SUBPOPULAÇÕES LINFOCITÁRIAS NO DIAGNÓSTICO DA SÍNDROME DE SJÖGREN PRIMÁRIA

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Tese para obtenção do grau de Doutor em Medicina

na Especialidade de Investigação Clínica

na Faculdade de Ciências Médicas | NOVA Medical School da Universidade NOVA de Lisboa

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Prefácio | Enquadramento

A Síndrome de Sjögren é uma doença crónica que apresenta considerável variabilidade clínica, não existindo nenhuma característica específica que, isoladamente, permita o seu diagnóstico. Na SSP este é feito considerando o conjunto de manifestações clínicas e alterações laboratoriais no contexto específico de cada indivíduo [1].

As manifestações típicas da doença, nomeadamente secura oral e ocular (síndrome Sicca) são pouco específicas e poderem surgir no contexto de outras doenças locais ou sistémicas, e são comuns na população mais envelhecida [2]. Manifestações sistémicas, que ocorrem em cerca de 50% dos doentes, frequentemente no início da doença, podem também alertar para a presença da SSP [1].

O diagnóstico clínico, baseia-se na confirmação objetiva da redução da capacidade secretória lacrimal ou salivar, e na confirmação obrigatória da presença de um distúrbio imunológico subjacente. Assim, perante a suspeita clínica, os principais elementos para confirmar a SSP são a presença de anticorpos anti-SSA e anti-SSB [3], e infiltrados linfocitários focais na biópsia de glândula salivar minor (labial) [4,5].

Nem sempre é fácil confirmar o diagnóstico, sobretudo nas fases precoces da doença, em que a fraca associação entre as queixas secas e os testes objetivos dificulta a avaliação da função secretória [1,6].

São desejáveis novos biomarcadores que traduzam a disfunção imune presente na doença. Exemplos promissores são a caracterização do perfil linfocitário por citometria de fluxo [7,8], a identificação de novos autoanticorpos [9] e a ecografia das glândulas salivares[10].

Enquanto o diagnóstico é o corolário de um processo clínico que avalia a probabilidade de um determinado indivíduo ter a doença, no seu contexto específico, a classificação pretende definir presença ou ausência da doença de acordo com caraterísticas pré-estabelecidas [11].

A correta identificação dos indivíduos com doença, quer cumpram ou não os critérios de classificação, tem implicações no prognóstico, na terapêutica e no plano de seguimento [12]. Indivíduos com doença estabelecida ou em progressão beneficiarão do reconhecimento precoce e intervenção mais ativa, com recurso a terapêuticas imunomoduladoras. Por outro lado, indivíduos com doença indiferenciada estável ou sem

doença autoimune não necessitarão de ser expostos a estas terapêuticas e poderão ser tranquilizados quanto ao melhor prognóstico do seu quadro clínico.

Ao longo dos anos, elaboraram-se vários conjuntos de Critérios de Classificação para a SSP, sendo os do *American-European Consensus Group* (AECG) [13] os mais extensamente utilizados, com mais de 1500 referências [14]. Dada a necessidade de se obterem critérios consensuais, o consórcio *International Sjögren's Syndrome Criteria Working Group* desenvolveu um novo sistema de classificação em 2016, conhecido como Critérios de Classificação ACR/EULAR, sendo composto apenas por elementos objetivos [15].

Apesar da elevada sensibilidade e especificidade de ambos os critérios, AECG e ACR/EULAR, nem todos os doentes com um diagnóstico clínico de SSP irão cumprir critérios de classificação [16]. Uma vez que os critérios ACR/EULAR são baseados na pontuação de elementos objetivos, a sua adaptação poderá ser exequível caso novos testes diagnósticos demonstrem mais-valia [14]. Na eventualidade de se atingir uma sensibilidade e especificidade perfeitas (ambas de 100%), os critérios de classificação seriam equivalentes a critérios de diagnóstico, pois identificariam corretamente todos os casos individuais [17].

A presença de alterações típicas em várias subpopulações linfocitárias na SSP [18–20] poderá contribuir para o diagnóstico e monitorização da atividade da doença. Além disso, ao contrário de métodos como a biópsia glandular, a caraterização das populações linfocitárias é um método não invasivo que pode ser repetido ao longo do tempo, de modo a melhor caraterizar a dinâmica da distribuição destas células ao longo da evolução da doença.

O objetivo principal a que me propus ao elaborar este Projeto de Investigação foi a exploração das subpopulações linfocitárias, caraterizadas por citometria de fluxo, como biomarcador auxiliar no diagnóstico e classificação da SSP.

De acordo com o Artigo 4.º do Diário da República, 2.ª série, N.º 111, de 9 de Junho de 2015 (Regulamento n.º 320/2015) e com o Artigo 19.º do Diário da República, 2.ª série, n.º 153, de 7 de Agosto de 2015 (Regulamento n.º 519/2015), os resultados apresentados e discutidos nesta tese foram publicados, aceites ou submetidos para publicação nas seguintes revistas científicas com arbitragem científica:

- **Barcelos F**, Martins C, Papoila A, Geraldes C, Cardigos J, Nunes G, Lopes T, Alves N, Vaz-Patto J, Branco J, Borrego LM. Association between memory B-cells and clinical and immunological features of primary Sjögren's syndrome and Sicca patients. *Rheumatol Int* 38, 1063–1073 (2018). https://doi.org/10.1007/s00296-018-4018-0.

- **Barcelos F**, Martins C, Monteiro R, Cardigos J, Prussiani T, Sítima M, Alves N, Vaz-Patto J, Branco JC, Borrego LM. Association between EBV serological patterns and lymphocytic profile of SjS patients support a virally triggered autoimmune epithelitis. *Sci Rep* **11,** 4082 (2021). https://doi.org/10.1038/s41598-021-83550-0.

