti-Ro/La and Scl-70 lymphocytic interstitial pneumonia
   Ourania D Argyropoulou, **Loukas G Chatzis**, Dimitra Rontogianni, Athanasios G Tzioufas, Clin Exp Rheumatol. 2019 May-Jun;37 Suppl 118(3):249-251. Epub 2019 Jul 19.

2. Cavitary lung lesions in an immunosuppressed patient.
   Chatzis L, Apostolidi E, Chatzis S. Int J Infect Dis. 2020 Jul;96:365-366. doi: 10.1016/j.ijid.2020.05.032. Epub 2020 May 16.

3. Sjögren's Syndrome: The Clinical Spectrum of Male Patients.
   Chatzis L, Pezoulas VC, Ferro F, Gandolfo S, Donati V, Binutti M, Callegher SZ, Venetsanopoulou A, Zampeli E, Mavrommati M, Argyropoulou OD, Michalopoulos G, Voulgari PV, Exarchos T, Baldini C, Skopouli FN, Fotiadis DI, De Vita S, Moutsopoulos HM, Tzioufas AG, Goules AV. J Clin Med. 2020 Aug 12;9(8):2620. doi: 10.3390/jcm9082620.

4. Primary Sjögren's Syndrome of Early and Late Onset: Distinct Clinical Phenotypes and Lymphoma Development.
   Goules AV, Argyropoulou OD, Pezoulas VC, **Chatzis L**, Critselis E, Gandolfo S, Ferro F, Binutti M, Donati V, Zandonella Callegher S, Venetsanopoulou A, Zampeli E, Mavrommati M, Voulgari PV, Exarchos T, Mavragani CP, Baldini C, Skopouli FN, Fotiadis DI, De Vita S, Moutsopoulos HM, Tzioufas AG.
   Front Immunol. 2020 Oct 19;11:594096. doi: 10.3389/fimmu.2020.594096. eCollection 2020.

5. Cryoglobulinemic vasculitis in primary Sjögren's Syndrome: Clinical presentation, association with lymphoma and comparison with Hepatitis C-related disease.
   Argyropoulou OD, Pezoulas V, **Chatzis L**, Critselis E, Gandolfo S, Ferro F, Quartuccio L, Donati V, Treppo E, Bassoli CR, Venetsanopoulou A, Zampeli E, Mavrommati M, Voulgari PV, Exarchos TE, Mavragani CP, Baldini C, Skopouli FN, Galli M, Fotiadis DI, De Vita S, Moutsopoulos HM, Tzioufas AG, Goules AV.
   Semin Arthritis Rheum. 2020 Oct;50(5):846-853. doi: 10.1016/j.semarthrit.2020.07.013.

6. T cell lymphoma in the setting of Sjögren's syndrome: T cells gone bad? Report of five cases from a single centre cohort.
   Stergiou IE, Papageorgiou A, **Chatzis LG**, Tzioufas AG, Voulgarelis M, Goules A.
   Clin Exp Rheumatol. 2020 Jul-Aug;38 Suppl 126(4):125-129. Epub 2020 Sep 30.

7. One year in review 2021: Sjögren's syndrome.
   Cafaro G, Bursi R, **Chatzis LG**, Fulvio G, Ferro F, Bartoloni E, Baldini C.Clin Exp Rheumatol. 2021 Nov-Dec;39 Suppl 133(6):3-13.

8. New frontiers in precision medicine for Sjogren's syndrome.
   Chatzis L, Vlachoyiannopoulos PG, Tzioufas AG, Goules AV.
   Expert Rev Clin Immunol. 2021 Feb;17(2):127-141.

9. Combined seronegativity in Sjögren's syndrome.
   Chatzis LG, Pezoulas V, Voulgari PV, Baldini C, Exarchos TP, Fotiadis DI, Mavragani CP, Skopouli FN, Moutsopoulos HM, Tzioufas AG, Goules AV.
   Clin Exp Rheumatol. 2021 Nov-Dec;39 Suppl 133(6):80-84.

10. Searching for the "X factor" in Sjögren's syndrome female predilection.
    Chatzis LG, Goules AV, Tzioufas AG.
    Clin Exp Rheumatol. 2021 Nov-Dec;39 Suppl 133(6):206-214.

11. A biomarker for lymphoma development in Sjogren's syndrome: Salivary gland focus score.
    Chatzis L, Goules AV, Pezoulas V, Baldini C, Gandolfo S, Skopouli FN, Exarchos TP, Kapsogeorgou EK, Donati V, Voulgari PV, Mavragani CP, Gorgoulis V, De Vita S, Fotiadis D, Voulgarelis M, Moutsopoulos HM, Tzioufas AG.
    J Autoimmun. 2021 Jul;121:102648.

12. SARS-CoV-2 Antigenemia as a Confounding Factor in Immunodiagnostic Assays: A Case Study.
    Belogiannis K, Florou VA, Fragkou PC, Ferous S, **Chatzis L**, Polyzou A, Lagopati N, Vassilakos D, Kittas C, Tzioufas AG, Tsiodras S, Sourvinos G, Gorgoulis VG.
    Viruses. 2021 Jun 14;13(6):1143.

13. An open label trial of anakinra to prevent respiratory failure in COVID-19.
    Kyriazopoulou E, Panagopoulos P, Metallidis S, Dalekos GN, Poulakou G, Gatselis N, Karakike E, Saridaki M, Loli G, Stefos A, Prasianaki D, Georgiadou S, Tsachouridou O, Petrakis V, Tsiakos K, Kosmidou M, Lygoura V, Dareioti M, Milionis H, Papanikolaou IC, Akinosoglou K, Myrodia DM, Gravvani A, Stamou A, Gkavogianni T, Katrini K, Marantos T, Trontzas IP, Syrigos K, **Chatzis L**, Chatzis S, Vechlidis N, Avgoustou C, Chalvatzis S, Kyprianou M, van der Meer JW, Eugen-Olsen J, Netea MG, Giamarellos-Bourboulis EJ.
    Elife. 2021 Mar 8;10:e66125.

14. A federated AI strategy for the classification of patients with Mucosa Associated Lymphoma Tissue (MALT) lymphoma across multiple harmonized cohorts.
    Pezoulas VC, Kalatzis F, Exarchos TP, **Chatzis L**, Gandolfo S, Goules A, De Vita S, Tzioufas AG, Fotiadis DI.
    Annu Int Conf IEEE Eng Med Biol Soc. 2021 Nov;2021:1666-1669.

15. Akt Signaling Pathway Is Activated in the Minor Salivary Glands of Patients with Primary Sjögren's Syndrome.
    Stergiou IE, **Chatzis L**, Papanikolaou A, Giannouli S, Tzioufas AG, Voulgarelis M, Kapsogeorgou EK.
    Int J Mol Sci. 2021 Dec 14;22(24):13441.

16. Serum, but Not Saliva, CXCL13 Levels Associate With Infiltrating CXCL13+ Cells in the Minor Salivary Gland Lesions and Other Histologic Parameters in Patients With Sjögren's Syndrome.
    Chatzis L, Goules AV, Stergiou IE, Voulgarelis M, Tzioufas AG, Kapsogeorgou EK.
    Front Immunol. 2021 Aug 17;12:705079.

17. A prospective multicenter study assessing humoral immunogenicity and safety of the mRNA SARS-CoV-2 vaccines in Greek patients with systemic autoimmune and autoinflammatory rheumatic diseases.
    Tzioufas AG, Bakasis AD, Goules AV, Bitzogli K, Cinoku II, **Chatzis LG**, Argyropoulou OD, Venetsanopoulou AI, Mavrommati M, Stergiou IE, Pezoulas V, Voulgari PV, Katsimpari C, Katechis S, Gazi S, Katsifis G, Sfontouris CI, Georgountzos AI, Liossis SN, Papagoras C, Fotiadis DI, Skopouli FN, Vlachoyiannopoulos PG, Moutsopoulos HM.
    J Autoimmun. 2021 Dec;125:102743.

18. Sjögren's syndrome: one year in review 2022.
Manfrè V, **Chatzis LG**, Cafaro G, Fonzetti S, Calvacchi S, Fulvio G, Navarro Garcia IC, La Rocca G, Ferro F, Perricone C, Bartoloni E, Baldini C.
Clin Exp Rheumatol. 2022 Dec;40(12):2211-2224.

19. Liver Fibrosis in Primary Sjögren's Syndrome.
Androutsakos T, Voulgaris TA, Bakasis AD, Koutsompina ML, **Chatzis L**, Argyropoulou OD, Pezoulas V, Fotiadis DI, Papatheodoridis G, Tzioufas AG, Goules AV.
Front Immunol. 2022 Jun 15;13:889021.

20. Programmed Cell Death Protein 1 Axis Inhibition in Viral Infections: Clinical Data and Therapeutic Opportunities.
Tsiakos K, Gavrielatou N, Vathiotis IA, **Chatzis L**, Chatzis S, Poulakou G, Kotteas E, Syrigos NK.
Vaccines (Basel). 2022 Oct 7;10(10):1673.

21. Primary Sarcoidosis of the Adipose Tissue: A New Variant of Sarcoidosis.
Tsiakos K, Zoupas I, Vamvakaris I, Poulakou G, Syrigos K, **Chatzis L**.
Mayo Clin Proc. 2022 Jul;97(7):1403-1405.

22. The clinical phenotype of primary Sjögren's syndrome patients with lymphadenopathy.
Stergiou IE, **Chatzis LG**, Pezoulas VC, Baldini C, Fotiadis DI, Voulgarelis M, Tzioufas AG, Goules AV.
Clin Exp Rheumatol. 2022 Dec;40(12):2357-2362.

23. Patient-centered approaches for patients with systemic autoimmune rheumatic diseases: development and evolution.
Vlachoyiannopoulos PG, **Chatzis LG**, Goules AV, Argyropoulou OD, Englezopoulou A, Stergiou I, Voulgarelis M, Tsanakas P, Exarchos T, Gorgoulis VV, Fotiadis DI, Tzioufas AG.
Expert Rev Clin Immunol. 2022 Feb;18(2):125-133.

24. Clinical and laboratory findings of primary Sjögren's syndrome patients without sicca symptoms.

Chatzis LG, Koulouri V, Baldini C, Pezoulas VC, Voulgari PV, Skopouli FN, Fotiadis DI, Tzioufas AG, Goules AV.
Clin Exp Rheumatol. 2022 Dec;40(12):2298-2302.

25. Clinical picture, outcome and predictive factors of lymphoma in primary Sjögren's syndrome: results from a harmonized dataset (1981-2021).
Chatzis LG, Stergiou IE, Goules AV, Pezoulas V, Tsourouflis G, Fotiadis D, Tzioufas AG, Voulgarelis M.
Rheumatology (Oxford). 2022 Aug 30;61(9):3576-3585.

26. The Role of the Akt Signaling Pathway in Sjögren's Syndrome.
Kapsogeorgou EK, Stergiou IE, **Chatzis L**, Voulgarelis M, Vlachoyiannopoulos PG.
Mediterr J Rheumatol. 2023 Mar 31;34(1):113-116. doi: 10.31138/mjr.34.1.113. eCollection 2023 Mar.

27. Predicting lymphoma in Sjögren's syndrome and the pathogenetic role of parotid microenvironment through precise parotid swelling recording.
De Vita S, Isola M, Baldini C, Goules AV, **Chatzis LG**, Quartuccio L, Zabotti A, Giovannini I, Donati V, Ferro F, Rizzo MT, Manfrè V, Pegolo E, Voulgarelis M, Zaja F, Fanin R, Masaoutis C, Rontogianni D, Fotiadis DI, Ponzoni M, Tzioufas AG.
Rheumatology (Oxford). 2023 Apr 3;62(4):1586-1593.

28. Addressing the clinical unmet needs in primary Sjögren's Syndrome through the sharing, harmonization and federated analysis of 21 European cohorts.
Pezoulas VC, Goules A, Kalatzis F, **Chatzis L**, Kourou KD, Venetsanopoulou A, Exarchos TP, Gandolfo S, Votis K, Zampeli E, Burmeister J, May T, Marcelino Pérez M, Lishchuk I, Chondrogiannis T, Andronikou V, Varvarigou T, Filipovic N, Tsiknakis M, Baldini C, Bombardieri M, Bootsma H, Bowman SJ, Soyfoo MS, Parisis D, Delporte C, Devauchelle-Pensec V, Pers JO, Dörner T, Bartoloni E, Gerli R, Giacomelli R, Jonsson R, Ng WF, Priori R, Ramos-Casals M, Sivils K, Skopouli F, Torsten W, A G van Roon J, Xavier M, De Vita S, Tzioufas AG, Fotiadis DI.
Comput Struct Biotechnol J. 2022 Jan 7;20:471-484.

INVITED SPEAKER

- **Invited speaker** at the 19th scientific meeting of rheumatologists of northwestern Greece on the "Pathophysiology of Enthesitis" held in Ioannina, 20-21 January 2017.

- **Invited speaker** in the first symposium “Contemporary sight in inflammation and autoimmunity” presenting a puzzling case of autoimmune disease (ASIA syndrome) held in Alexandroupoli, 20-21 September 2019

- **Invited speaker** in the first symposium of Messinia Rheumatology days on the subject of “The role of interleukin 27”, held in Kalamata, 8-10 October 2020.

- **Invited speaker** in the thirteen symposium of ΕΠΕΜΥ (Scientific community for the musculoskeletal health) on the subject of “Clinical Phenotypes in systemic autoimmune diseases) held in Halkidiki, 2- 5 September 2021

- **Invited speaker** in the second symposium of Messinia Rheumatology days on the subject of “Clinical, molecular, and histologic stratification of patients with Sjogren’s Syndrome” held in Kalamata, 13-15 October 2022

- **Invited speaker** at the 55° Congreso Argentino de Reumatología on the subject of “Precision Medicine in Sjogren's Syndrome” held in Buenos Aires, 12-15 October 2022

- **Invited speaker** at the 28th Hellenic rheumatology symposium at a roundtable on the subject of “Important publications in the last 2 years from Greek Research Centers” held in Athens, 8-11 December 2022

- **Invited speaker** along with prof Athanasios Tzioufas at the 28th Hellenic rheumatology symposium at a meet the experts session on “Sjogren’s Syndrome” held in Athens, 8-11 December 2022

- **Invited speaker** at the 25th scientific meeting of rheumatologists of northwestern Greece on the subject of "Biomarkers at the histology level of autoimmune epithelitis. Present and future", held in Ioannina, 20-21 January 2023

- **Invited participant** at the "4th international workshop – the precision medicine in profiling patients with autoimmune diseases: regarding therapy. From pathology to treatment, what evidence in rheumatic and autoimmune diseases?" in the working group for Sjogren Syndrome held in Rome, 17-18 February 2023

- **Invited speaker** in the second symposium "Contemporary sight in inflammation and autoimmunity" presenting a puzzling case of an autoiflammatory disease "A new mutation of the PSTPIP1 gene causes a periodic fever syndrome mimicking Stills disease with novel vascular characteristics. Molecular and functional studies." held in Alexandroupoli, 22-23 September 2023

- **Invited speaker** in the first 1st Annual Conference for Sjögren patients on the subject of "NeceSSity. Patients' contribution from clinician perspective" and the "Unmet needs from the perspective of clinicians" held in Athens, 13-14 October 2023

- **Keynote speaker** in the postgraduate (Masters of Science) program of the Athens Dentistry School titled "Pathology, Surgery and Radiology of the oral cavity" on the subject "oral involvement in the autoimmune diseases: Description and pathogenesis"

ORAL PRESENTATIONS OF SCIENTIFIC WORK

- **Oral presenter** in EULAR 2019 held in Madrid, Spain on the subject of "COMPARISON OF CLINICAL PHENOTYPE, SEROLOGICAL CHARACTERISTICS AND HISTOLOGIC FEATURES OF MALES VS FEMALES PATIENTS WITH PRIMARY SJÖGREN'S SYNDROME (PSS)"

- **Oral presenter** in EULAR 2020 held online on the subject of "The differences between SS patients with combined seronegativity and anti-Ro positivity",3-6 June 2020

- **Oral presenter** in EULAR 2021 held online on the subject of "Severity of labial minor salivary gland focus score and future lymphoma development in Sjögren's Syndrome", 2-5th June 2021

- **Oral presenter** in EULAR 2021 held online on the subject of "Saliva and serum levels of CXCL13: association with the severity of salivary gland lesions and lymphoma in patients with Sjögren's syndrome (SS)", 2-5th June 2021

- **Oral presenter** in EULAR 2021 held online on the subject of "Sjögren's Syndrome associated lymphomas: Clinical description and 10-year survival", 2-5th June 2021

- **Oral presenter** in the 15th International Symposium on Sjögren's Syndrome held in Rome on the subject of "TRANSITIONAL CD5+ B CELLS ARE ASSOCIATED WITH AUTOREACTIVITY IN PSS PATIENTS", 7-10 September 2022

- **Oral presenter** in the 15th International Symposium on Sjögren's Syndrome held in Rome on the subject of "CYTOKINE/CHEMOKINE EXPRESSION IN PRIMARY SJÖGREN'S SYNDROME PATIENTS", 7-10 September 2022

- **Oral presenter** in the fourteen symposium of ΕΠΕΜΥ held in Rhodes on the subject of "DEEP SPATIAL PROFILING OF SJÖGREN SYNDROME PATIENTS BY IMAGING MASS CYTOMETRY: PRELIMINARY RESULTS", 7-10 September 2022

Didactic work

- **2017-today**: Review of the literature lectures and research meetings.
  Department of Pathophysiology, UKNA, Athens, Greece.
- **2021-today**: Clinical training and lectures of medical students (at the 6th year of training).
  Department of Pathophysiology, UKNA, Athens, Greece.
- **2023**: Interuniversity Rheumatologic classes
  Sjogren Syndrome: Epidemiology - Diagnosis - Clinical Phenotype
- **2022**: CIVIS, Athens, Greece

Title: B cell “The good the bad and the ugly”.

- **2020-2022**: “Internal medicine” of the postgraduate program “Internal medicine, Surgery and Oral dental radiology, Dental Department of NKUA, Athens, Greece

  Title: Differential diagnosis of arthritis: from pathogenesis to clinical practice.

RESEARCH PROJECT PARTICIPATIONS

- Participation in the multicenter research work of the **Hellenic Nephrology Society** under the title: "Managing Phosphorus and Magnesium Levels in Pre-Dialysis Patients with Chronic Kidney Disease: The HSN-01 study”.

-Participation in the multicenter research project entitled "Observational study of the response of Epoetin Theta (EPORATIO) as well as the clinical and inflammatory factors affecting the response to treatment in patients with anemia of chronic renal disease undergoing hemodialysis or before, in the everyday clinical practice in Greece: The REPORT study".

-Participation in the multicenter research project entitled Study of Safety and Efficacy of Multiple Doses of CFZ533 in Two Distinct Populations of Patients With Sjögren's Syndrome (TWINSS)

- Co-facilitated along with Prof Athanasios Tzioufas the Thematic 8 (Chronic inflammatory and autoimmune diseases) of CIVIS Health Hub with 5 Participating centers: University of Athens (UOA), Université libre de Bruxelles (ULB), University Hospital Tubingen (UKT), University of Glasgow (UG), “Autónoma de Madrid” University (UAM). In the context of the program we organized 2 webinars and a summer school in Athens (15-19/06/2022).

-**Abstracts presenter** in the ACR meeting (2019) in Atlanta, GA with the following titles:

"Comparison of Clinical Phenotype, Serological Characteristics and Histologic Features Between Males and Females Patients with Primary Sjögren’s Syndrome (pSS)"

"Data Driven Prediction Lymphoma Model and 10-year Overall Survival Rates of a Large Harmonized Cohort of Patients with Primary Sjögren’s Syndrome Associated Lymphomas"

-Oral Communication at the “7th Panhellenic Congress of Medical Biochemistry - 3rd Symposium of Laboratory Hematology and Blood Donation” titled "Pathological values of T-high sensitivity troponin in patients with diagnoses other than acute myocardial infarction (OEM)”.

-Attendance in the European Society of Hypertension Summer School From 7th September to 13th September 2018, in Les Diablerets, Switzerland

-Good Clinical Practice certificate holder after successfully completing the “GCP Compliance Training for HCPs V2.0” (11/09/2019)

-Harvard medical school certificate of achievement in HMX Fundamentals Immunology (Final exam score: 100%, Overall score: 97%)

- Harvard medical school certificate of achievement in HMX Fundamentals Physiology
(Final exam score: 88%, Overall score: 93%)

Languages

Greek Native speaker

English Excellent
-C2 proficiency, previously known as Cambridge English Proficiency (CPE), University of Cambridge, Grade “A”.
-Certificate of Proficiency in English (ECPE) – University of Michigan

French Basic skills

Functional systems (Technical Skills & Competences)

- Excellent use of Microsoft Office (Word, Excel, Power Point, Access, Visio).
- Apple – Mac os, iOS
- Linux

Social Skills & Competences

- Team spirit: Gained with group work and research activities pursued in the context of my studies as well as my professional career.
- Communication skills: Working in several different medical environments has provided me the chance to successfully adjust in every circumstance and developed my communication skills and cooperative attitude.

Interests & Hobbies

-Taking part in sports (basketball, soccer, winter ski).

-Travelling around the world.

-Music enthusiast with semi-professional engagement.

Driving License:

Available

Acknowlegments

The culmination of this research journey has been made possible through the invaluable contributions, unwavering support, and the collective efforts of numerous individuals and organizations. In expressing my profound gratitude, I wish to acknowledge those who have played pivotal roles in shaping this work.

Words cannot express my gratitude to the chair of my committee, my supervisor and my professor, Athanasio G Tzioufa. I am profoundly grateful for the guidance, mentorship, and wisdom you have generously shared with me throughout this research endeavor. Your dedication to nurturing intellectual growth, your patience in addressing my inquiries, and your unwavering commitment to excellence have been my guiding light. Your belief in the potential of this research has been an immense source of motivation. He was the one who inspired me to the unbeaten path of autoimmunity.

I extend my heartfelt appreciation to my esteemed committee members for their critical insights, expert advice, and rigorous evaluation of this work. Professor Panayiotis Vlachoyiannopoulos, your extraordinary ability to think outside of the box, coupled with your profound knowledge of immunology, has been a constant source of inspiration throughout this research endeavor. Your artistic skills, combined with your scientific prowess, have not only enriched my perspective but have also been imperative in shaping the direction of this work. Associate Professor Efstathia Kapsogeorgou, your pivotal role as our laboratory expert cannot be overstated. You have been the bedrock upon which all laboratory work for this thesis has been built. Your organizational prowess, dedication, and meticulous attention to detail have ensured the successful execution of countless experiments and research activities integral to this doctoral thesis.

I could never leave out Assistant Professor Andrea Goule, whose countless hours discussing in theory and in practice every forwarding step made a substantial contribution to my thesis project, deserving special recognition. Throughout the course of this endeavor, he invested countless hours engaging in in-depth discussions that spanned both the clinical and laboratory perspective. These conversations, often extending into late nights and early mornings, were not just moments of academic exchange but transformative experiences.

I would also like to thank Professor Jacque Olivier Pers who taught me one simple but unbelievably valuable skill. How to conceptualize, organize, conduct, and write a comprehensive experimental work on Sjogren Syndrome.

I wish to extend my sincere gratitude to Professor Dimitrios Fotiadis and Mr. Vasilis Pezoulas for their invaluable contributions in the fields of bioinformatics and data analysis within our collaborative projects. Their expertise has played an indispensable role in the successful outcome of this endeavor, and their unwavering dedication to advancing our research and identifying the most crucial and independent variables has been truly instrumental.

I would also like to express my deep appreciation to our esteemed Sjögren's syndrome collaborators from Greece, including Professor Moutsopoulos, Professor Skopouli, Professor Mavragani, and Professor Voulgari, as well as our international partners, such as Professor Baldini and Professor De Vita, who generously shared their cohort data. Their collaborative efforts were pivotal in establishing a large cohort, which formed the foundation of numerous projects within this thesis. On the same prism, I would also like to thank Ariel Argyropoulou for her valuable support in taking care of patients with Sjogren's Syndrome.

I am immensely grateful to my girlfriend Savia for her boundless love but most importantly her remarkable patience. Her enduring encouragement and understanding have been my rock during the many trials of this academic journey that had a serious impact on the time spent together. Her presence has made this achievement all the more meaningful.

To my uncle, who has been my silent pillar of support and an invaluable secret contributor to all of my projects.

To my parents, Grigorio and Evangelia, and my sibling, Stamatios, your boundless love, unwavering support, and belief in my abilities have been the cornerstone of my academic journey. Your sacrifices, encouragement during moments of self-doubt, and your shared sense of accomplishment today are a testament to our shared dedication. Dad, your integrity, tireless work ethic and your compassionate care for your patients have set the highest standards for me, struggling to follow in your footsteps. Mom, your commitment to our family and your boundless kindness have shaped the person I am today being a constant source of inspiration. Together, you have made me who I am today, and I will forever cherish your love and guidance.

Abbreviations

A20: Tumor necrosis factor alpha-induced *protein* 3 (TNFAIP3),

aCCP: Anti–cyclic citrullinated peptide,

ACE2: angiotensin converting enzyme 2,

ACTH: adrenocorticotropic hormone,

AECG: American-European Consensus Group (classification criteria for Sjögren's syndrome)

AMPK: AMP-activated kinase,

ANCA: anti-neutrophil cytoplasmic antibody,

BAFF: B-cell activating factor,

Bax: Bcl-2 associated X protein,

Bcl: B-cell lymphoma,

BCR: B-cell receptor,

BTK: Bruton tyrosine kinase,

CCL: C-C motif ligand,

CD: cluster of differentiation,

CDR3: complementarity-determining region,

CMV: cytomegalovirus,

CTLA4: cytotoxic T-lymphocyte-associated protein 4,

CXCL: C-X-C motif ligand,

DAMPs: danger-associated molecular patterns,

DMARDs: Disease-Modifying Antirheumatic Drugs

DHEA: Dehydroepiandrosterone,

eGCLS: Ectopic germinal center like structures

EGF: epithelial growth factor,

EBER: Epstein-Barr virus encoded small RNA,

EBNA2: Epstein-Barr virus nuclear antigen 2,

EBV: Epstein-Barr virus,

ENA: Extractable Nuclear Antigens

eQTL: expression quantitative trait loci,

EULAR: European League Against Rheumatism

EWAS: epigenome-wide association study,

ESR: erythrocyte sedimentation rate,

ESSDAI: EULAR Sjögren's syndrome disease activity index

ESSPRI: EULAR Sjögren's Syndrome Patient-Reported Index

Fas (or CD95): tumor necrosis factor receptor superfamily member 6,

FasL: Fas ligand,

FDc: Follicular dendritic cells

FGF: fibroblast growth factor,

FoxP3: Forkhead box protein 3,

GC: Germinal Center

GH: growth hormone,

GN: glomerulonephritis

GWAS: genome-wide association study,

GM-CSF: granulocyte-macrophage colony-stimulating factor,

HCV: hepatitis C virus,

HHV: human herpes virus,

HGF: hepatocyte growth factor,

HIF1a: hypoxia induced factor 1 subunit a,

HIV: human immunodeficiency virus,

HPA axis: hypothalamo – pituitary – adrenocortical axis,

HTLV-1: human T-lymphotropic virus 1,

ICAM: intercellular adhesion molecule,

ICOS: inducible T-cell co-stimulator,

ICOSL: ICOS-ligand,

IFN: interferon,

IGF-1: insulin-like growth factor 1,

IkB-ζ: NFkB inhibitor ζ,

IL: interleukin,

IP3: inositol 1,4,5-triphosphate,

IP3R: IP3 receptor,

IRAK-1: intereukin-1 receptor associated kinase,

JAK: Janus kinase,

LFA-1: lymphocyte function-associated antigen 1,

LG: lacrimal gland,

M3R: muscarinic acetylcholine receptor M3,

MALT: mucosa associated lymphoid tissue,

MHC: major histocompatibility complex,

MICA: MHC class I polypeptide-related sequence A,

miRNA: microRNA

MMPs: matrix metalloproteinases,

MSG: minor salivary gland,

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells,

NGF: nerve growth factor,

OSDI: Ocular Surface Disease Index

PAMPs: pathogen-associated molecular patterns,

PBMC: peripheral blood mononuclear cell,

PDC: Plasmacytoid Dendritic Cells

PNS: peripheral nervous disease

PPARγ: peroxisome-proliferator-activated receptor-γ,

PRO: Patients' reported outcomes

PRR: pattern recognition receptor,

pSS: Primary Sjogren Syndrome

RA: Rheumatoid Arthritis,

RF: Rheumatoid Factor

RING: really interesting new gene,

SG: salivary gland,

SLE: Systemic Lupus Erythematosus,

SGECs: salivary gland epithelial cells,

SGUS: Salivary Gland Ultrasound

SS: Sjogren's Syndrome

TACI: transmembrane activator and CAML interactor,

TSLP: Serum thymic stromal lymphopoietin

SNARE: soluble N-ethyl-maleimide-sensitive factor attachment protein receptors,

STAT: signal transducer and activator of transcription,

STING: stimulator of interferon genes,

TCA: tricarboxylic acid,

TCR: T-cell receptor,

TGF: transforming growth factor,

TLR: toll-like receptor,

TNFa: tumor necrosis factor a,

UWS: Unstimulated Whole Saliva

UPR: unfolded protein response,

VCAM: vascular cell adhesion molecule,

VIP: vasoactive intestinal peptide

TABLE OF CONTENTS

Abstract

Introduction: Primary Sjögren's syndrome is a complex, autoimmune disease with distinct clinical phenotypes and variable outcomes. The systemic form of the disease is characterized by immune complex-mediated manifestations and is complicated by lymphoma as a result of a polyclonal B cell hyperactivity that is evolving into B cell malignancy. The clinical phenotype of Sjögren's syndrome can vary from person to person, and its symptoms can range from mild to severe. Since Sjögren's syndrome can have such a diverse range of clinical manifestations, one of the primary goals of research in Sjogren is to revolutionize the way we stratify and classify pSS patients based on clinical phenotypes and novel useful biomarkers.

Methods: Cumulative clinical, laboratory and histologic data of consecutive SS patients who fulfilled the 2016 ACR/EULAR classification criteria and were followed up, in 5 centers from Greece (University of **A**thens, **H**arokopio University, University of **I**oannina) and Italy (University of **P**isa, University of **U**dine) (**UPAHI** group), were collected, integrated, and analyzed in a unified dataset according to each project of the PhD thesis [both study group(s) and control group(s)]. For the prognostic models the Fast-Correlation based feature selection (FCBF) algorithm was applied on the unified datasets to identify potentially independent variables for constructing a logistic regression (LR) model with the appropriate feature of interest as an outcome. Statistical analysis for categorical data was performed by $\chi 2$ test with Yates correction or Fisher exact when cell counts <5 patients and for numerical data t test or Mann-Whitney, after Shapiro-Wilk normality test. CXCL13 levels at serum and saliva samples were measured by a commercially available ELISA (sensitivity: 1 pg/ml; Abcam) according to manufacturer's instructions.

Results: Male SS patients carry an increased risk of lymphoma development. Although statistics showed no difference in classical lymphoma predictors compared to females, data-driven analysis revealed gender and lymphadenopathy as independent lymphoma-associated features. Furthermore, Focus score evaluated at the time of SS diagnosis, is an independent lymphoma associated risk factor and may serve as a predictive biomarker for the early diagnosis of SS-associated lymphomas. A second minor salivary gland biopsy is patients with a FS ≥ 4, 4 years after SS diagnosis and in those with FS < 4 and a history of SGE, at 9-years, may contribute to an earlier lymphoma diagnosis and a better prognosis. Patients with combined seronegativity [triple seronegativity anti-Ro/SSA(-), anti-La/ SSB(-), RF(-) and ANA(+) and quadruple seronegativity [anti-Ro/SSA(-), anti-La/SSB(-), RF(-) and ANA(-)] accounts for almost 9% of total SS population and is associated with a milder clinical phenotype, partly attributed to the absence of rheumatoid factor. Serum levels of CXCL13 were associated with

histologic, serologic, and clinical features indicative of more severe pSS while saliva levels did not reveal clinically significant correlations. MALT lymphomas constitute the majority of lymphomas (92/121, 76.0%) followed by diffuse large B-cell lymphomas (DLBCL) (11/121, 9.0%) and nodal marginal zone lymphomas (NMZL) (8/121, 7%). The 10-year overall survival and event free survival rates were 79% and 45.5% for MALT, 40.9% and 24.2% for DLBCL and 46% and 31% for NMZL. Cryoglobulinemia, focus score and the total EULAR SS Disease Activity Index (ESSDAI) composite index at SS diagnosis were proven independent MALT lymphoma predictors. In addition, it was shown that patients presenting non-lymphoma related stable lymphadenopathy constitute a subgroup of younger individuals with B-cell hyperactivity while pSS patients without sicca complaints constitute a distinct phenotype involving younger patients, sharing common immunopathologic mechanisms with typical sicca patients.

Conclusion: In the context of Sjögren's syndrome (SS), the presence of male gender, a high focus score, non-lymphoma stable lymphadenopathy, and cryoglobulinemia collectively represent a cluster of characteristics that often manifest in SS patients with a more severe clinical phenotype related to future lymphoma development. Conversely, seronegativity and a low focus score tend to be associated with a milder clinical presentation. Conversely patients who exhibit seronegativity and a low focus score tend to experience a milder clinical manifestation of the disease. This practice of categorizing patients based on easily attainable clinical parameters, a process referred to as clinical phenotyping, holds considerable value for healthcare practitioners entrusted with the care of individuals diagnosed with Sjögren's Syndrome. Such categorization aids in risk assessment and contributes to more informed clinical management decisions. This PhD thesis is centered on the clinical phenotyping of patients with Sjögren's Syndrome.

Περίληψη

Εισαγωγή: Το πρωτοπαθές σύνδρομο Sjögren είναι μια σύνθετη, αυτοάνοση νόσος με διακριτούς κλινικούς φαινότυπους και ποικίλες κλινικές εκδηλώσεις. Η συστηματική μορφή της νόσου χαρακτηρίζεται από εκδηλώσεις που προκαλούνται από την παρουσία ανοσοσύμπλεγματων και μπορεί να επιπλακεί από την ανάπτυξη λεμφώματος ως αποτέλεσμα της πολυκλωνικής υπερδραστηριότητας του Β κυττάρου που σταδιακά εξελίσσεται σε λέμφωμα. Ο κλινικός φαινότυπος του συνδρόμου Sjögren μπορεί να διαφέρει από άτομο σε άτομο και τα συμπτώματά του μπορεί να κυμαίνονται από ήπια έως πολύ σοβαρά. Δεδομένου ότι το σύνδρομο Sjögren μπορεί να έχει τόσο ποικίλο φάσμα κλινικών εκδηλώσεων, ένας από τους πρωταρχικούς στόχους της έρευνας στο Sjogren είναι να αλλάξει τον τρόπο με τον οποίο διαστρωματώνουμε και ταξινομούμε τους ασθενείς με pSS με βάση νέους κλινικούς φαινοτύπους και χρήσιμους βιοδείκτες.

Μέθοδοι: Συλλέχθηκαν, ενσωματώθηκαν και αναλύθηκαν σε μία ενιαία κοορτή τα συσσωρευτικά κλινικά, εργαστηριακά και ιστολογικά δεδομένα συνεχόμενων ασθενών με SS, οι οποίοι πληρούν τα κριτήρια ταξινόμησης της ACR/EULAR του 2016 για το σύνδρομο Sjogren και παρακολουθούνται σε 5 κέντρα ανά την Ελλάδα (Πανεπιστήμιο Αθηνών, Πανεπιστήμιο Χαροκοπείου, Πανεπιστήμιο Ιωαννίνων) και την Ιταλία (Πανεπιστήμιο Πίζα, Πανεπιστήμιο Ουντίνε) (ομάδα UPAHI), σύμφωνα με κάθε μελέτη της διδακτορικής διατριβής [τόσο στην πειραματική ομάδα όσο και στην ομάδα ελέγχου]. Για τα προγνωστικά μοντέλα, εφαρμόστηκε ο αλγόριθμος Fast-Correlation based feature selection (FCBF) στα ενιαία σύνολα δεδομένων για την αναγνώριση πιθανών ανεξάρτητων μεταβλητών για την κατασκευή ενός μοντέλου λογιστικής παλινδρόμησης (LR) με την κατάλληλη μεταβλητή ενδιαφέροντος ως αποτέλεσμα (outcome). Η στατιστική ανάλυση για κατηγορικά δεδομένα πραγματοποιήθηκε με το τεστ χ2 με διόρθωση Yates ή Fisher exact test όταν ο αριθμός μεταβλητών ήταν <5, και για αριθμητικά δεδομένα με το t τεστ ή το Mann-Whitney, μετά από έλεγχος της κανονικότητας των δεδομένων Shapiro-Wilk. Τα επίπεδα του CXCL13 στα δείγματα αίματος και σάλιου μετρήθηκαν με εμπορικά διαθέσιμη ELISA (ευαισθησία: 1 pg/ml; Abcam) σύμφωνα με τις οδηγίες του κατασκευαστή.

Αποτελέσματα: Οι άνδρες ασθενείς με SS διατρέχουν αυξημένο κίνδυνο ανάπτυξης λεμφώματος. Αν και η ανάλυση δεν ανέδειξε διαφορά στους κλασσικούς προγνωστικούς παράγοντες ανάπτυξης λεμφώματος σε σύγκριση με τις γυναίκες, η data-driven analysis ανάλυση αποκάλυψε το φύλο και τη λεμφαδενοπάθεια ως ανεξάρτητα χαρακτηριστικά που σχετίζονται με το λέμφωμα. Επιπλέον, το Focus score που αξιολογήθηκε τη στιγμή της διάγνωσης, είναι ένας ανεξάρτητος παράγοντας κινδύνου που σχετίζεται με το λέμφωμα και μπορεί να χρησιμεύσει ως προγνωστικός βιοδείκτης για

την έγκαιρη διάγνωση των λεμφωμάτων που σχετίζονται με SS. Μια δεύτερη βιοψία σιελογόνων αδένων σε ασθενείς με FS ≥ 4, 4 χρόνια μετά τη διάγνωση SS και σε αυτούς με FS < 4 και ιστορικό SGE, στα 9 χρόνια, μπορεί να συμβάλει σε μια πρώιμη διάγνωση λεμφώματος και σε καλύτερη πρόγνωση. Ασθενείς με συνδυασμένη οροαρνητικότητα [τριπλή οροαρνητικότητα anti-Ro/SSA(-), anti-La/ SSB(-), RF(-) και ANA(+) και τετραπλή οροαρνητικότητα [anti-Ro/SSA(-), anti-La Τα /SSB(-), RF(-) και ANA(-)] αντιπροσωπεύουν σχεδόν το 9% του συνολικού πληθυσμού SS και σχετίζεται με έναν ηπιότερο κλινικό φαινότυπο, που εν μέρει αποδίδεται στην απουσία ρευματοειδούς παράγοντα. Τα επίπεδα του CXCL13 στον ορό συσχετίστηκαν με ιστολογικά, ορολογικά και κλινικά χαρακτηριστικά ενδεικτικά πιο σοβαρού SS, ενώ τα επίπεδα του CXCL13 στη σίελο δεν αποκάλυψαν κλινικά σημαντικές συσχετίσεις. Τα λεμφώματα MALT αποτελούν την πλειοψηφία των λεμφωμάτων (92/121, 76,0%) ακολουθούμενα από διάχυτα λεμφώματα μεγάλων Β-κυττάρων (DLBCL) (11/121, 9,0%) και λεμφώματα οριακής ζώνης (NMZL) (8/121, 7%) . Τα ποσοστά 10ετούς συνολικής επιβίωσης (OS) και επιβίωσης χωρίς συμβάν (EFS) ήταν 79% και 45,5% για το MALT, 40,9% και 24,2% για το DLBCL και 46% και 31% για το NMZL. Η κρυοσφαιριναιμία, το Focus Score και ο συνολικός δείκτης ενεργότητας της νόσου (ESSDAI) στη διάγνωση του συνδρόμου αποτελούν ανεξάρτητους προγνωστικούς παράγοντες ανάπτυξης λεμφώματος MALT. Επιπλέον, φάνηκε ότι οι ασθενείς που παρουσιάζουν σταθερή λεμφαδενοπάθεια που δεν σχετίζεται με το λέμφωμα αποτελούν μια υποομάδα νεότερων ατόμων με υπερενεργοποίηση Β-κυττάρων, ενώ οι ασθενείς με SS χωρίς συμπτώματα ξηρότητας αποτελούν έναν ξεχωριστό φαινότυπο που περιλαμβάνει νεότερους ασθενείς, που μοιράζονται κοινούς ανοσοπαθολογικούς μηχανισμούς με τυπικούς ασθενείς με συμπτώματα ξηρότητας.

Συμπέρασμα: Στο πλαίσιο του συνδρόμου Sjögren, το ανδρικό φύλο, το υψηλό Focus Score (>=4), η λεμφαδενοπάθεια που δεν σχετίζεται με λέμφωμα και η κρυοσφαιριναιμία αντιπροσωπεύουν συλλογικά εκδηλώσεις που εμφανίζονται σε ασθενείς με SS με πιο σοβαρό κλινικό φαινότυπο και αυξημένη πιθανότητα ανάπτυξης λεμφώματος στο μέλλον. Από την άλλη πλευρά, η οροαρνητικότητα και το χαμηλό Focus score τείνουν να σχετίζονται με ηπιότερη κλινική εικόνα. Αυτή η κατηγοριοποίηση των ασθενών με βάση εύκολα προσβάσιμες κλινικές παραμέτρους, μια διαδικασία που αναφέρεται ως κλινική φαινοτύπηση, έχει σημαντική αξία για τους επαγγελματίες υγείας που παρακολουθούν ασθενείς με σύνδρομο Sjögren. Η παρούσα διδακτορική διατριβή επικεντρώνεται στον κλινικό φαινότυπο των ασθενών με σύνδρομο Sjögren’s.

Chapter 1: Historical Perspectives

1.1 Sjogren's syndrome before Henrick Sjogren

Although the names of many ancestral physicians are etched in medical history through eponymous descriptions, Sjogren syndrome stands out in its richness and historical complexity relating to its author. Not only does the disease syndrome bear the name of the great ophthalmologist that devoted his life to its description and recognition, but his precise diagnostic methods are still in use today, including his belief, later to be confirmed, that "sicca symptoms" can accompany rheumatoid arthritis and represent a systemic disease. More significant to note is that his work was rejected by his peers, a situation mirrored today in the experience of many Sjogren Syndrome patients whose complaints about their life restricting dryness symptoms are misunderstood, minimized, or dismissed [1]. Undoubtedly, patients with Sjogren syndrome existed before Henrik Sjögren's doctoral thesis, and historical traces of SS can be found in a number of case reports, especially toward the end of the nineteenth century.

The first report of a dry mouth can be traced back in 1868 when Dr A.G. Bartley requested assistance about a 77-year-old patient, with a question to the editor of the Medical Times and Gazzete of London. She had experienced hyposalivation, loss of taste, and tooth decay, even though she felt "perfectly well and cheery". In 1882, German ophthalmologist Theodor Karl Gustav von Leber (1840–1917) described an entity called "keratitis filamentosa" (filamentous keratitis), a condition characterized by the formation of filaments/threads that adhere to the corneal surface causing a foreign body sensation, in a tear-deficient dry eye [2]. However, in his report, corneal filaments were associated with a virus infection, known as herpes keratitis infection [3]. Following those reports, a number of cases about patients with dryness of the mouth and eyes were published. Notably, the term xerostomia was coined by Dr WB Hadden, a compound word borrowing from the Greek words ξηρός (xero)= dry and στόμα (stomia)= mouth, while presenting a case report to his colleagues at the Clinical Society of London on March 9, 1888. His narrative description of the 65-year-old woman who complained of a dry mouth for several months was vivid and an apt description of a typical Sjogren's syndrome patient. Her symptoms included difficulty in swallowing, frequent fluid intake, and being unable to cry, though her overall health status was fine. Dr. Haddon described her

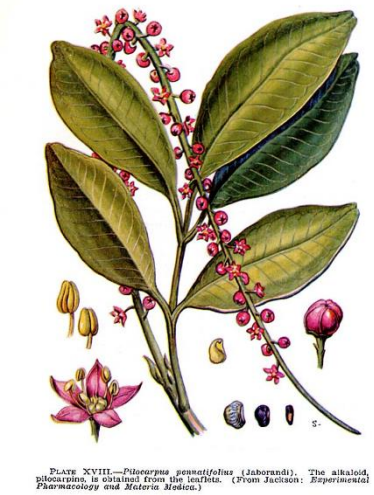
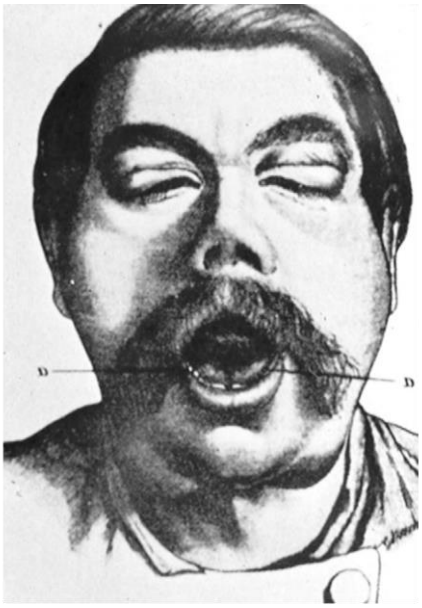


PLATE XVIII.—*Pilocarpus pennatifolius* (Jaborandi). The alkaloid, pilocarpine, is obtained from the leaflets. (From Jackson: *Experimental Pharmacology and Materia Medica.*)

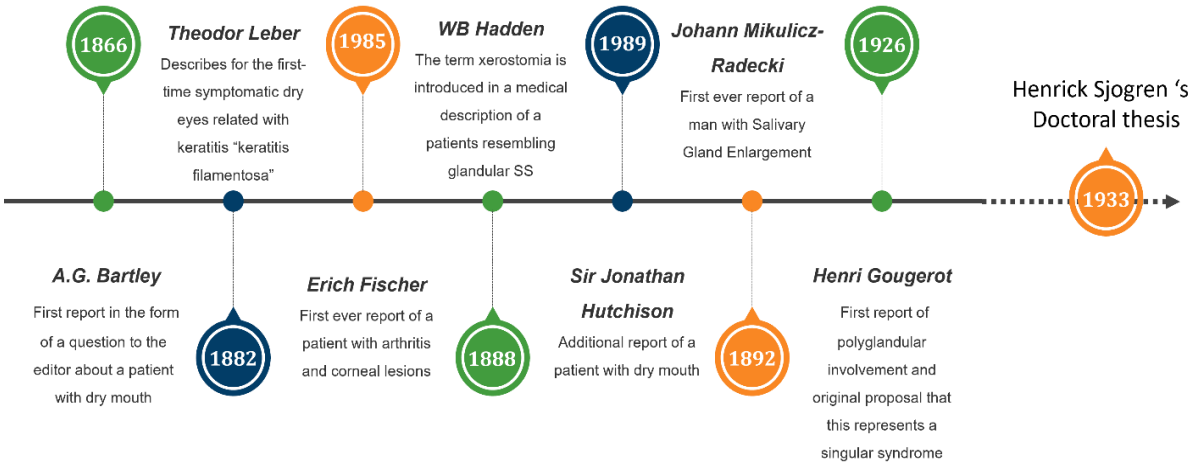
Pilocarpus pennatifolus (Jaborandi) derived form the Greek Pilo (hat) + carpus (fruit). About 0.5-0.8% of the total weight of the leaves is pilocarpine.

tongue as “red, devoid of epithelium and cracked in all directions like a crocodile’s skin”. He continued, “No tears appeared when she tried to cry.” Intriguingly, after she was treated with the use of an alkaloid called tincture of jaborandi three times a day, her mouth dryness improved significantly. The active substance in of jaborandi tincture is pilocarpine, the first FDA approved symptomatic treatment of dryness in SS, more than a century later. [4]. It is highly likely that Dr WB Hadden was treating a patient with Sjogren’s syndrome. Similarly, Sir Jonathan Hutchison (1828-1913) of London, best known for his description of 'Hutchison teeth' in congenital syphilis described a patient with dry mouth and Dr Erich Fischer from Germany reported in 1889 a woman with dryness induced corneal lesions and articular involvement in the form of arthritis [5].

Just a few years later, glandular enlargement was described by the Polish surgeon Dr. Johannes Freiherr von Mikulicz-Radecki (1850-1905) in a 42-year-old farmer who presented with painless swelling of the parotid, lacrimal, submandibular and sublingual glands, with massive infiltration of these glands by mononuclear centrocyte-like cells, a clinical constellation that still borrows his name [6]. The first presentation of the case report was given in 1888 while a more detailed description was published in 1892 in a special issue dedicated to the renowned surgeon, Theodor Billroth. The patient died 14 months later. It is not far-fetched to assume that he was suffering from MALT lymphoma. Less than 20 years later Otto Napp reported that these symptoms are not indicative of a single disease and can be seen in a variety of disorders such as sarcoidosis, lymphoma, tuberculosis, and plaque [7]. Despite the involvement of many physicians in the antecedent descriptions of Sjogren’s syndrome, it was Henri Gougerot whose name got attached to the disease. Gougerot was an eminent French dermatologist with prolific and valuable scientific contributions. He wrote in reference to 3 patients with multiglandular dryness involving the eyes, mouth, nose, and vagina together with salivary gland atrophy. His contribution is still honored in France where his name accompanies that of Sjogren’s in the nomenclature of the disease [8,9].

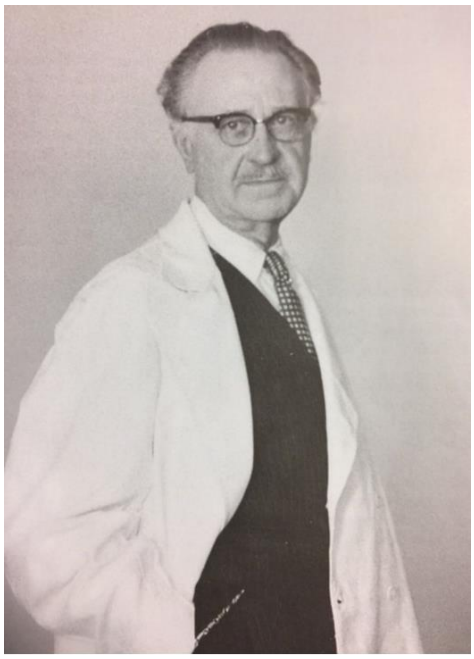


Parotid, lacrimal, submandibular and sublingual enlargement.
(Reprinted from Mikulicz)



Timeline of milestones that led to Henrick Sjogren seminal doctoral thesis

1.2 Henrick Sjogren



Henrick Sjogren

The modern notion of the systemic nature of the disease was elegantly built by Henrik Samuel Conrad Sjögren in 1933, and remains valid today. To be clear, Henrik Sjögren was not a rheumatologist but an ophthalmologist. He was born in Köping, a small town near Stockholm, on the 23rd of July 1899. He attended the medical school at the Karolinska Institutet, graduating as a medical doctor in 1927. The man who was later to see his name attached to the disease, encountered the first patient with dry eyes in 1930. It was a 49-year-old woman with glandular hypofunction and joint pain for about 6 years [10]. She was visiting Dr Sjogren because of eye discomfort. This pattern of symptoms intrigued the young ophthalmologist and, along with 4 other similar cases, he put together his initial publication in Hygiea, the Proceedings of the Swedish Medical Association [11]. In that article, with the help of his wife, Maria Hellgren, daughter of a well-known ophthalmologist, he introduced the neologism 'keratoconjunctivitis sicca,' which means inflammation of the conjunctiva resulting in ocular dryness. He also elegantly described the diagnostic methods used to stain the damaged epithelium by applying either 1% Bengal rose or a methylene blue staining. At that point Henrik Sjögren was well aware that each symptom separately, had been described before: inflammation in conjunctiva "keratitis filamentosa" by Leber, the clinical spectrum of a dry mouth by Dr WB Hadden, the combined multiorgan glandular hypofunction in the works of Henri Gougerot, and the possibility of articular involvement by Doctor Erich Fischer. Fascinated by what was reported, he continued working on similar cases, being able to collect 19 of

them that he published in his now classic 150-page doctoral thesis [12], written in German, the international language of science and letters in Sweden in the prewar era. All 19 patients were women, 13 had signs of arthritis (10 had arthritic changes demonstrated by X-ray), 2 had parotid gland enlargement and almost all were perimenopausal. Henrik Sjögren concluded that the constellation of symptoms constituted a novel nosological entity, with symptomatology far exceeding the ocular surface, thus pointing out the systemic nature of the disease. Besides the clinical details, the manuscript included a pathological part, with microscopic analysis of lacrimal glands and conjunctiva. In addition, autopsy results were presented from a patient that had died including a parotid gland examination showing a round cell infiltration.