- **Barcelos F**, Martins C, Madeira N, Ângelo-Dias M, Cardigos J, Alves N, Vaz-Patto J, Cunha-Branco J, Borrego LM. Lymphocyte subpopulations in Sjögren's syndrome are distinct in anti-SSA-positive patients and related to disease ativity. *Clin Rheumatol*. 2021 Jan 14. doi: 10.1007/s10067-020-05537-y. Epub ahead of print.

- Cardigos J, **Barcelos F**, Carvalho H, Hipólito D, Crisóstomo S, Vaz-Patto J, Alves N. Tear Meniscus and Corneal Sub-basal Nerve Plexus Assessment in Primary Sjögren Syndrome and Sicca Syndrome Patients. *Cornea*. 2019 Feb;38(2):221-228. doi: 10.1097/ICO.0000000000001800.

- **Barcelos F**, Hipólito-Fernandes D, Martins C, Ângelo-Dias M, Cardigos J, Monteiro R, Alves N, Vaz-Patto J, Cunha-Branco J, Borrego LM. Corneal Sub-basal Nerve Plexus Assessment and its Association with Phenotypic Features and Lymphocyte subsets in Sjögren's Syndrome. Aceite para publicação em *Acta Ophthalmologica 2021.* doi*:* 10.1111/AOS.14811

- **Barcelos F**, Brás-Geraldes C, Martins C, Papoila AL, Monteiro R, Cardigos J, Madeira N, Alves N, Vaz-Patto J, Cunha-Branco J, Borrego LM. Added value of lymphocyte subpopulations in the classification of Sjögren's syndrome. Submetido à revista *Rheumatology* (Oxford).

De acordo com a alínea e) do Artigo 19.º do Diário da República, 2.ª série, N.º 153, de 7 de agosto de 2015 (Regulamento n.º 519/2015), este Projeto de Investigação foi aprovado pelas comissões de ética do Instituto Português de Reumatologia, do Hospital CUF Descobertas, e da NOVA Medical School/Faculdade de Ciências Médicas (autorização n.º 17/2016/CEFCM).

Parte dos resultados obtidos foram apresentados nas seguintes reuniões científicas:

COMUNICAÇÕES – RESUMOS ACEITES PARA PUBLICAÇÃO

- **Barcelos F**, Martins C, Nunes G, Lopes T, Vaz Patto J, Amaral J, Branco JC, Borrego LM. AB0013 Is there immune disregulation in non-sjögren sicca syndrome? A study of blood lymphocyte subpopulations. Abstract accepted for publication. *Annals of the Rheumatic Diseases* 2017 Jun;76 (Suppl 2):1051.
  doi: 10.1136/annrheumdis-2017-eular.6367

- **Barcelos F**, Martins C, Nunes G, Lopes T, Vaz Patto J, Amaral J, Branco JC, Borrego LM. AB0134 Blood lymphocyte subsets according to the clinical profile in Sjögren's Syndrome. Abstract accepted for publication. *Annals of the Rheumatic Diseases* 2017;76:1094.
  doi: 10.1136/annrheumdis-2017-eular.3030

- **Barcelos F**, Martins C, Monteiro R, Geraldes C, Papoila AL, Cardigos J, Madeira N, Alves N, Vaz-Patto J, Branco J, Borrego LM. Helper and Cytotoxic Follicular T-Cells in Sjogren's Syndrome. *Annals of the Rheumatic Diseases*. 2019; 78: 382-382.
  doi: 10.1136/annrheumdis-2019-eular.5352

- **Barcelos F**, Martins C, Monteiro R, Prussiani T, Sitima M, Vaz-Patto J, Branco J, Borrego LM. EBV Serological Profile affects Circulating Lymphocytes in pSS. *Annals of the Rheumatic Diseases*. 2019; 78: 382-382.
  doi: 10.1136/annrheumdis-2019-eular.7972

- **Barcelos F**, Martins C, Geraldes C, Papoila AL, Cardigos J, Nunes G, Lopes T, Alves N, Vaz-Patto J, Branco J, Borrego LM. Clinical Associations and Diagnostic Potential Of Regulatory-Like B-Cells In Sjogren's Syndrome. *Annals of the Rheumatic Diseases*. 2019; 78: 1538-1539.
  doi: 10.1136/annrheumdis-2019-eular.2614

COMUNICAÇÕES ORAIS

- **Barcelos F**, Martins C, Papoila AL, Geraldes C, Cardigos J, Nunes G, Lopes T, Alves N, Vaz-Patto J, Branco JC, Borrego LM. Association between memory B-cells and phenotypic features of Sjögren's syndrome. *XX Congresso Português de Reumatologia* – Vilamoura, Portugal - maio de 2018.

- **Barcelos F**, Martins C, Geraldes C, Papoila A, Cardigos J, Nunes G, Lopes T, Alves N, Vaz-Patto J, Branco J, Borrego LM. Clinical associations and diagnostic potential of

- regulatory-like B-cells in Sjogren's syndrome. *XXI Congresso Português de Reumatologia* – maio de 2019. Centro de Congressos do Algarve, Vilamoura, Portugal.

- Perfil clínico e distribuição de supopulações linfocitárias na Síndrome de Sjögren são distintos em doentes anti-SSA+. **Barcelos F**, Madeira N, Dias M, Martins C, Cardigos J, Alves N, Vaz Patto J, Branco J, Borrego LM. XXVII *Jornadas Internacionais do Instituto Português de Reumatologia*, 5 a 6 de dezembro de 2019, Lisboa.

COMUNICAÇÕES *POSTER*

- **Barcelos F**, Martins C, Nunes G, Lopes T, Vaz Patto J, Amaral J, Branco JC, Borrego LM. Subpopulações linfocitárias e perfil clínico na Síndrome de Sjögren primária. Poster. *XIX Congresso Português de Reumatologia* – maio de 2017 – Herdade dos Salgados, Albufeira, Portugal.

- **Barcelos F**, Martins C, Vaz Patto J, Nunes G, Lopes T, Cardigos J, Alves N, Amaral J, Branco JC, Borrego LM. Existe desregulação imunitária na síndrome sicca não-Sjögren? Um estudo das subpopulações linfocitárias circulantes. Poster. *XIX Congresso Português de Reumatologia* – maio de 2017 – Herdade dos Salgados, Albufeira, Portugal.

- **Barcelos F**, Martins C, Vaz Patto J, Cardigos J, Alves N, Nunes G, Lopes T, Amaral J, Branco JC, Borrego LM. Caraterísticas clínicas e imunológicas da Síndrome de Sjögren precoce. Poster. *XIX Congresso Português de Reumatologia* – maio de 2017 – Herdade dos Salgados, Albufeira, Portugal.

- **Barcelos F**, Vaz Patto J, Martins C, Cardigos J, Alves N, Amaral J, Borrego LM, Branco JC. As várias faces da síndrome sicca. Poster. *XIX Congresso Português de Reumatologia* – maio de 2017 – Herdade dos Salgados, Albufeira, Portugal.

- **Barcelos F**, Martins C, Vaz Patto J, Nunes G, Lopes T, Amaral J, Cardigos J, Alves N, Branco JC, Borrego LM. Temporal evolution and differences in lymphocyte profiles in Sjögren's Syndrome SSA+. Poster. *XV Congresso da Sociedad Ibérica de Citometria* – maio de 2017 – Centro Cultural de Belém, Lisboa, Portugal.

- **Barcelos F**, Martins C, Nunes G, Lopes T, Vaz Patto J, Amaral J, Branco JC, Borrego LM. Is there immune disregulation in non-sjögren sicca syndrome? A study of blood lymphocyte subpopulations. Poster. *EULAR 2017 Congress* – junho de 2017 – Madrid, Espanha.

- **Barcelos F**, Martins C, Nunes G, Lopes T, Vaz Patto J, Amaral J, Branco JC, Borrego LM. Blood lymphocyte subsets according to the clinical profile in Sjögren's Syndrome. Poster. *EULAR 2017 Congress* – junho de 2017 – Madrid, Espanha.

- **Barcelos F**, Martins C, Monteiro R, Nunes G, Lopes T, Vaz Patto J, Branco JC, Borrego LM. Follicular CD8 T cells: correlation with circulating B cells and disease ativity in primary Sjögren's Syndrome SPR. Poster. *XX Congresso Português de Reumatologia* – 2 a 5 de maio de 2018 – Vilamoura, Portugal.

- **Barcelos F**, Martins C, Papoila AL, Geraldes C, Cardigos J, Nunes G, Lopes T, Alves N, Vaz-Patto J, Branco JC, Borrego LM. Association between memory B-cells and phenotypic features of Sjögren's syndrome. Poster. *EULAR 2018 Congress* – 13 a 16 de junho de 2018 – Amesterdão, Holanda.

- Monteiro R, Martins C, **Barcelos F**, Nunes G, Lopes T, Borrego LM. Follicular helper and follicular cytotoxic T cells in primary Sjögren's Syndrome: clues for an abnormal antiviral response as a pathogenic mechanism. Poster. *3rd International Congress of CiiEM-2018* - 20 a 22 de junho de 2018 – Caparica, Almada, Portugal.

- Monteiro R, Martins C, **Barcelos F**, Nunes G, Lopes T, Borrego LM. Follicular CD8 T cells in primary Sjögren's Syndrome: clues for viral aetiology? Poster. *XLIV SPI Annual Meeting* 2018 – 27 a 29 de junho de 2018 – Lisboa, Portugal.

- **Barcelos F**, Martins C, Monteiro R, Prussiani T, Sítima M, Cardigos J, Alves N, Vaz-Patto J, Branco J, Borrego LM. Epstein-Barr Virus serological profile affects circulating lymphocytes in Sjogren's Syndrome. *XXI Congresso Português de Reumatologia* – 1 a 4 de maio de 2019 – Centro de Congressos do Algarve, Vilamoura, Portugal.

- **Barcelos F**, Martins C, Monteiro R, Madeira N, Geraldes C, Papoila A, Cardigos J, Alves N, Vaz-Patto J, Branco J, Borrego LM. Helper and cytotoxic follicular T-cells in Sjögrens syndrome. *XXI Congresso Português de Reumatologia* – 1 a 4 de maio de 2019 – Centro de Congressos do Algarve, Vilamoura, Portugal.

- **Barcelos F**, Geraldes C, Martins C, Papoila A, Monteiro R, Cardigos J, Madeira N, Alves N, Vaz-Patto J, Borrego LM, Branco J. Valor acrescentado das subpopulações linfocitárias na classificação da Síndrome de Sjögren. *XXI Congresso Português de Reumatologia* – 1 a 4 de maio de 2019 – Centro de Congressos do Algarve, Vilamoura, Portugal.

- **Barcelos F**, Martins C, Monteiro R, Prussiani T, Sítima M, Cardigos J, Alves N, Vaz-Patto J, Branco J, Borrego LM. Epstein-Barr Virus serological profile affects circulating lymphocytes in Sjogrens Syndrome. Annual European Congress of Rheumatology EULAR 2019 – Madrid, Espanha – 12 a 15 de junho de 2019.

- **Barcelos F**, Martins C, Monteiro R, Madeira N, Geraldes C, Papoila A, Cardigos J, Alves N, Vaz-Patto J, Branco J, Borrego LM. Helper and cytotoxic follicular T-cells in Sjögrens syndrome. Annual European Congress of Rheumatology EULAR 2019 – Madrid, Espanha – 12 a 15 de junho de 2019.

No meio do caminho tinha uma pedra
tinha uma pedra no meio do caminho
tinha uma pedra
no meio do caminho tinha uma pedra.

Nunca me esquecerei desse acontecimento
na vida de minhas retinas tão fatigadas.
Nunca me esquecerei que no meio do caminho
tinha uma pedra
tinha uma pedra no meio do caminho
no meio do caminho tinha uma pedra.