Henrik Sjögren defended his doctoral thesis on the 8th of May, 1933, but even though many aspects were recognized for their scientific value, he also received harsh criticism by the board of examiners. He earned a grade of 1.5 out of 3, which disqualified him from a "Docent" (Teaching) grade that was a prerequisite for an academic career [13]. But this did not deter him in pursuing a medical and scientific path. Indeed, the publication of his thesis in Acta Ophthalmologica Scandinavica paved the way for his international recognition. In this, a very important milestone was the English translation of his thesis by the Australian Ophthalmologist Dr Bruce Hamilton in 1943, who later became close friends with Sjögren, in their shared passion for the study of the disease [14]. Henrik Sjögren continued enthusiastically to contribute to the field throughout his medical career. In 1951 he updated his first description of the disease by presenting a series of 80 patients with keratoconjunctivitis sicca, 50 of them with concomitant arthritis. In all, he published more than 70 papers related to keratoconjunctivitis sicca and participated in many international congresses, contributing significantly to the worldwide recognition of the disease. The eponym Sjogren Syndrome was given in 1936, by the eminent Hungarian ophthalmologist Stephan von Gròsz and has stood the test of time remarkably well. [15]. It is a well-deserved honor paid to an individual whose astuteness and painstaking attention to detail helped to define a novel and common nosological entity, despite the rejection and skepticism by his peers.

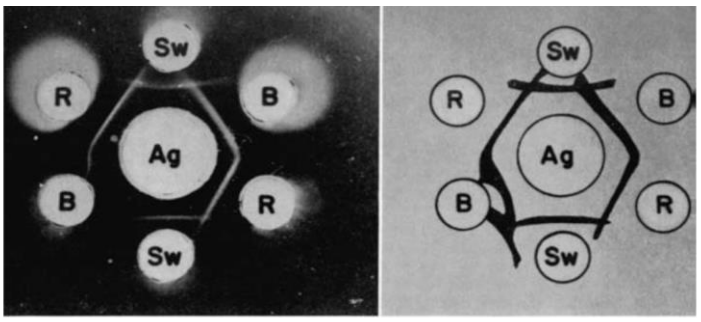
1.3 Sjogren's syndrome: an autoimmune disease

For the first half of the 20th century, Sjogren's syndrome was not considered an autoimmune disease (in most reports it was associated with an unknown viral intruder). Not until the pivotal work of Barrie R. Jones in the Lancet in 1958 that a new thinking was set in motion. Prompted by the raised gamma globulins found in patients with Sjogren's syndrome, reminiscent of Hashimoto's thyroiditis, he

detected circulating autoantibodies in the patient's serum against lacrimal and salivary gland extracts removed from cadavers [16]. Two years later, Joseph J. Bunim, from the National Institutes of Health (NIH), described in great detail a series of 40 patients with Sjogren's syndrome, pointing out the multiorgan involvement, the high titers of rheumatoid factor even in the absence of rheumatoid arthritis, and the presence of clinical features atypical of rheumatoid arthritis, such as vasculitis and neuropathy [17]. In 1965, Bloch, a student of Bunim, reported in collaboration with him sixty-two cases, introducing the distinction between primary Sjogren and secondary Sjogren's syndrome [18], a distinction that laid the foundation for all classification criteria, even as this has been under dispute lately. The same group of investigators at the NIH also examined the serological profile of Sjogren's syndrome patients, encountering raised levels of ANA antibodies (indirect immunofluorescence on rat liver tissue) and the presence of precipitating antibodies against 2 self-antigens, SjD and SjT, now recognized as Ro/SSA and La/SSB (Ouchterlony agar-diffusion technique) [19]. During that time, Dr Norman Talal, a tenacious authority on Sjogren's syndrome who has shaped our current view of the disease, became a senior investigator at the NIH and started working on the disease. His first publication was in cooperation with Dr. Joseph Bunim on the association of Sjogren's syndrome with the development of lymphoma. Talal trained more than 80 postdoctoral fellows (both at the NIH and at the San Francisco VA Medical Center). This core of highly trained and spirited international Sjogren experts engineered a worldwide knowledge wave about the disease [20].

Following the widespread recognition of the syndrome, the early endeavors at quantifying the pathological and inflammatory findings seen on minor salivary gland biopsies bore fruit with a number of publications. In 1968, Chisholm and Mason proposed a grading system with five grades 0-4, based on the presence of diffuse lymphocytic infiltration and/or lymphocytic foci, with a focus defined as an aggregate of at least 50 lymphocytes. Lower grades (0, 1, 2) represented absent, slight, or moderate infiltrates, while grades 3 and 4 indicated the presence of foci. The study was performed in 60 postmortem subjects and 40 patients with autoimmune diseases, 10 of them with Sjogren's syndrome [21]. More than a decade later, Greenspan and Daniels suggested the focus score (FS) concept, with the focus as the number of foci per 4 mm2 of normal-appearing tissue [22,23].

Toward the end of the 60s two different groups simultaneously described the presence of autoantibodies in the sera of patients with Sjogren's syndrome and Systemic Lupus Erythematosus that react with RNA protein antigens. Reichlin's group, using an immunodiffusion methodology, proposed the term Ro and La antigens, after the patients' names initials in whom the antibodies were originally identified, while Tan's group coined the terms Sjögren's syndrome-associated antigen A (SS-A) and Sjögren's syndrome-associated antigen B (SSB). Dr Tan and his team managed to "clone" the relevant cellular antigen using a technique called phage display library. [24-26]. Subsequently, Ro and La antigens proved to be antigenically identical to SSA and SSB respectively [27]. Credit should also be given to Reichlin and his colleagues for being the first to spot an association between the presence of anti/Ro-SSA antibodies and certain HLA-DR antigens.



Ouchterlony agar diffusion demonstrating for the first time the Ro antigen.

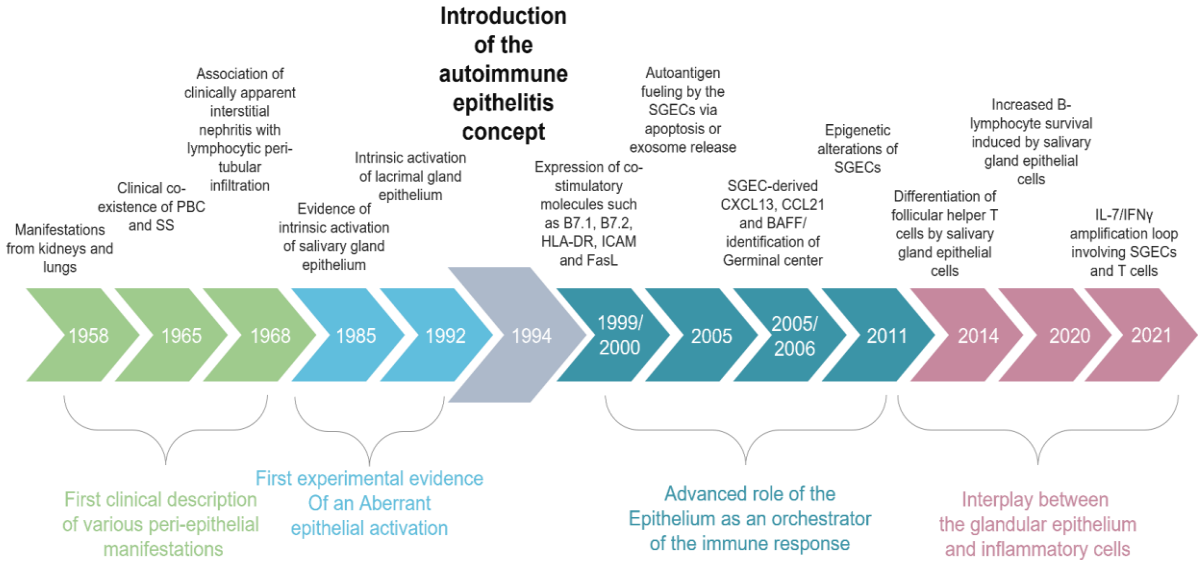
From the original publication of Clark G, Reichlin M, Tomasi TB in Journal of Immunology 1969

1.4 Sjogren's syndrome: An autoimmune epitheliitis

As our understanding of the disease improved during the 80s and 90s, numerous hypotheses about the origin and the primary pathogenetic mechanisms underpinning Sjogren's syndrome were put forward. Of them, the concept of a complex disease with a clinical spectrum beyond the ocular and oral surfaces that hovers around a dysfunctional and activated epithelium prevailed. The theory of Sjogren's syndrome as an autoimmune epithelitis was proposed in 1994 by Dr Haralampos Moutsopoulos, a distinguished researcher, clinician and teacher, trained by Norman Talal, who inculcated in him the love for Sjogren's. However, as it is always the case, Dr Moutsopoulos insightful proposal was an inspired and meticulous synthesis of a great deal of work done before by several investigators. The first clinical descriptions of epithelia involved in Sjogren's syndrome, besides the lacrimal and salivary glands, occurred in the decades of 50s and 60s, when it was shown that the lung (Peribronchial), kidney (Peritubular) and the liver (Pericholangial) epithelium can also be affected in Sjogren's syndrome[28-30]. The first signal of a phenotypic alteration involving the affected epithelium appeared in the work of Lindahl and his colleagues in 1985[31]. With the use of double staining immunohistochemistry, they demonstrated that epithelial cells in close proximity to dense lymphocytic infiltrates in minor salivary gland biopsies of Sjogren patients express the Class II major

histocompatibility complex (MHC) HLA-DR+, transforming partly into professional antigen presenting cells. This finding was further elaborated, expanded, and substantiated a few years later by researchers in the University of Brest, working with epithelial conjunctival cells. In this study, conjunctival cells from Sjogren's syndrome patients were analyzed for the presence of MHC class II molecules, La/SSB antigen, and heat-shock protein. It was proposed that through epithelial-cell injury there is a cell mobilization of La (SSB) protein from the nucleus to the surface that might be presented to T lymphocytes through HLA-DR molecules and/or heat shock proteins [32]. Dr Moutsopoulos, who was among the investigators, by combining the presented clinical and experimental findings arrived at the suggestion that, since a great deal of the extraglandular manifestations of the disease did involve many different epithelial tissues, the descriptive term "autoimmune epithelitis" would be a fitting one for the disease [33].

This transfer of focus upon the epithelium spurred several studies. By the beginning of the new millennium, the activated epithelium was shown to express many co-stimulatory molecules such as B7.1, B7.2, HLA-DR, ICAM and FasL, while it was also shown to act as a constant re-fuel source of autoantigens either by apoptosis or exosomes, further establishing its key role[34,35]. The setting up of long-term cell cultures from non-neoplastic SGECs facilitated the more in-depth analysis of the functions of Sjogren's epithelium, including enhanced environmental sensing through TLR expression and important epigenetic alterations[36-39]. During the last decade various co-culture studies have revealed the interplay of the epithelium with different invading inflammatory cells[40-42]. Last, has been the study of the role of regenerative potent Salivary Gland Stem cells showing less regenerative potential, with an acquired senescent phenotype[43].



Key Timelines of Sjogren Syndrome as an autoimmune epithelitis

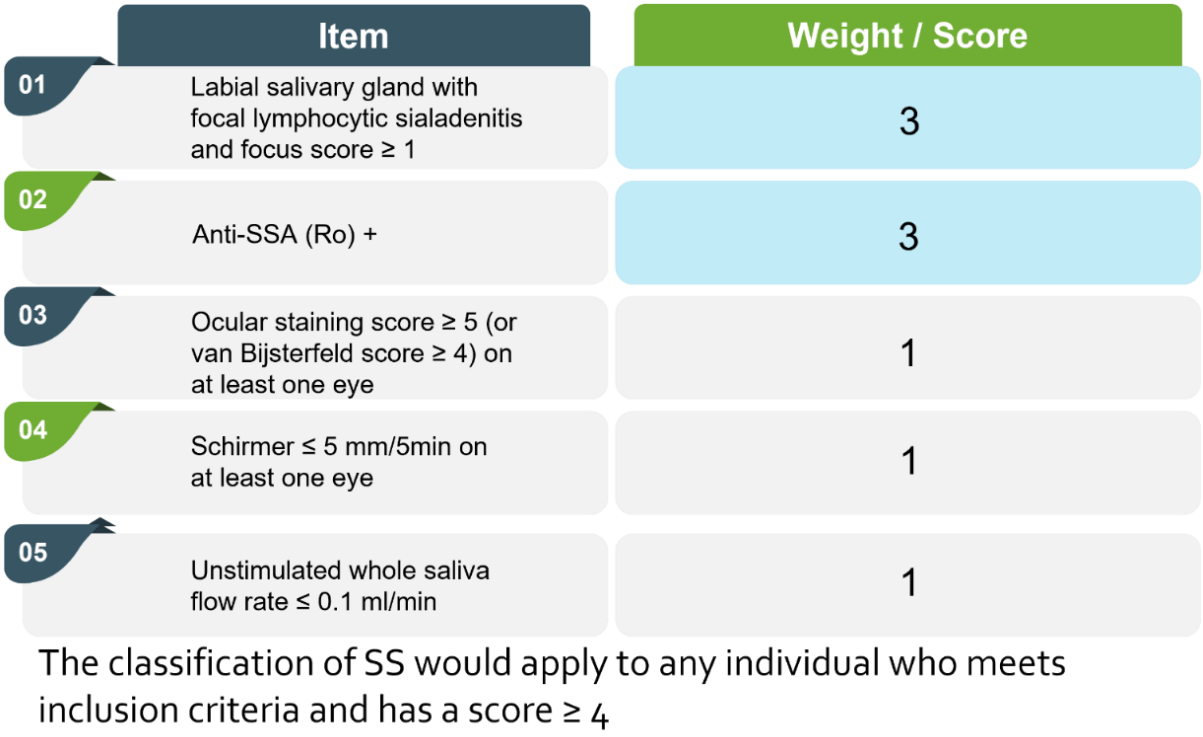
1.5 Establishing classification criteria for the disease.

In rheumatology, most conditions lack a diagnostic "gold standard", whether laboratory based or revealed by radiographic and histologic examinations. Therefore, sets of criteria have been introduced to classify patients into disease groups, for the purpose of routine clinical care or clinical research. These criteria, however, are not considered diagnostic, as they fail to encompass the many, unique clinical situations encountered among various rheumatologic conditions. In Sjogren, for example, the clinical heterogeneity of the disease, the co-existence of other rheumatologic diseases, the diverse autoantibody profile, the multidisciplinary nature of a patient's care team (rheumatologist, oral medicine doctor, ophthalmologist), the many imitators of the disease, and the histologic variability have made the formation of unanimously accepted criteria a challenging exercise. A good example of this was set in 1965 by Block and Bunim who proposed the first arbitrary definition of SS as a triad of keratoconjunctivitis sicca, xerostomia, and another connective tissue disease, with two of three components considered sufficient for diagnosis [18]. The first set of criteria however were proposed by Daniels and Talal in San Francisco in 1975, pointing out the importance of objective measurements of dryness "It is our opinion that subjectively evaluated "xerostomia" is not sufficient evidence of the presence of salivary gland involvement in SS [44]. Evaluation of patients suspected of having SS should include standardized estimation of salivary flow rate and an accessory salivary gland biopsy with determination of a semiquantitative focus score." Those criteria were updated in 1984, when a number of other criteria had also been proposed from different places with active scientific interest for Sjogren's: Copenhagen (1976), Japanese (1977), Greek (1979), California (1986)[45-49]. There was general agreement about the need for objective dryness measurements, but no consensus was reached regarding the methods and thresholds to be used for objective dryness measurements (both oral and ocular), on the value of autoantibodies, the terminology and the item weighting. Only two representations, the Greek and the Copenhagen, discriminated between primary and secondary Sjögren's syndrome. All the different sets of criteria were presented in the First International Symposium on Sjogren's syndrome in 1986, held in Denmark.

The first attempt to unify the criteria began by a multicenter study performed in Europe and supported by the Epidemiology Committee of the European Community (EEC-COMAC Epidemiology). The study began in 1989 and ended in 1993, with the publication of the Preliminary European Classification Criteria for SS [50]. In the methodology, instead of using a Delphi method, based on the identification of a consensus view across experts, the criteria were based on a real-world retrospective cohort, a practice used by the American College of Rheumatology for creating the classification criteria for Rheumatoid Arthritis. These criteria are considered the predecessor both in the form and the spirit of the ones used today and they received widespread acceptance. Pre-existing lymphoma, acquired

immunodeficiency syndrome, sarcoidosis and graft versus host disease were used as exclusion criteria. Secondary Sjogren was also included with its specific set of criteria. Following their introduction, the criteria were reinforced after a multinational validation study showed a high specificity (94%) along with a similarly high sensitivity (97,5%), the latter having been the Achilles heel of previous criteria. Despite the promising initial results, however, criticism followed especially about the classification of patients with "sicca symptoms" and no autoantibody or histologic evidence of the disease, lacking any sign of aberrant immune response. This discordance was overcome in the 2002 American–European Consensus Group (AECG) criteria, where either a positive minor salivary lip biopsy or positivity for serum anti-Ro/SS-A and/or anti-La/SS-B antibodies were considered prerequisites for the classification of the disease [51].

To avert the misclassification of asymptomatic or mildly symptomatic patients as falsely negative for SS and to further increase the specificity that would limit the exposure of individuals, falsely classified as SS, to biologics of uncertain safety in clinical trials, another set of criteria consisting of only three objective items was developed in 2012 [52]. These were developed employing the Sjögren's International Collaborative Clinical Alliance (SICCA) registry and are known as the 2012 ACR classification criteria. However, it is worth noticing that even though subjective symptoms were excluded due to their low specificity they were used as entry criteria for the enrollment into the SICCA registry. In 2016, in response to the confusion created by the use of 2 different sets of classification criteria and as a middle ground, the American College of Rheumatology [53] and the European Alliance of Associations for Rheumatology [54] jointly endorsed the newest set of criteria for the classification of primary Sjögren's syndrome. The methodology used was based on previous similar cooperative efforts between ACR and EULAR for the formation and validation of the 2010 ACR-EULAR criteria for rheumatoid arthritis and the 2013 for systemic sclerosis. It involves the generation of candidate features by an expert consensus team (Delphi method) followed by a multicriteria decision analysis and multivariate logistic regression model (conjoint analysis). These criteria are applicable to any patient with at least 1 symptom of ocular or oral dryness (based on AECG questions) or suspicion of SS due to systemic features, derived from the ESSDAI measure, with at least 1 positive domain item. There is an extensive list of exclusion criteria of diseases with overlapping clinical characteristics including: 1) history of head and neck radiation treatment, 2) active hepatitis C infection (with confirmation by polymerase chain reaction, 3) AIDS, 4) sarcoidosis, 5) amyloidosis, 6) graft-versus-host disease, 7) IgG4-related disease. In addition, they are not directed to secondary Sjogren's syndrome since these patients are not suited for SS clinical trials, thus they are beyond the main scope of the criteria[55].



Classifications Criteria of Sjogren Syndrome

Classification criteria	Senior investigator/ Year	Main elements	Major points of interest
Block and Bunim	Bunim JJ/1965	Triad of keratoconjunctivitis sicca, xerostomia, and another connective tissue disease with two of three components considered sufficient	• First ever set of criteria. • Central place of sicca symptoms • Presence of another • autoimmune disease • increases the probability for SS
San Francisco criteria	N. Talal/1975	For definite SS: Presence of a positive lip biopsy along with KCS	• Oral component of SS was considered present when the LSG biopsy with a focus score greater than 1. • Sjogren with an autoimmune disease were considered other entity that Sjogren with RA • Possible Sjogren • Sarcoidosis, malignant lymphoma, chronic myelogenous leukemia, amyloidosis, hemochromatosis were considered sicca causing diseases • Measurement of stimulated parotid flow

Copenhagen criteria	M Schiødt/1976	Simultaneous presence of objective keratoconjuctivitis sicca and xerostomia Dry eyes: 1. Shcirmer's test (≤ 10mm/5 min), 2. Break-up time (≤ 10s), 3. Van binjsterveld score (≥ 4, scale 0-9) Dry mouth: 1. Unstimulated whole sialometry (≤ 1.5m;/15min), 2. >1 Focus, 3. Salivary gland scintigraphy At least two of three abnormal tests for each organ	• Increased prevalence • Introduction in the criteria objective tests still used today • Whole salivary flow/ Rely on unstimulated tests • Discrimination between primary and secondary • Positive cutoff value for histopathology was one focus per lobe of salivary or lacrimal gland tissue
Japanese criteria	Ofuji T/1977	Dry eyes or dry mouth + One of the following: 1. Keratoconjuctivitis sicca (Rose Bengal ≥2 or Schirmer's <10mm/5min, and florescent test 1+) 2. Abnormal histology (More than one focus) 3. Abnormal findings on sialography	• Presence of possible and definite cases • Dry mouth or dry eyes are indispensable • Positive cutoff value for histopathology was one focus per lobe of salivary or lacrimal gland tissue • Criteria in Japanese • Represent another part of the world
Greek criteria	Moutsopoulos /1979	For definite SS fulfillment of 2/3 of the following criteria a) Xerophtalmia, b) Xerostomia, c) Parotid gland enlargement and a positive labial biopsy (≥2)	• Presence of possible and definite cases • For the definition of both xeropthalmia and xerostomia both subjective and objective tests were required • Discrimination between primary and secondary • For the first time use of a questionnaire for subjective symptom • Positive Schirmer's less than 5 mm/5 min and not 10, which was the standard then
California/San diego Criteria	F. Howelly/ 1986	For definite SS fulfillment of all 4 criteria: 1) objective evidence of keratoconjunctivis sicca, as documented by rose bengal or fluorescein dye staining 2) objective evidence of diminished salivary gland flow	• Presence of possible and definite cases • The use of Focus score as a value related to surface (per 4/mm^2) • Can identify only the tip of the iceberg (full-blown disease)

		3) minor salivary gland biopsy with a FS≥2, 4) evidence of a systemic autoimmune process, as manifested by the presence of autoantibodies, such as rheumatoid factor and/or anti- nuclear antibody	
Preliminary European Classification Criteria for SS	Pierre Youinou./ 1993	For definite SS fulfillment of 4 out of 6-item criteria: 1) Ocular symptoms 2) Oral symptoms 3) Ocular signs 4)Histopathologic features 5)Salivary gland involvement 6)Autoantibodies	• Presence of possible and definite cases • Use of a validated questionnaire for dryness symptoms • Focus score ≥1 (focus defined as an agglomeration of at least 50 mononuclear cells; focus score defined as the number of foci 4 mm^2 of glandular tissue) • Serum autoantibodies (including ANA) • Inclusion of patients without any sign of aberrant immune response
American-European Consensus Group	C Vitali/2002	6-item criteria: 1) Ocular symptoms 2) Oral symptoms 3) Ocular signs 4)Histopathologic features 5)Salivary gland involvement 6)Autoantibodies For SS: a) fulfillment of 4 out of 6 as long as either item IV [56] or VI (Serology) is positive b) The presence of any 3 of the 4 objective criteria items (that is, items 3, 4, 5, 6)	• Evidence of an autoimmune process • Exclusion of ANA antibodies from the autoantibodies section • Specified that the Schirmer test be performed with anesthesia • lissamine green and fluorescein were allowed as replacements for Rose Bengal dye • FS further elaborated: defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) • In the list of exclusion criteria Past head and neck radiation treatment Hepatitis C infection, use of anticholinergic drugs (since a time shorter than fourfold the half-life of the drug) were added
ACR classification criteria	T. E. DANIELS/2012	At least 2 of the following 3 objective features: 1. Positive serum anti-SSA/Ro and/or anti-SSB/La or (positive rheumatoid factor and ANA titer) 2. Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥1 focus/4 mm2 3. Keratoconjunctivitis sicca with ocular staining score ≥3 (assuming that	• Very high specificity • Exclusion of the symptoms of dry mouth and dry eye from the criteria set • As an alternative serologic item of anti-Ro/SSA and anti-La/SSB antibodies a positive ANA and RF test can be used • Ocular Staining Score (OSS) with a cut-off value of three points (from 0 to 12), which is less specific than the 4-point threshold of the van Bijsterveld score

		individual is not currently using daily eye drops for glaucoma and has not had corneal surgery or cosmetic eyelid surgery in the last 5 years)‡	
ACR/EULAR criteria	Xavier Mariette/ 2016	1. FS ≥ 1 foci/4 mm2 2. Anti-SSA/Ro-positive 3. Ocular Staining Score ≥ 5 (or van Bijsterveld score ≥ 4) 4. Schirmer's test ≤ 5 mm/5 min1 5. Unstimulated whole saliva flow rate ≤ 0.1 mL/min 1 and 2 is scored with 3 points while 3,4 and 5 with 1 SS requires a score of ≥ 4	• For the first time there is an entry criterion: at least one symptom of ocular or oral dryness or ESSDAI ≥ 1 • Exclusion of anti-SSB/La positivity from the criteria • No discrimination between primary and secondary SS • No use of salivary gland ultrasonography • Lymphoma is not included in the exclusion criteria

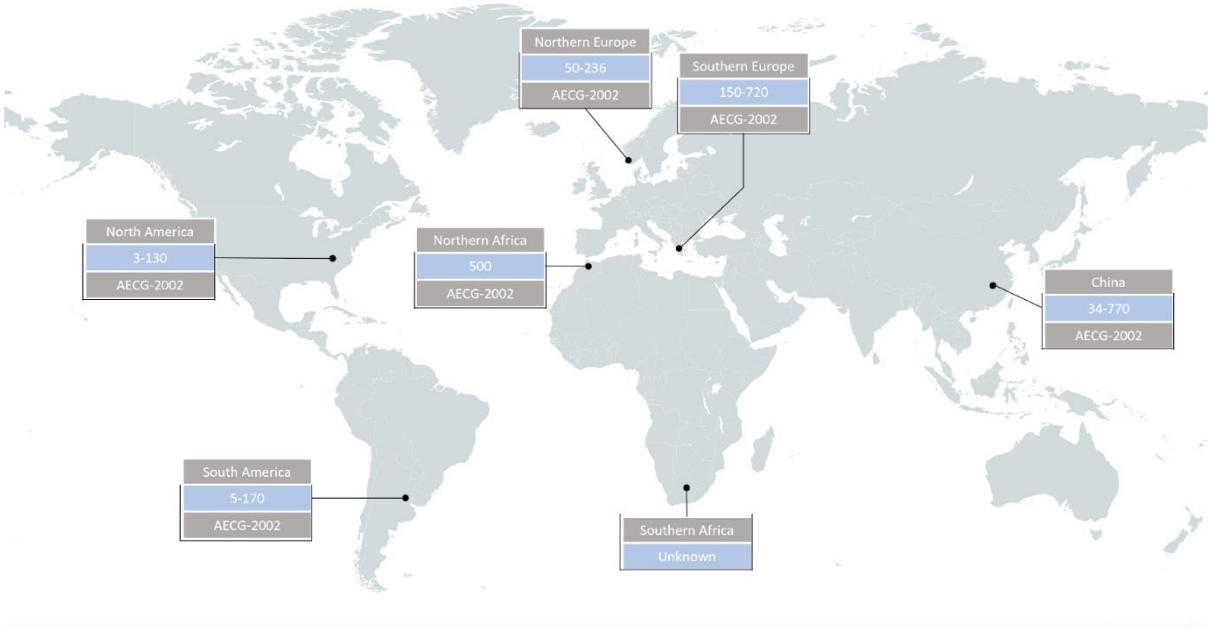
Chapter 2: Epidemiology

2.1 Prevalence and incidence

Primary Sjogren's syndrome is the second most common systemic autoimmune disease, after rheumatoid arthritis, with a prevalence close to that of systemic lupus erythematosus [57]. It is noted for its very strong female predilection, with a peak incidence in the perimenopausal period, around the 4th and 5th decade of life. The disease has a worldwide distribution and a remarkable geographic variability, regarding both its prevalence and its incidence. Because of its predominantly non-life-threatening nature, the number of undiagnosed cases in the general population is not negligible [58]. With prevalence being the proportion of the population with Sjogren's syndrome at a specified point in time and with the incidence being limited to new diagnoses made over time, each new incident case enters the prevalence pool. The noted difference in the reported prevalence (ranging from 0.01% to 4%) could be partly explained by the long-standing lack of universally accepted diagnostic criteria [1,59]. For example, pooled prevalence rates in studies that used the 1993 European Classification Criteria were reported to be 12-fold higher than the same culled from studies that used the 2002 AECG criteria [60]. However, considerable variation in prevalence persists between studies even when the same classification criteria are used, possibly implicating other cofounders, such as geographical variations, differences in the health care systems, difference in the genetic backgrounds, the race and ethnicity of patients or just diverse methodological strategies (for example, the age limit for inclusion). Reports of studies from Southern Europe show an estimated prevalence as high as 720 cases per 100.000 persons, while figures from the United States are as low as 3 cases per 100.000 [61-70]. Overall, it appears that the prevalence is higher in the Southern part of Europe and in East Asia. Intriguingly, a study evaluating the influence of race or ethnicity on the prevalence of SS in the Greater Paris area, showed a two-fold higher prevalence among individuals of a non-European background compared to those of a European background, exposing the difficulty in drawing reliable conclusions regarding the real prevalence of Sjogren by geographic region [53]. Similarly, data accrued from almost 8000 patients from 5 continents, part of the Big Data Sjogren Consortium, revealed an earlier disease diagnosis in black/African-Americans, contrasting with a dominance of sicca symptoms and a higher frequency of positive salivary biopsy in Hispanic and white patients [71]. Sicca symptoms had their lowest prevalence in Asian patients in whom pulmonary and cutaneous involvement is more common.

Symptoms characteristic of Sjogren's syndrome can also be found in patients with other autoimmune diseases, with rheumatoid arthritis and systemic lupus erythematosus showing in a

recent meta-analysis an estimated pooled prevalence of associated Sjogren's of 19.5% and 13.9%, respectively [72]. For systemic sclerosis the prevalence is around 14% and is associated with limited cutaneous systemic sclerosis, while data for other systemic diseases are scarce and spotty [73].



Global prevalence of Sjogren Syndrome per 100.000 people

2.2 Gender differences in Sjogren's syndrome

A shared characteristic of most autoimmune diseases is a strong sex difference in prevalence, with females generally much more frequently represented than males. Interestingly, the magnitude of this female predilection usually correlates with a disease's prevalence, since more common diseases show a higher female skewing. For example, autoimmune thyroiditis, the most common organ-specific autoimmune disorder, systemic lupus erythematosus [74] and Sjögren's syndrome (SS), the prototypical systemic autoimmune disorders, share the most striking female sex biases. For Sjogren's syndrome, even though the unbalanced gender ratio is common between all epidemiological studies, its exact quantification is problematic given the vast geographic disparity. For years, it was traditionally reported as 9 women to 1 male, but a number of newer highly populated studies set the bar even higher. The Big Data Sjögren Project Consortium, encompassing almost 8000 patients, found the female-to-male ratio to be around 14:1 [75], while a study specifically designed to decipher gender differences in Sjogren, consisting of almost 100 male SS patients from Greece and Italy, showed a ratio of around 20:1 [76]. However, other smaller reports from around the world have shown ratios ranging from 6 to 1 in the

USA and Norway to 27 to 1 in China [58,77]. Whatever the exact ratio, however, it seems that male patients constitute a specific subset of the disease with a different clinical phenotype from that of the ordinary female patient with SS, a trend observed in various autoimmune diseases. In fact, male Sjogren's syndrome patients appear to have a higher frequency of Raynaud's phenomenon, a higher prevalence of anti-La/SSB antibodies, and more importantly a stronger association with the development of lymphoma [78]. The higher frequency of lymphoma in men with SS has been shown to be related to gender, independently of other well-known lymphoma predictors[76].

The etiopathology underpinning the female predilection in autoimmune diseases remains obscure. To date, studies at the molecular level that might account for such differences have largely focused on sex hormones, while recently the inherent genetic imbalance between sex chromosomes (XX in females and XY in males), despite the imperfect epigenetic mechanisms of gene silencing created by nature to compensate for the genetic material inequality, has been also under intensive investigation [79-81]. Other factors including the exposome, the unique and versatile Y chromosome, maternal microchimerism and intrauterine influences have been postulated as other potential factors [81]. Thus, SS constitutes the most female predominant systemic autoimmune disease, and it presents the best research model to investigate and elucidate the mechanisms favoring a women bias.

Country	Year	Classification criteria	Female/male Ratio
Japan [82]	1999	Modified Japanese criteria	9.7:1
Spain [83]	2000	Preliminary European Classification Criteria for SS	10:1
Spain [84]	2004	Preliminary European Classification Criteria for SS	18:1
Greece [62]	2006	AECG	20:1
Spain [85]	2007	AECG	12:1
Turkey [86]	2009	AECG and European classification	10:1
Norway [87]	2011	AECG	6:1
China [88]	2015	AECG	27:1

USA [89]	2017	AECG	6:1
Worldwide [71]	2019	AECG	14:1
Australia [90]	2020	AECG	9:1
Greece and Italy [75]	2020	ACR/EULAR	20:1

Table: Female to male ration in Sjogren's Syndrome

2.3 Effect of age on Sjogren's syndrome

Sjogren's syndrome is commonly diagnosed between the ages of 40 and 60, but younger as well as older patients including children are not immune from the disease. The onset of the disease precedes the diagnosis by 5 or more years and that makes both the age at which the symptoms are first declared and the age at diagnosis important determinants of the disease. The former better represents the underlying pathogenetic process, while the latter signals the clinically evident threshold that brings the patient to medical attention. Numerous studies have explored the effect of both on the clinical phenotype of the disease. Cumulatively, it appears that younger patients have a richer autoantibodies profile, with more extraglandular manifestations and a higher probability of lymphoma, while older Sjogren's syndrome patients present with more "sicca" symptoms, a more prevalent lung involvement, and a higher frequency of lymphoma. Likely, SS associated lymphoma in older patients is brought about through different mechanisms than in younger counterparts and is associated with the multilevel effects of aging (hormonal, telomere shortening, CHIP accumulation, inflammaging, increased senescence etc.).

Sjogren's syndrome is exceedingly rare in children, but onset as early as 4 years old has been reported [91]. In the last few years, the publication of large cohort studies has revealed the median disease onset for childhood Sjogren to be around 10-14 years old. The clinical picture is distinct from that of adults, including more recurrent salivary gland enlargement, constitutional symptoms, nervous system involvement, and less prominent dryness symptoms [91-93]. It is worth noting that even within a disease onset at pre-puberty, the female sex prevails with a male: female ratio of 1:6-9. The more diverse clinical manifestations, in comparison to those in adults, necessitate the group's own diagnostic criteria and may account for the failure of pediatric Sjogren to be readily diagnosed [94].

Study	Year	Prevalence	Sample size	Patient group	Important findings
Studies on Disease Diagnosis					
Lin Wei et al. [95]	2022	10.8%	33 Young	Young<35	↑ IgG ↑ Anti-Ro/SSA ↑ Anti-La/SSB ↓ C4 levels

					↑CD4+ T-cell lymphopenia ↑ Renal involvement
Ramos-Casals et al. [85]	2008	14% Young 15% Old	137 Young 156 Old	Young<35 Old>70	**YOUNG** ↓ xerostomia ↓ ocular tests ↓ thyroiditis ↑ Anti-Ro/SSA ↑ Anti-La/SSB ↑ RF ↓ C4 levels ↑ SGE **OLD** ↓ SGE ↓ Arthralgias ↓ Raynaud ↓ Anti-Ro/SSA ↑ Pulmonary ↑ Anemia
Müçteba Enes Yayla et al [96]	2020	11.4%	35 Young	Young<35	↑ IgG ↓ C4 levels ↑ Anti-Ro/SSA ↑ Renal involvement ↑ Cutaneous involvement
Céline Anquetil et al [97]	2019	13.9%	55 Young	Young ⩽35	↑ IgG ↑ Anti-Ro/SSA ↑ Anti-La/SSB ↑ RF ↓ C4 levels ↑ SGE ↑ Lymphadenopathy ↑ Purpura ↑ Renal involvement
Hans-Jacob Haga and Roland Jonsson, [98]	1999	24% Young 35.8% Old	16 Young 24 Old	Young<45 Old>60	**YOUNG** ↑ IgG ↑ Anti-Ro/SSA ↑ Anti-La/SSB ↑ RF **OLD** ↓ IgG ↓ Anti-Ro/SSA ↓ Anti-La/SSB ↓ RF
Studies on Disease Onset					
Ramos-Casals et al. [99]	1998	9%	13 Young	Young<35	↑ Anti-Ro/SSA ↑ RF ↑ Monoclonality ↑ Lymphadenopathy

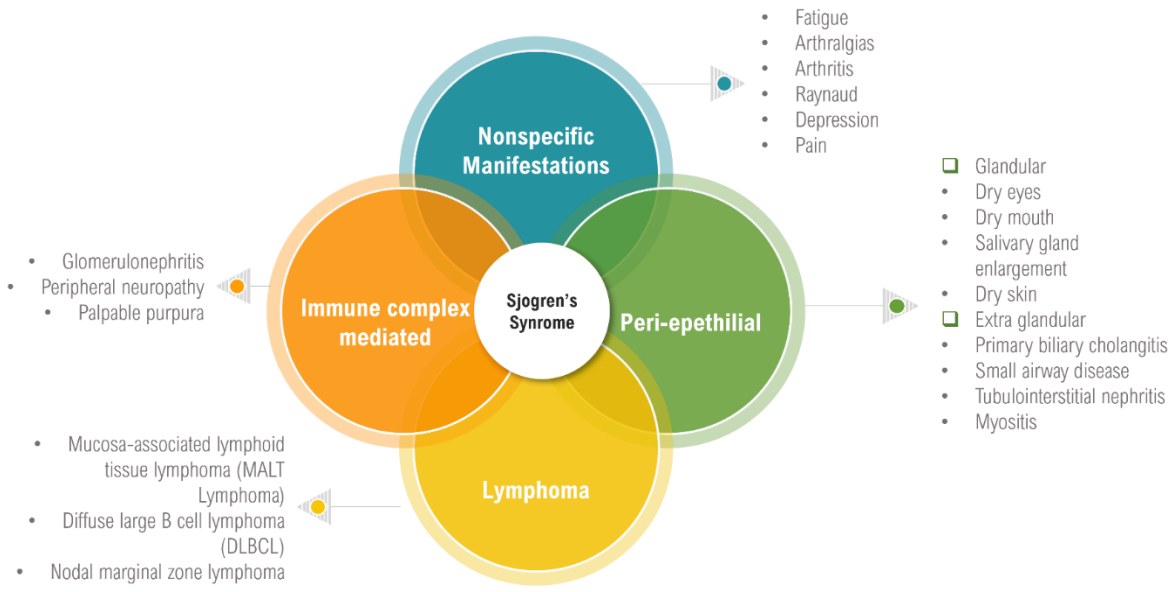
M García-Carrasco et al. [100]	1999	14%	31 Old	Old>70	No differences observed
M García-Carrasco et al. [101]	2002	15% Young 11% Old	63 Young 43 Old	Young<35 Old>70	**YOUNG** ↑ Anti-Ro/SSA ↑ RF ↑ Fever ↑ Lymphadenopathy **OLD** ↓ Anti-Ro/SSA ↓ Positive ocular tests
Tishler et al [102]	2001	20%	17 Old	Old>65	↑ Anti-Ro/SSA ↑ RF ↑ Saliva IL-6 levels
Costantino Botsios et al. [103]	2011	6% Old	21 Old	Old>65	No differences observed
Goules et al [104]	2020	19% Young 15% Old	379 Young 293 Old	Young ≤35 Old≥65	**YOUNG** ↑ IgG ↑ Anti-Ro/SSA ↑ Anti-La/SSB ↓ C4 levels ↑ Leukopenia ↑ Raynaud ↑ Lymphoma **OLD** ↑ Dry mouth ↑ Lung involvement ↑ Lymphoma

Table: Important disease related findings of patients according to their age of disease onset or diagnosis

Chapter 3: The multiple faces of the disease

3.1 Introduction

Sjogren's syndrome is a chronic, systemic autoimmune disease typified by an aberrant lymphocytic infiltration around epithelial structures, mainly of the lacrimal and salivary glands. Most SS patients have an indolent and benign course, with early clinical manifestations that might be nonspecific and often not severe enough for the individual to seek medical advice. The primary features of the disease are expressed by eyes and mouth dryness. However, the clinical spectrum of the disease extends far beyond the exocrine glands, affecting parenchymal organs and small-sized arteries, producing various extraglandular manifestations, making SS, as first proposed by Henrick Sjogren, a protean systemic disease. Adding to the complexity of the presenting features, around 5% of patients develop a Sjogren associated non-Hodgkin B-cell lymphoma, usually of a good prognosis. The insidious onset of symptoms, the complexity and heterogeneity of the numerous faces and facets of the disease, the profusion of a variety of participating medical specialists often unfamiliar with SS ranging from a dentist to a pulmonologist to a psychiatrist, and the decreased awareness of the disease from both the public and the healthcare community are some of the factors that enable it to evade timely recognition and diagnosis. To circumvent those problems and acquaint ourselves with the disease's clinical diversity of the disease, it might be helpful to assign its many manifestations into 4 major clusters, based on the implicated pathobiology: non-specific, peri-epithelial, immune complex mediated manifestations and lymphoma.



The clinical constellation of Sjogren Syndrome

3.2 Nonspecific manifestations

Chronic fatigue is one of the most common and debilitating symptoms of patients with Sjogren's syndrome, as well as perhaps being the most important of the complaints. The feeling of exhaustion, both mental and physical, overwhelming tiredness (even early in the day), easy fatigability and inability to concentrate can be found in up to 85% of patients, and in 40% they overshadow all the other clinical manifestations [105,106]. The severity of symptoms varies greatly between individuals, some reporting profound fatigue, but others volunteering few symptoms. Patients also report cognitive symptoms, such as subjective memory loss and difficulties with concentration, referred to as brain fog. It is critical to exclude other diagnoses (including sleep disorders) by implementing a thorough medical history, clinical examination and a basic laboratory panel before chronic fatigue is attributed to SS.

Objective fatigue measurement is challenging given the absence of suitable objective tests or markers. However, there is a large number of self-reported questionnaires, with the commonest being (PROFAD and Facit-F), that can reflect on the magnitude of the patients' symptoms, but also provide evidence of any possible improvement. Fatigue along with the symptoms of pain and dryness is also a part of the European League Against Rheumatism [54] Sjögren's Syndrome Patient Reported Index (ESSPRI), which is a validated questionnaire used in everyday clinical practice and also as an endpoint in clinical studies [107]. It is worthy of note that fatigue does not usually mirror the illness's path as measured by the systemic disease activity [108,109], but fairly often is associated with depression. However, whether it is the depressive state that exacerbates the feeling of exhaustion, or the other way around is debatable.

Pain is also a common distressing symptom in Sjogren's syndrome, running the gamut from a chronic, widespread musculoskeletal pain, typical of fibromyalgia, to an inflammatory arthritis. Even though the overlap of the clinical features of Sjogren's syndrome with those of fibromyalgia are hampering its diagnosis, fibromyalgia has been reported in about 20% of SS patients [110]. Sjögren's syndrome-associated fibromyalgia and primary fibromyalgia have a share in the number of tender points [111]. The reason for the increased incidence of fibromyalgia in rheumatic diseases compared to the general population is not well understood. Dysregulation of the central pain processing, disruption of the neuroendocrine stress axis and the altered neurotransmitter function have been implicated. Among all systemic autoimmune diseases, fibromyalgia is more prevalent in Sjögren's syndrome. A correct diagnosis of fibromyalgia is therefore necessary before instituting treatment with corticosteroids in Sjogren patients with symptoms simulating fibromyalgia.

Arthralgias, equally involving the large and small joints, may affect 50–75% of patients, while overt nonerosive synovitis is seen in a minority of cases (5-10%) [112]. In several studies, ultrasound was used to investigate subclinical synovitis, revealing mild or moderate synovial hypertrophy in 15% of patients, but its relation to the symptoms and their severity is unclear [113]. Jaccoud arthropathy in the proximal interphalangeal joints, wrists and knees, with reversible articular deformities has also been reported in Sjogren's syndrome.

Raynaud's phenomenon is a well demarcated color change of the distal aspects of fingers and toes induced by exposure to cold temperatures or cold water (for example, doing the dishes or swimming). It is observed in 13–40% of Sjogren's syndrome patients [114], especially in relation to anti-centromere antibody positivity [115]. The attack can be painful and usually reverts to normal in about 30 minutes. It notably lacks vascular complications and tends to be milder in Sjogren's syndrome compared to the same in other systemic autoimmune diseases.

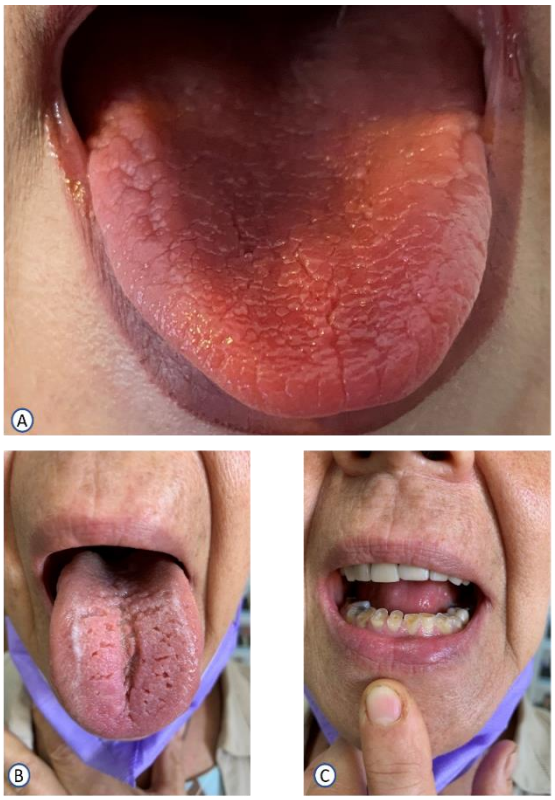
3.3 Periepithelial manifestations

Glandular

In Sjogren's syndrome the saliva's reduced quantity and quality accounts for the patients' sensation of dry mouth that substantially affects their quality of life. Diminished saliva production is a common and often ignored medical complaint associated with aging, a number of medical conditions, as well as with the side effects of some drugs. However, hyposalivation in Sjogren's syndrome, which is the main oral feature of the disease, is usually more severe reaching even complete absence of saliva, either in the unstimulated or the stimulated phase. In general, the amount of saliva produced cannot be accurately correlated with the patient's complaints, and that might be partly because SS

alters the protein profile and composition of saliva (decrease in salivary amylase and carbonic anhydrase [116], causing a stickier fluid that has lost its moisturizing capacity).

Patients express this feeling variably as difficulty in chewing and swallowing dry food without the added moisture of drinking liquids, or as dysgeusia, and an inability to speak uninterruptedly. They will tend to be thirsty and always carry a bottle of water during the day and keeping it by their bedside at night. In the morning xerostomia is even worse, with an accumulated thick viscous saliva necessitating a mouthwash. Patients have difficulty in wearing dentures and they may also complain of a sensation of oral burning or glossodynia (tongue soreness), probably the result of a secondary fungal infection.



The tongue in patients with Sjogren syndrome is dry and fissured (Panel A and B), Low quality frothy saliva may by evident in some cases (Panel B), Lack of salive results in denture issues (Panel C)

Physical examination reveals a dry, erythematous, sticky, oral mucosa, and atrophy of the filiform papillae. The tongue appears bright red, hyperlobulated and fissured. Dry and fissured lips are also features of the disease. Salivary pooling in the mouth floor may be absent or reduced, saliva may be frothy, and food residues may be observed in the oral cavity. Especially in patients with very low salivary flow and a long-lasting disease, the loss of the saliva's anti-microbial, buffering, cleansing, and lubricating properties leads to dental complications. There is an increase in dental carries as well as in noncarious tooth surface loss such as erosion, abrasion, and attrition. This happens because saliva is also responsible for the formation of pellicle and keeping the oral and plaque pH elevated and thus protecting the oral cavity from acids. In addition, a predisposition to oral candidiasis is observed in over one-third of patients [117,118]. It is usually seen in the atrophic variant, which is characterized by erythema and atrophy of the oral surfaces and loss of papillae on the dorsum of the tongue accounting for the oral sensitivity to spicy and acidic foods [116]. The classic presentation with the typical white exudates is less common but can occur in SS, especially after recent antibiotic exposure. Angular cheilitis is also common, in contrast to the rarely encountered ranula [119].

Most Sjogren's syndrome patients will develop one or more episodes of salivary gland swelling during their lifetime, usually involving the parotids. Approximately, one out of two will have recurrent or persistent swelling molding a microenvironment favorable to the development of lymphoma [120]. The swelling is usually unilateral, it subsides within a few days, and can also be bilateral. Chronic swelling is usually painless and the presence of a Sjogren associated lymphoma should always be investigated. Microbial infectious participation and the acuity of the episode determine the presence of pain. Less commonly, submandibular glands may become enlarged. However, it should be stressed that major salivary gland enlargement can be associated with other medical conditions, such as sarcoidosis, IgG4 related disease, parotid malignant tumors or a Warthin tumor, that need to be excluded before SS is implicated.

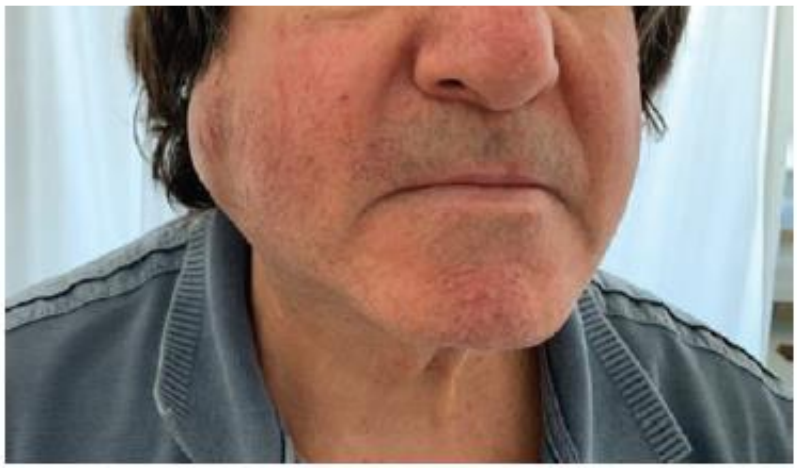
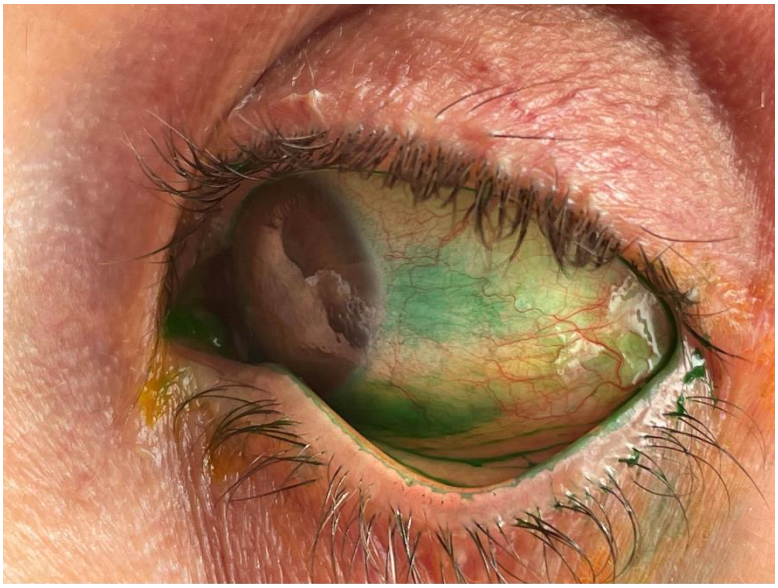


Image: Bilateral Salivary Gland Enlargement

Eye dryness is the other hallmark feature of Sjogren's syndrome. Diminished tear secretion, the result of the chronic inflammation of the lacrimal glands, leads to the destruction of the corneal and bulbar conjunctival epithelium, known as keratoconjunctivitis sicca, a termed coined by Henrick Sjogren. But as is the case with hyposalivation, diminished tear secretion is not the sole contributor to the problem. The altered tear composition (hyperosmolarity), the secretion of inflammatory cytokines, the damaged goblet cells, and the frictional damage from the blink mechanism together lead to the loss of the tear film [121]. In seeking relief from the symptoms of eye dryness and associated discomfort, it is likely that an ophthalmologist would be the first to see such a patient with Sjogren's syndrome. This is a golden opportunity for a prompt diagnosis of a systemic disease. Sadly, the data indicate that the majority will seek medical evaluation on average 10 years after the dryness first became a noticeable symptom [122]. The severity of eye involvement is sometimes so profound that a cluster of Sjogren's syndrome patients with only glandular involvement, ought to be seen by their ophthalmologists much more often than by their rheumatologists.