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Resumo

A Síndrome de Sjögren primária (SSP) é uma doença inflamatória crónica, de origem autoimune, que se carateriza pela infiltração linfocitária e lesão das glândulas exócrinas.

O diagnóstico diferencial entre uma síndrome seca de origem autoimune (SSP) ou não-autoimune (complexo Sicca) é o principal desafio no diagnóstico da SSP, tendo extrema importância no prognóstico e na terapêutica.

Atualmente, os principais elementos para confirmar o componente autoimune da SSP são a presença de anticorpos anti-SSA e anti-SSB e de infiltrados linfocitários focais na biópsia de glândula salivar *minor* (labial). São desejáveis novos biomarcadores que traduzam a disfunção imune presente na SSP, pois nem sempre é fácil confirmar o diagnóstico, sobretudo nas fases precoces da doença

A hiperatividade das células B, suportada por células T, é responsável por algumas das caraterísticas clínicas e imunológicas mais reconhecíveis da doença, sendo sugerido na literatura que a avaliação de perfis de linfócitos específicos pode constituir um elemento auxiliar no diagnóstico da SSP.

A partir destes pressupostos, este trabalho teve como objetivo principal explorar das subpopulações linfocitárias (caraterizadas por citometria de fluxo), e a sua utilização como biomarcadores auxiliares no diagnóstico e classificação da SSP. Explorámos também a distribuição das subpopulações linfocitárias com os seguintes objetivos secundários: avaliar a sua relação com o status serológico da infeção pelo vírus Epstein-Barr (EBV); avaliar a sua relação com o perfil serológico (anti-SSA) e eventual associação a caraterísticas fenotípicas da SSP; avaliar a relação da morfologia do plexo nervoso corneano sub-basal, quer com o perfil linfocitário, quer com a atividade da doença na SSP.

Para este efeito, foram incluídos 57 doentes com SSP classificados de acordo com os critérios do *American-European Consensus Group* (AECG) [13], 68 doentes com síndrome Sicca sem critérios de SSP, 20 doentes com Artrite Reumatoide (AR) e 24 indivíduos saudáveis, sem queixas secas.

A citometria de fluxo foi a metodologia utilizada na avaliação imunofenotípica das populações celulares, considerando também os ensaios funcionais, utilizados para a

avaliação da produção de citocinas. Na abordagem dos níveis séricos de anticorpos, em particular no contexto da infeção por EBV, recorreu-se a ensaios imunoenzimáticos clássicos.

Resumidamente, a avaliação imunofenotípica implicou a incubação de sangue periférico com um painel pré-validado de anticorpos monoclonais, otimizado para a caraterização de células T (incluindo várias subpopulações dentro das células T CD4 e T CD8, tais como células reguladoras e células foliculares), e de células B (assegurando a caraterização de diferentes estadios desde as células de transição a células de memória e plasmablastos, ou células reguladoras, através de estratégias distintas como a classificação Bm1-5, frequentemente aplicada neste contexto). Foi ainda estudada a capacidade de as células T secretarem citocinas como a IL-17 e a IL-21, após estimulação com PMA e ionomicina. O painel de anticorpos monoclonais incluiu os seguintes marcadores: CD3, CD4, CD8, CD19, CD24, CD25, CD27, CD38, CD45, CD45RA, CCR6, CCR7, CXCR3, CXCR5, anti-IgD, anti-IgM. Por fim, para a obtenção de contagens absolutas, utilizou-se uma estratégia de plataforma única, com a utilização de tubos TruCount™, com esferas calibradas.

A aquisição das amostras foi efetuada num citómetro BD FACS Calibur™ (BD Biosciences, San Jose, CA, EUA), equipado com dois lasers, permitindo a avaliação de quatro fluorescências distintas. O software de aquisição foi o MultiSet™ (BD Biosciences), e para efeitos de análise, foram usados ainda os softwares Cell Quest 3.3™ (BD Biosciences) e Infynicyt™ (Cytognos, Salamanca, Espanha). A análise estatística utilizou o programa Graph Pad Prism (GraphPad Software, San Diego, CA, EUA).

Em linha com a literatura, no nosso estudo, a classificação clássica IgD/CD27 verificou a existência de uma redução dos números absolutos e percentagens de células B de memória (sobretudo sem switch) e aumento das células B naïve em doentes com SSP. A avaliação das células B de acordo com a classificação Bm1-Bm5 confirmou também o aumento das subpopulações Bm2 e Bm2´, e a diminuição das eBm5 e Bm5. Na razão entre as células Bm2+Bm2' e as eBm5+Bm5 foi possível aplicar cut-offs na discriminação entre SSP e controlos, ainda que com menor sensibilidade e especificidade em relação aos anteriormente descritos.

Ainda assim, doentes com SSP com valores mais baixos células B de memória apresentavam maior frequência de caraterísticas fenotípicas da doença, como FS≥1, anticorpos anti-SSA, anticorpos anti-SSB, ANA e FR, e aumento das imunoglobulinas. Estes resultados, que se associam a menores valores de células B de memória e à disfunção autoimune na SSP estão de acordo com a hipótese de que a diferenciação das células B periféricas que migram para as glândulas salivares favorecem a formação de plasmócitos e a consequente produção de autoanticorpos.

Verificámos também que os doentes Sicca apresentaram percentagens de células B naïve e de memória semelhantes às de doentes com SSP, e distintas das dos controlos, enquanto que os valores absolutos destas populações foram superiores aos da SSP, mas inferiores aos controlos. Considerando o desafio diagnóstico dos doentes Sicca, o seu perfil linfocitário intermédio entre SSP e controlos, evidente nos doentes Sicca, sugere a presença de disfunção imunológica, podendo alguns deles corresponder a formas ligeiras ou precoces de SSP.