The patients may report itchiness and a burning sensation under the lids or may complain of grittiness and a foreign body sensation. Photophobia and photosensitive are also common complaints [123]. Symptoms tend to worsen by exposure to an air current, to dry air, to places that are air conditioned, during reading, or watching a computer screen causing the patients to try to improve the

lubrication of the ocular surfaces by increasing the frequency of blinking. Despite that, blurry vision is a factor limiting the daily activities of patients, including driving at night. Left untreated, severe forms of the disease might lead to corneal scarring ulcer or perforation. Men seem to be more prone to severe eye complications [124].


Advanced keratoconjunctivitis sicca in a patient with Sjögren syndrome visualized with lissamine green

Clinical signs include dilatation of the bulbar conjunctival vessels, pericorneal injection, irregularity of the corneal image, thick mucus in the inner canthus, and occasionally enlargement of the lacrimal glands. A mere suspicion of a dry eye calls for objective tests of dryness. These include the Schirmer's test, which is a quantitative measure of tear production, and staining of both the cornea with fluorescein (epithelial defects) and the conjunctiva with lissamine green or Rose Bengal (damaged epithelial cells), to assess the degree of damage to the ocular surface [125]. Lissamine green and Rose Bengal are more specific for Sjogren's syndrome, with the former being better tolerated [126].

Though less frequently, other exocrine glands can be involved causing diminished mucous gland secretions of the respiratory, cutaneous, and gastrointestinal tracts. Thus, patients may present with dry nose, dry throat or xerotrachea, leading to chronic dry cough and an urge to repeatedly clear their throat. Voice hoarseness, being the result of the coating of the vocal cords by thick mucus can also be observed [127]. The skin can similarly be involved with more than a third of patients experiencing dermal stinging and itching most often affecting the lower extremities and axillary creases [128,129]. Esophageal mucosal atrophy, atrophic gastritis, and rarely subclinical pancreatitis may also occur. Dyspareunia, vulvovaginal dryness, and pruritus are also common complaints by women with SS, contributing significantly to the morbidity of the disease [130]. Patients are often hesitant to report on their genital symptoms, obligating doctors to include relevant questions as part of the routine history taking, with an appropriate degree of sensitivity.

3.4 Extra-Glandular manifestations

Extraglandular periepithelial manifestations usually arise in the lungs, the kidneys and the liver. The prevalence of pulmonary involvement in SS varies significantly across a number of studies ranging from 9% to as high as 70% depending on the diagnostic methods used, the characteristic of the included patients and the definition of pulmonary involvement [131]. Increased prevalence has been reported in patients from China and in those diagnosed at an older age. In general, clinically significant pulmonary involvement is usually affecting about 10-20% of patients [132]. Importantly, however, pulmonary involvement in SS is associated with diminished quality of life and lower 5 and 10 year survival rates [133,134]. Less often, it can be the presenting symptom of SS [135]. Pulmonary involvement in SS is expressed predominantly as small airways disease or interstitial lung disease.

Small airway disease manifests as dry cough and exertional dyspnea, symptoms that are potentially aggravated by cigarette smoking. Histopathologically, pulmonary involvement is characterized by peribronchial/ peribronchiolar lymphocytic infiltration (lymphocytic bronchiolitis) with or without formation of lymphoid follicles in the bronchiolar walls (follicular bronchiolitis), causing small airways obstruction. Lung function testing indicates small airways involvement, with reduced maximal mid-expiratory flows. High-resolution CT (HRCT) reveals reticular or nodular abnormalities, mosaic attenuation, bronchial thickening, bronchiectasis, subsegmental atelectasis, and air trapping [136,137]. To improve the CT yield an inspiratory-to-expiratory approach is required. However, a normal CT does not rule out small airway disease. Even within its high prevalence, small airway disease is not a major contributor to the mortality nor to the morbidity of the disease.

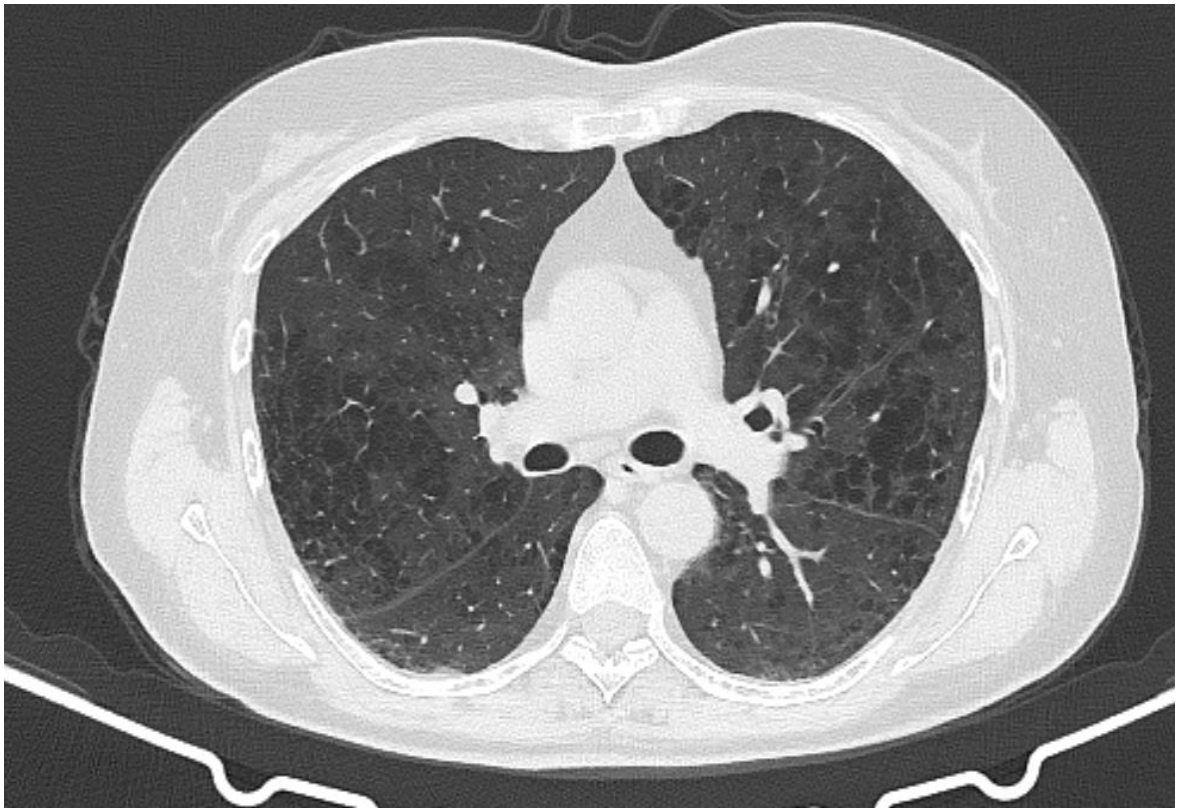
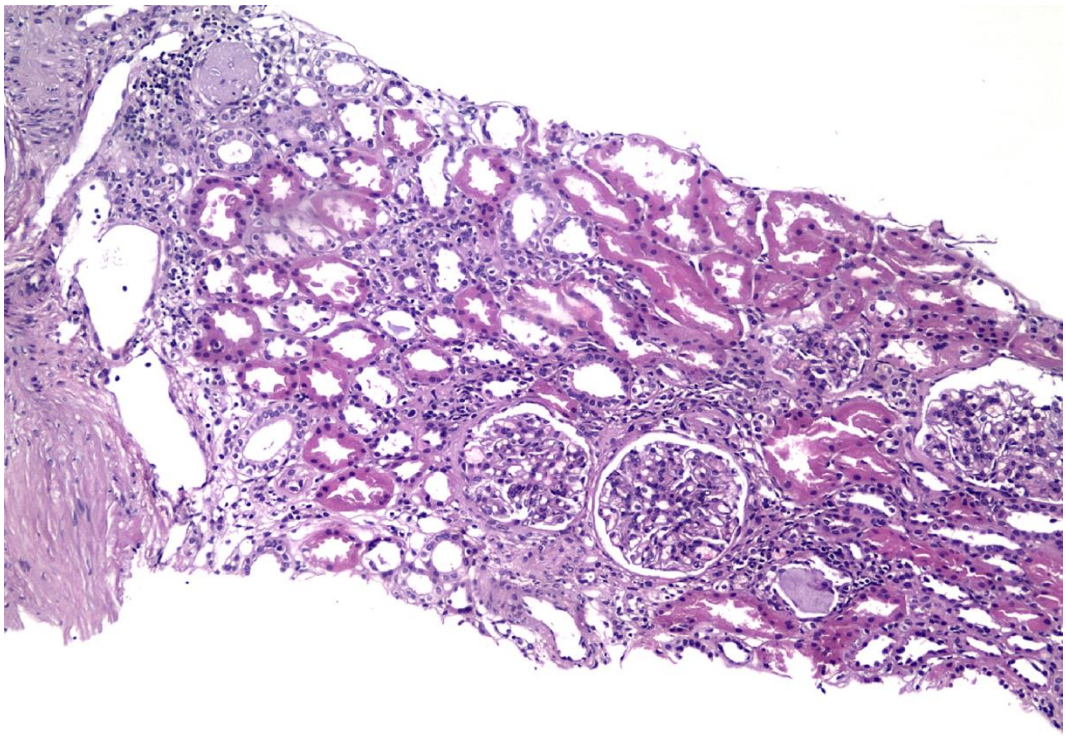


Image: Ct findings showing air trapping in a patient with Sjogren's Syndrome with small airways disease

Interstitial lung disease (ILD) manifests as dry cough with progressively worsening shortness of breath. Pulmonary function tests typically reveal a restrictive pattern, along with a decreased diffusing capacity of carbon monoxide (104) The most common histological and radiographic pattern of ILD is nonspecific interstitial pneumonia (NSIP), followed by usual interstitial pneumonia [138], cryptogenic organizing pneumonia (COP), and lymphoid interstitial pneumonia (LIP). Although LIP is not the commonest pattern of ILD in Sjogren, it has the most characteristic histologic picture, with marked cellular infiltration of the interstitium by lymphocytes, plasma cells, and sometimes germinal centers. Lymphocytic infiltration may even include the bronchiolar walls. LIP may be a precursor of bronchus-associated lymphoid tissue [139] lymphoma. Pulmonary function tests typically reveal a restrictive pattern, along with a decreased diffusing capacity of carbon monoxide [140]. High resolution CT in associated LD may show reticular or nodular infiltrates, ground-glass opacities, centrilobular nodules, bronchiectasis or honeycombing, whereas honeycombing is typically replaced by cyst formation in LIP [40]. Although lung biopsy is necessary in difficult cases, it is not part of every-day practice. Ito et al evaluated 33 cases (31 surgical lung biopsies and 2 autopsies) with primary Sjögren's syndrome and found evidence of NSIP in 20 (61%), non-Hodgkin's lymphoma in four (12%), diffuse bronchiolitis in 4 (12%), and amyloid in 2 (6%) [141]. Finally, rarely, pulmonary involvement in Sjögren's syndrome may cause lymphocytic pleuritis, pulmonary hypertension, and pulmonary amyloidosis.

Kidney involvement in SS can be either periepithelial or extraepithelial. Periepthilial involvement comes in the form of tubulointerstitial nephritis, as first described in 1968 [30]. Clinically significant disease occurs in 5% of patients, however a defective urine-concentrating ability can be found in over 20% of patients [142,143]. More than 50% of kidney biopsies of SS patients present inflammatory infiltrates in the interstitium [144]. The main histopathological finding in these patients is a diffuse or patchy lymphocytic infiltration of the kidney interstitium, with mainly peritubular involvement. Patients may develop chronic renal failure and low range proteinuria. Most of the patients with interstitial nephritis present with latent distal renal tubular acidosis (dRTA or type I RTA). Alpha-intercalated cells responsible for hydrogen ion secretion into the urine under the adjacent inflammatory stress become dysfunctional. It is a subclinical form of dRTA that can be only revealed after an acid-loading test. A smaller proportion of patients present with the clinically apparent form of complete distal RTA, which manifests as hyperchloremic metabolic acidosis with normal anion gap, a persistent alkaline urinary pH, hypokalemia, hypercalciuria, hyperphosphaturia and hyposthenuria. Increased urinary calcium excretion may lead to renal stones, nephrolithiasis, and/or nephrocalcinosis.



Interstitial nephritis with diffuse interstitial mononuclear cells infiltrate with interstitial fibrosis, tubular atrophy, and occasional tubulitis. Glomeruli have a normal structure (H&E)

Liver epithelium can also be affected in the context of Sjogren's syndrome. Its involvement can be variably expressed as mildly elevated biochemical tests, primary biliary cholangitis or autoimmune hepatitis. A non-significant cholestatic liver enzyme pattern is observed in 10-20% of patients [145,146]. Biopsy findings in those patients reveal a pericholangial lymphocytic infiltration injury, reminiscent of the periductal infiltrates of the minor salivary glands, which of course represents the disease's

pathologic signature. All Sjogren's syndrome patients, especially those with abnormal liver enzymes, should be screened for hepatitis C to rule out a sicca syndrome-mimicking disease. Antimitochondrial antibodies positivity is seen in about 8% of patients, while overt primary biliary cirrhosis, with a usually mild clinical course, features in less than 4% of patients [147,148]. Autoimmune hepatitis figures in 1–2% of patients, while the co-existence of primary sclerosing cholangitis is referenced in case reports only rarely [149]. Interestingly, the frequency of NAFLD (Non-alcoholic fatty liver disease) and liver fibrosis among SS patients matches the one in controls [150].

Hypothyroidism is encountered in around one third of patients with SS. Its primary cause is Hashimoto thyroiditis, an autoantibody mediated diffuse lymphocytic infiltrate around the thyroid gland. Of note, the condition, is actually also affecting 10% of the general population. This being the case, it is debatable whether the increased prevalence of Hashimoto thyroiditis in SS is another peri-epithelial manifestation of the disease or just the co-existence of another autoimmune disease in the context of poly-autoimmunity. Strikingly, however, a concomitant well defined autoimmune disease can be diagnosed in almost half of the patients, justifiably earning SS a place at the center of systemic autoimmunity [151,152].

3.5 Extraepithelial manifestations

Systemic involvement in Sjogren's syndrome can also be found outside of epithelial structures, typically in the form of an immune complex mediated inflammation of small caliber blood vessels. In this type III hypersensitivity reaction, according to the Gell and Coombs classification, complexes of rheumatoid factors and IgG are deposited preferentially in the skin, lungs and nerves causing an aberrant inflammatory response. These immune complexes have cryoprecipitatable properties, causing them to precipitate from the serum or plasma at temperatures below 37°C, and are known as cryoglobulins. There are 2 main types of cryoglobulins according to Brouet classification: a) Type I: there is a single immunoglobulin monoclonal component, usually in the setting of protein-secreting monoclonal gammopathies and b) Type II and III: their constituent Ig is not a single monoclonal Ig. In type II which is the commonest form in Sjogren's syndrome the actual Rheumatoid Factor is monoclonal (usually of the IgMκ type), while in type III both the rheumatoid factor and the IgG are polyclonal. The presence of cryoglobulinemia in SS is an important predictor of increased morbidity. One in three patients with cryoglobulinemia will develop a Sjogren associated B cell lymphoma within 5 years of the cryoglobulinemic vasculitis course. Compared to the HCV related cryoglobulinemia, the one in Sjogren

associated more often with lymphadenopathy, type II IgMκ cryoglobulins and lymphoma and less often with C4 hypocomplementemia and peripheral neuropathy [151].

Skin

Small vessel vasculitis characterizes the skin involvement of SS, although medium vessels can also be affected, though less frequently. The former can be clinically expressed as palpable purpura and less often as vesicles, pustules, and patches, while the latter, representing less than 5% of cases, shows up as livedo reticularis (racemosa) and ulcerative lesions of the lower extremities. Palpable purpura is the most common vasculitic skin lesion, occurring in almost 15% of SS patients. It usually follows a symmetric distribution over gravity-dependent areas, starting around the ankles and extending centripetally, but rarely spreading above the knees. Episodes of active vasculitis leave behind residual brownish hyperpigmented lesions noticeable at the inactive phase. Such a footprint of a previous vasculitic event should always prompt a directed investigation. Non-palpable (flat) purpura is often misdiagnosed as a vasculitic lesion, but has a different pathophysiology attributed to hypergammaglobulinemia with very high levels of polyclonal IgG. It should be clearly distinguished from palpable purpura due to vasculitis, since it imparts a mild non-lymphoma related clinical course [153]. Occasionally, palpable purpura is the first clinical sign of Sjogren's syndrome.

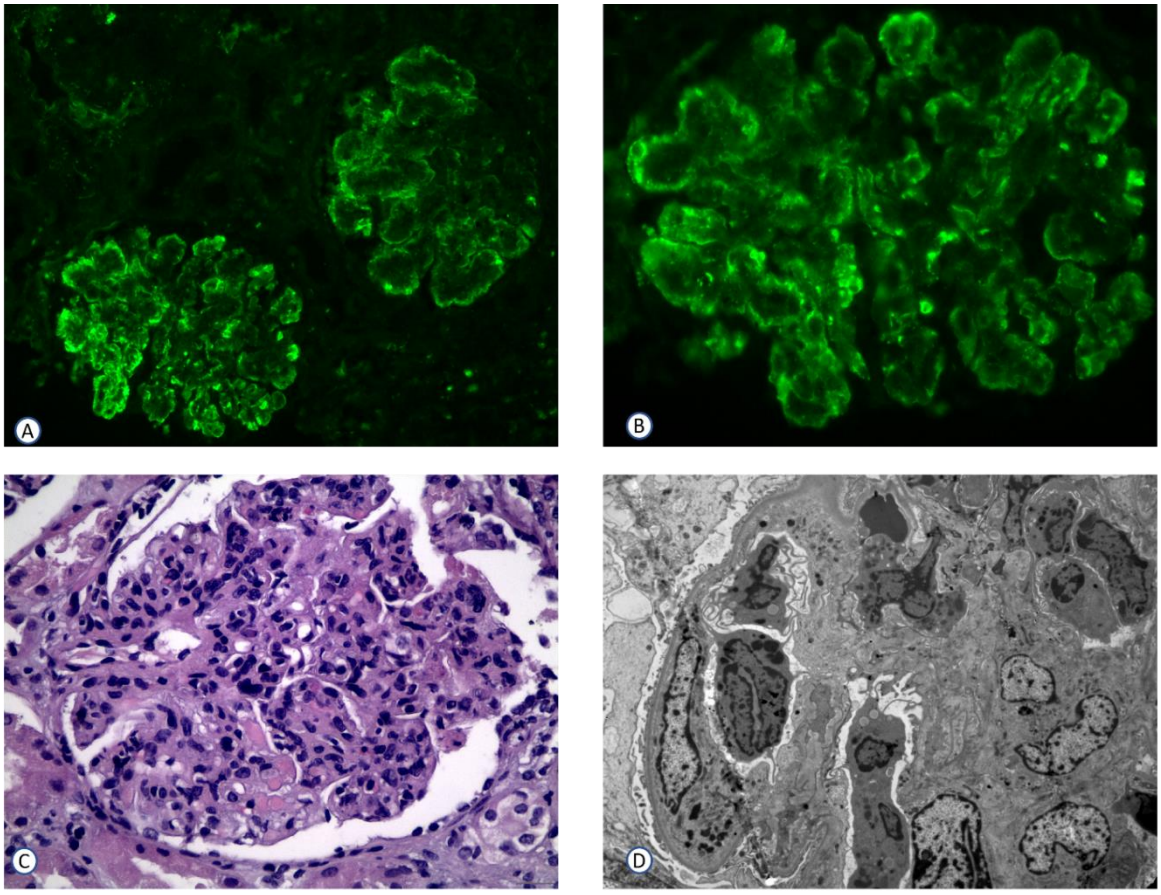
Nervous system

The prevalence of nervous system involvement in Sjögren's syndrome has been reported to be in the range of 2% to 15% [154-156]. Although it may present as both central (CNS) and peripheral involvement (PNS), CNS participation is an emerging and topic without convincing evidence of a causal association, beyond a limited number of case reports. Peripheral nervous involvement, on the other hand, is well ascertained and studied for decades and showing up in a variety of forms. Amongst them, axonal sensorimotor polyneuropathy and mononeuritis multiplex, both a result of vasculitis, are the most common types of involvement. Immune complexes are deposited in the vasa nervosum - the small arteries that supply the peripheral nerves, resulting in inflammation, vessel injury, and compromised blood supply to the nerves. In mononeuritis multiplex, two or more noncontiguous, asymmetrical nerves are damaged either concurrently or sequentially. Nerves of the lower extremities are usually affected first, including a peroneal neuropathy causing a foot drop. If the condition is left untreated, as it progresses the upper extremity can also be involved, with radial involvement causing a wrist drop.

On the other hand, axonal sensorimotor polyneuropathy is a symmetric, length-dependent painful process with sensorimotor symptoms and signs in the distal limbs, often referred to as a stocking-glove distribution. However, PNS involvement in Sjogren can be expressed in a variety of ways including a pure sensory neuropathy, demyelinating sensorimotor polyneuropathies, trigeminal neuropathy, radiculomyelopathy and autonomic neuropathy with anhidrosis. Pure sensory neuropathy is increasingly recognized in Sjogren's syndrome. It usually is in the form of small fiber neuropathy characterized by injury and loss selectively affecting small diameter sensory and/or autonomic axons. Patients with small fiber neuropathy complain of numbness, a mild burning dysesthesia or hyperesthesia, allodynia, and altered thermal perception. Against the plurality of symptoms, the disease is associated with a normal physical and neurologic examination, including nerve conduction studies. The diagnosis is made with a skin biopsy assessing the epidermal nerve fiber density (using a PGP 9.5 antibody for staining).

Kidney

Glomerulonephritis is another extraepithelial immune complex mediated manifestation observed in around 2% of patients with SS [157]. Renal features include the periepithelial injury and dysfunction of the renal tubules (previously discussed), as well as inflammation of the tiny filters in the kidneys (glomeruli), usually associated with cryoglobulinemia. Most often glomerulonephritis co-exists with palpable purpura, but a cutaneous involvement is not a constant feature. Hypertension and mild peripheral edema are related clinical facets, but glomerulonephritis is not usually associated with any noticeable symptoms, even though it can lead to long-term renal insufficiency. Warning signs of glomerular involvement, includes an active urine sediment with more than five RBCs of glomerular origin with or without cellular casts and proteinuria of more than 500 mg/day. However nephrotic range proteinuria suggesting an overt nephrotic syndrome is rarely encountered. Any glomerular in origin hematuria/proteinuria should prompt physicians a renal biopsy, confirming the diagnosis. The most frequent histologic types are that of a membranoproliferative, membranous, and mesangial glomerulonephritis. Glomerulonephritis has been associated with increased mortality and incidence of lymphoma in Sjogren's Syndrome patients [142].



Panel 1. Intense IgM granular expression of the subendothelial deposits along glomerular capillary walls (Immunofluorescence, IgM X200), Panel 2. Intense k light chain granular expression along glomerular capillary loops (Immunofluorescence, k light chain X200), Panel 3. Membranoproliferative pattern with accentuation of lobular glomerular architecture and "hyaline thrombi" into glomerular lumens (H&E X400), Panel 4. Mesangial cell expansion and cellularity, inflammatory cells into glomerular capillary lumens, endothelial cell activation along with segmental thickening of GBM and glomerular lumen obstruction (Electron microscopy, Uranyl acetate X2800).
Images provided by Liapis George(1st Department of Pathology, Medical School, National and Kapodistrian University of Athens, Laiko General Hospital, Athens, Greece.)

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3.6 Lymphoma

Normal Talal and Joseph Bunim after examining four patients with Sjogren's syndrome and lymphosarcoma (a term used for a malignant tumor of lymphatic tissue, now obsolete), concluded in 1964 that "in Sjogren's syndrome the chronic state of immunologic hyperactivity and the proliferation of immunologically competent cells producing abnormal tissue antibodies predispose to the relatively frequent development of malignant lymphoma". Today more than half a century later their hypothesis has proved to be the case, with its application now considerably expanded. Patients with Sjogren's Syndrome shoulder a stellar record with 6 to 19 times the likelihood of developing any kind of B cell lymphoma compared to the general population, a 30-fold increase in the risk for marginal zone lymphoma, and a 1000-fold increased risk developing a parotid gland extranodal marginal zone

lymphoma. Cumulatively, the relative risk for the development of lymphoma far exceeds that of any other systemic autoimmune disease, placing Sjogren at the crossroads of autoimmunity and lymphoproliferation, and thus rendering it a prime research model. Up until today, a key pathogenetic role is attributed to the overactivation of B cells as evidenced by the polyclonal hypergammaglobulinemia, the increased serum κ and λ chains, and the presence of a surfeit of serum autoantibodies. The abundant lymphocytic peri-epithelial lesions observed in the salivary glands or other affected organs, indicates a continuous interplay of B and T cells, resulting in B-cell hyperactivity that may eventually lead to their uncontrolled proliferation. This sustained antigen driven autoimmune B-cell propagation may escape the control of regulatory mechanisms, occasioning both indolent and aggressive B-cell lymphomas.

The cumulative incidence of B cell lymphomas in Sjogren's syndrome is 5% to 8%. The majority are mucosa-associated lymphoid tissue (MALT) lymphomas (55-75%), followed by Diffuse large B cell lymphomas (DLBCL) (15%) and nodal marginal zone lymphoma (NMZL) (10%). Less often we see follicular lymphomas and CLL/SSL. Compared to the B cell lymphomas, T cell lymphomas are less common in SS, especially after chemotherapeutic treatment or a bone marrow transplant. MALT lymphomas are encountered mainly in the minor or major (parotid, submandibular) salivary glands, although, cases associated with Sjogren have been reported in the lacrimal gland, orbital adnexa, eyelid, skin, thyroid, lung and stomach. Importantly, in 10-20% of MALT lymphoma cases multiple sites are simultaneously involved calling forand thus a complete work up (including thorough clinical examination, imaging evaluation, upper gastrointestinal endoscopy, thyroid ultrasound) in a patient with a diagnosis or suspicion of a lymphoma. One in five patients with lymphoma show bone marrow infiltration. If not diagnosed concurrently, MALT lymphomas usually follow Sjogren's diagnosis at a mean of 5 to 7.5 years. Diffuse large B-cell lymphomas may arise de novo or in the setting of a preexisting low-grade MALT, though, distinguishing between the two is problematic There are significant differences between the two predominant types of SS associated lymphomas regarding both the age at diagnosis (younger age in MALT, older in DLBCL) and the time point in the course of the disease at diagnosis (earlier in MALT, later in DLBCL). MALT lymphomas have a fairly good prognosis, with an 80% survival rate at 10 years, while both DLBCL and NMZLs exhibit a poorer prognosis with reduced survival rates at both 5 and 10 years. Intriguingly, the 10-year event free rate for MALT plummets to 45%, calling forth closer observation for optimum control.

A number of demographic, clinical, histologic and laboratory features have been related to future lymphoma development in Sjogren's syndrome. It has been shown that the best predictors of lymphoma, present at SS diagnosis, are cryoglobulinemia, the focus score and a high disease activity mirrored in an increased ESSDAI composite index. The highest odds ratio among all of nearly 6, is

attributed to cryoglobulinemia. Identification of predictors for lymphoma development in patients with SS is crucial to ensure a correct patient stratification. If a patient presents with, or develops, lymphoma predictors it is ought to be assigned to a high-risk group, necessitating, diligent follow up, frequent imaging and more aggressive treatment. Further large-scale studies are required to decipher the best treatment strategy for this cluster of SS patients.

Lymphoma predictors in SS

Major predictors

- Cryoglobulinemia
- Male gender
- Salivary gland enlargement (Persistent)
- High Focus Score in SS diagnosis

Minor predictors

Demographic

- Young age at SS onset (<35)
- Old age at SS onset (>65)

Clinical

- Persistent lymphadenopathy
- Splenomegaly
- Glomerulonephritis
- Raynaud's phenomenon

Serology

- Rheumatoid Factor
- Low C4 serum levels
- Anti/SSA and anti/SSB positivity

Blood

- Leukopenia
- Lymphopenia
- IgM kappa monoclonal protein

Histology

- Germinal centers
- Lymphoepithelial lesions

Chapter 4: Pathogenesis of Sjogren's syndrome

Like most systemic autoimmune disorders, the exact pathogenetic mechanisms that steer the immune system towards self-harm in SS have not been completely delineated. In general, a multistep process has been proposed to describe the general principles of the disease's development and evolution. The currently suggested model involves the following stages: a) glandular epithelium acquires an activated phenotype under the influence of various environmental triggers (e.g. infection, radiation, trauma), with viral infection being the most probable initial event, b) activated epithelial cells have the capacity to upregulate, redistribute, and release a variety of autoantigens and at the same time attract T and B lymphocytes at the sites of lesion where autoantigen presentation is taking place in genetically or epigenetically predisposed individuals, c) an interplay among epithelia, innate immunity through activation of plasmacytoid dendritic cells and adaptive immunity with the expansion of autoreactive B cells and autoantibody production, ensures fueling, perpetuation and maintenance of local autoimmune response d) tissue damage and secretory dysfunction occurs as a result of the ongoing inflammatory process and e) chronic antigenic stimulation may lead to the transition of a polyclonal/oligoclonal B cell component into a malignant lymphoma of B cell origin, most likely within the ectopic germinal center like structures (eGCLS) of the inflamed salivary glands. This short overview can designate the two key players of the disease's pathogenesis: the epithelial cell and immune cells.

4.1 The role of the epithelial cell

In the past 30 years, the central role of salivary gland epithelial cells (SGEC) in the development and maintenance of minor salivary gland (MSG) inflammation has been highlighted and can be summarized in 2 basic mechanisms: a) an activated phenotype characterized by expression of key molecules that: i) give epithelial cells properties of antigen-presenting cells ii) mediate homing and activation of immune cells into MSG, iii) promote survival, differentiation and proliferation of B cells, and b) release of autoantigens capable of fueling the local autoimmune response[158]. Therefore, "autoimmune epithelitis" has been proposed as an alternate term for Sjogren's syndrome, emphasizing the epithelium 's crucial and multidimensional pathogenetic role[33,159]. In particular, it has been shown that SGEC exhibit properties of non-professional antigen presenting cells, since they express high levels of HLA DR major histocompatibility complex (MHC) II molecules on their surface along with costimulatory molecules B7-1 (CD80), B7-2 (CD86) CD40, and adhesion molecules (ICAM-1, VCAM and E-selectin), displaying all the necessary elements of a functional immunologic synapse for autoantigen presentation[38,160-162]. In addition, another B7 family costimulatory molecule B7-H3

(CD276), has been recently found to be overexpressed, promoting inflammation and inducing apoptosis of epithelial cells by activating the NF-κB pathway[163]. Another role of SGEC is the production of cytokines such as IL-1a, IL-6, IL-7, IL-22, and TNF-a as well as a variety of chemokines[163-168]. Interestingly, CXCL12 and CXCL13, both produced from SGEC, are believed to contribute to the formation of eGCLS within the inflamed salivary glands[169-171]. In this line, epithelial cells have also been found to produce BAFF after IFNa exposure, thus regulating B cells longevity and maturation at the tissue level and creating a bridge between the innate and adaptive immunity in pSS[172,173]. Recently, it has been shown that the BAFF receptor, apart from B cells, is also expressed on the epithelial surface, further supporting their B cell fostering role[41,174]. The cellular lineage of SGEC, apart from their evolutionary role of covering, protecting, and secreting, also expresses pathogen recognition receptors on their surface, including Toll Like Receptors (TLRs), participating in innate immune responses[160,175]. However, in SS, a robust overexpression of TLRs and especially TLR 3 is apparent, hence its potential pathogenetic role has been a subject of extensive research. Toll like receptor 3 ligation in SS, may promote anoikis, a form of programmed apoptotic death, the production of several inflammatory cytokines or may lead to downregulation of PPARγ that mediates a strong activation of NF-kB and IL-1 pathways[176,177]. Similarly, TL3 stimulation in SS murine models has been found to accelerate the development of sialadenitis[178]. Finally, TLR-3 signaling in ex-vivo cultures of SGEC from SS patients leads to robust mRNA expression of the autoantigen Ro52/TRIM21 and less significant expression of other autoantigens of the Ro/La hYRNA ribonucleoprotein complex, linking innate immunity driven autoantigen overproduction with adaptive autoimmunity deleterious phenomena[139]. Furthermore, epithelial cells, apart from apparent secretory dysfunction, are prone to apoptotic death as well. SGEC of SS patients, although overexpressing FAS/FAS ligand (FasL) proteins, are relatively resistant to this kind of apoptosis and therefore a second hit such as the presence of a particular cytokine is required for the apoptotic process to be completed[179]. Additionally, FAS independent apoptosis may have also been documented through TL3 ligation and tumor necrosis factor related apoptosis-inducing ligand (TRAIL)[180].To this end, increased rate of epithelial apoptotic death in MSG of SS patients results in the release of apoptotic blebs loaded with specific autoantigens such as SSA/Ro and SSB/La, which can be subsequently uptaken by antigen-presenting cells, promoting in this way a local humoral autoimmune response and the production of autoantibodies. In addition, exosomes containing ubiquitous autoantigens such as Ro/SSA, La/SSB, and Sm ribonucleoproteins are secreted by SGEC and may serve as an additional source of autoantigens[181]. Finally, a recent study has shown the ability of SGEC to increase B cell survival, especially after TLR3 stimulation through still undefined soluble factors, even though this effect could not be reversed by targeting single cytokines such as IL-6, BAFF, or JAK molecules[41]. Epigenetics changes seem also to affect the activation status of the epithelium, as shown

by studies focusing on the epigenome and the differential methylation level of several genes [182-184]. Interestingly, MSG from SS patients exhibited DNA hypomethylation as demonstrated by IIF while La/SSB promoter of ex vivo cultures of SGEC was found hypomethylated and treatment with azacytidine, a hypomethylating agent, led to increased expression of La/SSB[185].

4.2 The role of immune cells

Lately, the contribution of innate immunity in the pathogenesis of Sjogren's syndrome has been studied extensively, focusing mainly on the role of interferons (IFNs) and plasmacytoid dendritic cells (pDCs) as the primary source of IFNα. Type I IFNs are known to modify the immune system and affect the function of many cell types. The presence of type I IFN-inducible genes was initially documented at the MSG level in SS patients, while type I IFN signature was also detected in PBMCs, B cells, and monocytes of peripheral blood along with elevated plasma levels of IFNα/β[186,187]. Interestingly, interferon type I and II induced protein activity was detected on ductal epithelial cells, while the inflammatory infiltrate surrounding the inflamed ducts produced type II only activity, supporting a dual role of the two types of interferons upon the diseased epithelium[188,189]. In accordance, pDCs have been identified in the MSG of SS patients and there is evidence that they can be activated through endogenous free DNA, transposons, or nucleic acid containing immune complexes. Recently, type III IFNs and especially interferon λ2 bearing similar immune modifying functions as type I IFNs, were found upregulated within the inflammatory sites of MSG due to TLR3 ligation of the epithelial cells[190]. Despite the fact that the presence of macrophages and IL-18^{+} cells in MSG of SS patients has been associated with a high focus score and the occurrence of well-known lymphoma risk factors and the fact that M2 type macrophages have been implicated in tissue destruction possible through the production of chitinases, the role of both macrophage types in SS pathogenesis remains to be elucidated [191-193].

The cardinal histopathologic lesion of SS is an abundant infiltrate of small lymphocytes surrounding the ductal epithelium. Although diverse, the inflammatory infiltrate's cellular composition is mainly composed of T and B cells, while less than 10% of the total immune cell population accounts for dendritic cells (conventional, plasmacytoid, or follicular), macrophages, and natural killer cells. A correlation between the type of lymphocytes and degree of inflammation has been reported in the literature. In mild lesions, T cells predominate, and these patients present primarily with peri-epithelial manifestations while more severe and extended lesions are characterized by the presence of B cells coupled with immune complex mediated manifestations[192]. Most T cells within the inflammatory infiltrate of the MSG of SS patients bear the CD4+ phenotype and most likely represent Th1 cells,

although Th2 and Th17 cells have also been observed[194]. IFN-γ-producing Th1 and interleukin 17 producing Th17 cells have been both detected in the glands of SS patients at the protein and mRNA level, while Th1 derived cytokines have been detected in the saliva of SS patients[195-198]. Interestingly, chemokines CXCL9 and ten that promote Th1 migration are produced by epithelial cells of SS patients. Apart from its immune modulating role, IFNγ has been found to impair tight junction function in vitro, a process that could reflect acinar dysfunction in SS and can induce apoptosis in SGEC, enhancing the Fas-FasL mediated apoptotic pathway[199]. Th17 cells are strongly activated and present in SS, augmenting the inflammatory response through IL-17A, IL17F and IL-22 cytokines among others[164,198]. Recently, it has been shown that $CD4^{+}IL17^{+}$ cells from PBMCs of SS patients are resistant to the effect of IL-27, an inhibitory cytokine of Th17 expression[200]. Interleukin 17 is also produced by the so-called double negative ($CD4^{-}CD8^{-}$) T cell population, implicated in the pathogenesis of various autoimmune diseases. In SS, double-negative T cells are present and are associated with more severe glandular involvement and a higher incidence of germinal centers[201,202]. On the contrary, Th2/IL-4^{+} cells are present in the MSG of SS patients, although with no difference compared to controls expect in situations of strong lymphocytic accumulation, while Th2-derived cytokines such as IL-10 and IL-4, have been found elevated in the saliva of patients with SS, also implying that Th2 cells may be involved at least to some extent in disease pathogenesis[194,203]. On the other hand, the number of CD8+ T cells, despite being low, remains stable as the lesion worsens and their exact role is unclear, even though their involvement in the cytotoxic induced epithelial cell death is apparent[192].

Various clinical, laboratory and histologic findings point out the significant contribution of B cell component in SS pathogenesis that appears to evolve gradually from a polyclonal B cell activation to a malignant transformation into non-Hodgkin's lymphoma of B cell origin: a) hypergammaglobulinemia and free serum light chains b) elevated serum levels of b2-microglobulin c) a plethora of autoantibodies d) presence of eGCLS within the MSG of SS patients, expressing AID and producing large quantities of high affinity autoantibodies e) increased frequency of monoclonal gammopathy and type II cryoglobulins and f) development of MALT lymphomas at the sites of pre-existing and chronic inflammation, most likely as a result of chronic antigenic stimulation[151,204-207]. Interestingly, more than 75% of all lymphomas detected in patients with SS are low grade, extranodal marginal zone lymphoma (MZL) of mucosa associated lymphoid tissue (MALT), revealing the potential key role marginal B cells play in disease progression. Marginal zone-specific B cell depletion in an animal model of SS had a positive impact on disease progression in the glands, supporting this notion[208]. Furthermore, marginal B cells are responsible for the majority of natural IgM positive memory cells found increased in the inflamed human salivary gland [209,210]. On the contrary, both IgM+ memory cells and class switched late memory cells are reduced in SS patients' peripheral blood either

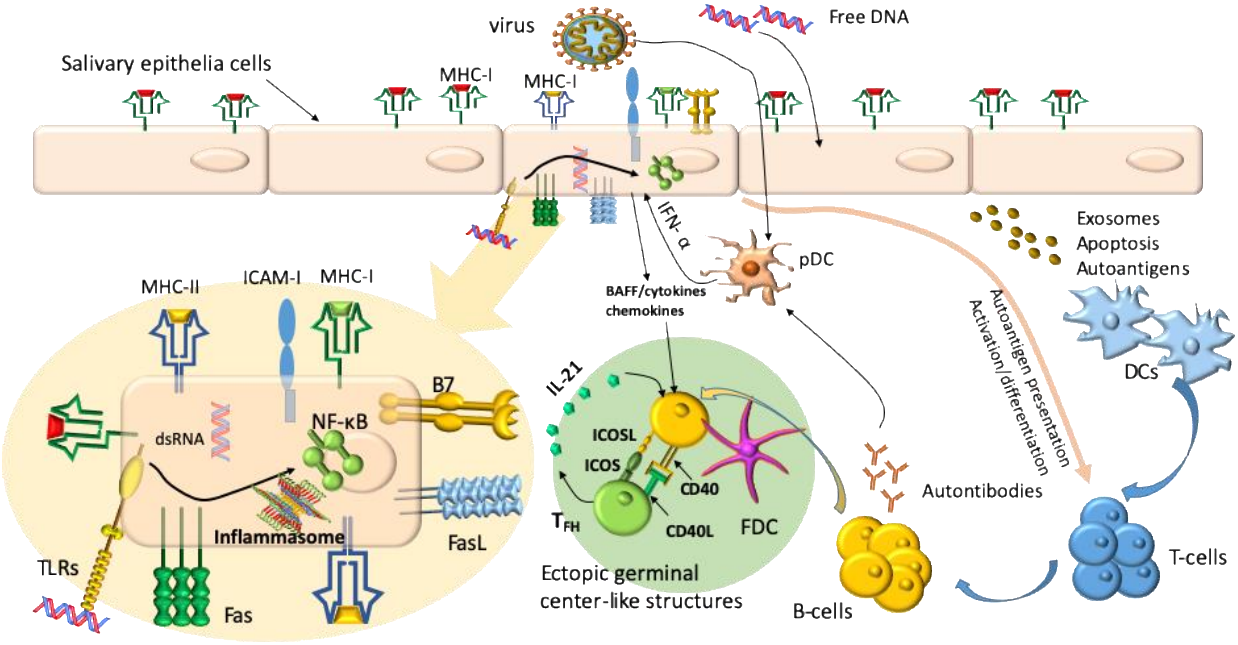
because of their homing in the glands or due to increased differentiation into plasma cells [211,212]. In addition, crucial for the development of the disease is the architectural formation of the inflammatory infiltrate which can resemble secondary lymphoid organs designating specific high throughput functions. These structures are called eGCLS, also known as "tertiary lymphoid organs," and are observed in 20-40% of SS patients, representing aggregates of specialized follicular T and B cells supported by a network of follicular dendritic cells (FDc). Although ectopic, it seems that these structures are fully functional and properly equipped with all the necessary molecular tools to contribute to B cell differentiation, isotype switch and affinity maturation. BAFF and BAFF receptors are present within the eGCLS, while simple B cell aggregates are distinct from eGCLS, being unable to express the key enzyme AID, as well [213,214]. Critical for the formation and maintenance of germinal centers are T follicular helper (Tfh) cells, found in the structures' periphery. They express CXCR5, ICOS, PD-1, and Bcl-6 and secrete IL-21, a key molecule for B cell differentiation into plasma cells[215,216]. Their identification in the inflamed gland is laborious and complicated because of the co-expression of CXCR5 in B cells impairing immunohistochemical studies, the loss of CXCR5 expression in the preparation of cells for flow cytometry analysis and finally owing to the downregulation of Bcl6 expression of the antigen-specific Tfh cells when the germinal center formation completes[217-219]. Finally, FDc lacking MHC-II expression, form a stable network for the development of eGCLS and express Fc receptors presenting opsonized antibody-antigen complexes to B cells[220]. Resident plasmablasts and long-lived plasma cells in the salivary glands can produce high amounts of autoantibodies (SSA/Ro and SSB/La) under the appropriate microenvironment influence through a milieu of cytokines and trophic agents, primarily BAFF and APRIL [221,222]. Besides the effector properties of B cells, it has been shown that there are B cells subpopulations in Sjögren's Syndrome mediating regulatory functions. They can stimulate the production of Treg cells and at the same time diminish the effect of pro-inflammatory Th1 and Th17 helper cells. These regulatory properties are supposedly mediated through the production of anti-inflammatory cytokine IL-10[223]. Pers et al. showed that B cells' immune regulatory properties are not an exclusive ability of certain B cell subpopulations, but the microenvironment can act as the defining factor. This is substantiated by the ability of a transitional B cell subset to secrete interleukin 10 under the appropriate conditions[224].

4.3 Lymphomagenesis in Sjogren

Sjogren's syndrome is a multifaceted disease with either glandular or extra-glandular/systemic manifestations. However, the most unique characteristic of SS among all organ specific or systemic

autoimmune disorders is the high-risk association with the development of B cell NHLs. More than 5% of SS patients will eventually develop a lymphoproliferative disorder that may complicate their clinical course and affect survival[225]. Interestingly, the common pathogenetic denominator of these two distinct clinical entities is evident either by the fact that various Sjogren specific manifestations are considered predictors for the development of lymphoma (e.g. salivary gland enlargement) or by the shared location of the major inflammatory target of Sjogren's and SS associated lymphomas, both positioned on the peri-epithelial mucosa. To this end, the prevailing hypothesis for lymphomagenesis in SS involves a continuum from a benign nonspecific B cell polyreactive population generated in the context of the local autoimmune process to a malignant monoclonal B cell component that dominates and invades the epithelia. This evolution involves multistep "hits" accumulated in the context of chronic antigenic stimulation and persistent inflammation, present in the salivary glands of SS patients[226]. The implicated antigen-driven process is supported by a study showing that more than 40% of MALT lymphomas in the salivary glands express immunoglobulin (Ig)VH-CDR3s hypermutated regions, homologous to stereotypic rheumatoid factors. This was less evident in specimens from MALT lymphomas of the stomach or lung[227]. A recent study from the same research group showed that all B cells from MALT lymphomas arising within the SS inflammatory lesions, express IgMk immunoglobulins carrying somatic mutations that increase IgG's affinity[228]. Besides, type 2 cryoglobulins that retain endogenous RF activity and have cryoprecipitable properties have been associated with heavier inflammatory infiltrates, more systemic disease, and future lymphoma development[151]. Different pathways involving BAFF/BAFF receptor, the inflammasome, and TNFAIP3/NF-kB have been implicated in the development of lymphoma, at the cellular and molecular level. Specifically, it has been shown that BAFF levels can serve as a predictor of lymphoma development with specific polymorphisms conferring greater risk, while the snip His159Tyr mutation in BAFF receptor has been associated with MALT lymphomas occurring at a younger age[173,229]. In addition, Baldini et al. investigated the role of the P2X7 receptor (P2X7R) acting as an activator of NLRP3 inflammasome in SS, showing that lymphomas of SS share an upstream cytokine production (IL-18) related to this pathway[230]. Finally, A20 protein encoded by the TNFAIP3 gene, regulating ubiquitination, seems to be a vital pathway inhibiting NF-kB activity. Several A20 polymorphisms have been associated with protein dysfunction and the development of several autoimmune disorders. In accordance, it has been shown that more than 75% of SS related MALT lymphomas are associated with mutations in the TNFAIP3 gene loci, implicating a possible role in lymphomagenesis[231]. All these findings suggest that in SS, B cell clones displaying RF specificity, with or without cryoprecipitable properties, pertain a survival and selection benefit through an antigen driven stimulus under the effect of various trophic agents and activated intracellular pathways. However, even an autoreactive hyperplastic B cell clone is not *a priori* malignant and

therefore additional mutations involving genes related to cell growth and proliferation such as oncogenes or tumor suppressor genes are required for a B cell to acquire the necessary traits and phenotype that will eventually complete the transformation process.



Chapter 5. Management of Sjogren's syndrome

SS syndrome should be conceptualized as a systemic autoimmune disease with the potential to affect almost any organ or tissue. To this end, SS patients should be followed up and treated only in specialized centers through a multidisciplinary approach that involves many specialists including rheumatologists, internists, ophthalmologists, dentists and oral medicine specialists, nephrologists, pulmonologists and pathologists [232].

5.1 General principles

Management of SS patients is complex and should follow specific steps and principles in order to control disease symptoms and prevent organ damage. The main goals are summarized as follows [232]:

- Estimate disease activity, damage, extent and the clinical phenotype for each patient separately, based on history, physical examination and special investigations. It is of great clinical importance to classify, in the best possible way by using well established clinical biomarkers, a patient into low or high risk group for developing the systemic form of the disease and lymphoma, in order to schedule the follow up intervals and the necessary work up.
- Inform and educate patients about all aspects of the disease (course, complications, prognosis) and consult them for efficient self-care (sicca manifestations, oral hygiene, regular dental visits etc.), healthy life style modifications (exercise, diet, smoking cessation) immunizations and concerns regarding pregnancy and conception.
- Discuss all therapeutic options with each patient individually and explain the efficacy and adverse events in order to reach a common treatment strategy.
- Escalate properly treatment modalities after considering each individual special characteristic including age, sex, co-morbidities, other medical conditions or medications and personal preference.
- Follow a strategy tailored to patients' need but in accordance to the international recommendations as follows [232,233]:
    - Non pharmacologic interventions and preventive measures
    - Local treatments
    - Conventional and organ based treatments
    - Targeted therapies [234,235]

5.2 Non-pharmacologic interventions and preventive measures

All SS patients should be clearly instructed to follow specific non-pharmacologic recommendations and preventive measures in order to improve the general health status and limit the complications of the disease as much as possible [232].

- Self-care for oral and ocular dryness

  SS patients with oral dryness should be instructed to maintain adequate hydration status by drinking sugar free liquids, avoiding irritant factors and sucrose and limiting as much as possible the use of medications causing dryness. In addition, intensive oral hygiene is mandatory to prevent dental carries and must be accompanied by regular visits to the dentist. Mechanical stimulation of salivary flow may be recommended by sucking sugar free candies or chewing gums.

  Patients with ocular dryness should introduce preventive measure for tear conservation in the everyday routine including avoidance of medications causing dryness and deteriorating environmental factors such as eye irritants and special pollutants. The use of physical barriers such as special types of sunglasses or side shields can be used by patients in certain environmental conditions.

- Lifestyle modifications

  Exercise, diet and smoking may affect the global health status of all individuals, including SS patients. Especially smoking cessation is an important modification that may also alleviate oral dryness symptom while it is mandatory for SS cases with respiratory involvement including xerotrachea, small airways disease and interstitial lung disease with or without bronchiectasis. Interestingly, SS patients with dry syndrome have been found to benefit from omega-3 fatty acids [236].

- Vaccinations

  Apart from the recommendations for the general population, SS patients should be instructed to be vaccinated for pneumococcus, seasonal flu, HBV, SARS-CoV2 and for herpes zoster with the recombinant (non-live) formulation. Live attenuated vaccines are in general contraindicated for immunocompromised patients, especially those who receive heavy

immunosuppression. Vaccination should be performed ideally before the onset of immunosuppression, otherwise and depending on the regimen and dosage (conventional vs biologic targeted treatments), treatment modifications can be applied to augment the immunogenicity of vaccines.

- Conception, pregnancy and lactation.

  Special counseling should be offered to the female SS patients of reproductive age who display anti-Ro/SSA (either Ro52 or Ro60) and anti-La/SS B positivity, receive disease modifying drugs or targeted therapies. The presence of anti-Ro/SSA and/or anti-La/SSB has been associated with very low risk of neonatal lupus and congenital heart block of the child (around 1%) [237] but history of previously affected child increases the risk to 15-20% for subsequent pregnancy [238]. Seropositive SS mothers should be monitored weekly or every other week by pulsed colored fetal Doppler echocardiography from 16th-28th week of gestation [239]. As a preventive measure, pregnant mothers with previous history of affected child with CHB should receive 400mg hydroxychloroquine daily to reduce the risk of recurrence, starting between 6th -10th week of gestation and during pregnancy [240]. In case of utero detection of first or second degree heart block, fluorinated glucocorticoids (oral 4mg dexamethasone or 3mg betamethasone) may be administered [241,242] at least until the 26th week (if reverted to normal sinus rhythm) and discontinued if progressed to third degree block. In second degree block, 2mg dexamethasone may be maintained through pregnancy even when the fetus has normal heart rate. In general, low dose of prednisone, azathioprine and hydroxychloroquine are allowed in contraception and during pregnancy or lactation. Other treatment modalities can be considered after assessing the benefit-risk and discuss with patients all options and adverse events.

5.3 Local treatments

For SS patients who have not achieved symptomatic relief of oral dryness with general measures or adequate salivary flow with mechanical stimulation, pharmacological stimulation with muscarinic agonists (sialagogues) may be considered [232,233]. Pilocarpine and cevimeline are the most frequently used medications, although the latter is not available in Europe. Pilocarpine can be administered orally at a dose of 5mg up to 4 times daily, after increasing the dose progressively to avoid side effects such as sweating. Similarly, the dose for cevimeline is 30mg orally three times daily. Randomized control trials have shown almost equal efficacy of both dugs. The main side effects are due to their cholinergic actions including nausea,

diarrhea, excessive sweating and urinary frequency. Side effects may be associated in some cases with limited tolerance and poor compliance. In SS patients who do not respond or are intolerant to muscarinic agonists, mucolytic or choleretic agents may be administered. Saliva substitution with artificial saliva is the final step for non-responders SS patients with persistent oral dryness. Several formulations are available including gels, sprays and rinses which can be used regularly and especially before sleep or on demand without significant adverse events or safety issues [232,233] (Table 9).

Ocular dryness should be managed with artificial tears and lubricants to replace volume and protect the cornea. The currently available formulations include ointments, gels or eyedrops that contain polymeric or viscosity agents such as hyaluronate or methylcellulose. In severe or refractory cases of ocular dryness, escalation of local treatment includes eyedrops with non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids (GC), cyclosporine (CyA) or serum tears. Local treatment with NSADs and GC should be followed for a short period, not exceeding 2-3 weeks due to the related adverse events that may occur such as keratitis, ulcerations, perforations and melts of the cornea, and always in collaboration with an eye specialist. If frequent cycles of local treatments with NSAIDs or GC are required to control eye dryness, then eyedrops formulation of CyA 0.05% can be introduced as an alternative. In non-responders and difficult cases, autologous serum tear drops may be also applied. Finally, extremely severe cases of ocular dryness may require combination of oral muscarinic agonists and special interventions with plug insertion to restore tear production [232,233] (Table 9).

Local treatments for Sjögren's syndrome

Therapeutic approach for oral dryness

1. Non-pharmacological stimulation
    a. Mechanical stimulants (sugar-free chewing gum)
    b. Gustatory stimulants (sugar-free acidic candies, lozenges, xylitol)
2. Pharmacological stimulation
    a. muscarinic agonists
        i. Pilocarpine
        ii. Cevimeline
    b. Rescue therapies
        i. choleretic agents
        ii. mucolytic agents
3. Saliva substitution (gels, sprays, rinses)

Therapeutic approach to ocular dryness

1. Volume replacement and lubrication
    a. artificial tears (eye drops)
    b. Lubricants (ointments, gels)
2. Topical NSAIDs/corticosteroids
3. Topical Cyclosporine
4. Autologous serum tear drops
5. Tear canal plug insertion

5.4 Systemic and organ-based treatment.

To treat systemic and organ-based manifestations, many drugs can be employed including glucocorticoids (GC), conventional disease modifying antirheumatic drugs (cDMARDs) (hydroxychloroquine, methotrexate, leflunomide, azathioprine, mycophenolate mofetil and cyclophosphamide) and targeted therapies such as rituximab and belimumab [232,233]. Glucocorticoids should be used at the lowest possible dose and for the shortest period while cDMARDs can be used as cortisone sparing drugs depending on the affected tissue/organ. Although no immunosuppressive agent has been proven to be superior compared to the others, the therapeutic strategy and recommendations are mainly tissue/organ based, considering also patients' comorbidities, age, sex and concurrent medications [232] (Table 10).

Organ based treatment modalities for Sjögren's syndrome

1. Glandular Involvement
    a. Glucocorticoids (0.3 mg/kg/day) for 2-3 weeks
    b. Rituximab (1 g x2, 15 days apart) or Belimumab (10 mg/kg at 0, 2 and 4 weeks and then every 4 weeks)
2. Articular Involvement
    a. Corticosteroids (5-7.5mg/daily)
    b. Hydroxychloroquine (200mg/daily)
    c. Methotrexate (10-15mg/weekly)
    d. Leflunomide (20mg/daily)
    e. Rituximab (1g x2, 15 days apart)
3. Cutaneous Involvement
    a. Glucocorticoids (0.3-1 mg/kg/day)

b. HQ plus MTX or colchicine or azathioprine or mycophenolate
c. Dapsone or Thalidomide or lenalidomide
d. Plasma exchange plus rituximab or cyclophosphamide

4. Pulmonary Involvement
    a. Short/long acting beta agonists plus short/long acting muscarinic antagonists (for small airways disease)
    b. Glucocorticoids (0.5-1 mg/kg/day) plus mycophenolate mofetil or azathioprine (for ILD)
    c. Rituximab or cyclophosphamide (for ILD)
5. Renal Involvement
    a. Alkali supplements, potassium alkali, thiazides (for Interstitial nephritis)
    b. Glucocorticoids (0.5-1 mg/kg/day) plus mycophenolate or azathioprine (for GN)
    c. Plasma exchange plus rituximab or cyclophosphamide (for GN)
6. CNS Involvement
    a. 1g iv pulses of methyl-prednisolone x 3 plus glucocorticoids (0.5-1 mg/kg/day) plus cyclophosphamide or rituximab
    b. 1g iv pulses of methyl-prednisolone x 5 days plus rituximab or mycophenolate mofetil or eculizumab
7. Peripheral neuropathy
    a. Glucocorticoids (0.5-1 mg/kg/day) plus cyclophosphamide or rituximab with or without iv 1g pulses of methyl prednisolone x 3
    b. IVIG
    c. Rituximab or cyclophosphamide
    d. Plasma exchanges
    e. calcium channel alpha 2 delta anticonvulsant agonists
8. Hematologic involvement
    a. Glucocorticoids (0.5-1 mg/kg/day)
    b. Rituximab
    c. IVIG
9. Cryoglobulinemic Vasculitis
    a. Glucocorticoids (0.5-1 mg/kg/day)
    b. Rituximab or cyclophosphamide
    c. Plasma exchanges
    d. Combination of belimumab and rituximab
10. SS related lymphomas
    a. Wait and see policy
    b. Rituximab and mustard
    c. R-CHOP

- Major salivary gland enlargement

SS patients may present with acute unilateral or bilateral parotid enlargement. Unilateral involvement requires a careful work up to exclude infection, especially if fever and pain are present. In case of SS related major salivary gland enlargement, NSAIDs should be used with caution for no more than 7 days to relief symptoms, and if not contraindicated. As an alternative or a second line treatment GC (prednisone) at 0.3 mg/kg daily (e.g. 20mg prednisone daily) may be administered for a week with tapering and discontinuation within 2-3 weeks from the beginning of treatment. For chronic persistent parotid enlargement exceeding 2 or 3 months, an underlying MALT lymphoma should be excluded and rituximab or belimumab may be considered.

- Articular Involvement

Patients with arthralgias should be clearly differentiated from those with frank synovitis. For episodic arthralgias, NSAIDs may be prescribed for a few days but in case of disabling and chronic arthralgias, hydroxychloroquine (HQ) is the drug of choice at a dose of 200mg/daily. Polyarthritis with synovitis can be treated initially with oral prednisone 5-7.5mg/daily orally in combination with HQ 200mg/daily for at least 3-4 months while in refractory cases methotrexate (10-15mg/daily) or leflunomide 20mg/daily may be added. For refractory cases, co-existence with rheumatoid arthritis should be ruled out and rituximab may be considered.

- Cutaneous Involvement

Annular erythema is a skin rash that may be also observed in subacute cutaneous lupus erythematosus and is related to the presence of anti-Ro/SSA autoantibodies. Limited rash is treated with topical GC but therapy should not last more than 2-3 weeks because of the risk of skin atrophy. Alternatively local treatments with tacrolimus can be applied. For extensive disease, combination therapy with GC at 0.3mg/kg/daily (e.g. 20mg prednisone) in tapering and HQ 200mg/daily can be employed. Methotrexate can be also added for refractory cases (10-15mg/weekly). Other options of cDMARDs include azathioprine, mycophenolate mofetil, dapsone and thalidomide/lenalidomide.

Purpura is another common cutaneous manifestation. Non-palpable petechial purpura of the lower extremities is due to hypergammaglobulinemia and is managed with non-pharmacologic measures such as prolonged standing avoidance and compression stocks. In refractory cases HQ may be considered. On the contrary, palpable purpura is usually a manifestation of cryoglobulinemic vasculitis (CV) that may be clinically expressed with other skin lesions such as urticaria, skin ulcerations and less commonly digital necrosis. The presence of palpable purpura should rise clinical suspicion for other organ involvement, especially glomerulonephritis and peripheral neuropathy as well as an underlying lymphoproliferative disorder. In case of limited palpable purpura without the involvement of other organs, a short course with low dose GC (0.3mg/kg/day) plus HQ 200 mg/d and colchicine 0.5 mg twice daily can be administered. Severe cutaneous lesions (ischemic ulcers, necrosis) in the context of CV should be treated aggressively with high dose GC (0.5-1mg/kg/daily), plasma exchanges and rituximab or iv pulses of cyclophosphamide (either 0.5g/15days for 6 pulses or 750mg-1g/m^2 BSA monthly for 6 pulses-maximum total dose of 1.5g) followed by maintenance therapy with either rituximab, mycophenolate mofetil or azathioprine, especially if the kidneys and peripheral nerves participate in the clinical picture of the disease.

Raynaud's phenomenon should be initially managed with non-pharmacologic preventive measures: avoidance of cold exposure and vasoconstrictive medications, smoking cessation and strategies to ensure warmth of digits (e.g. use of special gloves). First line treatment includes long acting dihydropyridine calcium channel blockers or alternatively phosphodiesterase type 5 inhibitors while in case of digital ulcers endothelin receptor antagonists may be administered.

- Pulmonary Involvement

For SS patients with small airways disease and exertional dyspnea, pharmacotherapy is tailored according to the symptoms severity and exacerbation rate. For mild symptomatic patients with low exacerbation rate, inhaled short acting beta agonists with or without short acting muscarinic antagonists may be administered on demand for episodic relief. In more severe and persistent cases, regular treatment with inhaled long acting beta agonists with or without long or short acting muscarinic antagonists should be designed while short acting beta agonists may be given as needed for symptomatic relief.

Interstitial lung disease is usually expressed as non-specific interstitial pneumonia (NSIP), lymphocytic interstitial pneumonia (LIP) and less commonly usual interstitial pneumonia [138]. Treatment choice

depends on symptoms severity, disease extent in terms of affected lung parenchyma on HRCT and the abnormalities on pulmonary function tests. For SS-ILD asymptomatic patients with limited disease and normal pulmonary function tests, no treatment may be initiated and patients can be re-evaluated after 3-6 months. For symptomatic patients, with more extended disease and impaired pulmonary function tests, GC (1mg/kg/day) combined with azathioprine or mycophenolate mofetil are administered. In rapidly progressive cases with worsening symptoms or extended lung disease, high GC doses should be combined with either rituximab or iv monthly pulses of cyclophosphamide.

- Renal Involvement

Interstitial nephritis may lead to latent or complete dRTA, proximal RTA, nephrogenic diabetes and nephrocalcinosis. Distal RTA is the most common clinical presentation and special attention should be taken to treat hypokalemia that in severe cases may be proven fatal. Alkali supplements at 1-2mEq/Kg divided in 4 doses daily can be prescribed to control hypercalciuria, renal stone formation and acidosis while for proximal RTA the required doses may reach 10-15mEq/kg daily due to increased renal losses. Thiazide diuretics may reduce the required alkali salts dose. Potassium alkali are indicated in cases of persistent hypokalemia. Immunosuppressive treatment has not been proven beneficial for RTA and IN [243] but a short course of GC may be considered in cases with acute decline of renal function and histologic evidence of tubulitis.

Glomerulonephritis (GN) is either mesangial or membranoproliferative histologic type is almost always part of CV. In mild forms of GN (e.g. mesangial GN) without impaired renal function, low dose of GC (0.5mg/kg/daily) combined with azathioprine or mycophenolate mofetil can be administered. For more active and severe forms of GN (e.g. membranoproliferative), high doses of GC (0.5-1mg/daily), plasmapheresis and rituximab or iv pulses cyclophosphamide should be given to control disease progression and prevent end stage renal disease.

- Neurologic Involvement

Peripheral nervous system disease (peripheral neuropathy) includes multiple polyneuropathy (mononeuritis multiplex), sensory and sensorimotor peripheral neuropathy (axonal PN), sensory ataxic neuropathy (ganglionopathy), chronic inflammatory demyelinating polyradiculopathy (CIDP) and small fiber neuropathy. Multiple polyneuropathy is mainly attributed to CV and should be treated in the

context of cryoglobulinemia with high GC doses (0.5-1mg/kg/daily) coupled with rituximab or iv pulses of cyclophosphamide, with or without plasma exchanges. Intravenous pulses of methylprednisolone (1g/daily for 3 consecutive days) may be considered especially in acute motor neuropathy. Axonal PN with mild sensory features may be treated symptomatically with calcium channel alpha 2 delta anticonvulsant agonists (gabapentin, pregabalin). For more persistent and disabling sensory symptoms or motor neuropathy, CV should be considered and investigated while treatment includes GC combined with rituximab or iv pulses of cyclophosphamide with or without iv pulses of methyl-prednisolone. First line treatment for ganglionopathy and CIDP is considered the early administration of IVIG while alternatives include rituximab and plasma exchange sessions. For rescue therapy pulses of iv methyl-prednisolone and cyclophosphamide may be considered. Finally, for small fiber neuropathy gabapentin and pregabalin may be beneficial.

Central nervous disease is rare in SS and includes CNS vasculitis and neuromyelitis optica spectrum disorder (NMOSD). For acute neurologic manifestations related to brain or spinal cord lesions, iv pulses of 1g methyl-prednisolone for 3-5 days followed by high doses of oral GC (1mg/kg/daily) and iv monthly pulses of cyclophosphamide is the treatment of choice. Azathioprine or mycophenolate mofetil can be used for maintenance therapy after the iv course of cyclophosphamide. Plasma exchanges may be also considered for severe cases. Acute demyelination episodes in the context of neuromyelitis optica spectrum disorder should be managed with iv pulses of 1g methyl-prednisolone for 5 days, followed by maintenance therapy with rituximab, mycophenolate mofetil, azathioprine or eculizumab. For refractory to GC cases, plasma exchanges may be performed.

- Hematologic manifestations

Hematologic abnormalities are relatively common among SS patients but rarely treatment is required. Anemia may be of various causes but frank warm autoimmune hemolytic anemia is infrequent and raises differential diagnostic dilemma with lupus. Warm autoimmune hemolytic anemia is managed by high doses of oral GC (1mg/kg/daily) plus rituximab. Similarly, immune mediated thrombocytopenia is not common and is treated with IVIG in case of active bleeding or very low platelets count (<20.000/μl) plus high dose of oral GC (1mg/kg/daily) and rituximab. Intravenous pulses of cyclophosphamide are rescue therapy for refractory cases. Finally, leukopenia/neutropenia is usually mild and responds very well in a short course of low dose GC (0.3mg/kg/day).

- Checkpoint inhibitors

The wide clinical use of immune checkpoint inhibition (ICI) therapy for cancer, has led to a variety of immune mediated adverse events including the post ICI sicca symptoms/SS like syndrome. Although the post ICI Sjögren's like syndrome shares common features with the idiopathic primary Sjögren's syndrome, the 2 entities are considered distinct. Management of the post-ICI Sjögren's like syndrome depends on the severity of symptoms, patient's performance status and comorbidities as well as the primary malignancy. For mild dryness manifestations, local treatment is the cornerstone of therapy while ICI may be continued as scheduled. For severe manifestations including sicca symptoms, arthritis and skin lesions, ICI should be discontinued and low to moderate doses of GC can be introduced. If manifestations are controlled, the ICI treatment can be resumed, otherwise it must be permanently discontinued [244].

- Lymphoma associated lymphoproliferation

The most common histologic types of SS associated non-Hodgkin's lymphomas covering more than 85% of cases, are MALT and DLBC lymphomas. MALT lymphomas display a relatively favorable prognosis as opposed to DLBC lymphomas [245]; thus for MALT lymphomas which are mainly expressed as salivary gland enlargement, a wait and see policy may be followed. Treatment may be instituted when other extra-nodal or nodal sites are involved implying disease dissemination, such as the bone marrow or the lymph nodes respectively. Regimens include either rituximab alone or rituximab coupled with chemotherapy (e.g. mustard). For refractory cases or DLBC lymphomas the R-CHOP is the first line of treatment. For all cases of SS associated lymphoproliferation, consultation by a specialized hematologists mandatory.

5.5 Targeted treatments

Several targeted biologic treatments have been tested in randomized control or open label studies to modify disease course, improve functional parameters of salivary and lachrymal glands and treat both glandular and extra-glandular manifestations [234,235] (Table 11). To this end, most of these studies have adopted as primary outcome the reduction of the overall disease activity expressed by the ESSDAI index and a decrease in ESSPRI representing the subjective components of the disease such as pain, fatigue and dryness. Several reasons may be responsible for the limited inefficacy of targeted therapies: i) the slowly progressive nature of the disease and the advanced stage at the time SS

diagnosis, ii) the short duration of most studies, iii) the limited knowledge of the key pathogenetic mechanisms, iv) the remarkable diversity of the clinical phenotypes of the disease, reflecting distinct endotypes and key molecular pathways and v) the attempt to reduce the overall disease activity rather than treat tissue specific manifestations [246].

- Rituximab

Rituximab has been tested in many studies without showing significant efficacy to control overall disease activity. In few of them, it has been found to improve fatigue, salivary flow rate, transiently sicca symptoms and disease activity due to effect on biologic domain of ESSDAI. However, rituximab has been effective for treating B cell immune complex mediated manifestations related to cryoglobulinemia including palpable purpura, glomerulonephritis and peripheral neuropathy. In addition, rituximab may be considered for articular manifestations, ILD, NMOSD and immune mediated hematologic manifestations [247-253].

- Belimumab

Belimumab, a monoclonal antibody against the BAFF, showed only modest reduction in dryness symptoms, parotid enlargement and arthralgias [254,255]. However, it was found to be effective in combination with rituximab to control refractory cases of cryoglobulinemic vasculitis [256,257].

Other targeted treatments that have been tested so far include: TNF inhibitors, anakinra, abatacept, tocilizumab, epratuzumab, ianalumab, iguratimod, seletasilib and baminericept. A brief summary of pros and cons emerging from clinical trials are presented in Table 11.

Table 11. Targeted treatments tested in Sjögren's syndrome

Targeted treatments	Pros	Cons	Refs
Rituximab (anti-CD20 mab)	• Effective for treating B cell mediated manifestations due to cryoglobulinemia • Can be combined with belimumab for refractory CV • Therapeutic option for arthritis, ILD, NMOSD and hematologic manifestations	• Improvement of disease activity, dryness, fatigue and salivary flow in some studies.	[247-253]
Belimumab (anti-BAFF-R mab)	• May be combined with rituximab for refractory CV	• Modest reduction only in dryness, parotid enlargement and arthralgias	[254-257]

TNF inhibitors (infliximab, etanercept)	_	• No efficacy in any aspect of the disease	258,259
Abatacept (CTLA4-Ig fusion protein)	• Evidence of biologic activity and improvement of disease related laboratory parameters	• Lack of clinical efficacy • Relatively higher rates of serious adverse events	138,260
Anakinra (IL-1 receptor antagonist)	_	• No effect in fatigue	261
Tocilizumab (anti-IL6R mab)	_	• Lack of efficacy	262
Ianalumab (anti-monoclonal BAFF-R mab, engineered for efficient ADCC)	• Decreased overall disease activity • Depletion of mature B cells over naive	• Infusion/injection related reactions	263,264
Epratuzumab (anti-CD22 mab)	• Limited efficacy in tear production, salivary flow and fatigue	• Significant improvement in limited proportion of patients	265,266
Iguratimod (small molecule inhibiting inflammatory pathways including NF-kB)	1) Improved fatigue and dryness 2) Decreased plasma cells	• No reduction in disease activity • High rates of adverse events	267
Baminercept (Lymphotoxin-β receptor fusion protein)	• Reduced B and T circulating cells • Decreased CXCL13 plasma levels	• No reduction in disease activity, salivary flow rate or dryness • Liver toxicity	268
Seletasilib (selective PI3kd inhibitor implicated in B cell signaling)	• Reduced inflammation in diseased salivary glands	• No reduction in disease activity salivary flow or tear production • High rates of adverse events	269

Chapter 6: Biomarkers

6.1 Traditional biomarkers

In the current era of better understanding diseases' pathogenesis, different epidemiologic and genetic patterns as well as disease-related complications, prognosis, and outcomes, the global medical community is hesitantly adopting a precision medicine approach in every aspect of diseases' treatment and prevention. To that end, it is necessary to cluster patients in groups that share the same disease defining characteristics to guide targeted treatments and group monitoring approaches. This strategy is impossible without measurable biologic parameters, known as biomarkers, that combined will allocate each patient in the correct group. Sjogren's syndrome being a common disease, with a wide spectrum of mild to life threatening complications, a complex pathogenesis, and easily obtainable disease-related biologic specimens (MSG biopsy, serum, saliva, tears) is an ideal disorder for such approach.

Several clinical, serologic, and histologic parameters have been widely used in Sjogren's syndrome for years to predict disease severity with systemic manifestations and/or lymphoma. Episodic parotid gland enlargement, either unilateral or bilateral, cryoglobulinemic vasculitis manifested as palpable purpura or necrotic skin ulcers in legs, and transient or persistent cervical lymphadenopathy have been described as independent risk factors for lymphoma development[270-273]. Another report adds splenomegaly as well[274]. Lately, it has been shown that age as well as gender are important determinants of disease course[76,97,275]. Young-onset SS patients have a more aggressive clinical phenotype involving SGE, Raynaud, leukopenia, and lymphoma, while older patients develop less frequently arthritis, more often interstitial lung disease, and display higher risk for lymphoma compared to middle aged SS patients. In addition, the study of large SS populations from multinational cohorts have allowed the clinical phenotype description of males with SS, proving that male patients develop more often SS associated lymphomas, implying that gender is an independent risk factor for lymphoproliferation. Finally, data from the Big Data Sjögren Project Consortium provided the first substantiated evidence about the influence of geolocation on the age SS is diagnosed, the female to male ratio and the prevalence of sicca symptoms[71].

Autoantibodies in SS are highly prevalent and characteristic of the disease. Autoantibodies, as in many autoimmune diseases, may precede the diagnosis of SS by at least 20 years[276]. In addition, the presence of anti-Ro/SSA autoantibody is one of the two major diagnostic criteria of the currently used 2016 ACR-EULAR Classification Criteria for Sjogren's[277]. These data imply that autoantibodies can be used as early biomarkers for disease diagnosis or prediction in the right clinical context (individuals with a strong family history of seropositive autoimmunity). In addition, the detection of anti-Ro and/or

anti-La antibodies in asymptomatic women of childbearing potential can serve as biomarkers for treatment intervention. Both autoantibodies are pathogenetically linked to heart rhythm abnormalities in the fetus, necessitating the use of hydroxychloroquine in the pregnant mother as a protective measure[278]. The presence of anti-Ro/SSA and/or anti-La/SSB autoantibodies in SS patients has been linked to a more severe systemic activity and longitudinal disease course[279,280]. Seronegativity defined as absence of anti-Ro/SSA and anti-La/SSB autoantibodies seems to be associated with less B cell manifestations, including lymphoma development[281]. Rare patients with anti-La/SSB positivity without anti-Ro/SSA antibodies constituting less than 3% of the SS population do not display differences in their clinical disease course[282,283]. ACA positive Sjogren is a subset of SS found in approximately 10% of SS population involving patients positive for anticentromere antibodies. These patients develop a more scleroderma-like phenotype with a higher frequency of Raynaud's phenomenon and a more severe labial inflammatory infiltrate[284-286]. Furthermore, the presence of anti-RNP antibodies in SS patients has been associated with increased muscular and pulmonary involvement[287]. Finally, 10% of SS patients with inflammatory polyarthritis have anti-CCP positivity obscuring the borders with rheumatoid arthritis. Serologic biomarkers also have a validated predictive role for lymphoma development[288,289]. The presence of type II serum cryoglobulins, low levels of serum complement C4, and rheumatoid factor activity have all been shown to be related to future lymphoma development[112,290,291]. Interestingly, John Ioannidis et al revealed the significance of C4 hypocomplementemia and type II cryoglobulins at the first rheumatologic visit, showcasing that these patients have a much higher probability for future development of a lymphoproliferative disorder and fatal outcome[292]. Doctors can also utilize simple hematologic parameters derived from a CBC as potential biomarkers for future lymphoma development. Neutropenia, leukopenia, lymphopenia, and particularly CD4 lymphopenia are all predictors for the development of Sjogren related malignant lymphoma[272,274,293-295].

The critical role the cellular infiltrate plays in the pathogenesis of the disease was analyzed in detail previously. Similarly, different aspects derived from the MSG pathology assessment can be used as biomarkers for disease progression. Particularly, more abundant inflammatory infiltrates, mirrored in an MSG focus score above 3, have been associated with a more extra glandular disease progression and future lymphoma development, implying the importance of the labial biopsy as a prognostic tool even in patients that other classification criteria suffice to reach a Sjogren's diagnoses[296]. The presence of germinal centers, found in approximately 25% of MSG biopsies, has also been supported to be an independent lymphoma predictor bearing a 5-16-fold increased probability of future lymphoproliferation[294,297,298]. However, these findings have been debated and are not always reproducible[299,300]. In addition, SS patients with eGCLS presence, suffer from more systemic disease

and a more prolific autoantibodies profile. Finally, the clinical associations of a B or T lymphocyte predominant MSG infiltrate were discussed previously.

6.2 Novel biomarkers

Aside from all the widely used biomarkers described above, new biotechnologies and more in-depth research of the mechanisms of SS that involve analysis of serum, MSG biopsy, saliva and tears have led to the introduction of several potentially novel biomarkers. The clinical and prognostic utility of these novel biomarkers is currently under investigation and further studies are needed to clarify their significance.

Recently, a series of new autoantibodies have been described in relation to SS[301]. Antibodies to muscarinic three receptor (Anti-M3R) are present in the vast majority of SS patients. Anti-M3R antibodies seem to bear a presumed pathogenetic role since they block the neurotransmission axis necessary for saliva production[302]. In addition, autoantibodies against aquaporin 5 (AQP5), a water-permeable channel highly expressed in the apical epithelium of the salivary acini, has been described and correlated with serologic and histologic features of the disease[303]. In addition, Anti-carbonic anhydrase II antibodies have been found in approximately one-fifth of SS patients carrying a presumed pathogenetic role in renal tubular acidosis (RTA)[304,305]. There are several other antibodies against proteins located at the salivary and lacrimal glands (i.e., autoantibodies to salivary protein-1, parotid secretory protein and carbonic anhydrase-VI) that have been associated with preclinical and early SS[306]. Noteworthy, are antibodies against MDM2, an inhibitor of p53 and retinoblastoma protein that have been found in a proportion of seronegative SS patients characterized by longer disease duration, heavier infiltration in the MSG and cytopenia[307]. Finally, anti-cofilin-1, anti-alpha-enolase and anti-RGI2 antibodies have been investigated using proteomic analysis as autoantibody biomarkers able to predict lymphoma development[308].

Apart from the detection of autoantibodies, serum can be used as a pool for several cytokines or chemokines related to various aspects of the disease. The role of chemokine CXCL13 in the formation of eGCLS is known and therefore it has been shown that CXCL13 serum levels correlate with SS activity. A recent study from Wan-Fai et al. revealed that elevated serum CXCL13 levels are associated with B cell hyperactivation, higher ESR and a moderate risk for lymphoma development[309]. In this line, elevated levels of CXCL13 and CCL21 in MSGs are associated with reactive lymphoid proliferation, while CXCL12 presence is mainly related to B cell lymphomas[170]. Gene expression of CCL19/CCR7 in minor salivary glands has also been recently shown to discriminate efficiently patients with SS from non-SS controls[310]. Previously, we stressed the importance of BAFF in the heterogenous B cell related manifestations of SS. Hence BAFF has been studied as a potential biomarker, reflecting B

cell activity. Mariette et al. in 2003 showed a correlation between BAFF levels and autoantibodies in SS while Jacques-Olivier Pers et al. described that 50% of SS patients display an intense BAFF driven B cell activation, predicting an inadequate response to CD20 depletion treatment such as rituximab[311,312]. Finally, it has been shown that serum thymic stromal lymphopoietin (TSLP), an IL-7 member cytokine involved in lymphomagenesis increases during SS course and as the disease evolves from a benign glandular disorder to a malignant monoclonal B cell condition, contrary to the expression by MSG epithelial cells where it is decreasing, findings that were recently validated by the study of independent cohorts[313,314].

Taking into consideration the recent pathophysiologic aspects of the disease, type I interferon inducible genes might also serve as potential biomarkers for SS activity and/or response to treatment. Interestingly, specific type I interferon inducible genes in blood of SS patients are upregulated and reflect disease activity as assessed by the ESSDAI score, with Interferon-α (IFN-α)-inducible protein 27 (IFI27) to be the most useful and specific biomarker for SS, among others[187]. Given that type I INFs can also induce BAFF production by the epithelial cells in MSG of SS patients and that both type I and II IFNs related proteins are present at the site of lesion, a reinforcing interplay between type I and II IFNs may be implicated in SS related lymphomagenesis [315]. Nezos et al. demonstrated that a high ratio of IFNγ to IFNα mRNA levels from an MSG biopsy is associated with the development of lymphoma and may serve as a biomarker[189].

The complex regulatory mechanisms that ensure gene expression and intercellular communication, have been also studied recently in the context of SS. Studies focusing on miRNAs involved in Ro/SSA and La/SSB expression in the MSG of SS patients, showed that microRNA (miRNA) family 200 may harbor regulating properties. Specifically, miRNA200b-5p levels were found to be reduced in patients with lymphoma or pre-lymphoma states while increasing levels may indicate MALT treatment response. In addition, multivariate analysis in pre-lymphoma patients distinguished miRNA200b-5p levels as the strongest independent predictor for future lymphoma development among classical risk factors[316]. A microRNA expression profile between SS patients and controls revealed microRNA “hsa-miR-768-3p” and “hsa-miR-574” as potential diagnostic biomarkers for SS related to glandular inflammation[317]. Recently, Alevizos et al. demonstrated that T cell derived exosomes carrying miR-142-3p can affect SGECs by modulating their secreting capabilities leading to dryness[318]. In addition, circular RNAs, long non-coding RNAs created by back-splicing of exons from a pre-mRNA, may act as a miRNA hub since they pack multiple miRNAs binding sites. Recently, circ-IQGAP2 and circ-ZC3H6 were found upregulated in SS correlating with clinical and histologic activity of SS, serving as potential biomarkers[319].

Sjogren syndrome is prominently characterized by an alteration in the normal function of the salivary and lacrimal glands, making saliva and tears a noninvasive, easily obtainable source of candidate biomarkers for the disease. A number of studies have analyzed the proteome of whole saliva and tears, including extracellular vesicles revealing many potential biomarkers that may distinguish SS patients from sicca controls. However, the results are still preliminary and not always reproducible since being significantly affected by inter and intra-subject variability. Overall, proteins related to various normal functions of salivation are usually found reduced in the saliva (histatins, PRPs, salivary alpha-amylase, carbonic anhydrase VI, etc.) while proteins related to inflammation and B cell activity are overexpressed (Beta-2 microglobulin, neutrophil elastase, calreticulin, S-100 proteins IL-1 related cytokines, etc.)[320-324]. Saliva proteomic analysis recently has focused on extracellular vesicles comprised mainly of exosomes. Specific miRNAs (ebv-miRBART13-3p) and Y-RNAs have been detected in the salivary exosomes of SS patients[325]. Recently, Karagianni et al. showed that epigenetic changes, primarily in the methylation of the DNA, can be detected in the saliva of SS patients representing potential epigenetic biomarkers for the diagnosis and monitoring of the disease[184]. Tear analysis has been less investigated probably due to the more strenuous collection of the biologic specimen. Liquid chromatography-mass spectrometry (LC-MS) of the tears of SS patients revealed overexpression of proteins involved in TNF-α signaling (CPNE1) and B cell survival and differentiation (PRDX3, TPD52) and ubiquitination (LMO7 and HUWE1)[321,326]. Finally, extracellular vesicles analysis of the tears revealed upregulation of various proteins related to different immune response related pathways.

Similarly, SS patients' oral microbiota, constituting the most proximal part of the gastrointestinal tract hosting numerous bacterial species, have been recently found in many studies significantly different than controls[327-330]. Oral dysbiosis may take part in the pathogenetic mechanisms involved, but it is more possible to be an epiphenomenon result of the reduced saliva secretion and function. In any case, the microbial abundance of certain species in SS patients compared to controls (i.e., Lactobacillus and Haemophilus) may act as biomarkers of the disease. In accordance, gut microbiome is also dysregulated in SS disclosing a higher presence of phylum Bacteroides species[331,332]. A Bacteroides subspecies (Bacteroides thetaiotaomicron) abundant in SS gut microbiome has been implicated in disease pathogenesis since heat-killed bacterial lysates of the microbe can bind to Ro positive serum[333].

Finally, salivary radiographic evaluation is standing on the verge between traditional and novel biomarkers. Numerous studies have been conducted showing that ultrasonographic findings are correlated with the presence of SS, worse salivary flow measurements, and even with disease-related manifestations such as cryoglobulinemic vasculitis and lymphoma[334,335]. Two studies have also demonstrated that incorporating ultrasonographic evaluation of the salivary glands as a minor

classification criterion of SS may improve their sensitivity with no loss in specificity[336,337]. Nonetheless, because of the lack of international consensus on ultrasonographic basic definitions and scores and the highly operator-dependence of the technique, no formal guidance for the use of salivary ultrasonography has been issued. In addition, parotid shear elastography has been suggested recently as an easy and non-invasive method to replace MSG biopsy in cases the latter is not feasible[338].

TRADITIONAL BIOMARKERS	NOVEL BIOMARKERS
Clinical	***Novel autoantibodies***
Salivary gland enlargement	***Cytokines and chemokines***
Palpable purpura	BAFF and BAFF receptor
Lymphadenopathy	CXCL13, CXCL12, CXC21
Splenomegaly	CXCL13, CXCL12, CXC21
Serological-Hematological	CCL19/CCR7
SS related autoantibodies	Thymic stromal lymphopoietin (TSLP)
Anti Ro/SSA	***Type I and II interferon signature genes***
Anti La/SSB	***Noncoding RNAs/Exosomes***
Rheumatoid Factor	miRNA200b-5p
Cryoglobulinemia	hsa-miR-768-3p, hsa-miR-574
C4 hypocomplementemia	miR-142-3p
Monoclonality	circ-IQGAP2, circ-ZC3H6
Other autoantibodies	***Saliva***
ACA	Proteins related to the function of saliva
U1RNP	Proteins related to inflammation
CCP	***Tears***
Cytopenias	Proteins involved in TNF-α signaling, B cell related and ubiquitination
MSG biopsy	***Oral and Gut microbiota***
Focus score	Lactobacillus
Germinal centers	Haemophilus
Lymphoepithelial lesions	Bacteroides,
Cell infilatrate	***Salivary gland ultrasonography***
Monoclonal population	**Genetic polymorphisms**
Cell infiltrate composition	BAFF His159Tyr snip mutation
Epidemiology	BAFF receptor polymorphisms
Age of SS onset	TNFAIP3/A20 polymorphisms
Gender	
Geolocation	

Chapter 7: The clinical unmet needs of the disease for further understanding the pathogenesis and treatment for the disease.

7.1 Unmet needs

While there has been significant progress in understanding and managing the disease, several clinical unmet needs persist:

a) **Improving Patients' Stratification and Classification**: One of the primary goals of research in Sjogren is to revolutionize the way we stratify and classify pSS patients based on clinical phenotypes. This effort has far-reaching implications, from tailoring personalized treatment plans to facilitating the recruitment of patients for clinical trials. Additionally, the harmonization of patient data plays a pivotal role in defining health policies, ensuring that medical interventions align with the diverse needs of patients.

b) **Validating and Discovering Biomarkers**: Biomarkers are at the forefront of modern medicine, and novel research endeavors are committed to both validating existing biomarkers for pSS and discovering new ones. This facet of the project carries profound implications for early diagnosis, disease monitoring, and treatment optimization.

c) **Predictive Modeling for Lymphomagenesis**: Lymphomagenesis is a critical concern for pSS patients, and we aim to develop cutting-edge prediction models to better understand and anticipate the risk factors and mechanisms associated with lymphoma development in pSS patients.

7.2 Ways to approach

Current research on both clinical and translational level of SS, disclosed a remarkable progress. In clinical grounds the most important step was the meticulous analysis of the wide clinical spectrum of the disease and the description of subsets of patients, along with their unique characteristics. The clinical phenotyping was mainly based on simple clinical, laboratory and histologic items with an intensive effort to link-specific features with the adverse outcome of the disease, that is the development of lymphoma. It was also evident that the activated B cell component characterizes the systemic form of the disease. To this end, simplified lymphoma prediction models were proposed, exploiting classical risk factors such as salivary gland enlargement, purpura, glomerulonephritis, peripheral neuropathy, cryoglobulinemia and hypocomplementemia. With the utilization of newly introduced harmonized cohorts, simple epidemiologic items, such as age and gender, proved to be

also important determining factors of clinical subgroups toward favorable or adverse outcomes. Indeed, the introduction of data-driven analysis revealed unique combinations of traditional lymphoma predictors among different SS subgroups. Thus, clinical research should proceed beyond the already existing and easily accessible clinical, serologic or histological indices, focusing on: a) the finding of approaches that offer the opportunity to identify patient clusters with homogenous characteristics toward lymphoma development or other rare adverse events such as glomerulonephritis, peripheral neuropathy, and systemic vasculitis, all leading to increased morbidity and low quality of life and b) patients' reported outcomes (PRO's) such as dryness, fatigue, pain, anxiety, or depression, that must be reconsidered and incorporated as primary research goals. In this line a novel methodological approach was introduced by Tarn et al. to stratify patients into different clusters linked to distinct clinical and biologic profiles [181]. In this direction, novel data-driven approaches, based on artificial intelligence, may handle a huge amount of information, unraveling new or hidden associations. To optimize the performance of these algorithms, it is necessary to utilize a vast quantity of data extrapolated from large cohorts. Therefore, several multinational cohorts should be analyzed using the same reference model, as in HarmonicSS project, in order to overcome obstacles related to diversity of data collection, recording and analysis. On the other hand, translational research through systems biology approaches provided important data referring to the potential underlying cellular and molecular mechanisms implıcated in SS and related lymphomagenesis. Studies focusing on the epithelial cell of the affected exocrine glands demonstrated its dynamic state that plays a central role in the attraction, activation, and differentiation of lymphocytes as well as in antigen presentation. Genetic polymorphisms, trophic agents such as BAFF, cytokines and chemokines have been also studied extensively in serum and MSG biopsies, showing the important role of B cells and eGCLS in disease pathogenesis. Beyond adaptive immune responses the role of innate immunity with type I and II IFNs was highlighted, particularly in the initiation and perpetuation of the inflammatory response within the MSG. Lately, work on saliva and tears has drawn much of attention, revealing an array of proteins mainly associated with inflammation and serving as a tool to better understand secretory dysfunction of epithelia in the context of SS. Finally, data on miRNAs pointed out the complex and multilevel regulation of gene expression involved in disease processes and lymphoma development. The actual purpose of such studies was to identify measurable parameters in various biologic specimens that could be used as potential biomarkers reflecting major underlying molecular phenomena and potential therapeutic targets. As pointed out in the opening lines of this section, future perspectives on this field are anticipated to: a) associate specific clinical phenotypes with distinct molecular events and disease endotypes b) dissect and study in depth those critical circuits that drive cell to cell communication involving chemokines, cytokines and exosome contents c) analyze

epigenetic and posttranscriptional modifications that may alter gene expression crucial for the pathogenesis of SS and associated lymphomas and d) reveal dynamic key molecules that play an active role – and not just representing innocent bystanders or epiphenomena – in sustaining disease process, lymphomagenesis and secretory epithelial dysfunction. In-depth study and understanding of the exact function of these molecules can really lead to the proposal of novel therapeutic targets to interrupt the vicious cycle of perpetuation that characterizes SS. In this line, several international projects are ongoing or are expected to be initiated, in an attempt to address the molecular clustering of SS patients taking into account the genetic, epigenetic and post-transcriptional imprint of pre-lymphoma.

7.3 Aims of the present Thesis

To address some of the aforementioned unmet needs, we have undertaken an evaluation encompassing the following key aspects:

1. Enhanced Patient Stratification: With a particular focus on male patients affected by Sjögren's syndrome.
2. Biomarker Validation: With a special emphasis on assessing the utility of the Focus Score as a predictor of lymphoma development.
3. Evaluating Combined Seronegativity: To understand its significance in the context of Sjögren's syndrome.
4. Serum CXCL13 Levels: Investigating its potential as a marker for lymphoma development in individuals with Sjögren's Syndrome.
5. Comprehensive Analysis of Lymphoma Predictive Factors as well as the survival of SS associated lymphomas: This entails creating and assessing the most extensive cohort of Sjögren's-associated lymphoma cases.
6. In-Depth Examination of the Clinical Phenotype: Focusing on primary Sjögren's syndrome with lymphadenopathy.
7. Clinical and Laboratory Findings: Assessing primary Sjögren's syndrome cases even in the absence of sicca symptoms.

Chapter 8: Sjögren's Syndrome: The Clinical Spectrum of Male Patients

8.1 Introduction

Sjögren's Syndrome (SS) is a complex and heterogeneous disorder that follows a strong trend of female predilection observed in many autoimmune diseases, ranking second after autoimmune thyroiditis [339]. The clinical spectrum of SS extends beyond the exocrine glands including also many extraglandular manifestations, either periepithelial (interstitial nephritis, interstitial lung disease, primary biliary cirrhosis) or extraepithelial B cell mediated manifestations (palpable purpura, peripheral neuropathy, glomerulonephritis), originated from various affected organs and tissues. Non-Hodgkin's lymphoma of MALT type is a serious complication of the disease with well-established risk factors such as cryoglobulinemia, parotid gland swelling, purpura and low C4 complement levels [112,340,341]. The exact pathogenetic mechanisms underlying the profound female predominance behind SS remain unclear. Studies focusing on the role of the different hormonal milieu between the two genders have been so far inconclusive [78]. Recently, the genetic imbalance created by the different sex chromosomes in each gender has drawn much attention. The presence of a second X chromosome, rich in immune related genes (FOXP3, TLR7, CD40LG etc), correlates with the clinical expression and pathogenesis of systemic autoimmune diseases [81]. The unique characteristics of X chromosome in terms of inactivation, escaping and skewing are implicated in the pathogenesis of systemic autoimmunity as reflected in the paradigm of males with Klinefelter syndrome (XXY) who exhibit a 36-fold increased risk of developing SS than males without (XY) [342]. Therefore, the clinical phenotyping of various subgroups of SS patients including males, is of major clinical importance, since it may reflect different underlying pathogenetic mechanisms associated with different clinical expressions, outcomes and response to treatment.

The literature on male gender in SS is scarce, involving very low numbers of male patients with heterogeneous classification criteria [343]. In addition, differences in study design and type of data analysis may account for different findings. However, in two recent studies with well-defined classification criteria and a rather small number of male patients, an association of male gender with lymphoma was described [280,344]. Nevertheless, whether male patients with SS have the same clinical phenotype, laboratory and histological characteristics, as well as, risk factors for lymphoma development as females is still unknown. In this retrospective study, we aimed to explore the differences in various aspects of SS expression between the 2 genders with a novel approach, including: (i) a well characterized cohort of males from a multicenter SS population, after careful matching with female controls, and (ii) the use of data driven approaches to identify lymphoma associated features.

8.2 Patients and Methods

8.2.1 Study Design

The current study was conducted in the context of the clinical phenotyping of the HarmonicSS project [345]. From a total study population of consecutive SS patients who fulfilled the 2016 ACR/EULAR classification criteria [55] and were followed up from May 1984 until March 2019, in 5 centers from Greece (University of **A**thens, **H**arokopio University, University of **I**oannina) and Italy (University of **P**isa, University of **U**dine) (**UPAHI** group), male patients were identified and their cumulative clinical, laboratory and histologic data were collected, integrated and analyzed in a unified dataset, based on the minimal criteria defined by the reference model of the HarmonicSS project [345]. For each male patient, 2 females were matched according to age of SS onset and disease duration from SS onset until last follow up, following a maximum of a 2-year deviation. SS onset was defined as the year the patient recalled disease related symptoms such as Raynaud's phenomenon, arthritis, sicca symptoms or salivary gland enlargement began while SS diagnosis was used instead, when SS onset could not be specified. Subjective symptoms and systemic organ involvement were based on ESSPRI and ESSDAI definitions respectively [346,347]. Minor salivary gland biopsies, laboratory and objective eye and oral tests were performed in the context of standard of care, according to physicians' judgment. The 2 groups were compared on the basis of clinical (dry mouth, dry eyes, parotid gland enlargement, Raynaud's phenomenon, lymphadenopathy, arthralgias, myalgias, arthritis, palpable purpura, liver involvement, kidney involvement, central and peripheral nervous involvement, lymphoma), laboratory (anti Ro/SSA antibodies, anti La/SSB antibodies, rheumatoid factor, cryoglobulinemia, low C4 complement levels, monoclonal gammopathy) and histologic (focus score, type of lymphoma) features. A subgroup analysis was also conducted to compare the Greek and Italian male patients. The study was approved by the local ethical committees of all the involved Hospitals and Institutions, after patients' informed consent and compliance according to the General Data Protection Regulations (GDPR).

8.2.2 Statistical analysis and data driven approach

Statistical analysis for categorical data was performed by $\chi 2$ test with Yates correction or Fisher exact when cell counts <5 patients and for numerical data t test or Mann-Whitney, after Shapiro-Wilk normality test. The Fast-Correlation based feature selection (FCBF) algorithm was applied on the unified dataset to identify potentially independent variables for constructing a logistic regression (LR) model with lymphoma as an outcome. The FCBC algorithm is a correlation based mathematical tool that identifies, among several potentially independent variables, those with the strongest association with the outcome of interest (e.g. lymphoma) and the weakest association amongst them, using as a

measure coefficient similarity. This subset of the FCBC derived potentially independent variables can be used for the logistic regression model to identify independent lymphoma associated factors [348]. A conventional 10-fold cross validation approach was applied to evaluate sensitivity, specificity, and accuracy of the combined FCBC/LR model. The implementation of the FCBF/based multivariable logistic regression approach and the statistical analysis was performed using Python 3.6 and GraphPad 7.0a.

8.3 Results

8.3.1 Patient characteristics

One thousand nine hundred and eighty-seven SS patients fulfilling the 2016 ACR/EULAR criteria were included in the total multicenter population. The number of patients from each of the 2 Mediterranean countries was similar: Greece n=980, Italy n=1007. Ninety-six (4,8%) males were identified and matched with 192 female controls according to disease duration and age at disease onset. All male patients of the study had normal physical examination regarding testicle size (length >3cm and volume >20 cc for each testicle) and male phenotypic characteristics (e.g. muscle mass, phallus size, sex hair, deep voice, absence of gynecomastia) and no family or personal history of infertility. The male to female ratio in the total population was approximately 1:20. The median age of males was 50.1 years (range 13-78) while the median age of females was 49.7 years (range 15-78) (Table 1). The median disease duration until last follow up was 6 years (range 0-28) for the male group and 7 years (range 0 - 26) for the females.

Table 1. Comparison of glandular, extraglandular, serological and histological features between male and female patients with Sjögren's syndrome (female control population was matched in an 1:2 ratio based on SS age onset and disease duration).

	Females (n= 192)	Males (n= 96)	p-value
BASIC CHARACTERISTICS			
Age onset of first symptom	49.7	50.1	0.7928
Median disease duration until last follow up (years)	7 (0-26)	6 (0-28)	0.2450
Greek patients	933	47	
Italian patients	958	49	
GLANDULAR MANIFESTATIONS			
Dry mouth	97% (186/191)	91% (87/96)	**0.0266**
Dry eyes	94% (180/191)	87% (83/95)	0.0747
Salivary gland enlargement	24%(45/186)	32% (31/96)	0.1900
Dry skin	9%(14/148)	7%(5/74)	0.6715
EXTRAGLANDULAR MANIFESTATIONS			
Chronic Fatigue	38%(57/148)	27%(21/77)	0.1733
Raynauds phenomenon	26% (48/185)	19% (18/94)	0.3290
Arthritis	9%(16/185)	10%(10/95)	0.7679
Arthralgia	52%(99/192)	47%(45/95)	0.5320
Myositis	0,5% (1/192)	2% (2/96)	0.2585
Interstitial renal disease	1%(2/190)	0%(0/95)	0.5540
Primary biliary cirrhosis	0%(0/192)	2%(2/96)	0.1103
Autoimmune hepatitis	0% (0/192)	2% (2/95)	0.1103
Interstitial Lung Disease	5%(9/192)	8%(8/96)	0.3309
SEROLOGY			
Rheumatoid Factor	47% (84/177)	51% (45/89)	0.7278
Anti-Ro	72% (136/190)	73% (69/95)	0.9628
Anti-La	34% (64/189)	50% (47/94)	**0.0128**
LOW C4	22%(38/175)	30%(27/89)	0.1657
Monoclonality	4%(7/192)	7%(7/96)	0.2866
Cryoglobulinemia	6%(8/135)	10%(7/73)	0.4877
HISTOLOGY			
Focus score ≥ 1	80% (90/113)	78%(42/54)	0.5425
Median FS in positive biopsies	1.383	1.477	0.2786

8.3.2 Comparison between males and females

Clinical manifestations were compared between the 2 groups (Tables 1, 2). Females had higher frequency of dry mouth compared to males (97.4% vs 90.6%, OR= 3.84, 95% CI: 1.29 to 10.46; p=0.0266) (Table 1). Dry eyes followed the same trend between males and females, without reaching statistical significance (94% vs 87% respectively, p=0.07) (Table 1). The prevalence of extraglandular manifestations including non-specific symptoms, liver, pulmonary and renal involvement, as well as, B cell associated manifestations such as palpable purpura, glomerulonephritis and peripheral nerve involvement did not differ significantly between males and females (Table 1 and 2, respectively). A statistically significant difference was identified in the frequency of lymphoma among males compared to females (18% vs 5.2 %, OR=4.89, 95% CI: 1.66 to 8.67; p=0.0014, respectively) with no observed difference in the classical risk factors of lymphoma development (Figure 1). To confirm the statistically

significant difference in the probability of a male patient to present with lymphoma, we applied the FCBF algorithm to identify potentially independent variables for lymphoma development on unified dataset, analyzing 33 distinct features (Table 3). Six variables including lymphadenopathy, salivary gland enlargement, anti-La antibodies, female gender, low C4, and monoclonal gammopathy were identified as potentially independent variables and the multivariable logistic regression model for lymphoma revealed lymphadenopathy as an independent positive risk factor (OR=6.31, 95%CI: 0.88 to 2.85; p=0.0003) and the female gender (OR=0.344, 95%CI: -0.31 to -1.92, p=0,01) as an independent negative risk factor, suggesting a positive relationship of male gender with lymphoma development (Table 3). The ROC curve for the FCBF/logistic regression model (FCBF/LR) is presented in Supplementary Figure 1 [accuracy=0.92, sensitivity=0.65, specificity=0.98, area under the curve (AUC)=0.87]. The histologic types of lymphomas did not differ between the 2 genders, with MALT lymphomas being the predominant type (Table 2). One male patient developed splenic marginal zone lymphoma and one female had small lymphocytic lymphoma. The frequency of anti-La (SSB) antibodies in the male group compared to females was significantly higher (50% vs 34%; OR=1.953, 95%CI=1.19 to 3.22; p=0.0128), as opposed to anti-Ro (SSA) and rheumatoid factor (Table 1). No difference was observed in focus score between male and female patients or in the proportion of positive biopsies between the two groups (Table 1). Although the total number of lymphoma patients was small, a comparison between male and female SS lymphoma patients was performed (supplementary Table 1). No statistically significant difference was found between the 2 groups in any clinical, laboratory or histologic parameter. However, male SS patients with lymphoma had older age at SS and lymphoma onset, longer median disease duration until lymphoma occurence and presented more frequently with lymphadenopathy (65% vs 50%), dry skin (27% vs 0%), serum monoclonality (23% vs 0%) and cryoglobulinemia (29% vs 0%) compared to SS females with lymphoma. The comparison between Greek and Italian male SS patients regarding clinical and serological parameters, classical risk factors of lymphoma, lymphoma, or lymphoma histologic subtypes, did not reveal differences, except chronic fatigue which was higher in Italians (47% for Italians vs 15% for Greeks, p=0,005).