As células T foliculares efectoras (Tfh) têm um importante papel na patogénese da SSP, onde foi descrito um aumento das células Tfh circulantes e uma expansão da sua diferenciação nas GS [21,22]. Ora, a marcada expansão de células Tfh e a simultânea presença de células Tfc nas glândulas salivares na SSP pode relacionar-se com mecanismos de resposta a um agente infecioso sialotrópico, como o EBV. Neste estudo, apesar dos números absolutos de células T $CXCR5^+$ estarem diminuídos na SSP, encontrámos um aumento das células T produtoras de IL-21, quer $CD4^+$ (Tfh), quer $CD8^+$ (Tfc). Além disso, as células Tfc estavam aumentadas nos doentes com SSP com maior atividade da doença, tendo sido possível identificar uma correlação positiva entre os números absolutos de células T $IL21^+CD8^+$ e o ESSDAI, sugerindo um papel destas células na patogénese da doença. Curiosamente, o aumento das células T produtoras de IL-21 lembra o perfil de uma infeção viral crónica, e de facto, em relação aos marcadores serológicos de EBV, os doentes com SSP apresentaram maior prevalência de anti-EBV EA-D IgG, em comparação com doentes com AR e controlos saudáveis.

Reconhecendo este aumento de prevalência de anti-EBV EA-D IgG e uma prevalência importante de anti-EBNA IgG em SSP, subdividimos estes doentes em 3 grupos de acordo com o padrão serológico para EBV. Doentes com serologia positiva para EBV apresentavam ESSDAI superior ao de doentes sem evidência de infeção (G3). Em doentes com perfil serológico de infeção recente/reativação de EBV, a frequência de doentes com PSS ativa era também tendencionalmente superior à dos doentes com infeção antiga. Do ponto de vista celular também se verificaram alterações, com os doentes com evidência de infeção por EBV, em especial infeção recente/reativação, a apresentarem maior representação das subpopulações T pro-inflamatórias Th1 e Tfh1, o que pode indicar a influência viral na diferenciação preferencial destas células. Adicionalmente, doentes com infeção por EBV recente/reativação apresentavam níveis superiores de células B de transição e de plasmablastos, o que pode indicar a influência do EBV na modulação das respostas imunes na SSP e na expressão clínica, conforme sugerido pela manifestação mais precoce da doença e maior frequência de doença ativa nestes indivíduos.

Na SSP, a presença de anticorpos anti-SSA também se associa a caraterísticas fenotípicas distintas, pelo que a investigação de diferenças nas populações linfocitárias nestes doentes permitiu identificar não só linfopénia T, mas também redução dos números

de células B, em paralelo com maiores proporções de células T $CD4^+$ e T $CD8^+$ produtoras de IL-21, uma acentuada redução das células B de memória e um aumento das B naïve B, em comparação com controlos saudáveis. Além disso, os doentes SSA^+SSP apresentaram maior atividade de doença, e verificou-se a existência de associações entre o ESSDAI e várias subpopulações linfocitárias, incluindo as populações T IL-21^+CD4^+, IL-21^+CD8^+ e Tregs. A correlação positiva entre o ESSDAI e as percentagens de células $CXCR5^+$Th1 (Tfh1), observada apenas nos doentes SSA^+SSP, e ainda mais significativa nos doentes com atividade de doença moderada/elevada (ESSDAI ≥5), sugere que os mecanismos patogénicos subjacentes à dinâmica das células T e às manifestações da doença diferem de acordo com a presença de anticorpos anti-SSA.

Considerando que são necessários novos biomarcadores que complementem a informação fornecida pelos testes convencionais, na abordagem da xeroftalmia, observámos que estes testes se mostraram alterados em ambos os grupos de indivíduos com queixas secas (SSP e Sicca). Na morfologia do plexo nervoso corneano sub-basal também se verificaram características distintivas, com as alterações do plexo nervoso corneano sub-basal a apresentaram uma boa associação com o teste de Schirmer e o tempo de quebra do filme lacrimal. A imagem da córnea por microscopia confocal demonstrou um bom desempenho na identificação de indivíduos com olho seco, apresentando-se como um instrumento promissor na avaliação objetiva da xeroftalmia, já que é uma metodologia não-invasiva e menos dependente de fatores ambientais e relacionados com o doente.

Em relação às alterações morfológicas do plexo nervoso corneano sub-basal atribuíveis ao olho seco, foram mais evidentes nos doentes com SSP com maior duração e atividade da doença, encontrando-se uma forte correlação negativa entre o ESSDAI e comprimento (CNFL) e a densidade das fibras (CNFD) em doentes com maior atividade da doença (ESSDAI ≥5), também com piores parâmetros oculares (menor CNFD e CNFL). Nos doentes com ESSDAI≥5, encontrou-se correlação positiva entre o CNFL e o número de Tregs, e uma forte correlação negativa entre as células Tfh1 e ambos os parâmetros morfológicos do plexo corneano (CNFL e CNFD). Correlações positivas entre o CNFL e células de memória ou células $CD24^{Hi}CD27^+$ foram também identificadas, havendo por outro lado correlação negativa entre a CNFL e células $IL21^+CD8^+$ T, que pode apoiar a influência da disfunção imune na lesão da córnea.

Finalmente, a avaliação da inclusão de subpopulações linfocitárias nos critérios AECG e ACR/EULAR permitiu reconhecer que algumas subpopulações demonstraram uma aceitável precisão na distinção entre SSP e Sicca. Ao serem incluídas no modelo multivariado para avaliar se melhoravam o desempenho dos critérios de classificação, populações como células B de memória com switch, ou células Bm1 e células Tfh,

permitiam aumentar o poder discriminativo dos critérios de classificação ACR/EULAR e AECG, respetivamente.

Em suma, este foi o primeiro estudo a identificar os valores de cut-off nas subpopulações de linfócitos B definidas pela classificação IgD/CD27, comprovando a existência de cut-offs em subpopulações de células B que claramente distinguem SSP de controlos saudáveis, que poderão vir a ser utilizados até em doentes Sicca, cujo perfil se assemelhe ao da SSP, podendo representar formas ligeiras ou precoces da doença.

Adicionalmente, demonstrámos que em doentes com SSP a presença de menores contagens de células B de memória está associada a uma evolução mais prolongada e a doença mais ativa, o que abre perspetivas para que a avaliação desta população celular possa ter também um papel prognóstico. O nosso estudo explora pela primeira vez também a associação entre os padrões serológicos do EBV e o perfil de subpopulações linfocitárias na SSP, suportando a presença de uma amplificação do ambiente folicular na SSP, que se traduz pela secreção aumentada de IL-21 por células T $CD4^+$ and $CD8^+$ (foliculares), com eventual associação ao background viral. Aliás, é a primeira abordagem das células Tfc ($CXCR5^+CD8^+$e $IL\text{-}21^+CD8^+$) no contexto de autoimunidade, com a associação entre estas células e a atividade da doença na SSP a sugerir o seu envolvimento na patogénese da epitelite autoimune.

Os nossos resultados indicam ainda que os doentes SSA^+SSP apresentam maior atividade de doença, incluindo marcadores de ativação B como hipergamaglobulinémia, FR e tumefação das GS. Além disso, a associação entre a atividade da doença e as células T $IL\text{-}21^+CD4^+$ e $IL\text{-}21^+CD8^+$ (foliculares), para além de poder traduzir o envolvimento destas células num fenótipo mais ativo da doença, poderá indicar um futuro papel da caraterização destas células no prognóstico e monitorização da doença.

Por outro lado, os nossos dados sugerem que a lesão dos nervos corneanos pode acompanhar a atividade sistémica da doença, e relacionar-se com a maior desregulação imune nestes doentes, indicando também uma possível evolução paralela do dano às fibras nervosas da córnea e da atividade imunológica da SSP. Deste modo, a avaliação morfológica do plexo nervoso corneano sub-basal em doentes com SSP pode fornecer indícios do grau de disfunção imunológica e da dinâmica das populações linfocitárias, assumindo-se o presente estudo como o primeiro na literatura a avaliar a relação entre as caraterísticas morfológicas do plexo corneano sub-basal e a distribuição das subpopulações linfocitárias na SSP.

Por último, o nosso estudo abre perspetivas para a utilização de populações linfocitárias nos critérios ACR/EULAR, com vista à melhoria do seu desempenho,

considerando os desafios que ainda se reconhecem atualmente no diagnóstico e classificação da SSP.

Abstract

Sjögren's syndrome (SjS) is a chronic systemic autoimmune disease, characterized by lymphocytic infiltration of the exocrine glands.

The main challenge in the diagnosis of SjS is the discrimination between sicca symptoms caused by autoimmune processes (SjS) from a non-autoimmune Sicca syndrome, which is extremely important for prognosis and therapy.

Presently, the main elements to confirm the autoimmune origin in a suspicious case of SjS are the presençe of anti-SSA and anti-SSB autoantibodies, and the demonstration of focal lymphocytic infiltration on histology of minor labial SG biopsy. New autoimmunity biomarkers are needed for SjS, since diagnosis is sometimes difficult, especially in the early phases of the disease.

The B-cell hyperactivity supported by T-cells is a Hallmark of SjS, and responsible for the most recognizable clinical and immunological features of the disease. It has been suggested that specific lymphocyte subset profiles, typical of SjS, may be helpful in the diagnosis of the disease.

Considering this, our main purpose was to characterize lymphocyte subsets through flow cytometry and explore their utility as a biomarker for the diagnosis and classification of SjS.

We also had complimentary objectives, namely: to evaluate the association between lymphocyte subsets distribution and the serological profile of EBV infection; to evaluate the association between anti-SSA positivity and clinical and immunological features of SjS, including lymphocyte subset profiles and disease activity; to assess the corneal sub-basal nerve plexus morphology and explore its association with lymphocyte subsets and disease activity in SjS.

We included 57 SjS patients, classified according to the *American-European Consensus Group* (AECG) criteria, 68 non-Sögren Sicca patients, 20 Rheumatoid arthritis patients, and 24 healthy non-sicca patients.

Flow cytometry procedures were used for the immunophenotypic evaluation of lymphocyte subsets, as well as for the functional protocols, through cytokine production

assessment. Regarding the evaluation of circulating antibodies, especially in the context of EBV infection, classic immunoenzymatic assays were used.

In brief, for the immunophenotyping protocols, peripheral blood samples collected in EDTA-coated tubes were processed incubated with a pre-validated panel of monoclonal antibodies (mAbs), optimized for characterization of T cells (including follicular and regulatory subsets of CD4+ and CD8 T-cells) and B-cells (assuring the characterization of distinct evolution stages from transitional to memory B cells and plasmablasts, with distinct strategies such as the Bm1-5 and the IgD/CD27 classification schemes, commonly used in autoimmunity context). We also assessed the production of IL-17 and IL-21 by T cells after stimulation with PMA and ionomycin. The mAb pane used consisted of: CD3, CD4, CD8, CD19, CD24, CD25, CD27, CD38, CD45, CD45RA, CCR6, CCR7, CXCR3, CXCR5, anti-IgD, anti-IgM. A single platform strategy was used to obtain absolute counts of all cell subsets (TruCount™ tubes with calibrated spheres).

All samples were acquired in a 4-colour cytometer (BD FACS-Calibur™ (BD Biosciences, San Jose, CA, EUA). MultiSet™ (BD Biosciences) software, complemented with CellQuest 3.3 (BD Biosciences) software were used for acquisition and analysis purposes and Infinicyt 2.0 (Cytognos S.L., Salamanca, Spain) software was also used for more differentiated subset analysis.

Statistical analysis was performed with Graph Pad Prism (GraphPad Software, San Diego, CA, EUA).