Table 2. Comparison of lymphoma and B cell associated manifestations between male and female patients with Sjögren's syndrome (female control population was matched in a 1:2 ratio based on SS age onset and disease duration).

FEATURE	Females (n=192)	Males (n=96)	p-value
Palpable purpura	9% (17/192)	10% (10/96)	0.8302
Vasculitic ulcer	2% (3/192)	1% (1/96)	1
Glomerulonephritis	0,5%(1/191)	3%(3/96)	0.1106
Peripheral nerve involvement	2% (4/192)	3% (3/96)	0.6895
Lymphadenopathy	9% (18/192)	17% (16/95)	0.0994
Central nervous system involvement	2% (3/192)	2% (2/96)	1

Lymphoma	5% (10/191)	18% (17/96)	**0.0013**
MALT	80% (8/10)	76% (13/17)	0.99
DLBCL	10% (1/10)	18% (3/17)	0.99

Table 3. FCBF-based multivariable logistic regression analysis for investigating the effect of gender on lymphoma development.

Prominent feature	Regression coefficient	Odds ratio	p-value	CI low	CI upper
Lymphadenopathy	1.869	6.549	0.0003*	2.456	17.482
SGE	0.689	2.006	0.129	0.853	4.724
Anti-La	0.682	1.989	0.11	0.884	4.477
Female Gender	-1.119	0.332	0.011*	0.148	0.742
Low C4 (< 20mg/dl)	0.465	1.599	0.337	0.629	4.069
Monoclonal gammopathy	0.537	1.728	0.512	0.353	8.592

* The strongest potentially independent variables identified by the FCBF algorithm to build the logistic regression model, after analysing the following initial features included in the dataset: Ethnicity, Gender, Disease duration (onset), Dry mouth, Dry eyes, Anti-Ro, Anti-La, RF, Focus score, Germinal centers in biopsy, Monoclonal gammopathy, SGE, Lymphadenopathy, Low C4, Dry skin, Chronic Fatigue, Arthralgias-myalgias, Arthritis, Raynaud's phenomenon, Palpable purpura, Vasculitic ulcer, Myositis, Peripheral Neuropathy, Peripheral Neuropathy,, CNS involvement, Liver–autoimmune hepatitis, Liver- PBC (Primary Biliary Cirrhosis), Lung – interstitial disease, Interstitial renal disease, Kidney/Glomerulonephritis, Heart(valvular disease), Cryoglobulinemia, Lymphoma

** < 0.05 (95% confidence interval): independent lymphoma associated features revealed by the logistic regression model

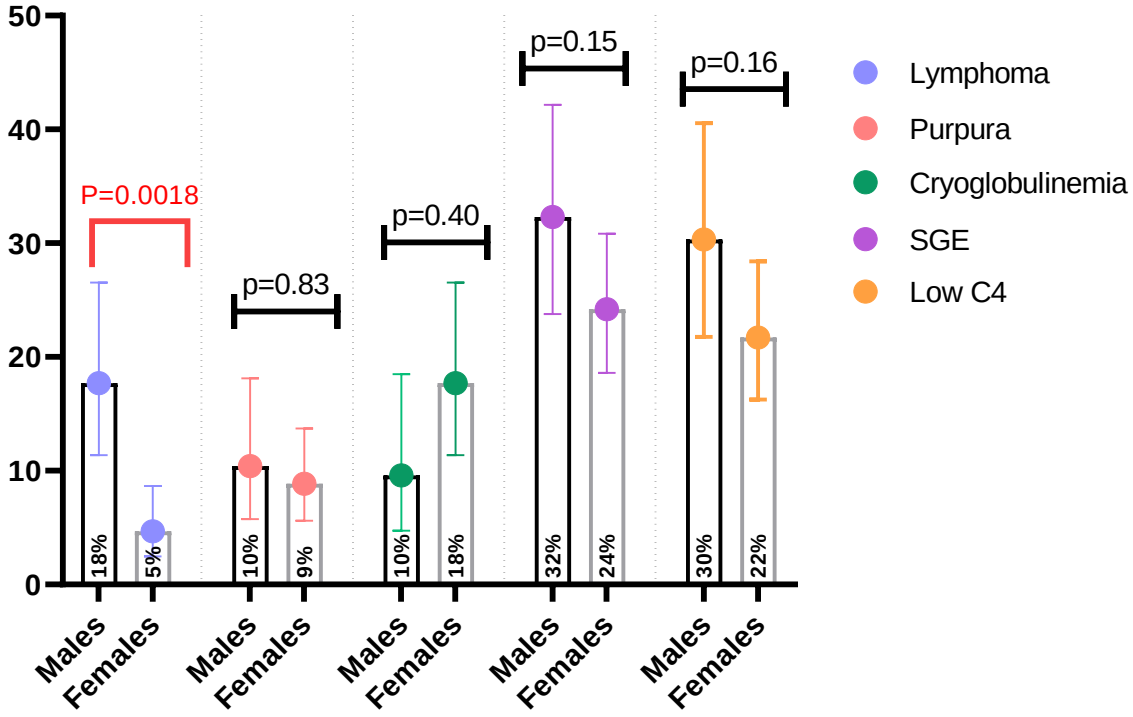


Figure 1. Comparison of lymphoma and classical risk factors between male and female patients with Sjögren's syndrome.

8.4 Discussion

This is the largest study ever conducted with well characterized SS male patients who fulfilled the 2016 EULAR/ACR criteria [55] and were compared to matched females, according to disease duration and age at disease onset. Only in one study, multivariate analysis adjusted for age and follow up time including 73 males was performed; that study showed only higher frequency of Raynaud's phenomenon and thyroiditis in females although the number of matched females and the follow up time were not mentioned [349]. The fact that in a previous study [344], both males and females had similar age at SS onset, allowed matching of the two groups on the basis of the above features, assuming that age and disease duration could potentially affect the clinical expression of the disease. The 20:1 female to male ratio in our study is considerably different from what is commonly described in many previous studies, ranging from 9:1 to 12:1 [60]. The median disease duration is sufficient to allow comparisons regarding the clinical phenotype, the serology and histology between male and female SS patients. Males have less frequently glandular manifestations but more commonly a double positivity of anti-La/SSB plus anti-Ro/SSA autoantibodies compared to females as shown previously [343,344]. In our study, we observed an increased prevalence of lymphoma in SS males compared to females, with no differences in the type of lymphoma or the distribution of traditional lymphoproliferative risk factors between the two genders, suggesting that male gender could be an independent risk factor for lymphoproliferative disorders in SS. The combined FCBF/LR algorithm also confirmed that male gender had a positive correlation with lymphoma development, since gender was identified as an informative feature.

The higher female to male ratio compared to previous studies can be attributed to the lack of consensus criteria for SS in the past and the small number of recruited patients, although recent studies based on more homogeneous cohorts seem to support the higher female to male ratio [343,344]. The observed differences in the clinical phenotype regarding the higher frequency of dry mouth and eyes in females, is reported for the first time. Given the subjective component of dryness, it may be associated with the clinical expression of the disease at the glandular level, the individual's perception and the peri-menopausal peak age of SS onset in women as probably modified by estrogens and possibly the epigenetic regulation of the X chromosome. Regarding anti-La/SSB positivity, it has been proposed as a variable increasing the risk of lymphoma [280] and its increased prevalence in males, further supports our finding for an association between male gender and lymphoma development. Interestingly, no other differences have been observed among other immunologic parameters including anti-Ro/SSA, RF and cryoglobulins. Anti-La/SSB positivity correlates with more intense inflammatory infiltrate of the minor salivary glands while several reports have shown that ectopic germinal centers within the inflamed minor salivary glands are active sites of anti-La/SSB production

[350-352]. In our study however, there were no differences in salivary gland focus score between males and females with FS≥1. Therefore, the explanation of this finding could be attributed to an anti-idiotypic response directed to anti-La/SSB in female patients which obscures the anti-La reactivity in these individuals [353].

According to the literature, only in few studies, male gender was associated with lymphoma in SS [280,344,354] and in two of them it was identified as an independent risk factor [280,354]. In both studies, the recruited number of lymphomas and male patients was rather small without appropriate female control matching. Interestingly, the overall proportion of lymphoma among female controls on the unified dataset was 5.2% which approximates the estimated prevalence of lymphoma among SS cohorts [355-357], given that more than 95% of SS patients are females. In the present study, the data driven multivariable logistic regression analysis pointed out male gender a lymphoma associated risk factor. The data driven FCBF/LR model is considered a novelty. In common practice, the selection of potentially independent variables for the logistic regression model, is usually based on univariate analysis positive findings and the possible biologic associations with the outcome of interest described in the literature, underestimating the independency criterion among the proposed variables. Inevitably, this approach carries a risk of bias selection and restricts the pool of potentially independent variables. In our case, the application of the FCBC algorithm handled and analyzed initially, 33 potentially independent variables in an unbiased manner and ended up with the 6 strongest-in order of magnitude- to construct the logistic regression model. Interestingly, cryoglobulins and purpura, classical risk factors of lymphoma, were not included in the set of the potentially independent variables, since the FCBF algorithm on the unified dataset ranked purpura and cryoglobulins in a lower position compared to low C4. This inconsistency could be attributed to the fact that cryoglobulins had a high percentage of missing values on the unified dataset and that low C4 is closely associated with palpable purpura (data not shown). Although no differences between males and females were observed in terms of classical risk factors of lymphoma including lymphadenopathy, data driven approaches utilized these variables in a rationale way, revealing lymphadenopathy and gender as prominent clinical features to identify high risk pSS patients who require close follow up.

The retrospective character of our study is an endogenous limitation. The inherent sex bias among systemic autoimmune disorders may misdiagnose and exclude male patients from SS diagnosis, underestimating the clinical diversity of the syndrome and obscuring any gender-related differences. The number of male patients, although the largest ever reported, is considered also a limitation, since larger cohorts with more male SS patients and longer disease duration may uncover more phenotypic differences between the two genders. Interestingly, all centers involved are, referral

centers for SS, managing complicated cases at the national level and therefore SS cases with milder severity might be underrepresented, especially males. The absence of karyotyping and sex hormone measurements could be also considered a limitation, but such studies are pending. To better understand the differences of various aspects of the disease between the 2 genders, other types of studies are required focusing not only on the hormonal state of patients but also on the role of the X and Y chromosomes that may shape and define the clinical phenotype of the disease by regulating the expression of the complex gene network implicated in pathogenesis. Despite the above-mentioned limitations, this study highlights the potentialities of data driven approaches in refining patient sex differences, opening new avenues for the investigation of the underlying biological basis of these differences as a prerequisite towards precision medicine in autoimmunity. The phenotypic differences among SS patients are most likely defined by several factors such as age, gender, race and ethnic group. In addition, environmental factors triggering the initial autoimmune response i.e. a viral infection may also affect the phenotypic expression of the disease. Single cohorts cannot reveal such differences because of the small number of patients. This limitation can be overcome only by a multicenter SS population. Large data gathering and employment of data mining tools may provide the opportunity to study in more detail the effect on phenotype of more than one factors (e.g. gender and young age of SS onset), creating distinct clinical clusters.

Chapter 9: A biomarker for lymphoma development in Sjogren's syndrome: Salivary gland focus score.

9.1 Introduction

The histopathologic signature of primary Sjögren's syndrome (SS) is the lymphocytic infiltrate that is localized around the ductal epithelium of the salivary and lacrimal glands, mediating oral and eye dryness respectively [21,22]. Early studies have linked the degree of lymphocytic infiltration in the labial minor salivary glands (LMSG) with the clinical phenotype and the disease autoantibody profile [358]. In addition, the lymphocytic inflammatory component within the LMSG appears to be pathogenetically related to lymphomagenesis either at the salivary glands or at the involved lymph nodes, supporting B-cell clonal expansion and participating actively in the development of mucosa-associated lymphoid tissue (MALT) and non-MALT lymphomas [359-361]. The typical histologic picture of focal sialadenitis is strongly suggestive of SS and has been incorporated as a parameter in most classification criteria, using various quantification scores of the focal lymphocytic infiltrate, Focus Score (FS) being one of them [55,362,363]. Indeed, a FS ≥1 can successfully distinguish SS patients from healthy controls or individuals with sicca symptoms which do not fulfill SS diagnostic criteria [22,23,362,363]. Apart from the diagnostic utility of focal sialadenitis, FS has also been employed to assess the severity and extension of inflammation in the LMSG [21,364,365].]. Only one study has explored the association of LMSG FS grading with lymphoma development thus far and it should be noted that the number of enrolled SS lymphoma and non-lymphoma patients was rather small. Furthermore, the LMSG FS threshold proposed was set arbitrarily (based on the mean of the lymphoma group) and the clinical manifestations were presented only by the EESDAI score [366].

Identification, validation, and use of clinically useful lymphoma predictors in the setting of SS has been a matter of extensive research over the past few decades, resulting in the introduction and establishment of several clinical and serologic lymphoma risk factors, including salivary gland enlargement (SGE), cryoglobulinemia and low C4 serum levels[112,367,368]. On the other hand, the predictive value of various histologic aspects of the LMSG such as: the LSMG FS, the presence of germinal centers and/or the presence of lymphoepithelial lesions have been less explored. The aim of this retrospective multicenter cohort study is to establish a presumed association between LMSG inflammation severity with lymphoma development, early lymphoma diagnosis and the spectrum of SS manifestations, using a simple and commonly used histologic biomarker, the LMSG FS. Based on the relation between LMSG FS at the time of SS diagnosis and the time interval to lymphoma diagnosis, a new LMSG FS threshold is proposed that classifies SS population into a high and a low LMSG FS

subgroup, guiding a follow up strategy centered around the major complication of SS, the development of MALT lymphoma.

9.2 Patients and methods

9.2.1 Study Design

A total population of 1997 consecutive SS patients in Greece and Italy (Universities of **A**thens, **P**isa, **U**dine, **H**arokopio and **I**oannina, **UPAHI** group) was evaluated, all of whom fulfill the 2016 ACR-EULAR criteria [55], and were followed up from May 1984 to May 2019. Of those, patients subjected to a LMSG were identified, to study the association of inflammation severity with lymphoma development and clinical phenotype. The final study group included: 1) all SS patients with FS≥ 1, and 2) SS lymphoma patients and non-lymphoma controls with at least 1 year from SS diagnosis until lymphoma diagnosis or last follow up respectively, to ensure the predictive value of FS for lymphoma development (Supplementary figure 1). Patients with known risk factors for lymphoma such as HCV infection, AIDS or a history of head and neck radiation treatment were excluded from the present study. A second lymphoma diagnostic biopsy was performed as follow up in high risk and long-standing cases presenting with recurrent or persistent SGE, purpura or generalized lymphadenopathy with or without cryoglobulinemia. All available SS diagnostic biopsies were re-evaluated by expert pathologists in each department to rule out lymphoma and the FS was re-assessed as follows: a) the total surface of the glandular tissue was initially calculated together with the total number of foci defined as lymphocytic aggregates with more than 50 mononuclear lymphoid cells by hematoxylin and eosin stain b) the FS was calculated as the ratio of total foci per 4 mm^2 of glandular tissue [21]. Histologic classification of lymphomas was performed according to the revised 2016 World Health Organization (WHO) criteria [369]. Cumulative clinical, serologic, and histologic data attributed to SS as defined by ESSDAI and/or ESSPRI [346,347], until lymphoma diagnosis or last follow up, were recorded. The type of pulmonary involvement (either small airway disease or interstitial lung disease) was defined by a pulmonary expert, in each center, after combining data from the clinical picture, medical history, radiology examination and pulmonary function tests. Leukopenia was defined as WBC (<3000/mmc) [272]. Data from all cohorts were collected following a common prespecified reference model as part of the HarmonicSS project, assuring that the final integrated dataset was highly homogenized. A sequential process was applied to identify a LMSG FS threshold classifying lymphoma patients into high and low LMSG FS subgroups, based on the maximum difference of duration from SS to lymphoma diagnosis between the two divided subgroups. Finally, high, and low FS SS patients were compared in terms of demographic, clinical and serologic features. Distinct lymphoma associated risk factors were explored for each subgroup. All SS patients were managed with the standard of care according to the attending

physicians' judgement. An approval was granted by the local ethical committees of all the Institutions involved, after obtaining patients' informed consent and in compliance to the general data protection regulations (GDPR).

9.2.2 Statistical analysis and data driven approaches

For categorical data, statistical analysis was performed using the chi-square test with Yates's correction. When cell counts were <5, Fischer's exact test was used instead. For continuous variables, the Shapiro-Wilk normality test was performed first, following an analysis either by utilizing the Mann-Whitney-Wilcoxon test or the t test. Additionally, the Kruskal-Willis test was performed for the comparison of medians among several groups. A data driven method was utilized based on the combination of the Fast Correlation Based Feature (FCBF) selection method with the Logistic Regression (LR) algorithm and was applied on the unified dataset of all patients and patient datasets with high and low LMSG FS for the identification of independent lymphoma associated risk factors, minimizing potential selection bias, as described previously [76]. In order to build the LR model, the FCBF algorithm was applied on the whole dataset, to identify potentially independent lymphoma associated risk factors. Subsequently, a random downsampling with replacement strategy was applied to deal with the reduced lymphoma to non-lymphoma patients' ratio, which is expected to significantly reduce the sensitivity of the LR model. According to the proposed strategy, the lymphoma patients were matched through a 1:1 ratio with non-lymphoma controls according to age, gender, and disease duration. To reduce the randomness during the downsampling of the control group, the overall process was repeated 100 times and the results of the FCBF combined with the LR method were averaged across the 100 executions, each representing a randomly chosen matched control group following a 10-fold cross validation procedure. A sequential process was applied across subgroups of lymphoma patients with FS values $\geq x$ and $< x$, where $x \in [2,12]$, so as to identify the critical FS threshold with statistically significant difference between the time intervals from SS diagnosis to lymphoma diagnosis, according to Mann-Whitney-Wilcoxon test between the 2 divided subgroups. The implementation of the FCBF-based multivariable logistic regression approach and the statistical analysis was performed using Python 3.6 and R software 4.0.3.

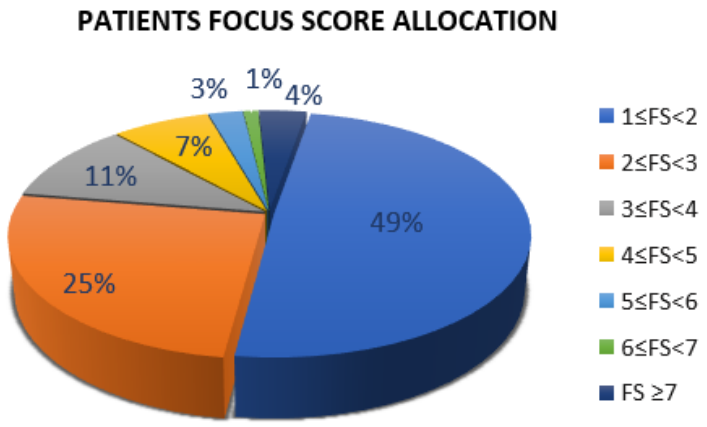
9.3 Results

9.3.1 Patients' characteristics

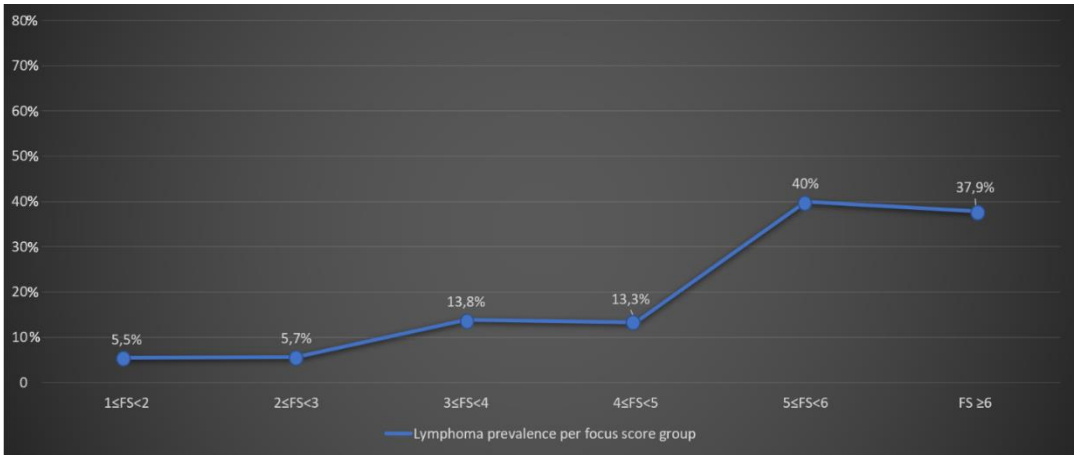
The final study group comprised of 618 SS patients with LMSG FS≥ 1 and at least 1-year interval from SS diagnosis to lymphoma diagnosis or the last follow up, 58 of whom had lymphoma and 560 were non-lymphoma controls (Supplementary Figure 1). Out of the 58 lymphomas, 5 were of non-MALT type and included 2 diffuse large B cell (DLBCL), 1 marginal zone splenic lymphoma, 1 nodal marginal zone lymphoma and 1 follicular lymphoma. Non-MALT lymphoma cases were diagnosed through a lymph node biopsy performed due to persistent lymphadenopathy. A unified dataset of 618 SS patients with 30 clinical, laboratory and histologic features was constructed. The median age of SS diagnosis was 53 (range:15 – 80) years old, the female to male ratio was 20:1 and the median disease duration was 6 (range: 1-35) years. The median age of SS symptom onset was 50 (range:15 – 78). The distribution of patients per FS escalating group is presented in Figure 1A, with almost half (49%) of the study group SS patients having a FS between one and two (1≤FS<2), followed by a declining percentage of patients accounted for every subsequent escalating FS group (Figure 1A). The lymphoma prevalence per FS group is illustrated in Figure 1B. Interestingly, no statistically significant difference regarding disease duration was found among the various FS escalating groups (p= 0.1603, Kruskal-Wallis test) (Supplementary Figure 2).

Figure 1. A. Pie chart graphical illustration showing the allocation of patients focus score B. Lymphoma prevalence per focus score group

A



B



9.3.2 Focus score is an independent lymphoma associated risk factor

The unified dataset of 618 patients x 30 features/variables was utilized to explore the association of FS in the diagnostic LMSG biopsy with lymphoma development. As described previously, an FCBF/LR algorithm was applied on the whole dataset which included 58 lymphoma SS patients and 560 non-lymphoma SS controls. The FCBF pre-selected cryoglobulinemia, history of persistent SGE, FS, persistent generalized lymphadenopathy, age at SS diagnosis, autoimmune thyroiditis, and dry mouth, as those features displayed the highest statistical correlation with lymphoma and the lowest amongst them and therefore most suitable to construct the LR model. To overcome the very low lymphoma: non-lymphoma patients' ratio (<1:10), the LR model was trained with the down sampling matching strategy in 1:1 ratio and the average results of 100 rounds/runs identified cryoglobulinemia, SGE and FS as a continuous variable as the final independent lymphoma associated risk factors. (Table 1) (model performance: sensitivity=75%, specificity=77% accuracy=75%, area under the curve/AUC=85%,

Supplementary Figure 3). Lymphoma prevalence per each FS graded group is shown in Figure 1B, confirming that the risk of lymphoproliferation is increasing with higher FS values. The plots showing the probability (odds) of lymphoma over non-lymphoma based on the LR model constructed from this specific dataset are presented in Supplementary Figure 4. It is clearly shown that the risk of lymphoma increases (odds>1) as FS exceeds 4 in patients with no cryoglobulinemia or SGE and is maximum in patients with cryoglobulinemia, SGE and high FS values.

A comparison between lymphoma and non-lymphoma control patients revealed that the median FS in non-lymphoma group was 1.8 (range: 1-12) while patients developing a lymphoproliferative disorder had a median diagnostic biopsy FS of 3 (range: 1-12) ($p<0.001$, OR= 0.731, 95% CI: 2.220-2.518). The median disease duration from SS diagnosis to last follow up (non-lymphoma control group) or lymphoma diagnosis (lymphoma group) was 6 (range: 1-27 and 1-21 respectively) years for both groups, with no statistically significant difference ($p=0.8597$) (Supplementary Table 1). Furthermore, in line with previous studies that explore the effect of age of SS diagnosis on the clinical expression of the disease, a younger age of diagnosis was found in lymphoma compared to non-lymphoma patients (46 vs 53, $p<0.0001$), implying that patients diagnosed early should be more closely followed up [104].

***Table 1**. FCBF-based multivariable logistic regression analysis results with lymphoma as an outcome in 618 SS patients with FS≥1.*

Prominent feature*	Regression coefficient	Odds ratio	p-value	CI low	CI upper
Cryoglobulinemia	1.73	5.928	0.021 **	1.457	24.558
SGE	1.577	5.078	0.008 **	1.849	13.975
Focus Score	0.27	1.316	0.049 **	1.034	1.677
Lymphadenopathy	0.739	2.239	0.29	0.661	7.713
Age at SS diagnosis	-0.033,	0.967	0.146	0.935	1.0
Autoimmune thyroiditis	-0.046	0.995	0.701	0.323	3.081
Dry mouth	0.387	0.995	0.654	0.258	9.679

* The strongest potentially independent variables identified by the FCBF algorithm to construct the logistic regression model, after analysing initially the following features included in the unified dataset: Gender, Age at SS, Dry mouth, Dry eyes, Anti-Ro, Anti-La, ANA, Focus Score, history of SGE, history of generalized Lymphadenopathy, Rheumatoid Factor, monoclonal gammopathy, Low C4(<20), Arthralgia-myalgia, Arthritis, Raynaud, Palpable purpura, Myositis, PNS-vasculitic, CNS involvement, Liver involvement – autoimmune hepatitis, Liver involvement- PBC, Lung involvement – interstitial disease, Lung involvement – small airway disease, Interstitial renal disease, Kidney involvement -GN-biopsy, Autoimmune thyroiditis, Cryoglobulinemia.

**< 0.05 (95% confidence interval): final independent lymphoma associated factors

9.3.3 Focus score threshold based on the time interval from SS diagnosis to lymphoma diagnosis

The time interval between SS diagnosis and lymphoma diagnosis was calculated for all lymphoma patients (except one patient whose disease duration was not available). Notably, SS lymphoma patients with FS above or equal to 1 and below 2, had a median time interval of 9 (range: 2-21) years,

representing the largest group among all groups (Figure 1B). A statistically significant negative correlation was found between FS and the time to lymphoma diagnosis according to Pearson coefficient (r=-0.32, p=0.015) (Figure 2A). A sequential process was performed in the lymphoma dataset of 57 patients combining FS values and time interval from SS to lymphoma diagnosis as described previously, to identify that FS threshold revealing a statistically significant difference according to Mann-Whitney test, in the time interval between the divided subgroups for each FS value/threshold (above or equal vs below a FS threshold) (Supplementary Table 2). This analysis revealed that FS ≥4 is the only threshold which could separate lymphoma patients in such a way that those with FS ≥4 (n=23) had a statistically significant shorter time interval compared to those with FS<4 (n=35) [4 (range: 1-15) vs 9 (range: 2-21) years, respectively, p=0,008]. To explore the effect of classifying a total SS population using FS = 4 as a threshold for lymphoma development rates, we employed a Kaplan-Meier/ Mantel-Cox log-rank analysis with lymphoma as an outcome/event. High and low FS subgroups included 23 (20 MALTLs) and 35 (33 MALTLs) lymphomas, respectively. Kaplan-Meier/ Mantel-Cox log-rank analysis showed that SS patients with FS ≥4 (n=90) had statistically significant higher lymphoma rates compared to those with FS<4 (n=528), exhibiting a 5-year overall event free rates of 80,5% vs 96,9% respectively (p long-rank <0.0001) (Figure 2B).

Figure 2. A. Pearson correlation between the time from SS until lymphoma diagnosis in SS lymphoma patients. B. Kaplan Meier analysis of lymphoma development in the total SS population with FS ≥4 vs FS<4.

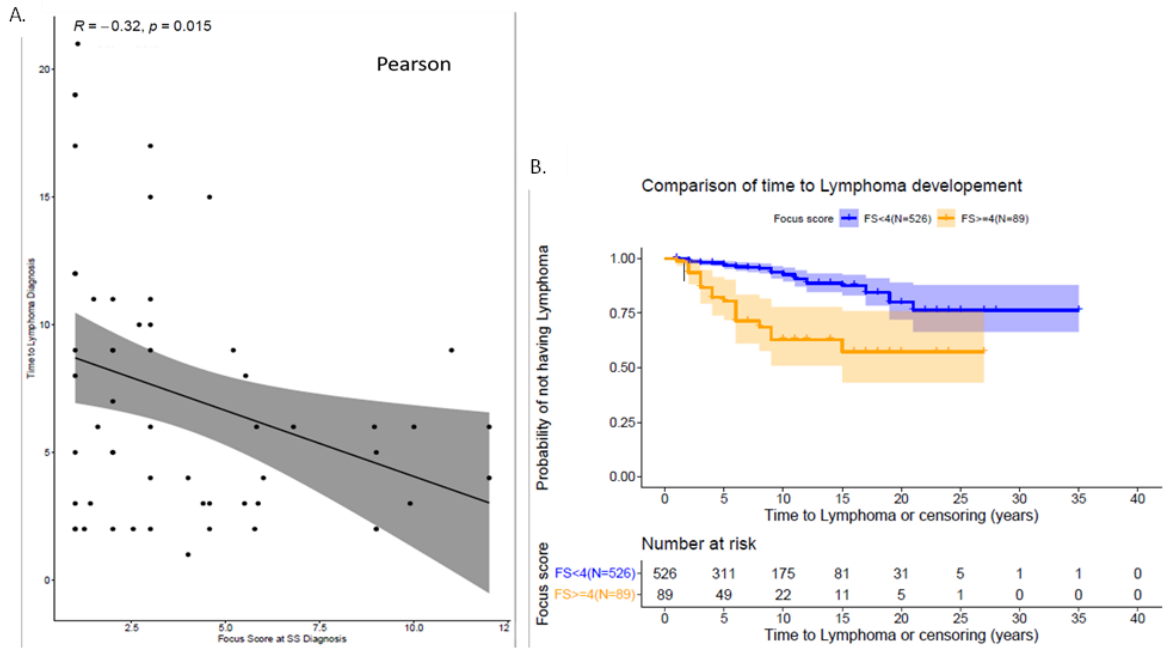


***Table 2**. Comparison of glandular, non-specific, extra-glandular and serologic features between patients with high (*FS≥4*) and low (*1≤FS<4*) focus score*

FEATURE	1≤FS<4 (n=528)	FS≥4 (n=90)	p-value
Demographics			
Median age at disease diagnosis (range)	53 (15-80)	49 (17-80)	0.08
Median disease duration from SS diagnosis (range)	6 (1-35)	7 (1-27)	0.55
Females	505/528 (95,6)	86/90 (95,5)	1
Males	23/528 (4,4)	4/90 (4,5)	1
GLANDULAR MANIFESTATIONS			
Dry mouth	92,5% (484/523)	97,7% (87/89)	0.103
Dry eyes	92,6% (489/528)	96,6% (87/90)	0.253
Salivary gland enlargement	26,9% (141/524)	46,0% (41/89)	**0.0004**
NON-SPECIFIC MANIFESTATIONS			
Raynaud's phenomenon	28,1% (147/523)	25,5% (23/90)	0.709
Arthritis	15,8% (80/506)	16,6% (15/90)	0.961
Arthralgia	58,0% (305/525)	54,4% (49/90)	0.594
Palpable purpura	9,8% (52/528)	14,4% (13/90)	0.259
Lymphadenopathy	13,2% (64/482)	24,6% (20/81)	**0.012**
ORGAN SPECIFIC MANIFESTATIONS			
Interstitial renal disease	2.6% (13/484)	2,4% (2/82)	1
Glomerulonephritis	1,7% (9/528)	2.2% (2/89)	0.664
Primary biliary *cholangitis*	1.7% (9/528)	1.1% (1/90)	1
Autoimmune hepatitis	0.6% (3/431)	0% (0/87)	1
Small airway disease	2.5% (13/528)	5.4 % (9/166)	0.100
Interstitial Lung Disease	5,6% (30/528)	7,7% (7/90)	0.593
Autoimmune thyroiditis	37,5% (115/306)	19,0% (12/63)	**0.007**
LYMPHOMA	6,6% (35/527)	25,8% (23/89)	**<0.0001**
SEROLOGY			
Antinuclear antibodies	88,8% (454/511)	97,7% (87/89)	**0.006**
Rheumatoid Factor	46,7% (229/490)	72,0% (62/86)	**<0.0001**

Anti-Ro	73,5% (378/514)	78,6% (70/89)	0.374
Anti-La	36,5% (186/509)	50,5% (45/89)	**0.01**
LOW C4	33,2% (151/454)	41,7% (33/79)	0.180
Monoclonal gammopathy	8,2% (36/438)	16,6% (12/72)	**0.03**
Cryoglobulinemia	10,8% (34/317)	22,2% (16/72)	**0.01**

9.3.4 The clinical phenotype of SS patients with high (≥4) and low (<4) LMSG FS.

From the 618 SS patients with positive LMSG biopsy, 90 (14,5 %) had FS≥4 and 528 (85,4%) FS <4. The demographic features of SS patients of this study are shown in Table 2. No statistical differences were identified between the two groups regarding gender, age, and disease duration from SS diagnosis.

SS patients with FS ≥4 presented more frequently with SGE (46% vs 26,9% p=0.0004, OR=2.32, 95% CI: 1.481-3.6971), longstanding lymphadenopathy (24,6% vs 13,2% p=0.012, OR= 2,14, 95% CI: 1.223-3.712), monoclonal gammopathy (16,6% vs 8.2% p=0.03, OR=2.23 95% CI: 1.131-4.503) and lymphoma (25,8% vs 6,6% p<0.0001, OR= 4.89, 95% CI: 2.780-8.625) in comparison to SS patients with FS between 1 and 4. No statistically significant difference was found regarding the age at the time of lymphoma diagnosis between high and low FS patients [median age: 51 (range: 30-74) vs 56,5 (range: 34-72) years old respectively, p=0,24]. Non-MALT lymphomas were distributed as follows: 1 DLBC, 1 nodal and 1 splenic belonged to the high FS group and 1 DLBC and 1 follicular to the low FS group. A higher prevalence of antinuclear antibodies (ANA) (97,7% vs 88,8% p=0.006, OR=5.46, 95% CI: 1.478-23.16, anti La/SSB antibodies (50,5% vs 36,5% p=0.01, OR=1.77, 95% CI: 1.123-2.813) and rheumatoid factor (RF) (72% vs 46,7% p<0,0001, OR=2.94, 95% CI: 1.802-4.920) were observed in the high FS group compared to patients with low FS. On the contrary, autoimmune thyroiditis was more prevalent in patients with low FS, compared to patients with high FS (19% vs 37,5% p=0.007, OR=0.39, 95% CI: 0.2043-0.7546). A detailed comparison of the clinical, laboratory, and serologic features between the 2 groups is presented in Table 2.

9.3.5 Lymphoma associated risk factors in high (≥4) and low (<4) LMSG FS subgroups of SS patients

An analysis was undertaken to identify specific lymphoma associated risk factors for both high (≥4) and low (<4) FS subgroups of SS patients, in order to achieve early lymphoma diagnosis. Therefore, an FCBF/LR model with down sampling matching strategy in 1:1 ratio between lymphoma and non-

lymphoma controls on both datasets was performed. The combined FCBF/LR algorithm when applied on the high FS subgroup revealed FS as the only independent lymphoma associated risk factor. This finding suggests that all SS patients with FS ≥4 can be evaluated for early lymphoma diagnosis around 4 years from SS diagnosis, corresponding to the median duration from SS to lymphoma diagnosis among SS lymphoma patients with FS ≥4 (Table 3) (model performance: sensitivity=74%, specificity=78% accuracy=75%, area under the curve/AUC=85%, Supplementary Figure 5). On the contrary, for the low FS subgroup, SGE was found to be the only independent lymphoma associated risk factor (Table 4) (model performance: sensitivity=74%, specificity=74% accuracy=75%, area under the curve/AUC=82%, Supplementary Figure 6). Interestingly, in the low FS group, 141 SS patients with a history of SGE developed 24 of the total 33 (72%) MALT lymphomas. Due to the higher number of low FS patients, early lymphoma diagnosis could be accomplished by a second LMSG biopsy limited in patients with history of SGE and should be performed around a 9-year interval from SS diagnosis, which corresponds to the median time of lymphoma diagnosis in SS lymphoma patients with a FS< 4.

***Table 3**. FCBF-based multivariable logistic regression analysis results with lymphoma as an outcome in high Focus Score group (FS≥4).*

Prominent feature*	Regression coefficient	Odds ratio	p-value	CI low	CI upper
Cryoglobulinemia	0.917	2.571	0.421	0.288	24.681
Low serum C4	1.306	3.836	0.147	0.701	21.575
Rheumatoid Factor	1.027	2.877	0.282	0.484	17.654
Salivary gland enlargement	0.815	2.34	0.345	0.47	11.777
Focus score	0.333	1.409	0.035**	1.104	1.803
Monoclonal gammopathy	0.298	1.399	0.779	0.142	15.134
Raynaud's phenomenon	0.517	1.75	0.561	0.321	9.746

* The strongest potentially independent variables identified by the FCBF algorithm to construct the logistic regression model, after analysing initially the following features included in the unified dataset: Gender, Age at SS, Dry mouth, Dry eyes, Anti-Ro, Anti-La, ANA, Focus Score, history of SGE, history of generalized Lymphadenopathy, Rheumatoid Factor, monoclonal gammopathy, Low C4(<20), Arthralgia-myalgia, Arthritis, Raynaud, Palpable purpura, , Myositis, PNS-vasculitic, CNS involvement, Liver involvement – autoimmune hepatitis, Liver involvement- PBC, Lung involvement – interstitial disease, Lung involvement – small airway disease, Interstitial renal disease, Kidney involvement -GN-biopsy, Autoimmune thyroiditis, Cryoglobulinemia.

**< 0.05 (95% confidence interval): final independent lymphoma associated factors

***Table 4**. FCBF-based multivariable logistic regression analysis results with lymphoma as an outcome in low Focus Score group (FS<4).*

Prominent feature*	Regression coefficient	Odds ratio	p-value	CI low	CI upper
Cryoglobulinemia	1.325	3.92	0.147	0.69	23.134
SGE	3.92	5.213	0.02 **	1.541	17.779
Lymphadenopathy	0.788	2.32	0.35	0.493	11.281
Age at SS diagnosis	-0.037	0.964	0.236	0.92	1.01
Gender	0.4	1.603	0.748	0.149	20.147
Autoimmune thyroiditis	0.008	1.051	0.736	0.283	3.956

* The strongest potentially independent variables identified by the FCBF algorithm to construct the logistic regression model, after analysing initially the following features included in the unified dataset: Gender, Age at SS, Dry mouth, Dry eyes, Anti-Ro, Anti-La, ANA, Focus Score, history of SGE, history of generalized Lymphadenopathy, Rheumatoid Factor, monoclonal gammopathy, Low C4(<20), Arthralgia-myalgia, Arthritis, Raynaud, Palpable purpura, , Myositis, PNS-vasculitic, CNS involvement, Liver involvement – autoimmune hepatitis, Liver involvement- PBC, Lung involvement – interstitial disease, Lung involvement – small airway disease, Interstitial renal disease, Kidney involvement -GN-biopsy, Autoimmune thyroiditis, Cryoglobulinemia.

**< 0.05 (95% confidence interval): final independent lymphoma associated factors

9.4 Discussion

For a long time it has been known, that inflammation severity of the minor salivary gland biopsy is correlated with a more pronounced systemic disease and more autoantibodies [358]. In more recent years, our knowledge regarding the clinical significance of LMSG has expanded and several studies have suggested that: i. The cellular composition of the higher FS is different from that of low FS, with the former being characterized by B-cell predominance (FS), ii. The heavily infiltrated LMSGs contain

ectopic lymphoid structures that have previously been associated with future lymphoma development [294,370], and iii. the infiltrated LMSGs are active sites for both autoimmune and lymphoproliferative pathogenetic mechanisms [371,372]. Indeed, they possess the appropriate armamentarium to differentiate B-cells towards autoantibody-producing plasma cells [373] and also contain oligo or monoclonal B-cell populations [374] as well as molecular biomarkers that may serve as predictors for future lymphoma development [316,375]. The above observations indicate that inflammation intensity of salivary gland biopsy at the time of SS diagnosis may reflect the clinical picture of SS and moreover serve as a predictive feature for NHL development in this patient group. For the latter, two important elements should be considered: a. The LMSG does not usually change, even many years after the disease onset [376], with the exception of lymphoma development and 2. The vast majority of lymphomas in SS are derived from the mucosal associated lymphoid tissue (MALT) [377].

Previous studies aiming to address the predictive value of LMSG for lymphoma development were hindered by: a) the relatively small number of biopsies, and SS NHL patients b) the lack of strict criteria regarding tissue sampling and scoring in relation to lymphoma diagnosis and c) the lack of detailed clinical phenotype description. Nevertheless, Gerli et al. after studying 82 patients, supported that FS was related to extra-glandular manifestations [378] while Carubbi et al. after evaluating 383 biopsies pointed out the prognostic power of FS on lymphoma development [379]. In addition, Risselada et al. were the first to show that FS≥3 at the time of SS diagnosis contributes significantly to the risk of subsequent lymphoma development, although the number of SS NHL patients was rather low [366]. However, none of the aforementioned studies focused on FS grading, proposing a key FS threshold for SS patients with positive LMSG biopsy, linked to early lymphoma diagnosis or a more severe clinical disease phenotype. Therefore, the clinical value of FS in the LMSG as a predicting factor of lymphoma development and a strategy to link the biopsy with early detection of lymphoma in SS, are still unmet need.

To address these questions, we investigated the largest number of salivary gland biopsies, studied so far, consisting of 618 patients and followed a specific study design approach, characterized by several novel points: i) an intergraded cohort of highly homogenized patients from different ethnic groups (Greece and Italy) following a common clinical and laboratory reference model ii) strict application of the 2016 ACR/EULAR criteria and participation of a large number of patients with FS≥1, iii) the recruited lymphoma patients and non-lymphoma controls had at least 1 year time distance from lymphoma diagnosis or last follow up respectively, to ensure the predictive power of FS on lymphoma development, iv) lymphoma associated risk factors were explored by data driven approaches, analyzing 30 different clinical and laboratory features related to SS , minimizing, therefore,

selection bias, and v) the investigation of a follow up strategy for early lymphoma diagnosis was correlated with the time interval from SS diagnosis until lymphoma diagnosis for the first time in the literature, as well as the introduction of a FS threshold to further stratify all SS patients. The main findings of our study are highlighted as follows: a) the LMSG FS was proven an independent lymphoma associated risk factor as a continuous variable, suggesting a positive quantitative correlation between FS level and lymphoma development, b) FS is inversely correlated with the time interval from SS to lymphoma diagnosis, c) almost half of the SS population with positive LMSG biopsy has FS values equal or above 1 and below 2, with a long time interval elapsing until the potential lymphoma diagnosis, d) the FS threshold of 4 maximizes the difference in the time interval from SS diagnosis to lymphoma diagnosis between patients with FS ≥ 4 and those with FS<4, defining high and low FS subgroups suggesting essentially a follow up strategy for early lymphoma diagnosis that can be applied in all SS patients and e) provide more clinically useful insights into disease phenotypes, since B cell clinical manifestations have been accumulated in the high FS subgroup, including various risk factors for lymphoma development, justifying, therefore the observed higher lymphoma prevalence and short time interval to lymphoma diagnosis. On the other hand, the low FS subgroup is characterized by a statistically significant higher prevalence of autoimmune thyroiditis, a finding that clusters this lesion in the autoimmune epithelitis spectrum.

The biologic significance of FS in relation to lymphoma development is further supported by the fact that typical risk factors such as SGE and cryoglobulinemia, tightly associated with the B cell component and the lymphomagenesis process, also serve as independent lymphoma predictors in our study.

The inverse correlation between FS and the time interval from SS diagnosis to lymphoma diagnosis, reported also for the first time, suggests that FS at diagnosis may reflect different stages in the continuum of biologic events towards lymphoproliferation, irrespectively of the histologic type of lymphomas, although non-MALT cases are very few in the present study. Thus, FS apart from the diagnostic and predictive power for lymphoma, is also a useful biomarker to assess the evolution and progression of lymphomagenesis during time. Indeed, the FS grade 4, was found to successfully separate SS lymphoma patients into high and low FS subgroups providing the maximum possible discrimination regarding the time interval until lymphoma diagnosis. This finding in combination with the increasing odds for lymphoma development above FS=4, allowed us to propose this threshold as the most informative for the physician to structure a follow up strategy for all SS patients by performing a second LMSG biopsy at different time points, considering the time interval until lymphoma diagnosis. Lymphomagenesis is a multistep and long-standing process, especially for MALT lymphomas which are characterized by an insidious course. Thus, it is reasonable to expect that most MALT lymphomas are

present within the LMSG, around the 4- and 9-year time interval from SS diagnosis for high and low FS subgroups respectively, and before the time point of clinically apparent manifestations of lymphomas. Unless there is a strong clinical suspicion for an underlying lymphoma, we recommend proceeding with a second LMSG biopsy only in specific subsets of SS patients, including all patients with a FS≥4, 4 years after SS diagnosis, and in those with FS<4 and a history of SGE, at 9-years, contributing to an early lymphoma diagnosis. Although more SS patients may be biopsied, an early lymphoma diagnosis seems to supersede the very low risk of LMSG biopsy related complications[380]. On the other hand, the benefit of early lymphoma diagnosis is clearly attested by the prognostic power of MALT IPI score, which incorporates both age at lymphoma diagnosis and the Ann Arbor staging, and can efficiently discriminate patients with poor, intermediate, or good event free and overall survival [381]. Therefore, a delayed diagnosis might reflect a worse malt IPI score and clinical outcomes. Irrespective of the MALT IPI however, a survival study including more than 9800 MALT patients clearly stated that age is a significant prognostic factor in a hazard model specialized for MALT lymphomas[382]. Needless to say, that the FS threshold and the proposed follow up strategy must be validated in other independent and if possible prospective cohorts.

It is also noteworthy that the low FS subgroup had higher frequency of autoimmune thyroiditis as opposed to the high FS subgroup, a finding that is also reported for the first time. It has been suggested that thyroid involvement could represent a part of the so-called peri-epithelial extra-salivary manifestations in the context of SS, since shared pathophysiologic mechanisms are implicated for both entities [383,384]. This particular finding is also in line with previous studies supporting that patients with autoimmune thyroiditis and mild inflammatory lesions in LMSG biopsy dominated by T cells experience mild glandular disease, most likely due to robust immunoregulatory mechanisms limiting the autoimmune response to the epithelial structures [385 386].

Limitations of this study include the relatively small number of lymphoma patients and the retrospective nature of the study. Prospective studies recruiting larger number of patients are expected to provide further insights on how lymphomagenesis evolves and what the real benefit is for SS patients when a second LMSG is performed blindly, regarding early lymphoma diagnosis. Higher numbers of lymphoma patients will also enrich the distribution of lymphoma according to each FS subgroup and offer the opportunity to study the less common forms of non-MALTs which carry a worse prognosis. In addition, the estimation of the inflammatory burden within the LMSG tissue based on FS and limited to hematoxylin and eosin staining may obscure other histologic parameters and to some extent may be subjective especially regarding the number of foci as perceived by the oral pathologists.

In summary, FS in LMSG biopsy is an independent lymphoma associated risk factor with the capacity to reflect the different stages of lymphomagenesis through the time interval from SS diagnosis

to lymphoma occurrence, serving as an excellent biomarker for prediction and early lymphoma diagnosis.

Chapter 10: Combined seronegativity in Sjögren's Syndrome

10.1 Introduction

Sjogren's syndrome (SS) is accompanied by plethora of autoantibodies as a result of B cell aberrant activation [387,388], with anti-Ro/SSA, anti-La/SSB, rheumatoid factor (RF) and antinuclear antibodies (ANA) being the most frequently encountered [358,389]. Anti-Ro/SSA antibody, is present in 50-75% of SS patients and in approximately half of them, anti-La/SSB antibody is also detected [390]. Anti-La/SSB antibodies almost always coexist with anti Ro/SSA and less than 2% of SS patients are anti-La/SSB monopositive, with a phenotype ranging between seronegative and seropositive patients[282,283]. The prevalence of rheumatoid factor reaches approximately 50% in SS and have been recognized as lymphoma predictor [151,391]. Other autoantibodies have been also reported to define unique clinical phenotypes of SS but are detected in low prevalence [387]. Preceding studies have explored the phenotype of partly seronegative patients (defined as anti-Ro/SSA and anti-La/SSB negative antibodies) versus seropositive controls (defined as positive for anti-Ro/SSA with or without anti-La/SSB antibodies), exhibiting differences regarding the age at SS diagnosis, sicca manifestations, specific extraglandular manifestations, and lymphoma [281,392]. In these studies, however, RF and ANA antibodies differed between study groups, obscuring the true effect of anti-Ro/SSA and/or anti-La/SSB antibodies on disease phenotype. Recently, it was shown that the absence of SS associated autoantibodies is related to less hematologic malignancies, although lymphomas were not included in the analysis [393]. Prompted by these findings, we sought to explore the clinical landscape of SS patients with autoantibody paucity and investigate the effect of RF on the clinical expression of the disease.

10.2 Patients and methods

Study population included 1723 consecutive SS patients who fulfilled the 2016 EULAR/ACR criteria and were followed between May 1984 and January 2021, in 4 clinical centers ([Universities of Pisa, Athens, Harokopio and Ioannina]. Patients with unknown autoantibody profile were excluded. Two study groups were identified: a) patients with triple seronegativity [anti-Ro/SSA (-), anti-La/SSB (-), RF(-) and ANA (+)](3pl) and b) patients with quadruple seronegativity [anti-Ro/SSA (-), anti-La/SSB (-), RF(-) and ANA (-)](4pl). Each study group was compared with 2 distinct SS control groups, matched according to gender, age at disease diagnosis and disease duration from SS diagnosis to last follow up, in a 1:1 ratio, and in terms of cumulative clinical, laboratory and histologic features: i) randomly chosen classic anti-Ro/SSA seropositive SS controls, independently of the RF and anti-La/SSB status [anti-Ro/SSA(+), ANA(+), anti-La/SSB (+or-), RF (+or-)] [SS(+)] and ii) classic seropositive anti-Ro/SSA SS controls with negative RF [anti-Ro/SSA (+), ANA (+), anti-La/SSB (+or-), and RF (-)] [SS(+)/RF(-)] to investigate the effect of RF on clinical phenotype of the disease and especially lymphoma. All data were collected retrospectively from medical records, following a common prespecified reference model, as part of the HarmonicSS project. Systemic organ involvement and all clinical variables were defined as previously described by the ESSDAI domains and glandular dryness according to EULAR validated questionnaires [13, 14]. Salivary gland biopsies were evaluated by 2 independent and highly experienced in SS pathologists. Autoantibodies were detected in all centers as part of standard of care accordingly. The study was approved by all local ethical committees after obtaining patients' informed consent and in compliance with general data protection regulations (GDPR) of the European Union. Statistical analysis for categorical data was performed by Fisher exact test or chi-square test and numerical data with the Mann-Whitney or t-test using Python 3.6.

10.3 Results

10.3.1 Patients' characteristics

Final population comprised of 1569 patients with full SS related autoantibody profile; 135 triple seronegative patients and 72 quadruple seronegative patients were identified, constituting 8.6% and 4.6% respectively. Seronegative SS patients from each study group were compared with i) 135 and 72 classic anti-Ro/SSA seropositive SS control patients respectively [SS(+)], of whom 84 (62.2%) and 34 (47.2%) had positive RF respectively and ii) 135 and 72 SS controls respectively, with RF(-)/anti-Ro/SSA seropositivity [SS(+)/RF(-)]. The median age at SS diagnosis was 60 years old (range: 26-80) for 3pl

negative patients and 56 years old (range: 11-74) for 4pl negative patients while disease duration was 3 years for both groups (range: 0-28 and 0-24, respectively). Each study group included five male patients. The demographic features of SS control groups are presented in Tables 1 and 2.

10.3.2 Comparison of triple and quadruple negative patients with distinct SS control groups

- Anti-Ro/SSA(+) SS controls independently of RF

Demographic, clinical, laboratory and histologic features were compared between the two study groups and their respective anti-Ro/SSA(+), ANA(+) RF(+or-) SS control groups [SS(+)]. Demographic characteristics were similar between comparing groups (Table 1). A statistically significantly lower frequency of peripheral nervous involvement (0% vs 7.2% p=0,002) was observed in 3pl negative SS patients compared to SS(+) controls. Similarly, purpura and lymphoma were less prevalent among 3pl negative patients, without though reaching statistical significance (Table 1). Quadruple negative patients presented less frequently with persistent lymphadenopathy (1.5% VS 16.9 p=0.004) and lymphoma (0% vs 9.8% p=0,006) compared to SS(+) controls (Table 2). Similarly, 4pl negative SS patients developed less often salivary gland enlargement, low C4 hypocomplementemia, arthritis, purpura and cryoglobulinemia, without statistically significant difference.

- SS controls with RF negativity and anti-Ro/SSA positivity

Demographic, clinical, laboratory and histologic features were compared between the two study groups and SS(+)/RF(-) control groups. Demographic characteristics were similar between comparing groups (Table 1). A statistically significantly higher frequency of dry mouth was found on 3pl negative patients compared to controls (96.2% vs 88% p=0,02) whereas SS(+)/RF(-) control patients displayed higher prevalence of interstitial renal disease (0% vs 5.2% p= 0,01). Quadruple negative patients presented more frequently with dry eyes (98.6% vs 87.5% p=0,01) and autoimmune thyroiditis (44.1% vs 17.1% p=0,02) while their SS(+) RF(-) SS controls displayed a stronger association with persistent lymphadenopathy (1.5% vs 15.3% p=0,008) (Table 2).

10.3.3 Comparison of triple and quadruple negative patients

Demographic, clinical, laboratory and histologic features were compared between 3pl and 4pl negative patients. The median disease duration of comparing groups was similar, while 4pl negative patients showed an earlier age at SS diagnosis than 3pl negative patients (Table 3). A statistically significantly higher frequency of persistent lymphadenopathy (12.5% % vs 1.5% p=0,01), minor salivary gland biopsy focus score (2% % vs 1.1% p=0,01) and lymphoma (5.9% % vs 0% p=0,05) was associated with 3pl negative patients. Quadruple negative patients displayed an increased prevalence of autoimmune thyroiditis and peripheral nervous disease, without reaching a statistically significant difference (Table 3).

Table 1. Comparison of clinical and laboratory features between 3pl negative patients and the two controls groups

FEATURE/ CLINICAL MANIFESTATION	3PL NEGATIVES **n= 135**	Ro (+) CONTROLS n= 135	P VALUE	RF (-), Ro (+) CONTROLS n=135	P VALUE
DEMOGRAPHICS					
Median age at disease diagnosis, (range)	60, (26-80)	60, (26-79)	0.97	60, (28-83)	0.81
Median disease duration from SS diagnosis to last follow up, (range)	3, (0-28)	3, (0-25)	0.75	3, (0-28)	0.86
GLANDULAR AND NON SPECIFIC MANIFESTATIONS					
Dry mouth	96.2% (128/133)	91.8% (1324/135)	0.20	88% (118/134)	**0.02**
Dry eyes	94.8% (128/135)	91.8% (124/135)	0.46	90.3% (122/135)	0.24
Salivary gland enlargement	22.3% (30/134)	24.6% (33/134)	0.77	14.1% (19/134)	0.11
Raynaud's phenomenon	34.3% (44/128)	31.7% (39/123)	0.75	30.6% (38/124)	0.61
Arthralgias	53.3% (71/133)	56.7% (76/134)	0.58	60% (81/135)	0..33
Arthritis	15.5% (19/122)	13.6% (16/117)	0.81	12.9% (15/116)	0.69
EXTRAEPITHELIAL MANIFESTATIONS					

Glomerulonephritis	0.7% (1/132)	0.7% (1/135)	1	2.2% (3/135)	0.62
Interstitial Lung Disease	7.7% (10/129)	7.8% (10/127)	0.84	4.8% (6/125)	0.47
Autoimmune hepatitis	0% (0/111)	0.8% (1/112)	1	0.9% (1/104)	0.48
Peripheral nervous disease	0% (0/124)	7.2% (8/110)	**0.002**	2.5% (3/116)	0.11
Palpable purpura	3.7% (5/135)	9.6% (13/135)	0.08	1.4% (2/135)	0.44
Persistent lymphadenopathy	12.5% (16/127)	11.7% (13/111)	0.99	8.8% (11/125)	0.44
PERIEPITHELIAL MANIFESTATIONS					
Tubulointerstitial nephritis	0% (0/132)	1.5% (2/133)	0.49	5.2% (7/134)	**0.01**
Small Airway disease	7.2% (9/124)	7.7% (9/116)	0.92	7.7% (9/116)	0.92
Primary biliary cholangitis	3.7% (5/135)	1.4% (2/135)	0.44	1.4% (2/134)	0.44
Autoimmune thyroiditis	30.5% (26/85)	29.6% (24/81)	0.97	28.3% (23/81)	0.88
FOCUS SCORE (range)	2 (1-9)	1.83 (0-12)	0.28	2 (0,25-12)	0.06
SEROLOGY					
Rheumatoid Factor	0% (0/135)	62.2% (84/135)	<0.0001	0% (0/135)	1
Anti-Ro	0% (0/135)	89.6% (135/135)	<0.0001	100% (135/135)	<0.0001
Anti-La	0% (0/135)	40% (54/135)	<0.0001	30.3% (41/135)	<0.0001
LOW C4	23.1% (28/121)	23.1% (38/116)	0.13	23.3% (28/120)	0.90
Monoclonality	7.2% (9/124)	11% (13/118)	0.42	7.5% (9/120)	0.86
Cryoglobulinemia	6.8% (6/88)	10.2% (8/78)	0.62	6.4% (5/77)	0.81
Anti-nuclear antibody	100% (135/135)	100% (135/135)	1	100% (135/135)	1
LYMPHOMA	5.9% (8/135)	10.3% (14/135)	**0.26**	1.4% (2/135)	0.1

Table 2. Comparison of clinical and laboratory features between 4pl negative patients and the two controls groups

FEATURE/ CLINICAL MANIFESTATION	4PL NEGATIVES n= 72	Ro (+) CONTROLS n= 72	P VALUE	RF (-), Ro (+) CONTROLS n=135	P VALUE
DEMOGRAPHICS					
Median age at disease diagnosis, (range)	56, (11-74)	56, (10-77)	0.91	56, (10-76)	0.89
Median disease duration from SS diagnosis to last follow up, (range)	4, (0-24)	4, (0-23)	0.96	3, (0-21)	0.49
GLANDULAR AND NON SPECIFIC MANIFESTATIONS					
Dry mouth	94.3% (67/71)	94.2% (67/71)	1	88% (64/72)	0.36
Dry eyes	98.6% (71/72)	91.6% (66/72)	0.11	87.5% (63/72)	**0.01**
Salivary gland enlargement	16.6% (12/72)	30.5% (22/72)	0.07	20.8% (15/72)	0.66
Raynaud's phenomenon	25.3% (16/63)	27.9% (19/68)	0.98	27.2 (18/66)	0.96
Arthralgias	54.1% (39/72)	61.1% (44/72)	0.61	58.3% (42/73)	0.73
Arthritis	6.4% (4/62)	18.7% (12/64)	0.06	9.8% (6/61)	0.52
EXTRAEPITHELIAL MANIFESTATIONS					
Glomerulonephritis	1.4% (1/71)	2.8% (2/71)	1	1.4% (1/71)	1
Interstitial Lung Disease	3% (2/65)	7.1% (5/70)	0.44	1.5% (1/66)	0.61
Autoimmune hepatitis	0% (0/62)	1.9% (1/52)	0.45	1.7% (1/57)	0.47
Peripheral nervous disease	3.2% (2/61)	3% (2/65)	1	3.3% (2/60)	1

Palpable purpura	4.1% (3/72)	12.5% (9/72)	0.13	0% (0/72)	0.24
Persistent lymphadenopathy	1.5% (1/65)	16.9% (11/65)	**0.004**	15.3% (10/65)	**0.008**
PERIEPITHELIAL MANIFESTATIONS					
Tubulointerstitial nephritis	2.8% (2/71)	2.7% (2/72)	1	1.4% (1/71)	1
Small Airway disease	5% (3/60)	7.4% (5/67)	0.72	8% (5/62)	0.71
Primary biliary cholangitis	1.3% (1/72)	1.3% (1/72)	1	1.4% (1/72)	1
Autoimmune thyroiditis	44.1% (19/43)	29.0% (9/31)	0.31	17.1% (6/35)	**0.02**
FOCUS SCORE (range)	1.1 (1-4)	2 (0-8.7)	0.07	1.33 (0-12)	0.83
SEROLOGY					
Rheumatoid Factor	0% (0/72)	47.2% (34/72)	<0.0001	0% (0/72)	1
Anti-Ro	0% (0/72)	100% (72/72)	<0.0001	100% (72/72)	<0.0001
Anti-La	0% (0/72)	47.2% (34/72)	<0.0001	34.7% (25/72)	<0.0001
LOW C4	23.3% (14/60)	39.0% (25/64)	0.09	33.8% (21/62)	0.27
Monoclonality	3.2% (2/62)	7.4% (5/67)	0.44	8% (5/62)	0.43
Cryoglobulinemia	6.8% (3/44)	13.8% (5/36)	0.45	13.5% (5/37)	0.45
Anti-nuclear antibody	0% (0/72)	100% (72/72))	<0.0001	100% (70/72)	<0.0001
LYMPHOMA	0% (0/72)	9.8% (7/71)	**0.006**	1.3% (1/72)	1

10.4 Discussion

Double anti-Ro/SSA and anti-La/SSB seronegativity has been a subject of intense clinical research [281,392]. However, little is known about triple and quadrable combined seronegativity, enclosing

the significant contribution of RF as the crossroad among B cell hyperactivity, monoclonality and lymphomagenesis, in the clinical phenotype of the disease. Our study gathers some unique features: a) it is conducted in 4 highly specialized clinical centers for SS from 2 different countries, b) integrated data are manually harmonized based on a common reference model, and c) it is focused on 3pl and 4pl seronegative SS patients for the first time in the literature, and d) two sequential comparisons have been performed with and without RF contribution, to explore their potential effect on clinical phenotype.

The first interesting findings of our study is the prevalence of patients with combined seronegativity reaching almost 9% of total SS population. This subset of patients is not negligible and is characterized by complete absence of autoantibodies, as typical serum biomarkers of autoimmunity. Second, it seems that both 3pl and 4pl negative patients adopt a milder clinical phenotype with less B cell mediated manifestations. Interestingly, 4pl negative patients have a tendency to present more frequently dry eyes, autoimmune thyroiditis and interstitial renal disease, especially when compared to SS(+)/RF(-) controls. These particular clinical manifestations, share as common underlying pathogenetic mechanism, the typical peri-pethelial inflammatory infiltration of the affected organs. This finding, in combination with the absence of autoantibodies, raises thoughts that 4pl negative SS patients might unleash strong local and systemic immunoregulatory mechanisms, capable of restricting the autoimmune response confined to the epithelial structures, avoiding in this way generalized and wide-spread immune responses against ubiquitous self-antigens. Third, our study reveals that 3pl seronegative patients have a worse prognosis compared to 4pl with a higher probability of developing persistent lymphadenopathy and lymphoma combined with a higher salivary gland focus score [394]. This finding is of clinical significance highlighting the fact that among seronegative patients those with positive antinuclear antibodies are expected to develop more severe disease manifestations as a consequence of a more generalized systemic autoimmune response. However, we should stress out that these patients were not matched, and our results may be affected by the

confounding factor of age, that can alter the clinical trajectory of SS patients [104]. Fourth, the similar prevalence of lymphoma between seronegative SS patients and SS(+)/RF(-) controls as opposed to SS(+) controls, could imply a central role of RF in the evolution towards lymphomagenesis. Indeed, RF have been previously proposed as independent lymphoma predictor [391]. However, the clinical expression of the disease should not be interpreted only from the side of the effector arm (e.g. autoantibodies) but the counter immunoregulation should be also considered, and therefore not all RF+ SS patients are finally expected to develop a worse clinical phenotype. In the literature, only one study after our initial publication [395] has focused on 4pl negative SS patients, showing no differences between seropositive and seronegative patients, apart from hypergammaglobulinemia [393]. In that study it was noteworthy the high proportion of 4pl negative SS patients, the omission of lymphoma as clinical feature, the tendency of these patients to develop autoimmune thyroiditis confirming our results and the absence of matched SS seropositive control groups, with and without RF(-) SS patients.

The present study is limited by the retrospective nature of the design, the relatively limited number of recruited triple and quadruple seronegative patients, the short period of follow up and the inherent physicians' hesitancy to perform further diagnostic work up for SS in suspicious individuals with negative autoantibody profile. In conclusion, SS patients with autoantibody paucity, display a mild clinical picture dominated by glandular and peri-epithelial manifestations while B cell symptoms are less apparent.

Chapter 11: Serum, but not saliva, CXCL13 levels associate with infiltrating CXCL13+ cells in the minor salivary gland lesions and lymphoma in patients with Sjögren's syndrome.

11.1. Introduction

Non-Hodgkin's lymphomas (NHL) of B cell origin are often developed in the setting of autoimmune diseases [396]. Primary Sjögren's syndrome (pSS) is the disorder with the highest prevalence of NHL (5-10%) among autoimmune diseases [225]. In fact, NHL represents the major adverse outcome of the disease, affecting both morbidity and mortality [112,340,397]. Although the underlying pathogenetic mechanisms of SS-related lymphomagenesis are not defined, it is considered a multistep, antigen-driven process arising from incessant chronic B cell activation at the inflammatory lesions [398,399]. In favor of this, the vast majority of SS- related NHLs are mucosa-associated lymphoid tissue (MALT) lymphomas, located at the salivary glands, whereas their development associates with intense inflammatory responses, as attested by the degree of infiltration, certain inflammatory cells, such as macrophages, and organization of the lymphocytic infiltrates to ectopic germinal centers (eGC) within the affected minor salivary glands (MSG) [400].

CXCL13 (C-X-C motif chemokine ligand 13) is a chemokine expressed by follicular dendritic cells (FDC), stromal cells, monocytes and macrophages, primarily in secondary lymphoid organs, such as the liver, the spleen and the lymph nodes [401,402]. Through interaction with the G protein-coupled chemokine receptor CXCR5, which is expressed on B and T follicular helper cells, CXCL13 keeps a vital role on the development and organization of lymphoid tissues [401-405]. CXCL13 has been mechanistically linked to several disorders, including autoimmune diseases and hematologic malignancies [406-415]. In pSS, CXCL13 expression has been implicated in SS pathogenesis, including the formation of eGCs in the minor salivary gland (MSG) lesions and the related process leading to lymphomagenesis [170,399,416]. Increased CXCL13 serum levels have been associated with NHL, pSS disease activity and MSG histologic features, and in a recent study they were linked to increased lymphoma risk [417-421]. Interestingly, serum, but not saliva, levels of CXCL13 were found elevated in Asian-Indian pSS patients [422], questioning whether elevated CXCL13 serum levels in patients with extended and/or organized to eGCs inflammatory MSG lesions originate from the affected glands.

Prompt by these findings, we sought to validate the clinical utility of CXCL13 in pSS by examining its expression in paired samples of serum, saliva and MSG biopsies from patients with pSS (with or without NHL), sicca-controls, and healthy individuals. In addition, we investigated possible associations with various histologic, serologic and clinical disease parameters, which have been previously

identified as adverse prognostic factors for NHL development, including severity of MSG autoimmune infiltrates and GC formation, high EULAR SS disease activity index (ESSDAI) score, salivary gland enlargement (SGE), purpura, vasculitis, leukopenia, cryoglobulinemia, hypocomplementemia, autoantibodies against Ro/La, and rheumatoid factor [112,340,355,386,400,423-426].

11.2. Materials & methods

11.2.1 Patients. Paired samples of serum and saliva were obtained from forty-five pSS patients [363,427], of whom fifteen had NHL (SSL subgroup), eleven sicca-complaining individuals with no infiltrates in diagnostic MSG biopsy and negative autoantibody profile (sicca-controls; SC subgroup), ten healthy controls (HC subgroup) and six patients with non-SS associated NHL [NHL subgroup; mean age 59.5, range 34.84, two with diffuse large B-cell lymphoma (DLCBL) and one each with primary mediastinal large B-cell lymphoma, follicular lymphoma, Hodgkin lymphoma and Richter's transformation in chronic lymphocytic leukemia (CLL)]. Patients' characteristics are summarized in Table-1. In 22 of 45 pSS patients (seven with NHL) and all sicca-controls, paired MSG biopsy specimens were available, and the expression of CXCL13 was examined immunohistochemically. The pSS patients without evidence of NHL at the time of serum, saliva and MSG sampling (SS subgroup, n=30) included twenty-three low-risk (median follow-up time 3.7 years, range: 0.0-23.3 years) and seven high-risk (median follow-up time 1.7 years, range: 0.0-3.0 years) for future lymphoma development as defined previously (31, 32). This subset of pSS patients (SS subgroup) was further classified according to lesion severity as arbitrary defined [386] by focus (FS) and Tarpley (TS) biopsy scores (mild: FS:1-1.79, TS:1, intermediate: FS:1.8-3.5, TS:2 and severe: FS: 3.6-11, TS: 3-4) and included ten patients with mild, twelve with intermediate and eight with severe lesions at MSGs. The SSL subgroup consisted of twelve MALT lymphomas (two located at parotid glands and the rest at MSGs) and one each with DLCBL, follicular lymphoma and CLL. Sampling was performed at SSL diagnosis in one patient, and on 4.5 years (median; range: 0.5-18 years) after SSL diagnosis in the rest. Seven SSL patients had been treated with anti-CD20 therapy 4 years (median; range 2-8 years) before sampling, whereas the patient with DLBCL received Rituximab, Cyclophosphamide, Doxorubicin hydrochloride, Vincristine, Prednisolone (R-CHOP) 7 years prior. In the SS subgroup (pSS patients without evidence of NHL), two received steroids, one hydroxychloroquine, two pilocarpine, two hydroxychloroquine and pilocarpine, two steroids, pilocarpine and hydroxychloroquine, one azathioprine and two hydroxychloroquine and methotrexate. CXCL13 levels were also evaluated in sequential sera of six additional pSS patients obtained before NHL onset (pre-lymphoma; median time to lymphoma diagnosis 3.63 years, range 3.0-9.33 years) and on lymphoma onset (median age on pre-lymphoma serum sampling 56 years, range: 29-67) to study the CXCL13 kinetics towards lymphomagenesis.

Medical records were retrospectively evaluated for various clinical, laboratory and histological parameters of SS and lymphoma, including MSG biopsy scoring, ESSDAI, arthralgias, arthritis, Raynaud's phenomenon, SGE, palpable purpura, vasculitis, lung involvement, as attested by pulmonary-function tests and X-ray and/or computed-tomography scans, renal involvement (persistent proteinuria/glomerular hematuria and verification by renal biopsy), liver involvement (liver-biopsy indicative of primary biliary cirrhosis), peripheral neuropathy as attested by nerve-conduction studies, anti-Ro/SSA and/or anti-La/SSB autoantibodies, rheumatoid factor, hypocomplementemia (C4<16mg/dL and C3<75mg/dL), hypergammaglobulinemia (IgG gammaglobulins>2 g/L), anemia (hemoglobin < 12g/dL), leukopenia (white-blood-cell count<4000/mm^3), lymphopenia (lymphocyte count<1000/mm^3) and neutropenia (neutrophil count<1500/mm^3).

Features		Controls			SS patients	
		Healthy *(n=10)*	Sicca *(n=11)*	NHL *(n=6)*	SS *(n=30)*	SSL *(n=15)*
General	Age (years), median (range)	48 (38-72)	50 (43-78)	59.5 (34-84)	60 (27-79)	70 (53-78)
	Men/women	0/10	2/9	4/2	3/27	1/14
	Disease Duration (years), median (range)	NA	NA	0.5 (0.5-8.0)	10 (0.25-34.0)	17.0 (4-37.0)
Histological *(MSG biopsy)*	Biopsy focus score *(number of lymphocytic foci/4mm²)*, median (range)	NA	0 (0.0-0.5)	NA	2.40 (1.0-10.44)	3.33 (1.0-10.0)
	Tarpley biopsy score, median (range)	NA	0	NA	2 (1-3)	3 (1-3)
	Germinal center formation "No,(%)"	NA	0 (0)	NA	8 (26.7)	4 (26.7)
Clinical	Arthralgias "No,(%)"	NA	1 (9)	NR	21 (70.0)	13 (86.7)
	Arthritis "No,(%)"	NA	0 (0)	NR	4 (13.3)	3 (20.0)
	SG enlargement (SGE) "No,(%)"	NA	0 (0)	NA	11 (36.7)	9 (60.0)
	Raynaud's phenomenon "No,(%)"	NA	0 (0)	NR	7 (23.3)	7 (46.7)
	Parenchymal organ involvement "No,(%)"	NA	NA	NA	5 (16.7)	4 (26.7)
	Lung involvement "No,(%)"	NA	NA	NA	*2 (6.7)*	*4 (26.7)*
	Renal involvement "No,(%)"	NA	NA	NA	*0 (0)*	*0 (0)*
	Liver involvement "No,(%)"	NA	NA	NA	*3 (10.0)*	*0 (0)*
	Indicative of vasculitic involvement "No,(%)"	NA	NA	NA	1 (3.3)	6 (40.0)
	Palpable purpura "No,(%)"	NA	NA	NR	*1 (3.3)*	*4 (26.7)*
	Vasculitis (%)"No,(%)"	NA	NA	NR	*0 (0.0)*	*0 (0.0)*
	Glomerulonephritis "No(%)	NA	NA	NR	*0 (0)*	*0 (0)*
	Peripheral neuropathy "No,(%)"	NA	NA	NR	*1 (3.3)*	*2 (13.3)*
	ESSDAI score, median (range)	NA	NA	NA	3.5 (0-15)	19 (12-25)
Laboratory	Anti-Ro/SSA and/or La/SSB positive "No,(%)"	0 (0)	0 (0)	NA	25 (83.3)	13 (86.7)
	Anti-Ro/SSA positive "No,(%)"	*0 (0)*	0 (0)	NA	*25 (83.3)*	*13 (86.7)*
	Anti-La(SSB) positive "No,(%)"	*0 (0)*	0 (0)	NA	*13 (43.3)*	*8 (53.3)*
	Rheumatoid Factor positive "No,(%)"	0*(0)*	0 (0)	NA	15 (50.0)	13 (86.7)
	C3-levels, median (range)	NR	NR	NR	111.5 (53-160)	102.0 (86-123)
	C4-levels, median (range)	NR	NR	NR	20.5 (7.0-45.6)	14 (1.0-22.4)
	C4- hypocomplementemia "No,(%)"	NR	NR	NR	*7 (23.3)*	*10 (66.7)*
	Cryoglobulinemia "No,(%)"	NA	NA	NR	0 (0.0)	5 (33.3)
	Hypergammaglobulinemia "No(%)"	NA	NA	NR	12 (40.0)	5 (33.3)
	Leukopenia "No,(%)"	NA	NA	NR	1 (3.3)	1 (6.7)
Treatment	Steroids, "No(%)"	NA	NA	0	2 (6.7)	0
	Hydroxychloroquine, "No(%)"	NA	NA	0	1 (3.3)	0
	Pilocarpine, "No(%)"	NA	NA	0	2 (6.7)	0
	Hydroxychloroquine & pilocarpine, "No(%)"	NA	NA	0	2 (6.7)	0
	Steroids, pilocarpine & hydroxychloroquine, "No(%)"	NA	NA	0	2 (6.7)	0
	Azathioprine, "No(%)"	NA	NA	0	1 (3.3)	0
	Hydroxychloroquine & methotrexate, "No(%)"	NA	NA	0	2 (6.7)	0
NHL-related treatment administrated prior to sampling						
	Anti-CD20 *(median 4years before sampling; range 2-8)*, No(%)	NA	NA	0	0	7 (46.6)
	R-CHOP *(DLBCL-pSS patient 7years before sampling)*, No(%)	NA	NA	0	0	1 (6.7)

NA, not applicable.
NR, not recorded.
R-CHOP, Rituximab, Cyclophosphamide, Doxorubicin hydrochloride, Vincristine, Prednisolone.

Paired MSG, serum and saliva samples from all participants were collected and stored according to the standard operations procedures of the HARMONICSS European-funded multi-centric protocol (H2020-SC1-2016; Grant Agreement No.: 731944). All available paired specimens collected during the past four years in the Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens (NKUA), Greece were included in the study. Samples from all participants were collected after signed informed consent and the study was performed in the context of the HARMONICSS research protocol, which was approved by the Bioethics Committee of School of Medicine, NKUA, Greece on 20/07/2017.

11.2.2. Evaluation of serum and saliva CXCL13 levels.

CXCL13 levels at serum and saliva samples were measured by a commercially available ELISA (sensitivity: 1 pg/ml; Abcam) according to manufacturer's instructions.

11.2.3. CXCL13 expression and GC formation at the MSG lesions. The expression of CXCL13 and the organization of MSG lymphoid infiltrates into eGCs were evaluated by a standard immunohistochemical technique [428] using antibodies against specific markers in serial sections. The presence of ectopic lymphoid structures in MSG lesions was evaluated by both hematoxylin and eosin staining and immunostaining in serial sections with antibodies recognizing specific markers of T, B and follicular dendritic cells, namely CD3 (rabbit mAb, Cell-Marque, Rocklin, California, USA), CD20 [mouse monoclonal antibody (mAb) L26, Dako, Denmark)] and CD21 (rabbit mAb, EP3093, Abcam, Cambridge, UK), respectively, as well as other molecules that characterize GCs, such as Bcl6 (mouse mAb PG-B6p, Dako) and AICDA (rabbit mAb ERP23436-45, Abcam). In pSS patients without available MSG biopsy specimen at the time of serum and saliva sampling, eGCs were recorded according to their presence in the diagnostic MSG biopsy. A monoclonal CXCL13 expression was detected by immunostaining with a rabbit monoclonal antibody (mAb) antibody (ERP23400-92, Abcam). Briefly, the immunohistochemical procedure was as follows: after deparaffinization, MSG sections (4μm) were blocked for endogenous peroxidase activity by a 20-min incubation in 0.5% H_2O_2 and antigens were retrieved by microwaving in Tris/EDTA solution, pH:9.0, for 15-min. To block non-specific antibody binding, slides were incubated in TBS buffer supplemented with 10% normal non-immune fetal bovine serum for 15-min, followed by overnight incubation at 4^{o}C with primary antibodies and the application of the EnVision system (Dako) recognizing mouse and rabbit antibodies as second antibody and development system. Negative-controls used in each MSG tissue sample was staining with irrelevant isotype-matched antibodies and no addition of primary antibody, whereas staining of tonsil with all primary antibodies was routinely used as positive control in each experiment. CXCL13+ cells in MSG tissues were blindly counted field-by-field in each section (consisted of at least four MSG-lobules) by two independent observers (EKK, LC) and expressed as number of cells per mm^2 of tissue.

11.2.4. Statistical Analyses. Differences in CXCL13 serum or saliva levels among the various subgroups of pSS patients or pSS patients, sicca-controls, healthy individuals and non-SS NHL controls were analyzed by the non-parametric Kruskal-Wallis test and subsequent post-hoc Dunn's multiple comparisons test to identify differences between specific pairs of groups. Significant differences in the CXCL13 serum or saliva levels between patients expressing or not various clinical, histological and serological markers were analyzed by the non-parametric Mann-Whitney test, and potential associations with continuous variables by Spearman's rank correlation test. The over-time change of CXCL13 serum levels in sequential pre-lymphoma and lymphoma (on diagnosis) sera samples was analyzed by Wilcoxon's matched pairs test. To evaluate disease features, including serum or saliva CXCL13 levels, associated with NHL development or high risk to develop NHL univariate analysis was performed. Categorical variables were compared by the Pearson chi-square or the Fisher exact test, when appropriate. To identify independent factors associated with NHL in SS, all variables associated with it with a p-value less than 0.1 in univariate analysis were further evaluated by multivariate binary logistic regression analysis with backward stepwise elimination. GraphPad Prism-5 (GraphPad Software, San Diego, CA, USA), Python 3.6 and SPSS-17 (Computing Resource Centre, Santa Monica, CA, USA) software were used. Statistical significance was defined as a p-value of less than 0.05 for all comparisons; p-values were 2-tailed. Only the statistically significant differences are reported.

11.3. Results

11.3.1. CXCL13 expression in MSG inflammatory lesions is associated with CD21+-FDC network.

Except one patient with intermediate infiltrates, who was at high risk for NHL development, CXCL13-positive cells were detected in areas of CD21+-FDCs networks within MSGs (Figure-1A). CXCL13-positive cells were observed within MSG inflammatory lesions in nine of fifteen pSS patients without evidence of NHL and in two of seven SSL patients who were immunohistochemically examined. CXCL13 staining was not observed in any of the pSS patients with mild infiltrates (n=5), while all sicca-controls were also negative for CXCL13 staining. The number of infiltrating CXCL13-positive cells per tissue area (mm^2) was significantly different between pSS patients and sicca-controls (median, range: 0.25, 0.00-11.14 and 0.00, 0.00-0.00, respectively; p=0.0064). Among distinct pSS subgroups, the number of CXCL13-positive cells per tissue area was significantly higher in pSS patients with severe MSG infiltrates (median, range: 5.59, 0.46-11.14) compared to those with mild lesions (0.0, 0.0-0.0, p=0.0025) or SSL (0.00, 0.00-3.63, p=0.021) (Figure 1B,C), as well as in pSS patients at high risk to develop lymphoma compared to those at low risk (4.18, 0.28-8.54 and 0.00, 0.00-11.14, respectively; p=0.016).

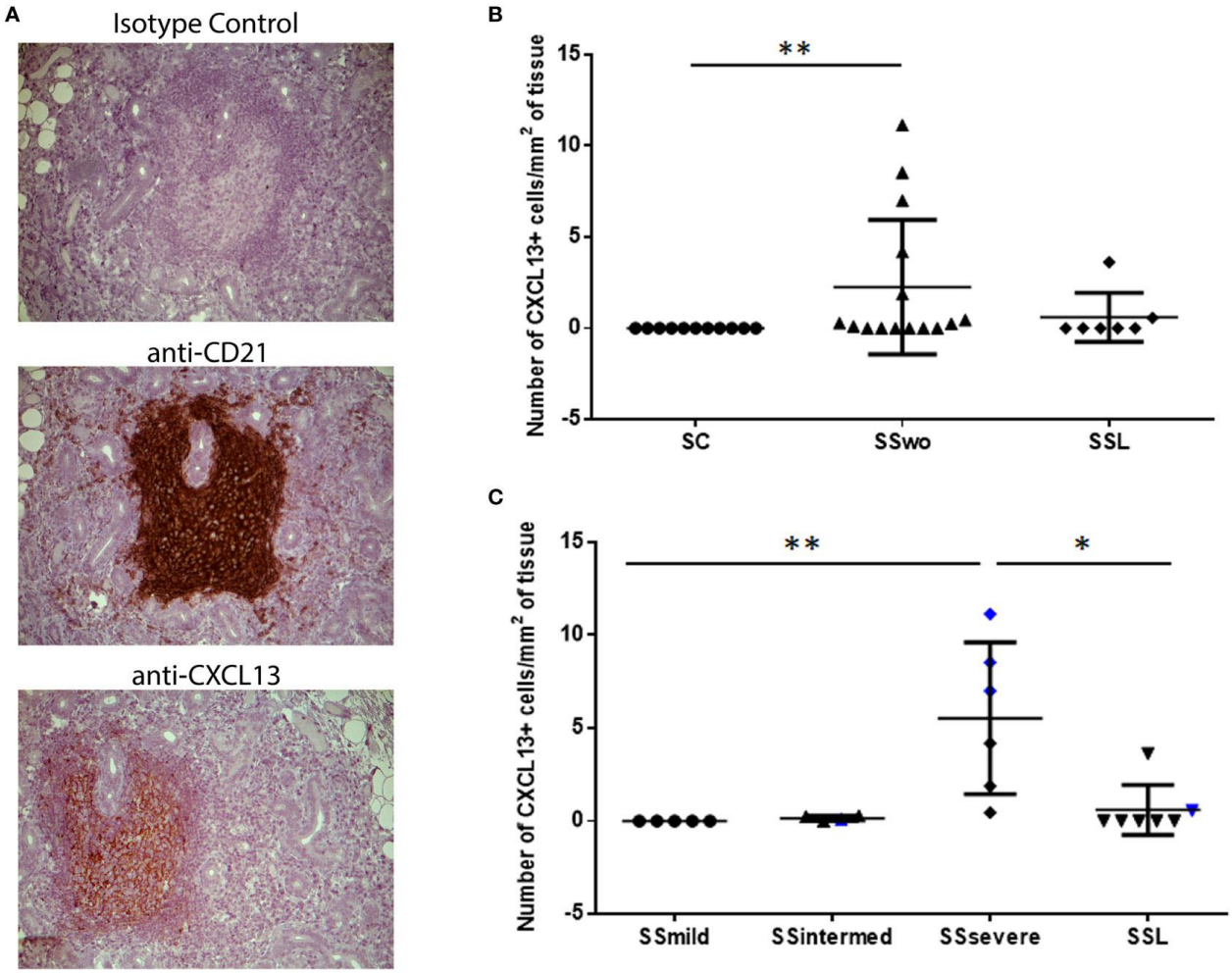


*FIGURE 1 Levels of CXCL13 expression in the MSG tissues of pSS patients and sicca-complaining controls. (A) CXCL13+-cells are detected in areas of CD21+-FDC networks in the MSG tissues of pSS patients. Representative pictures of immunohistochemical staining with isotype antibody (negative control), anti-CD21 (CD21) and anti-CXCL13 (CXCL13) antibody in MSG sections from a pSS patient with severe infiltrates and germinal center formation are shown. Original magnification: x20. (B) Dot plot displaying the number of CXCL13+-cells per tissue area (mm²) in the MSG tissues of sicca-complaining controls (SC), pSS patients without evidence of NHL (SS) and pSS patients with NHL (SSL). (C) Dot plot displaying the number of CXCL13+-cells per tissue area (mm²) in the MSG tissues of the various subgroups of pSS patients without evidence of NHL, as classified according to lesion severity to those with mild (SSmild), intermediate (SSintermediate) and severe (SSsevere) infiltrates, as well as pSS patients with NHL (SSL). Counts in MSG tissues with ectopic germinal centers (eGCs) are designated by blue color. Comparisons in (B, C) were performed by the non-parametric Kruskal-Wallis test. P-values are designated by asterisks (*p < 0.05, **p < 0.01), whereas horizontal bars represent the mean value of the group. Only statistically significant associations are indicated.*

11.3.2. Serum, but not saliva, CXCL13 levels are elevated in pSS patients compared to controls and associate with histologic features indicative of severe MSG inflammatory responses.

CXCL13 serum levels were significantly increased in pSS patients with or without NHL (median: 72.02 pg/ml and 87.00 pg/ml, respectively) compared to sicca-complaining controls (30.23 pg/ml; p=0.011 and p=0.0008 for pSS patients without or with NHL, respectively) and healthy individuals (17.56 pg/ml; p=0.012 and p=0.001, respectively) (Figure 2A). Although CXCL13 serum levels in patients with non-pSS associated NHLs (39.85 pg/ml) were lower than those with pSS patients (with or without NHL), they didn't reach statistical significance. On the other hand, CXCL13 saliva levels were increased in SSL patients (34.92 pg/ml) compared to non-SS NHLs (6.92 pg/ml, p=0.0065), but were not significantly different from those in other study groups (18.57, 18.70 and 14.19 pg/ml in pSS, sicca-complaining controls and healthy individuals, respectively) (Figure 2B).

CXCL13 serum levels were positively associated with saliva ones (r=0.368, p=0.014) (Figure 2C), and were correlated with the number of infiltrating CXCL13-positive cells per tissue area in the MSG lesions (r=0.534, p=0.011) (Figure 2D), a correlation that was further strengthened after excluding from the analysis pSS patients with NHL (r=0.797, p=0.0007). Again, CXCL13 serum, but not saliva, levels were found to correlate with MSG biopsy focus score (number of lymphocytic foci per 4 mm^2 of tissue) (r=0.644, p< 0.0001) (Figure 2E). Both serum and saliva CXCL13 levels were significantly higher in pSS patients with eGCs in autoimmune MSG lesions (median 121.6 and 48.76 pg/ml, respectively) compared to those without (61.33 pg/ml, p=0.003 and 25.52 pg/ml, p=0.013, respectively). Among pSS patients with distinct MSG lesion severity, those with severe infiltrates were found to express significantly higher CXCL13 serum levels compared to those with mild lesions (105.7 pg/ml *vs* 34.63 pg/ml, respectively, p=0.012), whereas saliva levels did not differ significantly among the three pSS subgroups.

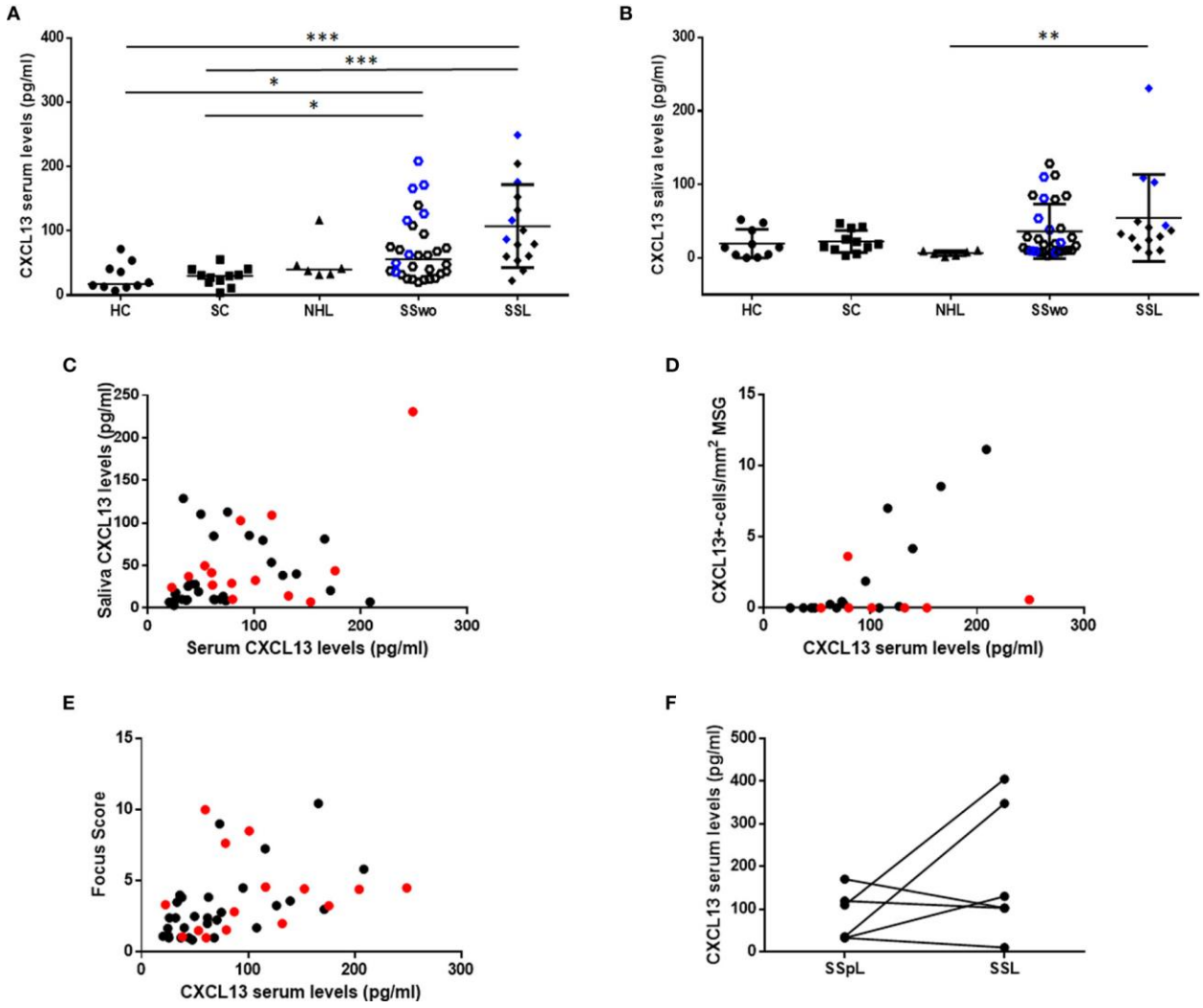


FIGURE 2 CXCL13 serum and saliva levels. (A) Dot plot displaying Kruskal-Wallis analysis of CXCL13 serum levels in healthy individuals (HC), sicca-complaining controls (SC), non-SS NHLs (NHL), pSS patients without evidence of NHL (SS) and pSS patients with NHL (SSL). (B) Dot plot representing Kruskal-Wallis analysis of CXCL13 saliva levels in healthy individuals (HC), sicca-complaining controls (SC), non-SS NHLs (NHL), pSS patients without evidence of NHL (SS) and pSS patients with NHL (SSL). P-values are designated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001), whereas horizontal bars represent the mean value of the group. Only statistically significant associations are indicated. CXCL13 serum and saliva levels from patients with ectopic germinal centers (eGCs) in the MSG infiltrates in panels (A, B) are highlighted by blue color. (C–E) Spearman's rank correlation analysis of associations between: (C) serum and saliva CXCL13 levels (r=0.368, p=0.014), (D) serum CXCL13 levels and number of CXCL13+-cells per tissue area (mm^2) in MSG tissues (r=0.534, p=0.011), (E) serum CXCL13 levels and biopsy focus score (r=0.644, p< 0.0001) in pSS patients. Red color designates samples obtained from pSS patients with NHLs. (F) Wilcoxon's matched-pair analyses of CXCL13 levels in sequential serum samples

from 6 pre-lymphoma pSS patients (SSpL) that transitioned to NHL (SSL) did not reveal any significant changes in CXCL13 expression levels before and on NHL diagnosis.

11.3.3. CXCL13 levels correlate with clinical and laboratory parameters indicative of adverse outcome and/or NHL.

CXCL13 serum levels were significantly increased in pSS patients with rheumatoid factor (105.0 pg/ml *vs* 53.72 pg/ml in patients with rheumatoid factor *vs* those without, respectively, p=0.0009), hypocomplementemia (74.96 pg/ml *vs* 40.26 pg/ml, respectively, p=0.002), high disease activity defined as ESSDAI score≥5 (83.29 pg/ml *vs* 44.57 pg/ml, respectively, p=0.024) and NHL (87.00 pg/ml *vs* 55.87 pg/ml respectively, p=0.036), and marginally higher in pSS patients with hypergammaglobulinemia (121.8 pg/ml *vs* 65.05 pg/ml, respectively, p=0.073). Both CXCL13 serum and saliva levels were significantly increased in high risk pSS patients for NHL development compared to those in low risk (median serum concentration: 95.31 pg/ml *vs* 44.57 pg/ml, p: 0.025; median saliva concentration 53.56 pg/ml *vs* 10.31 pg/ml in patients at high and low risk, respectively, p= 0.019), whereas CXCL13 saliva levels were marginally higher in patients with high disease activity (37.23 pg/ml *vs* 16.55 pg/ml in patients with ESSDAI score≥5 *vs* those with ESSDAI<5, p=0.057). Lastly, CXCL13 serum levels were inversely correlated with disease duration (r=-0.2977, p=0.05) (Figure 3).

Since CXCL13 serum levels were found to correlate with various clinical parameters previously associated with the NHL prediction in pSS, we subsequently investigated whether CXCL13 levels in serum and/or saliva associate with NHL in pSS. Univariate analysis revealed that pSS-related NHL in our cohort significantly correlates with higher CXCL13 serum levels (p=0.036), age (p=0.007), disease duration (p=0.071), rheumatoid factor (p=0.04), hypocomplementemia (p=0.012), cryoglobulinemia (p=0.012), purpura (p=0.036), and high disease activity score (ESSDAI ≥5; p=0.052). Age (p=0.003) and hypocomplementemia (p=0.005) remained as independent parameters associated with NHL in multivariate analysis.

Finally, in an attempt to evaluate whether CXCL13 serum levels change upon transition to lymphoma, we estimated its levels in sequential sera of six pSS patients before and at NHL onset. Although the CXCL13 serum levels were not found to statistically change upon transition to lymphoma, we noticed an increase in half patients (Figure 2F).

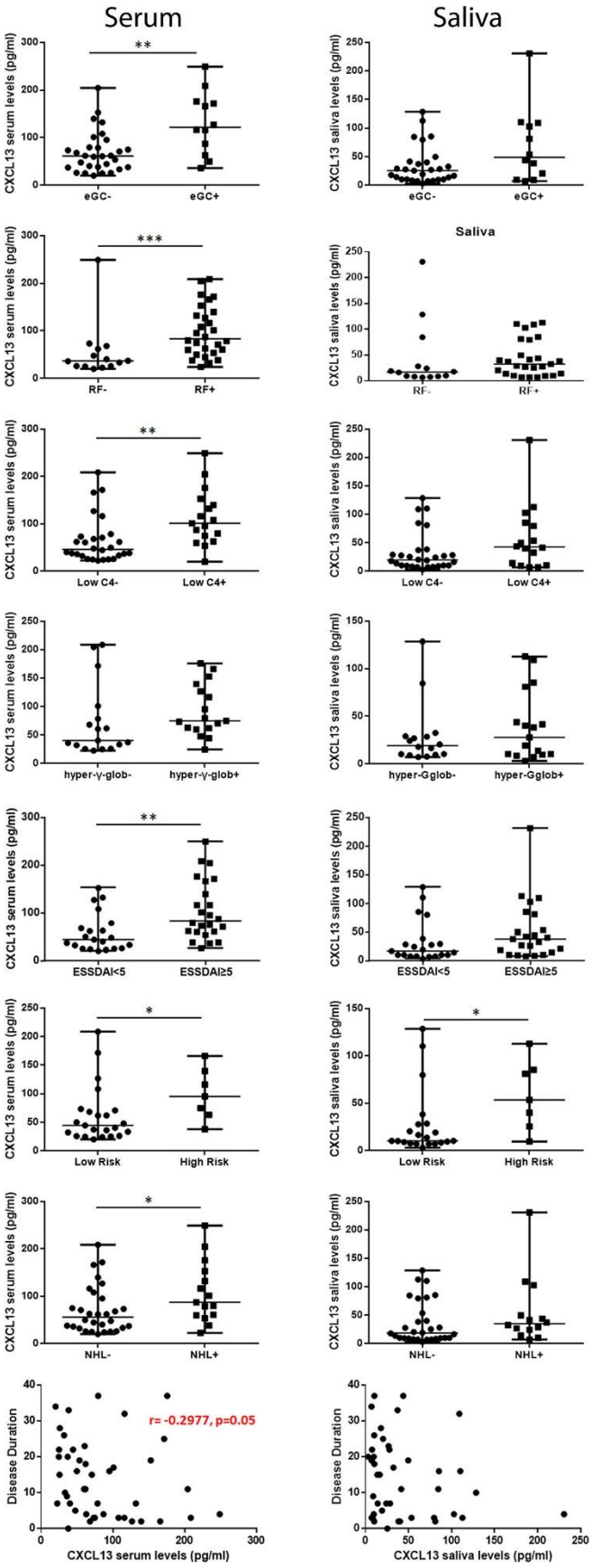


FIGURE 3 Association of CXCL13 serum and saliva levels with histologic, laboratory and clinical features. Mann-Whitney non-parametric analysis revealed that CXCL13 serum levels were significantly increased in pSS patients with ectopic germinal centers (eGCs) in the MSG infiltrates, presence of rheumatoid factor (RF), C4-hypocomplementemia (Low C4), hypergammaglobulinemia (hyper-γ-glob), high ESSDAI score (ESSDAI≥5), high risk to develop NHL (high risk) and NHL, whereas they were inversely correlated with disease duration. CXCL13 saliva levels were significantly increased in patients at high risk to develop NHL. P-values are designated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001), whereas horizontal bars represent the median value of the group.

11.4. Discussion

Our findings confirm previously published data indicating that CXCL13 serum levels in pSS patients are elevated compared to sicca-complaining controls or healthy individuals and associate with the severity of MSGs infiltrates, their organization in eGCs, increased risk for NHL development and NHL itself [417,418,420]. This is in agreement with the long-known central role of CXCL13 in the recruitment of B cells in secondary lymphoid tissues, the trafficking of B and T follicular cells in GCs and their compartmentalization, and therefore, in B cell responses, antibody production, and lymphomagenesis [399,401-403,429,430]. Increased number of infiltrating CXCL13-positive cells was found in severe MSG lesions of pSS patients and in high risk pSS patients for lymphoma development. This strongly implicates CXCL13 in lymphomagenesis associated with the disease, which has been long suspected (7, 10). Serum CXCL13 has been identified as a biomarker of GC activity and production of antibodies to vaccines [431,432], as well as of systemic immune activation and disease activity in infection and autoimmune diseases [406,407,410,430,433]. In this context, the association of CXCL13 serum levels in pSS patients with markers of B cell activation, such as rheumatoid factor, hypergammaglobulinemia, hypocomplementemia, and high disease activity (ESSDAI score) was anticipated. Elevated CXCL13 levels have been linked to prediction, presence, prognosis and/or therapeutic response of NHLs [434-438]. Although our findings along with previous studies [418,420], reveal significantly increased CXCL13 serum levels in pSS patients with NHL compared to those without, or in high risk patients for NHL development compared to low risk, all studies failed to identify CXCL13 serum levels as an independent lymphoma predictor. In the current study, we also evaluated CXCL13 serum levels in six patients with NHLs. Even though, CXCL13 serum levels in NHLs were lower than in SSLs, it did not reach statistical significance, possibly due to the small sample size. Furthermore, although these results need to be confirmed in larger cohorts, CXCL13 serum levels before lymphoma onset did not change significantly upon transition to lymphoma, suggesting that this chemokine may be upregulated before NHL clinical onset and therefore, implicated in earlier stages of lymphomagenesis.