Using the classic IgD/CD27 classification, we have confirmed the decrease in memory B cell absolute counts and percentages (especial the unswitched subset) and the increase in naïve B cells in SjS, in line with the literature.

B cell characterization according to the Bm1-Bm5 classification also confirmed the increase of Bm2 and Bm2´ subsets and the decrease of eBm5 and Bm5 cells. We calculated the cut-off in the Bm2+Bm2'/eBm5+Bm5 ratio that better discriminated between SjS and controls, but obtained a lower sensitivity and specificity compared to other authors.

Nevertheless, SjS with lower levels of memory B cells presented more frequently typical phenotypic features ao the disease, such as focus score 1, anti-SSA and anti-SSB antibodies, ANA, rheumatoid factor (RF), and increased immunoglobulin. These results, connecting the immune dysfunction to lower levels of memory B-cells support the hypothesis that the peripheral B cell that migrates to the salivary gland differentiate preferentially in plasma cells that consequently produce autoantibodies.

Curiously, Sicca patients presented percentages of memory and naïve B-cells similar to SjS and clearly distinct from controls, whereas absolute counts were in between SjS and controls. These results suggest that some Sicca patients have indeed immune dysfunction and, therefore, may correspond to mil dor early forms of SjS.

Follicular helper T cells (Tfh) play a prominent role in SjS pathogenesis, and an increase in their circulating numbers has been described in SjS, as well as an increase in their differentiation within the salivary glands têm [21,22]. This Tfh expansion, associated with the presence of cytotoxic follicular T cells (Tfc) resembles the anti-viral response to syalotropic virus such as EBV. Indeed, regarding the EBV serologic markers, SjS patients presented a higher prevalence of anti-EBV EA-D IgG, compared to RA patients and controls.

In our study, despite lower absolute counts of $CXCR5^+$ T cells in SjS, we found an increase in IL-21-producing $CD4^+$ (Tfh) and $CD8^+$ (Tfc) T cells. Additionally, Tfc cells were increased in SjS patients with greater disease activity, with a positive correlation between the ESSDAI and the absolute counts of $IL21^+CD8^+$ T cells, suggesting a role of this cells in the disease pathogenesis.

Considering the increased prevalence of anti-EBV EA-D IgG in SjS, as well as a considerable prevalence of anti-EBNA IgG, we sorted these patients into 3 groups, according to the EBV serological profile. Patients with positive EBV serology (G1+G2) had higher ESSDAI compared to patients without evidence of EBV infection (G3). Considering patients with positive EBV serology, SjS disease activity was more frequent in patients with a profile of recent infection/reactivation, compared to patients with latent infection.

Patients with a profile of recent infection/reactivation also presented increased pro-inflammatory Th1 e Tfh1, which may suggest the influence of the viral infection in the differentiation of these cells. These patients also presented increased levels of transitional B cells and plasmablasts, suggesting the influence of EBV in the modulation of the immune response in SjS, and consequently in the clinical profile, since these patients also presented an earlier onset and more frequently an active phase of the disease.

The presence of anti-SSA antibodies in SjS is associated with distinct clinical and serological features. In these patients, we also identified lymphocyte subsets disturbances.

Despite decreased T and B cells absolute counts, we identified an increase in IL-21-producing $CD4^+$ and $CD8^+$ T cells, a pronounced decrease in memory B cells and an increase in naïve B cells, compared to controls.

Also, SSA^+SSP patients presented higher disease activity, and we found an association between the ESSDAI and several lymphocyte subsets, including $IL\text{-}21^+CD4^+$, $IL\text{-}21^+CD8^+$ and Tregs. The positive correlation between the ESSDAI and $CXCR5^+$Th1 (Tfh1) cells was observed only in SSA^+SSP patients, with more significance when considering patients with a moderate/high level of disease activity (ESSDAI ≥5). These results suggest that the pathogenic mechanisms underlying the T cell's dynamics and disease activity are distinct according to the presence or absence of anti-SSA antibodies.

Confocal microscopy is a non-invasive method that may complement the routine objective ocular tests. The assessment of the corneal nerve plexus morphology has a good performance in the identification of dry eye patients.

We explored this promising biomarker in our patients with dry eye (SjS and Sicca) and found a good correlation with conventional dry eye tests in these patients

Morphologic changes in the corneal sub-basal plexus were more pronounced in SjS with longer disease evolution, as well as in patients with higher disease activity. In that regard, we found the worst também corneal never parameters (lower length and density) and a strong negative correlation between the ESSDAI and the nerve length (CNFL) and density (CNFD) in patients with moderate/high disease activity (ESSDAI ≥5). In the same group of patients, a positive correlation was also found between the CNFL and the absolute counts of Tregs, and a strong negative correlation between Tfh1 and both corneal parameters.

A positive correlation was also found between the CNFL and $CD24^{Hi}CD27^{+}$ B-cells, and a negative correlation was identified between the CNFL and $IL21^{+}CD8^{+}$ T cells.

Taken together, this data may suggest the influence of immune dysfunction in corneal damage.

Finally, we assessed the added value of the inclusion of lymphocyte subsets in the AECG and ACR/EULAR criteria, identifying some subsets that showed an acceptable performance in the discrimination between SjS and Sicca. When added to the multivariate model of the criteria, switched memory B cells improved the discriminative power of the ACR/EULAR criteria, whereas Bm1 and Tfh cells improved the discriminative power AECG.

In conclusion, our study was the first to identify cut-off values in lymphocyte subsets classified according to the IgD/CD27 classification. We have identified B cell subsets that clearly distinguish SjS from healthy controls, and that may even be helpful in the identification of Sicca patients with SjS-like lymphocyte profile, and therefore may have early or mild forms of SjS.

Additionally, we have shown that lower memory B cells counts in SjS are associated with longer disease evolution and more active disease. Therefore, assessing these cells may have prognostic value in SjS.