One unanswered question is the origin of the elevated CXCL13 serum levels in pSS. FDCs and macrophages in liver, spleen and lymph nodes are considered as the major producers of CXCL13 (9, 10). However, the correlation of CXCL13 serum levels with the number of CXCL13-positive cells within the MSG inflammatory lesions found in this study and with several histologic parameters, including the degree of MSG inflammation and the presence of eGCs, shown in this and previous studies [416,417,420], suggest that elevated CXCL13 serum levels in pSS patients arise due to elevated production in salivary glands. This is in agreement with previous findings linking local and systemic autoimmune responses in pSS [378,386,439], as well as results in other autoimmune diseases supporting that CXCL13

serum levels reflect local inflammation in the affected organs [410,440]. On the other hand, we have previously observed that the incidence of infiltrating FDCs in MSG lesions of pSS patients decreases with lesions severity, suggesting that they may migrate in the lymph nodes to orchestrate systemic autoimmune responses [386]. In this context, they may be also implicated in increased CXCL13 serum levels in pSS patients, by driving its production upon migration to the secondary lymphoid organs.

Intriguingly, CXCL13 saliva levels were not proved to strongly associate with disease characteristics although they were elevated in patients with eGCs in MSG autoimmune lesions and in high risk pSS patients for NHL development. This is in agreement with a previous study reporting that CXCL13 serum, but not saliva, levels may have diagnostic utility in an Asian-Indian patient cohort [422]. Although CXCL13 saliva levels correlate with serum levels and eGC formation in MSG autoimmune lesions, there is no association with the number of infiltrating CXCL13-positive cells or the extend of inflammatory lesions in MSGs of pSS patients. Thus, it paradoxically seems that CXCL13 saliva levels do not reflect its local production in MSG tissues, possibly due to rapid degradation by saliva proteases.

The major advantage of this study is the parallel evaluation of CXCL13 in paired serum, saliva and MSG tissues, allowing the cross-examination of associations with disease aspects. Although the size of study cohort is rather small, not permitting elaborated analyses, we found that CXCL13 serum, but not saliva, levels are associated with disease characteristics indicative of systemic active disease and lymphoma, supporting its role in disease pathogenesis. However, as previously reported [418,420], we were unable to identify CXCL13 as an independent parameter associated with lymphoma development, a fact that hampers its use as a biomarker.

Chapter 12: Clinical picture, outcome and predictive factors of lymphoma in primary Sjögren's syndrome: results from a harmonized dataset (1981-2021).

12.1 Introduction

Primary Sjögren Syndrome (pSS) is a systemic autoimmune disease of unknown etiology[441]. It is characterized by lymphocytic infiltration of various epithelial structures, mainly affecting the exocrine salivary and lacrimal glands leading to oral and eye dryness respectively. The clinical spectrum of the disease is broad involving tissues far exceeding the glandular and peri-epithelial level. B cell related immune complex mediated vasculitis, often in the form of cryoglobulinemic vasculitis, is also evident in the skin, the kidneys and the peripheral nerves, leading to serious multiorgan manifestations[387]. The central pathogenetic role of B cells is also supported by the polyclonal hypergammaglobulinemia and the presence of a plethora of serum autoantibodies[442]. The abundant lymphocytic peri-epithelial lesions observed in the salivary glands or other affected organs, indicates a continuous interplay of B and T cells, resulting in B-cell hyperactivity and potentially to their uncontrolled proliferation. This sustained antigen driven autoimmune B-cell propagation may escape the control of proper regulatory mechanisms, occasioning both indolent and aggressive B-cell lymphomas which are observed in approximately 5% of pSS patients, impacting significantly on their morbidity and mortality [225].

The association of pSS with different lymphoma subtypes, including low grade, extranodal marginal zone lymphoma (MZL) of mucosa associated lymphoid tissue (MALT) and high grade, diffuse large B-cell lymphoma (DLBCL) was first reported in 1963 and is a well-recognized complication [443]. Other histologic types of B cell and even T cell lymphomas associated with pSS have been described but they are rare and do not occupy a significant place in the lymphomagenesis landscape of pSS [444].

In the present study, we sought to define all the lymphoproliferative disorders associated with the disease in the largest one-center cohort of well characterized pSS patients. In this, the dataset of patients was extensively harmonized in an attempt to define in detail the treatment strategies selected during the observation period, the response, progression, or relapse of the lymphomas, as well as the survival and prognosis of the pSS-lymphoma patients who have a long follow up. Finally, we present a data driven prediction model defining simple clinical features at the time of pSS diagnosis that are associated with an increased risk for lymphoma development.

12.2 Patients and methods

12.2.1 Study cohort

The medical records of eight hundred seventy-eight patients with pSS who were followed up in our department (Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens) from September 1981 to July 2021 were reviewed for the purpose of this study. All patients fulfilled the 2016 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria 7. Patients had been followed at regular intervals of 6-12 months or more frequently if their condition required it. One hundred and twenty-one patients with an added diagnosis of Non-Hodgkin Lymphoma (NHL) as per the 2016 World Health Organization (WHO) revised classification criteria for lymphoma were identified from the cohort and enrolled in the study 8. All lymphoma diagnoses were made either concomitantly with (40 patients) or followed the diagnosis of pSS and were histologically confirmed. One patient whose lymphoma diagnosis preceded the pSS diagnosis by 5 years was excluded.

12.2.2. Data Collection, harmonization, and definition of Outcomes

The clinical profile of pSS representing cumulative data preceding the diagnosis of lymphoma was recorded. Glandular elements, nonspecific elements , extra-glandular manifestations as well as the serologic profile of pSS patients is reported. The recording of subjective symptoms and systemic organ involvement was based on EULAR SS Patient Reported Index (ESSPRI) and EULAR SS Disease Activity Index (ESSDAI) definitions, respectively [107,445]. Minor salivary gland biopsies, laboratory and objective eye and oral tests were performed usually at the time of diagnosis, in the context of standard of care, according to physicians' judgment. All minor salivary gland biopsies were evaluated blindly by specialized pathologists quantifying the degree of periepithelial inflammation based on a widely used grading score, the Focus Score (FS)[21]. When the pathologist suspected a lymphoproliferative disorder on the hematoxylin-eosin staining of the lip biopsy specimen, a more extensive immunochemistry and molecular analysis was performed in search of monoclonality, eliminating the possibility of lymphoma. The date of pSS onset is defined as the approximate year the patient recalled pSS related

manifestations. The clinical spectrum of pSS associated lymphoproliferative disorders was also recorded in detail. Upon lymphoma diagnosis, parameters related to staging of lymphoma, localization of disease, performance status, hematologic parameters and demographic data were collected and analyzed[381,446]. All clinical data were collected and stored in a manually harmonized manner, using a common reference model which was developed as part of the HarmonicSS project (Grant agreement: 731944) and was adjusted accordingly to host in detail the lymphoproliferative features. Therefore, a retrospectively homogenous harmonized dataset was created for the whole project.

Previous pharmacologic treatments related to pSS were recorded. ESSDAI was also calculated, primarily retrospectively, at pSS and again at lymphoma diagnosis as a means to illustrate and compare the activity of the autoimmune disease from both ends of the clinical spectrum. A comparison of the 2 ESSDAI scores was performed after eliminating the effect of the lymphadenopathy and lymphoma domain and after excluding patients with simultaneous pSS and lymphoma diagnosis. ESSDAI calculations were based on a detailed analysis of patients records by a rheumatologist experienced in pSS.

Complete remission (CR), partial remission (PR), stable disease (SD), progression of disease (PD) and relapse of lymphoma were defined based on the International Working Group consensus response evaluation criteria in lymphoma (RECIL 2017)[447]. Several lymphomas progressed and required therapy despite an initial "watch and wait" approach. The development of a second lymphoproliferative disorder with distinct morphologic appearance and molecular and cellular genetics was also recorded in rare cases. According to the National Cancer Institute (NCI) criteria, overall survival (OS) was calculated from the lymphoma diagnosis date to the time of patient's demise or until the last medical visit and event-free survival (EFS) was estimated as the duration from the time of lymphoma diagnosis up to the time of the first event or the last recorded medical follow-up. An event was defined as a disease progression, lymphoma relapse, treatment failure (PR, SD or PD requiring treatment), histologic transformation, starting treatment after a watch and wait approach, the development of a 2nd lymphoma or death from any cause. The study was approved by the local ethical committee of Laiko general hospital (20/02/2017-70/03/14006), after obtaining patients' or closest relatives (in cases of patient's demise or incapacity) informed consent and in compliance with the General Data Protection Regulations (GDPR).

12.2.3 Data driven lymphoma prediction model for MALT lymphomas

Another aim of the study was to apply innovative data driven analysis in an attempt to define specific predictors of MALT lymphoma based on readily available parameters at the time of Sjogren's diagnosis. Patients who developed lymphoma within a year from pSS diagnosis were excluded from the analysis. Only MALT lymphoma patients were identified and matched in an 1:2 ratio, with non-lymphoma pSS control patients according to age, gender and disease duration from pSS diagnosis to lymphoma development (lymphoma group) or last follow up (non-lymphoma pSS group). Clinical, laboratory, histologic data as well as the ESSDAI scores at the time of pSS diagnosis were recorded and compared between lymphoma and non-lymphoma patients. Independent lymphoma predictors were identified by a data driven Fast Correlation Based Feature (FCBF) selection /Logistic Regression (LR) algorithm as described previously[76].

12.2.4 Statistical analysis

Statistical analysis for categorical data was performed by $\chi 2$(chi square) test with Yates correction or Fisher exact when cell counts involved <5 patients/items while for numerical data using the t test or Mann-Whitney methods, after implementing the Shapiro-Wilk normality test. OS and EFS curves were constructed using the classic Kaplan-Meier method and potential statistical difference between the plots was calculated using the log-rank test. The FCBF selection algorithm was applied only on the MALT lymphoma database and their non lymphoma controls to identify potentially independent variables, present at pSS diagnosis, for constructing a logistic regression (LR) model with MALT lymphoma development as the outcome of interest. A description of the FCBF algorithm and the validation process used is provided in the supplementary. The implementation of the FCBF/based multivariable logistic regression approach and all statistical analysis was performed using Python version 3.6 and GraphPad Prism version 8.0.1.

12.3 Results

12.3.1 Patients' characteristics

The incidence of NHL among patients with pSS was 16,7% (121/878). Of the 121 pSS-lymphoma cases MALT lymphoma was the commonest histologic type (92/121, 76%) followed by DLBCL (11/121, 9%) and NMZL (8/121, 6.6%). The remaining 10 patients had various lymphomas of B [follicular (3 cases), Lymphoplasmacytic (3 cases), Small lymphocytic lymphoma (SLL)] and T cell origin (peripheral T cell lymphoma not otherwise specified, primary cutaneous T cell lymphoma, angioimmunoblastic T-cell lymphoma). The anticipated predominance of female patients in our cohort was rounded off by 8 male patients. The median time interval between the diagnosis of pSS and that of lymphoma was 4 years (range 0-30), while the onset of SS symptoms preceded its diagnosis by a median of a single year (range 0-21). The median age of lymphoma patients was 59 years (range: 29-82) while the median follow up was 10 years (range: 0-38) and the median disease duration was 14 years (range: 1-45). MALT lymphomas developed earlier in the course of pSS compared to DLBCL (4 years vs 8 years p=0.05) and in younger patients (57 years old vs 71 years old, p=0.01). The demographic characteristics of the total patient population and of each main histologic type are shown in Table 1.

Table 1. Sjögren syndrome manifestations and pharmacologic treatments before lymphoma diagnosis (n=121)

Glandular Manifestations	
Dry eyes	91,7% (111/121)
Dry mouth	95,0% (115/121)
Salivary Gland enlargement	66,1% (80/121)
Parotid only enlargement	92,5% (74/80)
Submandibular only enlargement	1,25% (1/80)
Parotid and submandibular enlargement	6,25% (5/80)
Permanent enlargement	77,5% (62/80)
Intermittent enlargement	22,5% (18/80)
Lacrimal Gland enlargement	5,7% (7/121)
Nonspecific manifestations	
Arthralgias	63,6% (77/121)
Arthritis	19,8% (24/121)
Raynaud	37,1% (45/121)
Chronic fatigue	74,3% (90/121)
Extraglandular manifestations	
Palpable purpura	34,7% (42/121)
Primary biliary cholangitis	0,8% (1/121)
Autoimmune hepatitis	0% (0/121)
Glomerulonephritis	4,9% (6/121)
Interstitial nephritis	3,3% (4/121)
Interstitial lung disease	8,2% (10/121)
Small airway disease	4,1% (5/121)
PNS involvement	9,9% (12/121)
CNS involvement	1,6% (2/121)

Autoimmune thyroiditis	33,0% (33/100)
Serology	
Anti-Ro/SSA	80,1% (97/121)
Anti-La/SSB	47,9% (58/121)
Rheumatoid Factor	75,8% (88/116)
Antinuclear antibodies	90% (109/121)
C4 hypocomplementemia	69,8% (81/116)
Cryoglobulinemia	38,7% (43/111)
Hypergammaglobulinemia	70,2% (85/121)
Hypogammaglobulinemia	3,3% (4/121)
Sjögren related pharmacologic treatments	
Pilocarpine	14,9% (19/121)
Corticosteroids	34,7% (42/121)
Hydroxychloroquine	49,5% (60/121)
DMARD (Methotrexate-Azathioprine-Cyclosporine-Mycophenolate mofetil-D penicillamine)	14% (18/121)
Cyclophosphamide	5,7% (7/121)
Rituximab	4,9% (6/121)
Other (Anakinra-Infliximab)	1,6% (2/121)
No pharmacologic treatment	33,8% (41/121)

12.3.2 pSS patients' features

Glandular manifestations of the pSS associated lymphoma patients included dry mouth (95%), dry eyes (91,7%) and SGE (66.1%). Parotids was the site most frequently enlarged and present in 74 patients, whereas isolated submandibular involvement was noticed in a single patient. Five patients had both parotid and submandibular enlargement during their disease course. Permanent SGE was found in the majority of patients with parotid swelling (77,5%), while episodic enlargement with intermediate complete remission was noticed in the remaining. The serologic profile consisted of anti-Ro/SSA (80,1%), anti La/SSB (47,9%), antinuclear antibodies (90%), the Rheumatoid Factor (75,8%), C4 hypocomplementemia (69,8%) in addition to the presence of cryoglobulinemia (38,7%), hypergammaglobulinemia (70,2%), hypogammaglobulinemia (3,3%). Extra-glandular manifestations included palpable purpura (34,7%), peripheral nervous involvement (9,9%), primary biliary cholangitis (0,8%), interstitial lung disease (8,2%), small airway disease (4,1%), interstitial kidney disease (3,3%,) and glomerulonephritis (4,9%). Seropositive autoimmune thyroiditis was concurrently present in 33% of lymphoma patients (Table 1).

The median ESSDAI score for the 121 lymphoma patients at the time of their pSS diagnosis was 9 (0-44) but had increased to 18 (2-50) by the time the lymphoma diagnosis was made. A description of

the number of patients with activity in each domain is presented in table 2. A comparison between the ESSDAIs at the two timepoints for MALT lymphoma patients after excluding both patients with concomitant pSS and lymphoma diagnoses and the effect of the domain lymphadenopathy and lymphoma, revealed that patients with lymphoma had a statistically significant more active disease. The glandular and the biological domains showed a statistically significant increase in their ESSDAI value at lymphoma diagnosis compared to pSS diagnosis earlier (Figure 1).

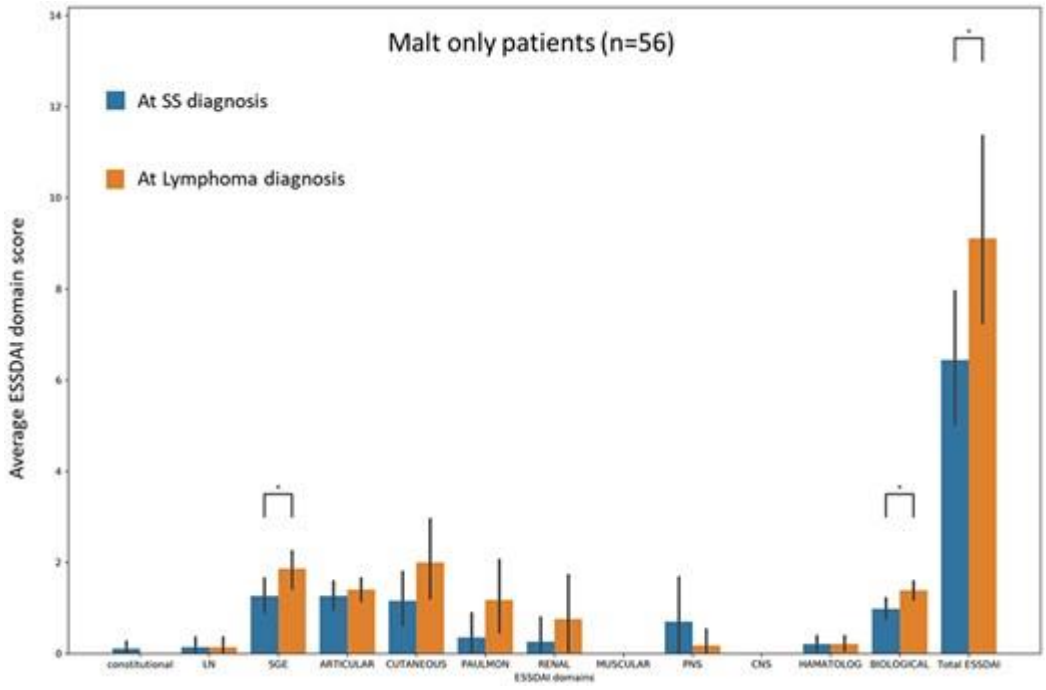


Figure 1. Change in the ESSDAI score from Sjogren to lymphoma diagnosis

12.3.3 Lymphoma patients' profile

MALT lymphomas

Of the 121 lymphoma patients, 92 (76%) developed MALT lymphoma. The majority involved the minor salivary glands (51/92), while 35 had biopsy proven parotid gland disease. Seven patients had histologic evidence of stomach involvement (3 Helicobacter pylori positive and 4 Helicobacter pylori negative) while 5 patients had lacrimal gland and lung disease. Thymus, eyelid-ocular adnexa, soft palate, and skin involvement was found in a single patient. Fourteen patients had biopsy proven lymphoma in at least 2 extra-lymphoid tissues/organs with no association with future event development (35.7% vs 38.4%, p=0.99). A detailed description is shown in table 4.

Nodal involvement after clinical and radiological assessment was found in 20.6% (19/92) of MALT lymphoma patients and was mainly regional (12 cervical, 2 cervical and axillary, 1 cervical, axillary, and supraclavicular, 1 supraclavicular, 2 axillary, 1 inguinal) while splenomegaly was observed only in 5 patients, all of them with bone marrow involvement. In total, lymphoma dissemination in the bone marrow was encountered in 20.6% (19/92). Staging according to the Ann Arbor classification revealed that the majority of MALT lymphoma patients was diagnosed having limited disease (stage I, II) whereas advanced stage IV disease was detected in less than 30%. No lymphoma was classified as stage III.

Anemia was the most common hematologic abnormality (59.7%, 55/92), followed by leukopenia (23.9%, 22/92) and thrombocytopenia (4.3% 4/92). Electrophoresis and immunofixation revealed evidence of a monoclonal component in 37 patients (40.6%, 37/91) with IgM kappa being the predominant monoclonal protein (78% 29/37) followed by IgG kappa (8.1% 3/37), IgA kappa (2.7% 3/37) and IgG lambda (5,2% 2/37).

A “watch and wait” approach was adopted in the subset of MALT lymphoma patients not fulfilling treatment initiation criteria, while patients requiring treatment received radiation, immunotherapy, chemotherapy, combination of a chemotherapy regimen and immunotherapy (Supplementary table 1). The watch and wait group with close follow up included 37 patients, while 25 patients were treated with single agent infusion of Rituximab, 19 received a Rituximab based immunochemotherapy and 5 patients received a chemotherapy-based drug treatment. Furthermore, two patients received treatment with radiation and 1 received a combination of radiation and Rituximab. Interestingly, a unilateral parotid surgery resection along with radiation was successfully exploited for the treatment of isolated parotid enlargement due to MALT lymphoma development in one patient.

DLBCL

The second most frequent type of NHL in our pSS cohort was DLBCL found in 9.0% (11/121). We were able to define the histologic subtype based on the WHO 2016 classification in 9 out of 11 DLBCLs, with 4 being germinal center B cell (GCB) subtype and 5 non-GCB subtype. Biopsy proven nodal involvement was confirmed in 90.9% (10/11) of DLBCLs. The most common sites were the neck (6) and the axillae (6) followed by the mediastinal (3), the abdomen (2) and the supraclavicular region (1). The patient without nodal involvement in imaging studies had malignant lymphoid infiltration confined to the lung. Similarly, extranodal disease was detected in 3 patients involving the major salivary gland, the lung, and the stomach. Bone marrow was infiltrated in 18% (2/11) while an enlarged spleen was found in 1

patient. Ann Arbor staging revealed 54% (6/11) having limited disease (stage I and II) while 46% (5/11) advanced disease (stage III and IV). Anemia at the time of lymphoma diagnosis was the most common hematologic disorder, as was the case with MALT lymphomas, found in 63% (7/11) followed by leukopenia in 27% (3/11). No DLBCL patients showed thrombocytopenia. IgM kappa monoclonality was evident in 4 patients (40% 4/10 – data available in 10 out of 11 patients). As expected with aggressive lymphomas, immediate pharmacologic treatment with a Rituximab-based immunochemotherapy regimen was offered in almost all patients diagnosed with a DLBCL (Supplementary table 1).

NMZL

Eight patients developed NMZL (6,8% 8/121). The axillary lymph nodes were the most common nodal site (n=4) followed by intraabdominal and inguinal nodes (n=3). As expected, no extranodal organs were involved. The spleen was found enlarged in 6 patients (75%, 6/8) and the bone marrow in 2 cases (25%, 2/8). The predominant Ann Arbor stage was III (75%, 6/8), while 2 patients with bone marrow involvement were stage IV. There were no patients with limited disease (stage I-II). Hematologic parameters at lymphoma diagnosis included anemia 50% (4/8), leukopenia 25% (2/8) and thrombocytopenia 12,5% (1/8). Two out of 7 patients with available data (28,5%) had a monoclonal band of the IgM kappa type in protein electrophoresis. Treatment modalities chosen were mainly cyclophosphamide centered (Supplementary table 1). One patient refused treatment.

	ALL PATIENTS (n=121)	**MALT Lymphoma** (n=92)	**DLBCL** (n=11)	**NMZL** (n=8)
Females/Males	113/8	85/7	11/0	7/1
Age at lymphoma diagnosis (median) (years)	58 (29-82)	57 (29-82)	71 (43-81)	54 (36-79)
Disease duration from SS onset to lymphoma diagnosis (median) (years)	8 (0-37)	7 (0-37)	14 (0-25)	13.5 (1-20)
Disease duration from SS diagnosis to lymphoma	4 (0-30)	4 (0-30)	8 (0-21)	6.5 (0-20)

diagnosis (median) (years)							
ECOG PS 1,0 % (no)	96.6% (117/121)	97.8 (90/92)		100 (11/11)		50% (6/8)	
B symptoms	6,8% (8/121)	3.2% (3/92)		9% (1/11)		12,5% (1/8)	
Nodal involvement	35,9% (42/121)	20.6% (19/92)		91% (10/11)		100% (8/8)	
Extranodal involvement	81,8% (99/121)	100% (92/92)		45,5% (5/11)		0% (0/8)	
Bone marrow involvement	23,9% (29/121)	20.6% (19/92)		27,3% (3/11)		25% (2/8)	
Bulky disease	0,8% (1/121)	0% (0/92)		18,2% (2/11)		0% 0	
Splenomegaly	11,5% (14/121)	5.4% (5/92)		18,2% (2/11)		75% (6/8)	
Ann Arbor stage							
I	56	53		2		0	
II	23	18		4		0	
III	10	0		2		6	
IV	31	21		3		2	
Anemia (Hb<12 g/dL)		59.7% (55/92)		63,6% (7/11)		50% (4/8)	
Leukopenia (WBC <4 x $10^3/\mu L$)		23.9% (22/92)		27,3% (3/11)		25% (2/8)	
Thrombocytopenia (PLTs <150 x $10^3/\mu L$)		4.3% (4/92)		0% (0/11)		12,5% (1/8)	
Monoclonality		40.6% (37/91)		40% (4/10)		28,6% (2/7)	
Prognostic Index Score		MALT-IPI		IPI		IPI	
		0	41.3% (36/87)	0		0	
		1	44.8% (39/87)	1	18,2% (2/11)	1	12,5% (1/8)
		2	9.1% (8/87)	2	45,5% (5/11)	2	12,5% (1/8)
		3	4.5% (4/857)	3	27,3% (3/11)	3	62,5% (5/8)

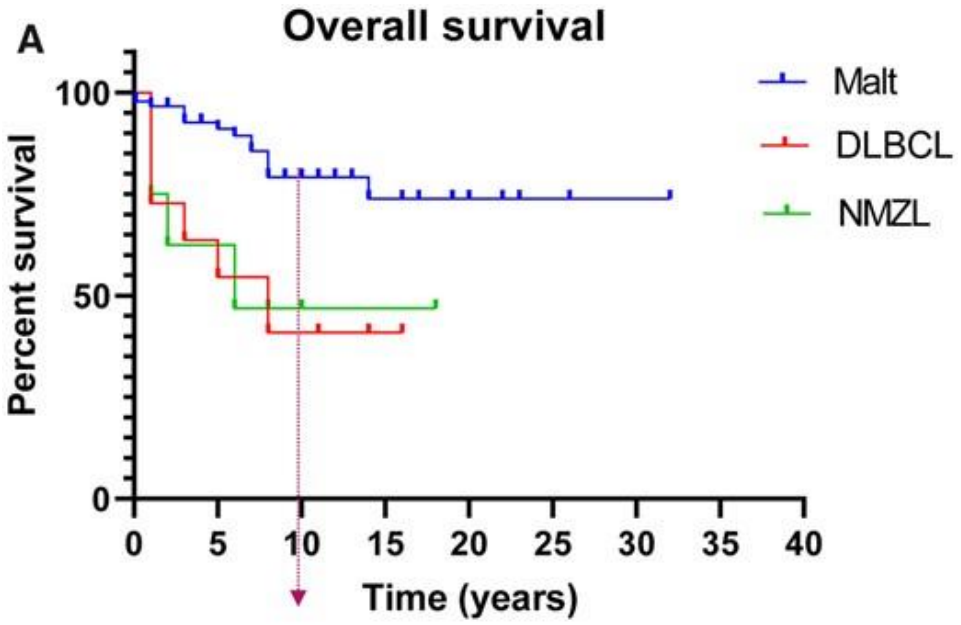
			4	9,1% (1/11)	4	12,5% (1/8)

Table 3. Lymphoma related characteristics of all patients

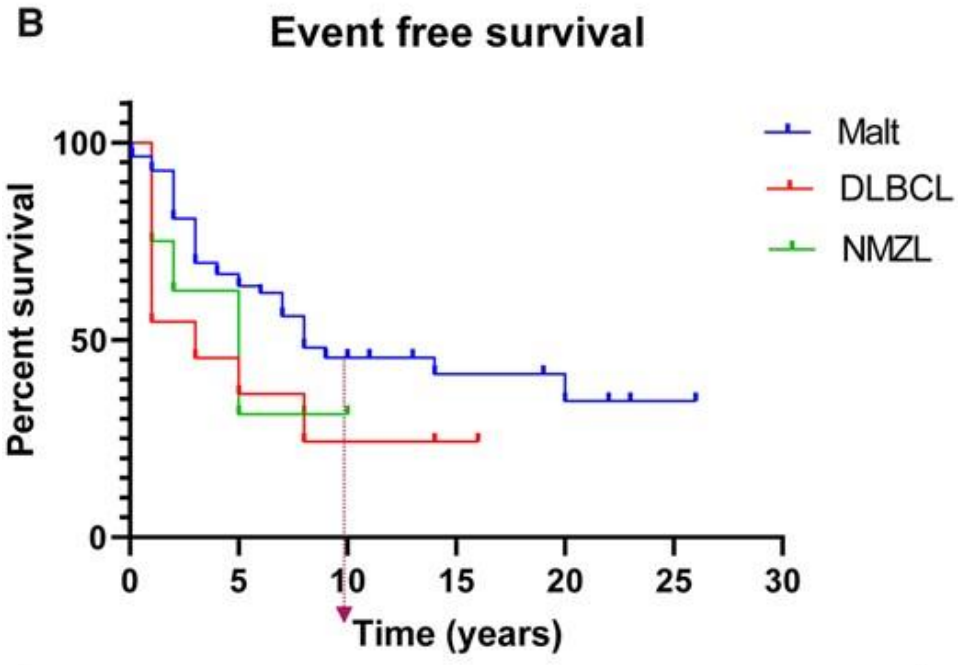
12.3.4 Outcomes and survival

In the total study population, the all-cause mortality was 22,3% (27/123). Fifty-four patients experienced an event as defined in the material and methods section. Six patients who initially received no treatment had disease progression necessitating drug therapy. Ten patients had partial or no response to the initial therapy, 17 patients had a relapse, 4 experienced a transformation to a more aggressive lymphoma, and 1 patient developed a second distinct lymphoma while in remission. Six patients died of their disease or of a treatment related complication during their initial evaluation and treatment, 6 during their salvage treatments and 13 patients died during their long follow up due to reasons unrelated to lymphoma diagnosis (6 cardiovascular, 2 solid tumor, 3 infection, 2 unknown). One patient died during his initial treatment for acute myeloid leukemia which was diagnosed during staging for a T cell lymphoma and one patient's cause of the death was unable to be determined. A detailed description of all the events in our cohort population, as well as the treatments provided are presented in Supplementary Table 2.

A Kaplan-Meier OS and EFS analysis showed MALT lymphomas have a 5-year OS of 91%, DLBCL 54.54% and NMZL 62.5% (Figure 2). The 10-year OS was estimated to be 79% for MALT lymphomas, 40,9% for DLBCL and 46% for NMZL. At 5 years the EFS of the MALT lymphoma population was 63.6%, 45.4% for the DLBCLs and 62.5% for the NMZL, while at 10 years, the EFS for MALT lymphoma patients drops to 45,5%, for DLBCL to 24,2% and for NMZL to 31%. The Mantel-Cox log-rank comparison of the OS curves revealed a statistically significant difference between the lymphoma subtypes (p=0.0016), a trend that was not maintained in the EFS curves (Figure 2).



10-year OS	Malt	DLBCL	NMZL
	79.140	40.909	46.875



10-year EFS	Malt	DLBCL	NMZL
	45.552	24.242	31.250

Figure 2. Overall and event-free Kaplan–Meier curves

12.3.5 MALT lymphoma prediction model

Fifty-seven patients with MALT lymphoma who were diagnosed at least one year after pSS diagnosis were identified and their features already present at at the time of the pSS diagnosis were compared with 114 non-lymphoma pSS controls. The former group were found to have a shorter interval from the pSS onset of symptoms to its diagnosis (2.65 vs 4.84 years, p=0.009), more frequent presence of SGE (49% vs 26%, p= 0.005), palpable purpura (23% vs 5%, p=0.0013), anti La/SSB antibodies (51% vs 34%, p= 0.0049), low serum C4 levels (63% vs 32%, p=0.0003), higher FS (median 3 vs 1,375), cryoglobulins (30% vs 17%, p<0.001) and a higher ESSDAI (6.51vs 2.66, p<0.001) compared to controls. By contrast, controls showed an increased prevalence of autoimmune thyroiditis (Table 5). The combined FCBF/LR analysis was applied on the unified dataset analyzing 37 distinct features including clinical serological and laboratory data. The 4 variables in terms of magnitude of order with the strongest association with lymphoma and the weakest association amongst them, as calculated by the FCBF algorithm, were: Cryoglobulinemia, total ESSDAI score, FS, and glomerulonephritis. Cryoglobulinemia, FS and the ESSDAI score were identified as the only independent lymphoma predictors with cryoglobulinemia displaying a very high odds ratio (Table 6). The performance of the FCBF/LR model was good with the following characteristics: Accuracy=0.75, Sensitivity=0.68, Specificity= 0.90 and an Average area under the curve (AUC)=0.84 (Supplementary Figure 1).

FEATURE	**Lymphoma(n=57)**	**Controls(n=114)**	**p-value**
BASIC CHARACTERISTICS			
Age of pSS Diagnosis	46,7 (years)	47.5(years)	0,66
Disease duration from pSS Diagnosis until lymphoma diagnosis or last follow up	8.15(years)	8.07(years)	0.8688
Disease duration from pSS onset to pSS diagnosis	2,65 (years)	4,84 (years)	**0,009**
Female gender	54	108	1
GLANDULAR MANIFESTATIONS			
Dry mouth	86%	84%	0.94
Dry eyes	85%	91%	0,43

Salivary gland enlargement	49%	26%	**0.005**
Lacrimal gland enlargement	3,5%	0	0.11
GENERAL MANIFESTATIONS			
Arthralgia	56%	67%	0.21
Arthritis	19%	21%	0.99
Raynaud's phenomenon	36%	31%	0.62
Palpable purpura	23%	5%	**0.0013**
ESSDAI at pSS diagnosis	6,51	2,66	**<0.001**
HISTOLOGY			
Focus score (median)	3	1,375	**<0.001**
SEROLOGY			
Rheumatoid Factor	77%	56%	0.02
Anti SSA/Ro antibodies	79%	81%	0.99
Anti SSB/La antibodies	51%	34%	**0.0049**
Low C4 serum levels	63%	32%	**0.0003**
ANA Positivity	5%	6%	1
Cryoglobulinemia	30%	17%	**<0.001**
ORGAN INVOLVEMENT			
Autoimmune thyroiditis	19%	48%	**0.004**
Peripheral nerve involvement	5%	0,8%	0.1
Lymphadenopathy	2%	5%	0.43
Autoimmune hepatitis	0%	0%	1
Primary biliary cholangitis	0%	0%	1
Small airway disease	0%	0%	1
Lung involvement	0.8%	4%	0.26

Interstitial renal disease	0%	0%	1
Glomerulonephritis	0.5%	0%	0.33

Table 4

Comparison of clinical, laboratory and histologic features present at pSS diagnosis between MALT lymphoma patients and non-lymphoma controls

Discussion

This is a single-center, retrospective study focusing on the clinical features of pSS associated NHLs and particularly MALT lymphomas. Although previous studies have attempted [205,270,292,293,355,391,448] to address similar clinical questions, our study design and cohort population presents some unique characteristics: first, our department is a national referral center for pSS and related lymphoproliferative disorders, with the opportunity to collect high quality data on both pSS-associated NHLs and pSS itself. Second, the long follow up and the large cohort size of pSS patients afforded us exposure into the broad clinical spectrum of pSS-associated NHLs including their initial presentation, evolution, management, and outcome. Third, the data accumulated throughout the years of follow up were harmonized in order to create a unified dataset, that can eventually be analyzed using data driven and artificial intelligence algorithms. Fourth, the ESSDAI of pSS MALT patients was calculated at the time of both the pSS and lymphoma diagnosis, after excluding the impact of lymphoma itself, to isolate the contribution made by pSS elements towards lymphomagenesis and fifth, a real prediction model exclusively for pSS MALT patients was developed, based on data driven algorithms, after carefully selecting pSS MALT patients whose diagnosis was made at least 1 year after pSS diagnosis, to parse overlapping clinical features between pSS itself and pSS MALT lymphomas. To this end, the major points of our study can be summarized as follows: a) a detailed description of all clinical aspects in both pSS itself and pSS-lymphoma patients in the largest cohort of pSS related lymphomas is provided, b) the role of pSS systemic disease activity in lymphomagenesis as reflected by the change in ESSDAI score between pSS and lymphoma diagnosis was addressed, c) the evolution of pSS elements towards lymphoma among pSS-MALT lymphoma patients was recorded, d) the 10 year survival and event free for all lymphoma subtypes were estimated and presented and e) independent risk factors for pSS-MALT lymphomas were identified.

In the current study, the high overall lymphoma incidence (16.8%) is intriguing but easily interpretable by the fact that our department serves as a referral center and the pSS cohort has a long

follow up time that given the disease's fairly good survival prospect is afforded a considerable length of time in which a lymphoma might arise. Most of pSS-lymphomas were of MALT type, with minor salivary or parotid gland localization, limited disease, and unexpectedly high rates of bone marrow and nodal (regional lymph nodes) involvement. The second most common type of DLBCLs were mainly confined to cervical and axillary lymph nodes and clinically expressed by extra-nodal involvement of major salivary glands, lung, and stomach with either limited or advanced stage. Such findings have been also reported by previous studies, although based on relatively small number of recruited patients[205,293,449]. The 10-year Kaplan-Meier for OS and EFS rates are a novelty of our study. The 5-year survival rates of pSS-MALT patients were similar to our previous work [355]but dropped by 12% at 10-year down to 80%, with the 10-year event free rate sitting at 45%. Taken together, the worse than expected event rate of pSS-MALT patients at long follow up would indicate the need for a closer observation to optimize the control of complications as they arise. By contrast, pSS-DLBCL patients exhibit a poor prognosis with reduced survival rates at both 5 and 10 years compared to the pSS-MALT lymphoma subgroup. Interestingly, death and clinically significant events in pSS-DLBC patients are anticipated to accumulate within the first 3 years after lymphoma diagnosis, since the 10-year survival curve shows a plateau after that time point. Finally, a real prediction model for pSS-MALT lymphoma is presented for the first time, using data driven approaches. ESSDAI at pSS diagnosis, FS and cryoglobulinemia were identified as independent predictors for MALTLs in pSS, confirming previous studies investigating lymphoma risk factors [151,292,367,391,394]. However, in those studies, the analysis of the pSS lymphoma group was applied to all histologic types as a block. Furthermore, the value of the ESSDAI at pSS diagnosis as an independent predictor was indicated in only one study [391].

With reference to the clinical features of pSS, pSS-lymphoma patients are marked by SGE, B cell mediated manifestations and have high prevalence of autoantibodies and markers of systemic activation. This particular phenotype has been also described by other groups[391,450-452]; In this line, pSS-lymphoma patients have remarkably higher ESSDAI score at pSS diagnosis compared to non-lymphoma pSS controls, indicating that high disease activity and severity of pSS, is most likely linked to the underlying lymphomagenesis process. Similarly, pSS-MALT lymphoma patients seem to display also high ESSDAI scores at pSS diagnosis. Importantly after calculating the ESSDAI change of these patients from pSS to lymphoma diagnosis by retaining the ESSDAI lymphoma domain similar to pSS diagnosis in order to eliminate the effect of lymphoma, it was found that glandular and biologic domains reflect the systemic burden carrying the transition to MALT lymphomas. This finding is described for the first time in the literature, although Briton et al have reported also increased risk for MALT lymphoma and overall hematologic malignancies in pSS patients with high ESSDAI score at pSS diagnosis[449]. It is noteworthy though that pSS-MALT lymphomas patients show much lower frequency of autoimmune

thyroiditis than controls, most likely due to insufficiency of the immune-regulatory mechanisms to restrict the disease in the epithelial structures, preventing systemic expansion. Given that MALT lymphomas represent the main histologic type of pSS-associated lymphomas, and that glandular and biologic domain are the major ESSDAI "carriers" towards lymphomagenesis, it would suggest that the microenvironment of the affected salivary glands and the B cell component respectively act as the main drivers of MALT lymphomagenesis in pSS.

Our study has enriched the clinical landscape of pSS-NHL, which constitutes a step to our understanding of the biology and pathogenesis of pSS-related lymphomagenesis. Although pSS hosts many lymphoma types, MALT lymphomas predominate, implying that the chronic antigenic stimulation and the B cell expansion are major and common contributors to the lymphomagenesis associated with pSS.[453]. However, given the multistep process of lymphomagenesis, there are still unknown cellular and molecular events that take place at the tissue level and define the clinical expression of lymphomagenesis, e.g., regional lymph nodes (DLBCLs, nodal-NLs) or salivary glands (MALT lymphomas). It would therefore be a reasonable suggestion that pSS is an excellent model of human lymphomagenesis for all lymphoma types (high- and low-grade NHL of B cell origin, T cell), considering the origin of the tissue structures and the dynamic state of B and T cells in their infiltration of the salivary glands[454].

In conclusion, pSS-associated lymphoma patients are characterized by high pSS systemic disease activity and B cell mediated manifestations. ESSDAI, cryoglobulinemia and high FS are considered MALT lymphoma predictors when present already at pSS diagnosis, while the biologic and glandular ESSDAI domains appear to reflect the lymphomagenesis course towards MALT lymphomas. The long-term prognosis of pSS-MALT lymphoma patients is favorable but close follow up is recommended toward the prompt recognition of clinically significant events linked to the underlying active lymphomagenesis process.

Chapter 13: The clinical phenotype of primary Sjögren's syndrome patients with lymphadenopathy.

13.1 INTRODUCTION

Primary Sjögren's Syndrome (pSS) is a chronic systemic autoimmune disease with a diverse clinical spectrum, ranging from a mild, benign exocrinopathy to a full-blown systemic disease[455]. The central role of B-cells in the pathogenesis of the disease is indicated by the rich autoantibody profile, the hypergammaglobulinemia and the increased risk for B-cell lymphoma development[33,387], mainly mucosa-associated lymphoid tissue (MALT) lymphomas with a rather favorable prognosis [456]. Approximately 10% of pSS patients display clinically apparent lymphadenopathy[457]. This clinical manifestation may be either related to concurrent lymphoma development or may constitute a benign reactive lymphoid tissue hyperplasia in the setting of autoimmunity. Chronic antigenic stimulation drives the lymphoid infiltration in the salivary glands (SGs) of pSS patients, with a more prominent B-cell component characterizing the severe lesions[192]; in accordance the presence of germinal center (GC)-like structures (GCLS) within SGs has been proposed as lymphoma predictor and has been associated with systemic disease activity [297,298,458]. Beyond the studied histopathology of lymph node enlargement in lymphoma cases, there is a paucity of data regarding the histology and the clinical significance of the "non-malignant" enlarged lymph nodes in the context of pSS per se. However, the reactive lymphoid hyperplasia with increased lymphoid follicles in the lymph nodes of pSS patients[459,460] points out chronic antigenic stimulation, that drives SG lymphoid expansion, as the common denominator between GCLS and pSS related "non-malignant" lymphadenopathy. The EULAR SS disease activity index (ESSDAI) has incorporated lymphadenopathy domain as one of the parameters contributing to the overall disease activity [461]. Reports on the subgroup of pSS patients with lymphadenopathy unrelated to lymphoma are lacking. Therefore, in the present study we describe the clinical features and the laboratory profile of this subset of pSS patients and explore possible differences in comparison with pSS patients without lymph node enlargement.

13.2 PATIENTS AND METHODS

After excluding patients with lymphadenopathy secondary to infections, malignancy including non-Hodgkin lymphomas (NHLs), and non-available data, the medical records of 1234 consecutive pSS patients from Greece and Italy were reviewed. All patients fulfilled the 2016 American College of

Rheumatology/EULAR criteria. Patients with lymphadenopathy were identified according to the relevant ESSDAI domain (12), as follows: a) lymph node enlargement with a diameter of ≥1cm in any nodal region or ≥2cm in the inguinal region on clinical examination, b) lymph node enlargement with a maximal diameter of ≥1cm in any nodal region or ≥2cm in the inguinal region, for patients with available ultrasound or CT, c) exclusion of infection or cancer, d) persistence of lymph node enlargement for at least 6 months, d) for patients with lymph node enlargement and clinical suspicion for lymphoma, histological examination of the affected node to exclude an underlying active lymphoproliferative disorder. Cumulative, clinical, laboratory, immunologic and histologic data were collected from all participants. pSS patients with lymphadenopathy were compared with 2 control groups: a) unmatched pSS patients without lymphadenopathy (Unmatched Non-Lymphadenopathy Group) (n=1069), and b) pSS patients without lymphadenopathy matched for age, sex, and disease duration, in an approximately 1:1 ratio (Matched Non-Lymphadenopathy Group) (n= 201). Statistical analysis for categorical data was performed by $\chi 2$ test, with Yates correction or Fisher exact when cell counts involved <5 patients/items, while for numerical data the t test or Mann–Whitney methods were used, after implementing the Shapiro–Wilk normality test. A p-value < 0.05 was considered statistically significant.

13.3 RESULTS

Of the 1234 pSS patients, 165 (13.37 %) developed stable lymphadenopathy during the disease course. More than 90% of lymphadenopathy cases involved the cervical region, while the rest included the axillaries and the hyperclavicular areas. Females prevailed in both the lymphadenopathy (95.7%) and the non-lymphadenopathy unmatched group (95.5%). The lymphadenopathy group was characterized by a younger age at pSS diagnosis (median 46 years old, range: 10-81 years) compared to the non-lymphadenopathy unmatched group (median 53 years old, range: 11-85 years) ($p<0.001$), as well as by a younger median age at disease onset [median 43 years old (range: 7-76 years) vs. 49 years old (range: 5-84 years), $p=0.001$]. Moreover, pSS patients with lymphadenopathy demonstrated shorter disease duration (median 7 years vs. 10 years, $p=0.008$), with no statistically significant differences in the reported symptoms of mouth and ocular dryness compared to their non-lymphadenopathy unmatched counterparts. Lymphadenopathy pSS patients presented more frequently with SG enlargement (SGE) (39% vs 21.6%, $p<0.001$), palpable purpura (14.5% vs 5.7%, $p<0.001$), peripheral nervous system (PNS) involvement (4.9% vs 1.5%, $p=0.012$), glomerulonephritis (4.8% vs 0.5%, $p<0.001$) and higher focus score (FS) at first SG biopsy (median 1.9 vs 1.3, $p=0.01$) compared to the

unmatched pSS controls. The serological profile of the lymphadenopathy group was characterized by higher frequencies of ANA (95.7% vs 89.2%, p=0.013), anti-Ro/SSA (87.2% vs 75.7%, p=0.001) and anti-La/SSB (47.2% vs 32.9%, p=0.0004) positivity. Regarding the hematological parameters, pSS patients with lymphadenopathy displayed higher prevalence of leukopenia (21.2% vs 7.9%, p<0.001), neutropenia (12.4% vs 6.8%, p=0.02) and lymphopenia (20.2% vs 8.3%, p<0.001) compared to the non-lymphadenopathy unmatched group (Table 1).

The comparison between the lymphadenopathy group and their matched non-lymphadenopathy control group, showed a median age at pSS diagnosis of 46 years old for both groups (range: 10-81 and 15-80 years old, respectively), while the median disease duration from pSS diagnosis to last follow-up was 4 (range 0-27 years) and 5 years (range 0-30 years), respectively. Patients with lymphadenopathy presented more frequently with SGE (39% vs 20%, p=0.0001), palpable purpura (14% vs 6%, p=0.01), PNS vasculitis (5% vs 0.5%, p=0.01), glomerulonephritis (4.8% vs 0.5%, p=0.01), and higher FS at first SG biopsy (median 1.91 vs 1.19, p=0.004) compared to their matched non-lymphadenopathy controls. No statistically significant difference was found between the 2 groups regarding ANA and anti-La/SSB positivity, but the lymphadenopathy pSS patients had still higher frequencies of anti-Ro/SSA (87% vs 74%, p=0.002) than the matched controls. Regarding the hematological parameters, leukopenia (21.2% vs 7.5%, p<0.001) and lymphopenia (20.2% vs 8.2%, p=0.004) were more often observed in the lymphadenopathy group compared to their matched controls (Table 2).

Table 1: Comparison of clinical and laboratory features of all pSS patients with (lymphadenopathy group) and without lymphadenopathy (unmatched non-lymphadenopathy group). SGE: salivary gland enlargement; FS: focus score

	Lymphadenopathy Group, %, n=165	**Unmatched Non-Lymphadenopathy Group, %, n=1069**	**p value**
DEMOGRAPHICS			
Female Sex	95.7% (158/165)	95.5% (1021/1069)	0.95
Median age at pSS diagnosis (years)	46	53	**<0.001**
Median age at pSS onset (years)	43	49	**0.001**

Median disease duration from pSS diagnosis to last follow up (years)	5	5	0.9
Median disease duration from pSS onset to last follow up (years)	7	10	**0.008**
GLANDULAR AND NON-SPECIFIC MANIFESTATIONS			
Dry mouth-subjective	92.1% (152/165)	93.1% (991/1064)	0.75
Dry eyes-subjective	95.1% (157/165)	93% (994/1068)	0.4
SGE	39% (64/164)	21.6% (229/1060)	**<0.001**
FS 1st biopsy	1.9	1.3	**0.017**
Raynaud's Phenomenon	18.2% (29/159)	24.3% (251/1029)	0.1
Arthralgias-Myalgias	63% (104/165)	63.6% (677/1063)	0.93
Splenomegaly	1.8% (1/54)	0.1% (1/717)	0.13
EXTRAEPITHELIAL MANIFESTATIONS			
Glomerulonephritis	4.8% (8/164)	0.5% (6/1064)	**<0.001**
Autoimmune Hepatitis	0.6% (1/159)	0.8% (7/798)	1
Peripheral Nervous Disease	4.9% (8/163)	1.5% (17/1065)	**0.012**
Palpable Purpura	14.5% (24/165)	5.7% (61/1069)	**<0.001**
PERIEPITHELIAL MANIFESTATIONS			
Interstitial Renal Disease	1.8% (3/163)	1.9% (21/1051)	1
Primary Biliary Cholangitis	1.8% (3/165)	2% (22/1069)	1
Sclerosing Cholangitis	0% (0/159)	1.2% (10/798)	0.38
SEROLOGY			
ANA+	95.7% (158/165)	89.2% (932/1044)	**0.013**
RF+	59.3% (95/160)	51.8% (517/997)	0.09
Anti-Ro	87.2% (144/165)	75.7% (792/1046)	**0.001**
Anti-La	47.2% (78/165)	32.9% (340/1033)	**<0.001**
Low C4	27.8% (44/158)	27% (273/1011)	0.89
Cryoglobulinemia	6.9% (10/144)	4.9% (30/605)	0.45

COMPLETE BLOOD COUNT			
Leukopenia	21.2% (35/165)	7.9% (85/1064)	**<0.001**
Neutropenia	12.4% (19/153)	6.8% (61/888)	0.02
Lymphopenia	20.2% (31/153)	8.3% (74/882)	**<0.001**

Table 2: Comparison of clinical and laboratory features of all pSS patients with (lymphadenopathy group) and without lymphadenopathy (matched non-lymphadenopathy group). SGE: salivary gland enlargement; FS: focus score

	Lymphadenopathy group, %, n=165	**Matched Non-Lymphadenopathy Group, %, n=201**	**p value**
DEMOGRAPHICS			
Female Sex	96% (158/165)	96% (194/201)	0.91
Median age at pSS diagnosis (years)	46	46	0.74
Median age at pSS onset (years)	43	42	0.57
Median disease duration from pSS diagnosis to last follow up (years)	5	4	0.65
Median disease duration from pSS onset to last follow up (years)	7	9	0.09
GLANDULAR AND NON-SPECIFIC MANIFESTATIONS			
Dry mouth-subjective	92% (152/165)	93% (187/200)	0.76
Dry eyes-subjective	95% (157/165)	91% (182/200)	0.18
SGE	39% (64/164)	20% (40/200)	**<0.001**
FS 1st biopsy	1.91	1.19	**0.004**
Raynaud's phenomenon	18% (19/159)	25% (49/192)	0.13
Arthralgias-myalgias	63% (104/165)	65% (132/201)	0.67

Splenomegaly	2% (1/54)	0% (0/144)	0.27
EXTRAEPITHELIAL MANIFESTATIONS			
Glomerulonephritis	4.8% (8/164)	0.5% (1/200)	**0.01**
Autoimmune Hepatitis	0.6% (1/159)	0% (0/140)	1
Peripheral Nervous Disease	5% (8/163)	0.5% (1/199)	**0.01**
Palpable purpura	14% (24/165)	6% (13/201)	**0.01**
PERIEPITHELIAL MANIFESTATIONS			
Interstitial Renal Disease	1.8% (3/163)	2% (4/197)	1
Primary Biliary Cholangitis	1.8% (3/165)	2.9% (6/201)	0.52
Sclerosing cholangitis	0% (0/159)	1.4%(2/140)	0.21
SEROLOGY			
ANA+	95% (158/165)	90% (176/195)	0.07
RF+	59% (95/160)	50% (93/186)	0.1
Anti-Ro	87% (144/165)	74% (146/197)	**0.002**
Anti-La	47% (78/165)	37% (73/87)	0.06
Low C4	27% (44/158)	24% (46/191)	0.49
Cryoglobulinemia	6.9% (10/144)	3.8% (4/105)	0.4
COMPLETE BLOOD COUNT			
Leukopenia	21.2% (35/165)	7.5% (15/199)	**<0.001**
Neutropenia	12.4% (19/153)	5.6% (9/159)	0.05
Lymphopenia	20.2% (31/153)	8.2% (13/157)	**0.004**

13.4 DISCUSSION

Autoimmunity associated lymphadenopathy shows marked clinical and histological diversity. The majority of published histopathological studies include cases related to rheumatoid arthritis and systemic lupus erythematosus, while reports on pSS lymphadenopathy, unrelated to lymphoma, are scarcer. However, it seems that the common histopathological features of paracortical hyperplasia and lymphoid follicle expansion, overall termed reactive follicular hyperplasia, are shared in systemic

autoimmunity [459,462,463]. This follicular pattern is driven by a humoral immune reaction after persistent antigenic stimulation and subsequent proliferation of B-cells. A study by McCurley et al., investigating the lymph node histopathology in the setting of pSS, reported that the subset of pSS patients with lymphadenopathy may have either B cell lymphoma or reactive follicular hyperplasia [460]. In this context and given that pSS patients are at high risk for B-cell lymphomas, it appears of major clinical significance to study the phenotype of pSS patients with non-malignant lymphadenopathy attributed to the pathogenetic mechanisms of pSS itself and not to an active lymphoproliferative disorder. In general, patient stratification based on clinical phenotyping is an easy strategy to identify distinct patients' subgroups, to reveal disease endotypes, to optimize follow-up approaches and to tailor possible therapeutic interventions.

Our study, gathers some unique points, worthy to be mentioned: i) well characterized pSS patients with strictly defined lymphadenopathy by excluding lymphoma cases or other potential causes of lymph node enlargement, ii) since subcentimeter lymphadenopathy in pSS detected by ultrasound or CT tends to remain stable overtime and probably does not represent a poor prognosis factor for pSS [464], we have included patients with lymph node enlargement of a diameter >1cm, to assess whether the presence of this easily assessed clinical feature could lead to the description of a distinct subgroup of pSS patients with specific clinical characteristics, iii) relatively large cohort of patients with pSS-related lymphadenopathy, iv) harmonized clinical data among the participating centers based on the HarmonicSS reference model to ensure high quality of data collection and processing, and v) analysis and comparison with both unmatched and matched control pSS patients without lymphadenopathy.

The prevalence of lymphadenopathy has been reported in approximately 10% of pSS patients [457]. This is also confirmed by the current study, since 13% of pSS patients developed lymphadenopathy, not related to lymphoma, during the disease course. In the unmatched comparison, patients with lymphadenopathy were characterized by a younger age at both pSS onset and diagnosis, as well as by shorter disease duration. In addition, patients with lymphadenopathy displayed more frequently SGE, higher FS at their first SG biopsy, extra-epithelial immune-complex mediated manifestations, such as palpable purpura, PNS involvement and glomerulonephritis, leukopenia, and autoantibodies. Similar findings were observed after comparing with matched for age, sex, and disease duration pSS controls without lymphadenopathy, although among serological parameters only anti-Ro/SSA retained their higher frequency for the lymphadenopathy group.

It has been previously shown that pSS patients with early disease onset display a distinct clinical phenotype and present more often with lymphadenopathy [104]. The present study is in accordance with

this finding, showing that pSS patients with lymphadenopathy tend to be of younger age at pSS onset and diagnosis compared to pSS patients without lymphadenopathy. A previous study demonstrated that the extension of the SG inflammatory infiltration correlated with lymphadenopathy [465], while a more recent one reported that patients with a minor SG FS≥4 present more often with lymphadenopathy compared to those with FS<4 [394]. The present study confirms the correlation of lymphadenopathy with higher FS at first SG biopsy. In the only published study describing the characteristics of pSS patients with lymphadenopathy, Elefante et al. reported that pSS patients with palpable lymphadenopathy express a more severe disease phenotype characterized by younger age at pSS diagnosis, more frequently male gender, higher disease activity, SGE, low C3 levels, hypergammaglobulinemia, anti-Ro/SSA, anti-La/SSB and rheumatoid factor (RF) positivity, and cryoglobulinemia [464]. In the present study, we have accordingly demonstrated higher rates of SGE and anti-Ro/SSA positivity in the comparison between lymphadenopathy and matched non-lymphadenopathy patients, while higher rates of anti-La/SSB positivity were found only in the unmatched comparison. There were no differences between the lymphadenopathy and non-lymphadenopathy group regarding gender, RF positivity and cryoglobulinemia.

Vasculitis and lymphoma are considered life-threatening complications of pSS [112] and patients with lymphadenopathy seem to display a clinical phenotype with vasculitic complications and a variety of lymphoma associated adverse prognostic factors. Extraepithelial vasculitic manifestations are observed in 10-15% of pSS defining a more systemic form of the disease [112,292], and constitute major lymphoma predictors[205,466,467]. In the lymphadenopathy group the vasculitic manifestations of palpable purpura, PNS involvement and glomerulonephritis were more frequent compared to the non-lymphadenopathy group, both in the unmatched and matched patient comparison. Interestingly, no differences were observed regarding RF positivity, C4 hypocomplementemia and cryoglobulinemia. In addition, SGE is another strong prognostic factor for lymphoma[226], which was more prevalent in pSS patients with lymphadenopathy compared to their non-lymphadenopathy counterparts. Lymphopenia has been considered as a possible predictive factor for lymphoma development in some studies [293,451], though not confirmed in more recent ones [456,457]. Leukopenia and neutropenia have also been identified as possible predictive factors for lymphomagenesis [272,451,468]. In the lymphadenopathy group leukopenia and lymphopenia were shown to be more frequent compared to the matched non-lymphadenopathy group. Overall, it seems that pSS patients with lymphadenopathy accumulate features that are considered adverse prognostic factors for lymphoma development and should be carefully followed up by clinicians. In this line, previous studies have supported lymphadenopathy as predictive factor of lymphoma development [205,271,451,457,469], while activity in the lymphadenopathy domain of the ESSDAI at the time of pSS diagnosis has been associated with hematological cancer [470].

Since the aim of the present study was to describe the clinical phenotype of pSS patients with lymphadenopathy, we have not addressed the question of lymphoma risk. A future prospective study of this subgroup of patients could provide information on the specific additional characteristics that may contribute to higher propensity for lymphomagenesis in the lymphadenopathy subset. Given that pSS lymphadenopathy reflects the expansion and antigenic stimulation of the B-cell component in disease pathogenesis, this subset of patients may be ideal to study the molecular mechanisms implicated in the crossword between autoimmunity and lymphomagenesis, as well as in the transition from the local to the systemic level of autoimmune response. In conclusion, pSS patients presenting with lymphadenopathy constitute a distinct clinical subgroup characterized by younger age at pSS diagnosis, shorter disease duration, more frequent SGE, higher FS at first SG biopsy, prominent autoantibody positivity and vasculitic extraepithelial manifestations, suggesting a strong and systemic B cell activation. Studies focusing on this subset of patients may provide further insights into the systemic nature of the disease and the related lymphomagenesis.

Chapter 14: Clinical and laboratory findings of primary Sjogren's Syndrome patients without sicca symptoms

14.1 Introduction

Primary Sjögren's Syndrome (pSS) is a chronic, autoimmune systemic disease [455]. Excessive oral and eye mucosal dryness resulting from an aberrant lymphocytic infiltration of the affected exocrine glands is the disease prominent clinical hallmark, affecting adversely the quality of daily life of these patients [471]. Although oral and eye dryness dominate the clinical picture, the disease may present with a variety of clinical manifestations arising from other exocrine glands and parenchymal (extra-glandular) tissues [33,387], including B-cell mucosa-associated lymphoid tissue (MALT) lymphomas that in most cases have a favorable prognosis [472]. The feeling of ocular and oral dryness, referred to as sicca symptoms, is the leading cause of patients' first visit to a physician. Thus, subjective symptoms of dryness are part of the inclusion criteria of the 2016 American College of Rheumatology-European Alliance of Associations for Rheumatology Classification Criteria for pSS (ACR-EULAR) [55]. Oral and ocular dryness have been reported in over 90% of pSS cases in different cohorts, with female patients showing a higher prevalence of sicca symptoms than male patients [104,473-475]. It is also known that some pSS patients tend to undervalue their dryness related discomfort [476], while patients without symptoms of dryness may fulfill the ESSDAI definitions [55], a combination of systemic manifestations and laboratory abnormalities, heralding possible pSS underlying pathology. However, reports on this group of patients and their clinical phenotype are lacking. Hereafter, we describe the clinical picture of this subset of pSS patients and explore possible differences compared to the typical pSS patients with sicca symptoms.

14.2 Patients and Methods

The medical records of 1738 consecutive pSS patients followed up in four centers from Greece and Italy (Universities of Athens, Pisa, Harokopio and Ioannina) (PAHI group) were reviewed. All patients fulfilled the 2016 American College of Rheumatology/EULAR criteria. Those patients without sicca symptoms were identified and enrolled in this study (non-dryness Group) and the presenting manifestations that led the physicians to evaluate them for pSS were recorded [55]. Subjective sicca symptoms were reviewed based on the validated questionnaire proposed by the -European Consensus Group in 2002 [51]. Cumulative, clinical, laboratory, immunologic and histologic data were collected from all participants and non-dryness pSS patients were compared with 2 control groups: a) unmatched pSS

sicca control patients with both oral and ocular dryness (Unmatched Dryness Group) (n=1516) and b) matched according to age, sex and disease duration, pSS sicca control patients with both dry eyes and mouth (Matched Dryness Group), in a 1:2 ratio (n=76). Objective tests of oral dryness were not included in the present study because of the high number of missing values. Statistical analysis for categorical data was performed by $\chi 2$ test, with Yates correction or Fisher exact when cell counts involved <5 patients/items, while for numerical data the t test or Mann–Whitney methods were used, after implementing the Shapiro–Wilk normality test. A p-value < 0.05 was considered statistically significant.

14.3 Results

Of the 1738 pSS patients, 38 (2.19%) lacked subjective symptoms of ocular and oral dryness. The most common presenting clinical manifestation of the non-dryness group that led to further evaluation for pSS included arthralgias (47.4%), followed by parotid gland enlargement (23.6%), Raynaud's phenomenon (10.5%), persistent lymphadenopathy (10.5%), fatigue (10.5%), palpable purpura (5.3%), and pulmonary symptomatology of dry cough with or without exertional dyspnea (5.3%).

Female predominance was evident in both the non-dryness (97.4%) and unmatched dryness group (96%). However, non-dryness patients were younger than the typical sicca patients with a median age at pSS diagnosis of 40 (range 12-88) vs 53 (range 11-85) years old ($p<0.001$) and a median age at disease onset of 34 (range 9-88) vs 49 (range 5-83) years old ($p<0.001$), respectively. Patients without sicca manifestations were less likely to have objective findings of ocular dryness (55.6% vs 91.7%, $p<0.001$). Laboratory findings portrayed higher frequencies of anti-Ro/SSA (100% vs 79.7%, $p<0.001$) and antinuclear antibody positivity (ANA) (100% vs 90.4%, $p<0.001$), as well as neutropenia (20.8% vs 7.5%, $p=0.04$) and thrombocytopenia (13.8% vs 4.2%, $p=0.04$) (Table 1).

Regarding the analysis between the non-dryness group and their matched dryness controls, the median age at the time of pSS diagnosis was 40 years old for both groups (range: 12-88 years old for the non-dryness group and 15-85 years old for the dryness group respectively), while the median disease duration from pSS diagnosis to last follow-up was 4 years for both groups (range; 0-24 years old for the non-dryness Group and 0-28 years old for the dryness group, respectively). Compared to the matched dryness group, non-dryness pSS patients disclosed lower rates of positive ocular tests (55.6% vs 93.9%, $p<0.001$), as well as lower rates of lymphopenia (0 vs 17.3%, $p=0.049$). No other

statistical differences were found between the two groups regarding clinical, immunological, or histological parameters (Table 2).

Table 1. Comparison of clinical and laboratory features of all pSS patients with (unmatched dryness group) and without sicca manifestations (non-dryness group)

	Non-Dryness Group, %, n=38	Unmatched Dryness Group, %, n=1516	p-value
DEMOGRAPHICS			
Sex	97.4 (37/38)	96 (1456/1516)	1
Median age at disease diagnosis	40	53	**<0.001**
Median age at disease onset	34	49	**<0.001**
Median disease duration from SS diagnosis to last follow-up	4	5	0.26
GLANDULAR AND NON-SPECIFIC MANIFESTATIONS			
Ocular tests positivity %	55.6 (15/27)	91.7 (1220/1331)	**<0.001**
Salivary gland biopsy positivity %	93.9 (31/33)	89.1 (903/1013)	0.57
Focus score	2.12	2.1	0.48
Lymphoma %	**10.8 (4/37)**	10 (151/1515)	0.78
SGE %	26.3 (10/38)	30.4 (458/1505)	0.71
Raynaud's phenomenon %	23.7 (9/38)	24.3 (312/1284)	0.92
Arthralgias %	52.6 (20/38)	61.6 (929/1508)	0.34
Arthritis %	25.7 (9/35)	18.1 (225/1243)	0.35
EXTRAEPITHELIAL MANIFESTATIONS			
Glomerulonephritis	0 (0/38)	1.4 (21/1511)	1
Interstitial Lung Disease	8.1 (3/37)	4.2 (63/1514)	0.21
Autoimmune Hepatitis	4 (1/25)	0.7 (9/1277)	0.18
Peripheral Nervous Disease	**0 (0/31)**	3.8 (47/1239)	0.63
Central Nervous Disease	**0 (0/36)**	2 (26/1310)	1
Palpable purpura %	7.9 (3/38)	9.9 (150/1514)	1
Lymphadenopathy %	**22.6 (7/31)**	16.1 (197/1225)	0.47
PERIEPITHELIAL MANIFESTATIONS			
Tubulointerstitial Nephritis	5.3 (2/38)	2.2 (33/1498)	0.21
Small Airway Disease	6.3 (2/32)	3.8 (54/1429)	0.35
Primary Biliary Cholangitis	0 (0/38)	1.8 (28/1516)	1

SEROLOGY			
RF positivity %	58.3 (21/36)	58.1 (826/1422)	0.89
Anti-Ro/SSA positivity %	100 (38/38)	79.7 (1190/1493)	**<0.001**
Anti-La/SSB positivity %	54.1 (20/37)	37.2 (551/1482)	0.055
Low C4 serum levels %	23.3 (7/30)	28.8 (379/1316)	0.65
Monoclonal Gammopathy %	12.9 (4/31)	6.8 (46/674)	0.27
Cryoglobulinemia %	10.5 (2/19)	9.8 (91/926)	0.71
ANA positivity %	100 (36/36)	90.4 (1344/1486)	**0.04**
COMPLETE BLOOD COUNT			
Leukopenia %	20.7 (6/29)	12.9 (184/1426)	0.44
Lymphopenia %	0 (0/23)	13 (136/1048)	0.1
Neutropenia %	20.8 (5/24)	7.5 (79/1050)	0.04
Thrombocytopenia %	13.8 (4/29)	4.2 (58/1383)	0.04

Table 2. Comparison of clinical and laboratory features of matched pSS patients with (Matched Dryness Group) and without sicca manifestations (non-dryness Group)

	Non-Dryness Group, %, n=38	Matched Dryness Group, %, n=76	p-value
DEMOGRAPHICS			
Sex	97.4 (37/38)	97.4 (74/76)	1
Median age at disease diagnosis	40	40	0.95
Median age at disease onset	34	39	0.83
Median disease duration from SS diagnosis to last follow-up	4	4	0.98
GLANDULAR AND NON-SPECIFIC MANIFESTATIONS			
Ocular tests positivity %	55.6 (15/27)	93.9 (62/66)	**<0.001**
Salivary gland biopsy positivity %	93.9 (31/33)	89.8 (53/59)	0.71
Focus score	2.12	2.19	0.97
Lymphoma %	10.8 (4/37)	6.7 (5/75)	0.47
SGE %	26.3 (10/38)	30.3 (23/76)	0.83
Raynaud's phenomenon %	23.7 (9/38)	27.6 (21/76)	0.82
Arthralgias %	52.6 (20/38)	56.6 (43/76)	0.84

Arthritis %	25.7 (9/35)	17.6 (12/68)	0.48
EXTRAEPITHELIAL MANIFESTATIONS			
Glomerulonephritis	0 (0/38)	1.4 (1/74)	1
Interstitial Lung Disease	8.1 (3/37)	2.9 (2/70)	0.34
Autoimmune Hepatitis	4 (1/25)	2.9 (2/69)	1
Peripheral Nervous Disease	0 (0/31)	1.6 (1/64)	1
Central Nervous Disease	0 (0/36)	1.4 (1/69)	1
Palpable purpura %	7.9 (3/38)	18.4 (14/76)	0.17
Lymphadenopathy %	22.6 (7/31)	20.3 (13/64)	0.99
PERIEPITHELIAL MANIFESTATIONS			
Tubulointerstitial Nephritis	5.3 (2/38)	5.5 (4/73)	1
Small Airway Disease	6.3 (2/32)	3.1 (2/65)	0.6
Primary Biliary Cholangitis	0 (0/38)	1.3 (1/76)	1
SEROLOGY			
RF positivity %	58.3 (21/36)	64.3 (45/70)	0.7
Anti-Ro/SSA positivity %	100 (38/38)	90.8 (69/76)	0.09
Anti-La/SSB positivity %	54.1 (20/37)	50.7 (38/75)	0.89
Low C4 serum levels %	23.3 (7/30)	30.3 (20/66)	0.65
Monoclonal Gammopathy %	12.9 (4/31)	8.1 (5/62)	0.47
Cryoglobulinemia %	10.5 (2/19)	3.9 (2/51)	0.3
ANA positivity %	100 (36/36)	95.9 (71/74)	0.55
COMPLETE BLOOD COUNT			
Leukopenia %	20.7 (6/29)	26.1 (18/69)	0.76
Lymphopenia %	0 (0/23)	17.3 (9/52)	**0.049**
Neutropenia %	20.8 (5/24)	5.8 (3/52)	0.1
Thrombocytopenia %	13.8 (4/29)	3.1 (2/64)	0.07

14.4 Discussion

The leading clinical symptom of Sjogren's syndrome is the sensation of dry mouth and eyes, while very few pSS patients lack sicca manifestations. This was also evident in this study, since only 2.19% of pSS patients had no complaints of dryness of neither eyes nor mouth, constituting a unique cluster of pSS patients. Defining the different clinical phenotypes of the disease may facilitate patients'

stratification, uncover simple but clinically useful biomarkers, and identify the optimal therapy for each subgroup. Non-dryness patients may present with a distinct clinical picture consisting of both non-specific extraglandular manifestations such as arthralgias, Raynaud's phenomenon and persistent lymphadenopathy as well as SS specific manifestations of parotid gland enlargement and palpable purpura. This particular phenotype although rare is described for the first time in the literature and clinicians evaluating patients with systemic autoimmune disease should be aware of this subset of pSS patients. In this line, the "non dryness" group comprise the majority of discordant patients that fulfill the 2016 ACR/EULAR but not the 2002 AECG classification criteria, confirming the advantage and the increased sensitivity of the new set of criteria to capture more pSS cases [477].

It is interesting to point out that patients who lack dryness sensation either in the eyes or mouth are younger, both when the initial symptoms of the disease occur and when the diagnosis is reached. Being younger might also act as an important confounder when it comes to the perception of sicca symptoms. Younger patients tend to underrate their symptoms, accounting also for the fact that age is inversely correlated with a poor adherence to the use of lubricating eye drops [478]. A comparison of the non-dryness group with the unmatched population complaining of both dry eyes and mouth, revealed that non-dryness patients had higher frequency of anti-Ro/SSA and antinuclear antibodies as well as neutropenia and thrombocytopenia. However, the aforementioned differences could also be explained by the younger age of the non-dryness group, given that age is an important determinant of the pSS clinical picture, with younger patients being more "lupoid" dominated by systemic B cell manifestations [104]. In order to eliminate a confounding bias between the 2 groups, we employed an age, sex and disease duration 2:1 matching process.

Comparison of the non-dryness group with matched patients exhibiting both oral and eye dryness (matched dryness group), revealed two statistically significant differences. First, as anticipated, the non-dryness group presented with a lower frequency of positive ocular tests for dryness. However, it is intriguing that despite the absence of subjective eye dryness, approximately half of the non-dryness patients showed objective findings of ocular dryness (positive Schirmer's and/or ocular staining score tests). This suggests that the severity of dryness symptoms does not necessarily parallel the extent of the disease, and vice versa. The same is also shown for the salivary gland biopsies, where a focus score above or equal to 1 is not associated with the presence of neither dry eyes nor dry mouth [479]. It also implies that the application of Schirmer's test and/or ocular staining score even in non-sicca patients with high suspicion for SS may be proven diagnostically useful. In addition, objective testing (both oral and ocular) apart from offering a higher diagnostic sensitivity at the pre-clinical level, could offer an exceptional opportunity to study the early stages of the disease, at least at the tissue level in highly suspicious SS patients without apparent dryness. Furthermore, it seems reasonable to have

even non-dry pSS patients undergoing ophthalmologic evaluation for monitoring purposes, since SS-related dry eye has been shown to have worse progression compared to non-SS dry eye [480,481].

Patients in the dryness group showed lymphopenia more frequently compared to the non-dryness patients. Lymphopenia has been previously identified as a lymphoma predictor among pSS patients [482,483], though more recent studies have not included lymphopenia as strong risk factor for lymphoma [367,472]. Yazisiz et al have proposed lymphopenia as a high specificity/low sensitivity risk factor for lung involvement in pSS patients, among others [484]. However, as shown in Table 2, in our study there was no significant difference in the frequency of lymphoma or pulmonary manifestations between non dryness pSS patients and both control groups.

Finally, it is noteworthy that patients without sicca manifestations were similar to their dry counterparts in terms of salivary gland enlargement (SGE), focus score (FS), cryoglobulinemia, and lymphoma risk, indicating that the absence of sicca complaints does not necessarily reflect milder inflammatory process, weaker B lymphocytic activity and/or lower risk of lymphomagenesis. The similar clinical and histologic picture may imply differences either in the functional properties of the epithelium including its secretory capacity, epithelium polarity, tissue remodeling or in terms of the regulatory component within the inflammatory lesion that may ameliorate the intensity of tissue injury. In conclusion, non-dryness pSS patients constitute a rare clinical subset characterized by younger age, certain extra-glandular manifestations, parotid swelling and anti-Ro/SSA antibodies who share common immunopathologic mechanisms with the typical sicca pSS patients. However, given the rarity of the non-sicca SS patients, the findings of the present study require further validation in larger multicentric studies.

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Publications in the context of the PhD thesis

Reviews

1) New frontiers in precision medicine for Sjogren's syndrome.

Chatzis L, Vlachoyiannopoulos PG, Tzioufas AG, Goules AV.

Expert Rev Clin Immunol. 2021 Feb;17(2):127-141.

doi: 10.1080/1744666X.2021.1879641.

2) Searching for the "X factor" in Sjögren's syndrome female predilection.

Chatzis L, Goules AV, Tzioufas AG.

Clin Exp Rheumatol. 2021 Nov-Dec;39 Suppl 133(6):206-214.

doi: 10.55563/clinexprheumatol/88dyrn. Epub 2021 Oct 14.

Book Chapters

1) European Handbook of dermatological treatments (Chapter: Sjögren's Syndrome) (In press)

Chatzis L, Goules A, Tzioufas AG.

2) Rose and Mackay Textbook of Autoimmune Diseases (Chapter: Sjögren's syndrome) (In press)

Goules A, Kaklamanos A, **Chatzis L** , Tzioufas AG.

Original articles

1) Sjögren's Syndrome: The Clinical Spectrum of Male Patients.

Chatzis L, Pezoulas VC, Ferro F, Gandolfo S, Donati V, Binutti M, Callegher SZ, Venetsanopoulou A, Zampeli E, Mavrommati M, Argyropoulou OD, Michalopoulos G, Voulgari PV, Exarchos T, Baldini C, Skopouli FN, Fotiadis DI, De Vita S, Moutsopoulos HM, Tzioufas AG, Goules AV.

J Clin Med. 2020 Aug 12;9(8):2620.

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2) A biomarker for lymphoma development in Sjogren's syndrome: Salivary gland focus score.

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3) Serum, but Not Saliva, CXCL13 Levels Associate With Infiltrating CXCL13+ Cells in the Minor Salivary Gland Lesions and Other Histologic Parameters in Patients With Sjögren's Syndrome.

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New frontiers in precision medicine for Sjogren's syndrome

Loukas Chatzis, Panayiotis G Vlachoyiannopoulos, Athanasios G Tzioufas & Andreas V Goules

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Review

Searching for the "X factor" in Sjögren's syndrome female predilection

L.G. Chatzis, A.V. Goules, A.G. Tzioufas

Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, and Institute for Autoimmune Systemic and Neurological Diseases, Athens, Greece.

Loukas G. Chatzis, MD
Andreas V. Goules, MD, PhD
Athanasios G. Tzioufas, MD

Please address correspondence to:
Athanasios G. Tzioufas,
Department of Pathophysiology,
School of Medicine,
National and Kapodistrian
University of Athens,
Mikras Asias Street 75,
115 27 Athens, Greece.
E-mail: agtzi@med.uoa.gr

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Competing interests: none declared.

ABSTRACT

Sjögren's syndrome is typified by a strong female predilection which is also observed in other systemic autoimmune diseases. Although many factors may be contributing to this phenomenon, the exact underlying mechanisms remain unclear. Apart from the traditionally considered hormonal and environmental factors, lately the role of sex chromosomes and especially of the X chromosome has drawn much attention. In the current review, we focus on the inherent genetic imbalance between the sex chromosomes and their influence and role on gender-discordant disease presentation. To compensate for this imbalance, nature has created a defective epigenetic mechanism to silence the second rich in immune related genes X chromosome. Genes escaping silencing, transfer the genetic imbalance into the transcriptional and protein level, contributing to gender differences as reflected in functions of the innate and adaptive immunity. Under this prism, recent research data on SS, regarding specific immune X-linked loci are being presented and analysed. The "X Factor" in the search for an explication of women's predilection in autoimmunity, may lie behind these unique properties of the X chromosome.

Introduction

Autoimmune disorders (ADs) represent a range of organ specific or systemic diseases characterised by aberrant immune responses against self-antigens, leading to damage of tissues or organs. Even though single autoimmune diseases are rare, as a group they affect up to 6% of the industrialised general population (1) and are among the leading causes of morbidity and mortality in middle aged women (2, 3). Importantly, around 78% of those with an autoimmune disease are women (4). The magnitude of female predilection correlates with disease's prevalence since common diseases show a higher female skewing. For example, autoimmune thyroiditis, the most common organ specific autoimmune disorder, as well as systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS), the prototypical systemic autoimmune disorders, share the most striking female sex biases. Earlier epidemiologic studies supported a female to male ratio in SLE and SS of 9-11:1, but recent data shows the gender ratio in SS to be even higher at 20:1 (5). Therefore, SS constitutes the most female predominant systemic autoimmune disease, and it presents the best research model to investigate and elucidate the implicated mechanisms favouring a women bias (Table I).

Growing scientific evidence shows that the adult females mount stronger innate and adaptive immunological responses, reflected by a lower risk for serious infections and a better vaccine antibody response, although the pathophysiology has not been thoroughly worked out (6-11). The ongoing worldwide severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, showing a case mortality ratio of 1,7 males *versus* 1 female, is another pertinent example (12). In this line, when it comes to immune deregulation, the precise pathogenetic processes that govern autoimmune disorders' significant sexual dimorphism and predilection are still undetermined, concealing a crucial step both in understanding the fundamental underlying mechanisms of autoimmunity and tailoring a true precision medicine therapeutic approach. It is commonly believed that inherent differences between genders might contribute to this phenomenon. For decades, the unique female hormonal milieu was considered

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Article

Sjögren's Syndrome: The Clinical Spectrum of Male Patients

Loukas Chatzis [1], Vasileios C. Pezoulas [2], Francesco Ferro [3], Saviana Gandolfo [4], Valentina Donati [3], Marco Binutti [4], Sara Zandonella Callegher [4], Aliki Venetsanopoulou [1], Evangelia Zampeli [5], Maria Mavrommati [5], Ourania D. Argyropoulou [1], Giorgos Michalopoulos [1], Paraskevi V. Voulgari [6], Themis Exarchos [7], Chiara Baldini [3], Fotini N. Skopouli [5], Dimitrios I. Fotiadis [2,8], Salvatore De Vita [4], Haralampos M. Moutsopoulos [5,9], Athanasios G. Tzioufas [1,*] and Andreas V. Goules [1,*]

1 Pathophysiology Department, Athens School of Medicine, National and Kapodistrian University of Athens, 11527 Athens, Greece; lukechatzis@gmail.com (L.C.); alikivenetsanopoulou@yahoo.com (A.V.); ariel.argiropoulou@gmail.com (O.D.A.); gdmichalopoulos@gmail.com (G.M.)
2 Unit of Medical Technology and Intelligent Information Systems, University of Ioannina, 45110 Ioannina, Greece; bpezoulas@gmail.com (V.C.P.); dimitris.fotiadis30@gmail.com (D.I.F.)
3 Rheumatology Unit, Department of Clinical and Experimental Medicine, University of Pisa, 56100 Pisa, Italy; francescoferrodoc@gmail.com (F.F.); valentina.donati@gmail.com (V.D.); chiara.baldini74@gmail.com (C.B.)
4 Rheumatology Clinic, Department of Medical area, University of Udine, ASUFC, 33100 Udine, Italy; saviana.gandolfo@uniud.it (S.G.); m.binutti@stdelta.com (M.B.); sarazandonella@gmail.com (S.Z.C.); devita.salvatore@aoud.sanita.fvg.it (S.D.V.)
5 Department of Nutrition and Clinical Dietetics, Harokopio University of Athens, 176 71 Athens, Greece; zampelieva@gmail.com (E.Z.); mmavrommati@euroclinic.gr (M.M.); fskopouli@hua.gr (F.N.S.); hmoutsop@med.uoa.gr (H.M.M.)
6 Rheumatology Clinic, Department of Internal Medicine, Medical School, University of Ioannina, 45110 Ioannina, Greece; pvoulgar@uoi.gr
7 Department of Informatics, Ionian University, 491 00 Corfu, Greece; themis.exarchos@gmail.com
8 Department of Biomedical Research, Institute of Molecular Biology and Biotechnology, FORTH, 70013 Ioannina, Greece
9 Athens Academy of Athens, Chair Medical Sciences/Immunology, 10679 Athens, Greece
* Correspondence: agtzi@med.uoa.gr (A.G.T.); agoules@med.uoa.gr (A.V.G.)

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Abstract: Background: To compare the clinical, serological and histologic features between male and female patients with Sjögren's syndrome (SS) and explore the potential effect of gender on lymphoma development. Methods: From a multicenter population (Universities of Udine, Pisa and Athens, Harokopion and Ioannina (UPAHI)) consisting of consecutive SS patients fulfilling the 2016 ACR/EULAR criteria, male patients were identified, matched and compared with female controls. Data-driven multivariable logistic regression analysis was applied to identify independent lymphoma-associated factors. Results: From 1987 consecutive SS patients, 96 males and 192 matched female controls were identified and compared. Males had a higher frequency of lymphoma compared to females (18% vs. 5.2%, OR = 3.89, 95% CI: 1.66 to 8.67; $p = 0.0014$) and an increased prevalence of serum anti-La/SSB antibodies (50% vs. 34%, OR = 1.953, 95% CI: 1.19 to 3.25; $p = 0.0128$). No differences were observed in the frequencies of lymphoma predictors between the two genders. Data-driven multivariable logistic regression analysis revealed negative association of the female gender with lymphoma and positive association with lymphadenopathy. Conclusion: Male SS patients carry an increased risk of lymphoma development. Although statistics showed no difference in classical lymphoma predictors compared to females, data-driven analysis revealed gender and lymphadenopathy as independent lymphoma-associated features.

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A biomarker for lymphoma development in Sjogren's syndrome: Salivary gland focus score☆

Loukas Chatzis [a], Andreas V. Goules [a], Vasilis Pezoulas [b], Chiara Baldini [c], Saviana Gandolfo [d], Fotini N. Skopouli [e,f], Themis P. Exarchos [b], Efstathia K. Kapsogeorgou [a], Valentina Donati [g], Paraskevi V. Voulgari [h], Clio P. Mavragani [i], Vasilis Gorgoulis [j], Salvatore De Vita [d], Dimitrios Fotiadis [b,k], Michalis Voulgarelis [a], Haralampos M. Moutsopoulos [l,m], Athanasios G. Tzioufas [a,*]

[a] Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece
[b] Unit of Medical Technology and Intelligent Information Systems, University of Ioannina, Ioannina, Greece
[c] Rheumatology Unit, Department of Clinical and Experimental Medicine, University of Pisa, Italy
[d] Rheumatology Clinic, Department of Medical area, University of Udine, Udine, Italy
[e] Department of Nutrition and Clinical Dietetics, Harokopio University of Athens, Athens, Greece
[f] Department of Medicine and Clinical Immunology, Euroclinic of Athens, Athens, Greece
[g] Unit of Pathological Anatomy 2, Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy
[h] Rheumatology Clinic, Department of Internal Medicine, Medical School, University of Ioannina, Ioannina, Greece
[i] Department of Physiology, Medical School, National and Kapodistrian University of Athens, Athens, Greece
[j] Laboratory of Histology-Embryology Molecular Carcinogenesis Group Medical School National and Kapodistrian University of Athens, Athens, Greece
[k] Department of Biomedical Research, Institute of Molecular Biology and Biotechnology, FORTH, Ioannina, Greece
[l] Athens Academy of Athens, Chair Medical Sciences/Immunology, Greece
[m] Institute for Autoimmune Systemic and Neurological Diseases, Greece

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ABSTRACT

The aim of this study is to explore the role of labial minor salivary gland (LMSG) focus score (FS) in stratifying Sjögren's Syndrome (SS) patients, lymphoma development prediction and to facilitate early lymphoma diagnosis. In an integrated cohort of 1997 patients, 618 patients with FS $\geq$ 1 and at least one-year elapsing time interval from SS diagnosis to lymphoma diagnosis or last follow up were identified. Clinical, laboratory and serological features were recorded. A data driven logistic re-gression model was applied to identify independent lymphoma associated risk factors. Furthermore, a FS threshold maximizing the difference of time interval from SS until lymphoma diagnosis between high and low FS lymphoma subgroups was investigated, to develop a follow up strategy for early lymphoma diagnosis. Of the 618 patients, 560 were non-lymphoma SS patients while the other 58 had SS and lymphoma. FS, cryoglobulinemia and salivary gland enlargement (SGE) were proven to be independent lymphoma associated risk factors. Lymphoma patients with FS $\geq$ 4 had a statistically significant shorter time interval from SS to lymphoma diagnosis, compared to those with FS $<$ 4 (4 vs 9 years, respectively, $p = 0{,}008$). SS patients with FS $\geq$ 4 had more frequently B cell originated manifestations and lymphoma, while in patients with FS $<$ 4, autoimmune thyroiditis was more prevalent. In the latter group SGE was the only lymphoma independent risk factor. A second LMSG biopsy is patients with a FS $\geq$ 4, 4 years after SS diagnosis and in those with FS $<$ 4 and a history of SGE, at 9-years, may contribute to an early lymphoma diagnosis. Based on our results we conclude that LMSG FS, evaluated at the time of SS diagnosis, is an independent lymphoma associated risk factor and may serve as a predictive biomarker for the early diagnosis of SS-associated lymphomas.

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* Corresponding author. Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, Mikras Asias Str 75, 115 27, Athens, Greece.
E-mail address: agtzi@med.uoa.gr (A.G. Tzioufas).

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Serum, but Not Saliva, CXCL13 Levels Associate With Infiltrating CXCL13+ Cells in the Minor Salivary Gland Lesions and Other Histologic Parameters in Patients With Sjögren's Syndrome

Loukas Chatzis [1,2†], Andreas V. Goules [1,2†], Ioanna E. Stergiou [1,2], Michael Voulgarelis [1,2], Athanasios G. Tzioufas [1,2] and Efstathia K. Kapsogeorgou [1,2]*

[1] Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece, [2] Institute for Autoimmune Systemic and Neurological Diseases, Athens, Greece

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Alan Baer,
Johns Hopkins University,
United States

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Sapienza University of Rome, Italy
Michele Bombardieri,
Queen Mary University of London,
United Kingdom
Muhammad Shahnawaz Soyfoo,
Université libre de Bruxelles, Belgium

***Correspondence:**
Efstathia K. Kapsogeorgou
ekapso@med.uoa.gr

[†]These authors have contributed equally to this work and share first authorship

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Recent studies suggest that elevated CXCL13 serum levels in patients with primary Sjögren's syndrome (pSS) associate with minor salivary gland (MSG) histologic features, disease severity, as well as high-risk status for non-Hodgkin lymphoma (NHL) development and NHL itself. In contrast, limited discriminative value of CXCL13 saliva levels has been reported. Prompt by these reports, we sought to validate the clinical utility of CXCL13 by investigating potential correlations of serum and saliva levels with MSG histopathologic [including CXCL13+-cell number, severity of infiltrates and germinal center (GC) formation], serologic and clinical parameters, as well as NHL. CXCL13 levels were evaluated in paired serum and saliva specimens of 45 pSS patients (15 with NHL; pSS-associated NHL: SSL), 11 sicca-controls (sicca-complaining individuals with negative MSG biopsy and negative autoantibody profile), 10 healthy individuals (healthy-controls) and 6 non-SS-NHLs. CXCL13+-cells were measured in paired MSG-tissues of 22 of pSS patients studied (including 7 SSLs) and all sicca-controls. CXCL13 serum levels were significantly increased in pSS and SSL patients compared to sicca- and healthy-controls and were positively correlated with the CXCL13+-cell number and biopsy focus-score. Serum CXCL13 was significantly higher in pSS patients with GCs, rheumatoid factor, hypocomplementemia, high disease activity, NHL and in high-risk patients for NHL development. CXCL13 saliva levels were significantly increased in SSL patients (compared to non-SS-NHLs), patients with GCs and in high-risk for NHL patients. Univariate analysis revealed that CXCL13 serum, but not saliva, levels were associated with lymphoma, an association that did not survive multivariate analysis. Conclusively, our findings confirm that serum, but not saliva, levels of CXCL13 are associated with histologic, serologic and clinical features indicative of more severe pSS.

Keywords: Sjögren's syndrome, non-Hodgkin's lymphoma, CXCL13 chemokine, serum, saliva, minor salivary gland

Combined seronegativity in Sjögren's syndrome

L.G. Chatzis[1,2], V. Pezoulas[3], P.V. Voulgari[4], C. Baldini[5], T.P. Exarchos[3], D.I. Fotiadis[3,6], C.P. Mavragani[7], F.N. Skopouli[8,9], H.M. Moutsopoulos[2,10], A.G. Tzioufas[1,2], A.V. Goules[1,2]

[1]Dept. of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, Greece; [2]Institute for Autoimmune, Systemic and Neurological Diseases, Athens, Greece; [3]Unit of Medical Technology and Intelligent Information Systems, University of Ioannina, Greece; [4]Rheumatology Clinic, Dept. of Internal Medicine, Medical School, University of Ioannina, Greece; [5]Rheumatology Unit, Dept. of Clinical and Experimental Medicine, University of Pisa, Italy; [6]Dept. of Biomedical Research, Institute of Molecular Biology and Biotechnology, FORTH, Ioannina, Greece; [7]Dept. of Physiology, Medical School, National and Kapodistrian University of Athens, Greece; [8]Dept. of Nutrition and Clinical Dietetics, Harokopio University of Athens, Greece; [9]Dept. of Medicine and Clinical Immunology, Euroclinic of Athens, Greece; [10]Athens Academy of Athens, Chair Medical Sciences/Immunology, Greece.

Loukas G. Chatzis, MD
Vasilis Pezoulas, Eng
Paraskevi V. Voulgari, MD
Chiara Baldini, MD, PhD
Themis P. Exarchos, PhD
Dimitrios I. Fotiadis, PhD
Clio P. Mavragani, MD, PhD
Fotini N. Skopouli, MD, FRCP
Haralampos M. Moutsopoulos, MD, FACP, FRCP (hc), Master ACR
Athanasios G. Tzioufas, MD, PhD
Andreas V. Goules, MD, PhD

Please address correspondence to:
Andreas V. Goules,
Department of Pathophysiology,
School of Medicine, National and Kapodistrian University of Athens,
Mikras Asias Street 75,
115 27 Athens, Greece.
E-mail: agoules@med.uoa.gr

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ABSTRACT

Objective. *To describe the clinical spectrum of Sjögren's syndrome (SS) patients with combined seronegativity.*

Methods. *From a multicentre study population of consecutive SS patients fulfilling the 2016 ACR-EULAR classification criteria, patients with triple seronegativity [anti-Ro/SSA(-), anti-La/SSB(-), RF(-) and ANA(+)] and quadruple seronegativity [anti-Ro/SSA(-), anti-La/SSB(-), RF(-) and ANA(-)] were identified retrospectively. Both groups were matched in an 1:1 ratio with 2 distinct control SS groups: i) classic anti-Ro/SSA seropositive patients [SS(+)] and ii) classic anti-Ro/SSA seropositive patients with negative rheumatoid factor [SS(+)/RF(-)] to explore their effect on disease expression. Clinical, laboratory and, histologic features were compared. A comparison between triple and quadruple seronegative SS patients was also performed.*

Results. *One hundred thirty-five SS patients (8.6%) were identified as triple seronegative patients and 72 (4.5%) as quadruple. Triple seronegative patients had lower frequency of peripheral nervous involvement (0% vs. 7.2% p=0.002) compared to SS(+) controls and lower frequency of interstitial renal disease and higher prevalence of dry mouth than SS(+)/RF(-) controls. Quadruple seronegative patients presented less frequently with persistent lymphadenopathy (1.5% vs. 16.9 p=0.004) and lymphoma (0% vs. 9.8% p=0.006) compared to SS(+) controls and with lower prevalence of persistent lymphadenopathy (1.5% vs. 15.3% p=0.008) and higher frequency of dry eyes (98.6% vs. 87.5% p=0.01) and autoimmune thyroiditis (44.1% vs. 17.1% p=0.02) compared to SS(+)/RF(-) SS controls. Study groups comparative analysis revealed that triple seronegative patients had higher frequency of persistent lymphadenopathy and lymphoma, higher focus score and later age of SS diagnosis compared to quadruple seronegative patients.*

Conclusion. *Combined seronegativity accounts for almost 9% of total SS population and is associated with a milder clinical phenotype, partly attributed to the absence of rheumatoid factor.*

Introduction

Sjögren's syndrome (SS) is accompanied by plethora of autoantibodies as a result of B cell aberrant activation (1, 2), with anti-Ro/SSA, anti-La/SSB, rheumatoid factor (RF) and antinuclear antibodies (ANA) being the most frequently encountered (3, 4). Anti-Ro/SSA antibody, is present in 50-75% of SS patients and in approximately half of them, anti-La/SSB antibody is also detected (5). Anti-La/SSB antibodies almost always coexist with anti Ro/SSA and less than 2% of SS patients are anti-La/SSB monopositive, with a phenotype ranging between seronegative and seropositive patients (6, 7). The prevalence of rheumatoid factor reaches approximately 50% in SS and have been recognised as lymphoma predictor (8, 9). Other autoantibodies have been also reported to define unique clinical phenotypes of SS but are detected in low prevalence (1). Preceding studies have explored the phenotype of partly seronegative patients (defined as anti-Ro/SSA and anti-La/SSB negative antibodies) *versus* seropositive controls (defined as positive for anti-Ro/SSA with or without anti-La/SSB antibodies), exhibiting differences regarding the age at SS diagnosis, sicca manifestations, specific extraglandular manifestations, and lymphoma (10, 11). In these studies, however, RF and ANA antibodies differed between study groups, obscuring the true effect of anti-Ro/SSA and/

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Original article

Clinical picture, outcome and predictive factors of lymphoma in primary Sjögren's syndrome: results from a harmonized dataset (1981–2021)

Loukas G. Chatzis [1,2], Ioanna E. Stergiou [1], Andreas V. Goules[1,2], Vasilis Pezoulas[3], Gerasimos Tsourouflis[4], Dimitrios Fotiadis[3,5], Athanasios G. Tzioufas[1,2] and Michael Voulgarelis[1]

Abstract

Objectives. Primary Sjögren's Syndrome (pSS) carries the highest risk for non-Hodgkin's lymphoma (NHL) development among systemic autoimmune diseases. However, the paucity of data on the long-term survival of those patients and the lack of established predictors for each lymphoma histologic subtype prompted our present study.
Methods. We retrospectively analysed 121 patients diagnosed with NHL according to the WHO classification criteria. All patients fulfilled the 2016 ACR-EULAR classification criteria for pSS. Cumulative clinical, laboratory, radiologic, treatment regimens and histologic data were recorded, harmonized and analysed. Overall survival (OS) and event-free survival (EFS) curves were calculated. A mucosa-associated lymphoid tissue lymphoma (MALTL) prediction model was developed by applying innovative data-driven analysis of clinical features present at the time of pSS diagnosis.
Results. MALTLs constituted the majority of lymphomas (92/121, 76.0%) followed by diffuse large B-cell lymphomas (DLBCL) (11/121, 9.0%) and nodal marginal zone lymphomas (NMZL) (8/121, 7%). MALTLs show salivary glands localization, limited disease and often bone marrow and nodal involvement. The 10-year OS and EFS rates were 79% and 45.5% for MALTLs, 40.9% and 24.2% for DLBCL and 46% and 31% for NMZL. Cryoglobulinemia, focus score and the total EULAR SS Disease Activity Index (ESSDAI) composite index at pSS diagnosis were proven independent MALTL predictors. Even though MALTLs have a comparatively good survival outlook, they are accompanied by frequent events throughout their clinical course.
Conclusions. Common features of pSS, present at diagnosis, can predict future lymphomagenesis meriting a more intensive follow-up plan.

Key words: Sjögren's syndrome, lymphoma, MALT lymphoma, DLBCL, Focus score, ESSDAI, cryoglobulinemia, B-cell activation

Rheumatology key messages

- ESSDAI, cryoglobulinemia and high FS are considered MALTL predictors when present at pSS diagnosis.
- Biologic and glandular ESSDAI domains appear to reflect the lymphomagenesis course towards MALTLs.
- Long follow-up of pSS-MALTLs reveals a good survival outcome accompanied by frequent events.

[1]Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, [2]Institute for Autoimmune Systemic and Neurologic Diseases, Athens, [3]Unit of Medical Technology and Intelligent Information Systems, University of Ioannina, Ioannina, [4]Second Department of Propedeutic Surgery, Laiko General Hospital, Medical School, National and Kapodistrian University of Athens, Athens and [5]Department of Biomedical Research, Institute of Molecular Biology and Biotechnology, FORTH, Ioannina, Greece

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Correspondence to: Michael Voulgarelis, Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, Mikras Asias Str 75, 115 27 Athens, Greece.
E-mail: mvoulgar@med.uoa.gr

The clinical phenotype of primary Sjögren's syndrome patients with lymphadenopathy

I.E. Stergiou[1], L.G. Chatzis[1], V.C. Pezoulas[2], C. Baldini[3], D.I. Fotiadis[2], M. Voulgarelis[1], A.G. Tzioufas[1], A.V. Goules[1]

[1]Pathophysiology Department, Athens School of Medicine, National and Kapodistrian University of Athens, Greece; [2]Unit of Medical Technology and Intelligent Information Systems, University of Ioannina, Greece; [3]Rheumatology Unit, Department of Clinical and Experimental Medicine, University of Pisa, Italy.

Abstract

Objective

Previous cohort studies have shown that around 10% of patients with primary Sjögren's syndrome (pSS) develop lymphadenopathy during their disease course. However, no studies have described their clinical phenotype. The present study aims to describe the clinical manifestations and laboratory findings of pSS patients presenting long-standing lymphadenopathy.

Methods

From a total of 1234 consecutive pSS patients fulfilling the 2016 ACR-EULAR criteria, those with stable lymphadenopathy unrelated to lymphoma were identified (lymphadenopathy group). Their clinical data were collected and compared with 2 control groups: a) the remaining unmatched pSS patients without lymphadenopathy (unmatched non-lymphadenopathy group) and b) pSS patients without lymphadenopathy matched for age, sex, and disease duration, in an approximately 1:1 ratio (matched non-lymphadenopathy group).

Results

One hundred and sixty-five (13.37%) patients presented persistent, stable lymphadenopathy. They were characterised by younger age at both pSS onset and diagnosis, and by shorter disease duration. Compared to the unmatched non-lymphadenopathy group, patients with lymphadenopathy had more frequently salivary gland enlargement ($p<0.001$), higher focus score at first salivary gland biopsy ($p=0.017$), palpable purpura ($p<0.001$), peripheral nervous system involvement ($p=0.012$), glomerulonephritis ($p<0.001$), and leukopenia ($p<0.001$), while the results of the matched comparison were similar. Regarding the serological profile, the comparison with the unmatched group demonstrated higher frequency of ANA ($p=0.013$), anti-Ro/SSA ($p=0.001$), and anti-La/SSB ($p<0.001$) positivity for the lymphadenopathy group, while in the matched comparison only higher rates of anti-Ro/SSA positivity ($p=0.002$) remained statistically significant.

Conclusion

pSS patients presenting non-lymphoma related stable lymphadenopathy constitute a subgroup of younger individuals with B-cell hyperactivation.

Key words

Sjögren's syndrome, lymphadenopathy, autoimmunity, lymphoid hyperplasia

Clinical and laboratory findings of primary Sjögren's syndrome patients without sicca symptoms

L.G. Chatzis[1], V. Koulouri[1], C. Baldini[2], V.C. Pezoulas[3], P.V. Voulgari[4], F.N. Skopouli[5], D.I. Fotiadis[3,6], A.G. Tzioufas[1], A.V. Goules[1]

[1]Pathophysiology Department, Athens School of Medicine, National and Kapodistrian University of Athens, Athens, Greece; [2]Rheumatology Unit, Department of Clinical and Experimental Medicine, University of Pisa, Italy; [3]Unit of Medical Technology and Intelligent Information Systems, University of Ioannina, Greece; [4]Rheumatology Clinic, Department of Internal Medicine, Medical School, University of Ioannina, Greece; [5]Department of Nutrition and Clinical Dietetics, Harokopio University of Athens, Greece; [6]Department of Biomedical Research, Institute of Molecular Biology and Biotechnology, FORTH, Ioannina, Greece.

Abstract

Objective

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterised by oral and eye dryness. A minority of patients can present without dryness but studies on their clinico-laboratory manifestations are scarce. Our purpose was to describe the clinical phenotype of pSS patients lacking sicca symptoms.

Methods

From a total of 1738 consecutive pSS patients fulfilling the 2016 ACR-EULAR criteria, those who presented without sicca symptoms were identified (non-dryness group). Their medical data was collected and compared with 2 control groups: a) the remaining unmatched sicca pSS patients with both oral and eye dryness (unmatched dryness group) and b) matched sicca pSS patients according to age, sex, and disease duration, in 1:2 ratio (matched dryness group).

Results

Thirty-eight (2.19%) patients lacked sicca manifestations presenting mainly with arthralgias (47%), parotid enlargement (24%), Raynaud's phenomenon (11%) and persistent lymphadenopathy (11%) that led them to be evaluated for pSS. Non-dryness pSS patients were younger than the unmatched sicca controls, displaying a higher frequency of anti-Ro/SSA antibodies (100% vs. 79.7%, $p<0.001$), ANA positivity (100% vs. 90.4%, $p<0.001$), neutropenia (20.8% vs. 7.5%, $p=0.04$) and thrombocytopenia (13.8% vs. 4.2%, $p=0.04$). They also had lower frequency of positive ocular tests compared to both unmatched and matched dryness patients. No differences were found between non-dryness pSS patients and both control groups regarding focus score or any other extraglandular manifestation.

Conclusion

pSS patients without sicca complaints constitute a distinct phenotype involving younger patients, sharing common immunopathologic mechanisms with typical sicca patients.

Key words

Sjögren's syndrome, sicca symptoms, dry eyes, dry mouth