We have also explored, for the first time, the association between EBV serological patterns and the lymphocyte subset profile. The increased secretion of IL-21 by $CD4^{+}$ and $CD8^{+}$ (follicular) T cells supports an amplified follicular environment in SjS, which may be related to a viral background. This was the first study to assess Tfc ($CXCR5^{+}CD8^{+}$ and $IL-21^{+}CD8^{+}$) cells in the context of autoimmunity, and the association found between these

cells and disease activity in SjS suggests their involvement in the pathogenesis of autoimmune epithelitis.

Our results showed that $SSA^{+}SSP$ patients presented increased disease activity, including markers of B cell activation such as hipergamaglobulinémia, RF and salivary gland enlargement. Additionally, the association between disease activity and IL-$21^{+}CD4^{+}$ e IL-$21^{+}CD8^{+}$ (follicular) T cells may not only confirm the involvement of these cells in a more active disease profile but may also constitute a marker of prognosis and disease monitorization.

We also suggested that corneal nerve damage may reflect the systemic disease activity and traduce a greater immune dysregulation in these patients. An interesting correlation was found between the worst corneal nerve features and lymphocyte subsets, which may link immune dysfunction and ocular damage. Therefore, the assessment of the corneal nerve plexus morphology may be a non-invasive method to evaluate disease severity and potentially, disease activity. Since we were the first to associate lymphocyte subset profiles and corneal nerve morphology, further studies are needed to try to identify SjS specific corneal nerve patterns for clinical practice.

Finally, our study showed that the utilization of lymphocyte subset profiles may be a way of improving the ACR/EULAR criteria, considering that a more diverse set of objective items may help to overcome the difficulties in the diagnosis and classification of SjS.

Índice

Lista de Abreviaturas, Acrónimos e Símbolos

-	Expressão de antigénio negativa
+	Expressão de antigénio positiva
ACR	American College of Rheumatology
ANA	Anticorpo antinuclear
AR	Artrite Reumatoide
ASCs	Células secretoras de anticorpos (“Antibod-secreting cells”)
BAFF	*B cell-activating factor*
BCR	Recetor de célula B (*B cell receptor*)
Bm	Linfócito B Maduro
Breg	Linfócito B regulador
CCR6 C-C-	*chemokine receptor* 6
CD	*Cluster* de Diferenciação
CG	Centro germinativo
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR5 C-X-C	*chemokine receptor type* 5
DC	Célula dendrítica
Dim/Low	Baixa intensidade de expressão de antigénio
ESSDAI EULAR	Sjögren’s Syndrome Activity Index
EULAR	European League Against Rheumatism
FasL	Fas ligando
Foxp3	*Forkhead box* p3
FR	Fator reumatóide
GS	Glândula salivar
Hi	Alta intensidade de expressão de antigénio
HLA	*Human leukocyte antigen*
HLA-DR	*Human leukocyte antigen – antigen D related complex*

IDO	*Indoleamine 2,3-dioxygenase*
IFN	Interferão
Ig	Imunoglobulina
IL	Interleucina
LES	Lupus Eritematoso Sistémico
MALT	*Mucosa-associated lymphoid tissue*
MHC	Complexo *Major* de Histocompatibilidade
NK	célula *Natural Killer*
pDCs	Célula dendrítica plasmacitóide
SSA	*Sjögren's syndrome antigen* A
SSB	*Sjögren's syndrome antigen* B
SSP	Síndrome de Sjögren primária
Tc	Linfócito T citotóxico
TCR	Recetor de célula T (T cell receptor)
Tfc	Linfócito T folicular citotóxico
Tfh	Linfócito T folicular efetor ("helper")
TGF	*Transforming growth factor*
Th	Linfócito T efetor ("helper")
TNF	*Tumor necrosis factor*
Treg	Linfócito T regulador

CAPÍTULO I. Introdução

1. O SISTEMA IMUNITÁRIO

O Sistema Imunitário consiste num conjunto de células, moléculas e processos que interagem para proteger o organismo de agressões causadas por micróbios (bactérias, fungos, parasitas), vírus, toxinas, e células cancerígenas [23].

Para além das barreiras de proteção estruturais e químicas (pele, epitélio respiratório, digestivo, urogenital, etc), o sistema imunitário é composto por duas linhas de defesa: a imunidade inata e a imunidade adaptativa. As células do sistema imunitário inato e adaptativo cooperam em órgãos e locais de barreira para estabelecer imunidade específica [24].

1.1. Imunidade Inata

A imunidade inata representa a primeira linha de defesa do organismo perante agentes patogénicos. É independente de antigénio, ou seja, não-específico, e é ativado logo após o encontro com o antigénio estranho [25]. A imunidade inata não possui memória imunológica, sendo incapaz de reconhecer o mesmo agente patogénico caso este venha a ser encontrado posteriormente [23].

As células inatas fulcrais nos tecidos são os macrófagos, que através da fagocitose removem debris celulares, micro-organismos e substâncias estranhas. Os macrófagos residentes são células de grande longevidade, que se estabelecem nos seus tecidos-alvo ainda durante o período pré-natal, altamente especializados e com capacidade de se auto-renovarem [26,27]. Exemplos de macrófagos residentes são as células da microglia no cérebro, os macrófagos alveolar no pulmão, os macrófagos da polpa vermelha do baço, e as células de Kupfer no fígado [28–30].

As células dendríticas (DCs) são as células da imunidade inata que estabelecem a ligação com a imunidade adaptativa, através do seu papel de células apresentadoras de antigénios (APCs), fundamental para a ativação dos linfócitos T. Devido à sua capacidade única de transportar antigénios das zonas de mucosa para os gânglios linfáticos locais, as DC convencionais desempenham o papel crucial de apresentar antigénios para induzir as respostas T [31,32].

As células dendríticas plasmacitóides (pDCs) são um pequeno subtipo de DCs que infiltram os tecidos e secretam grandes quantidades de interferão (IFN) dos tipos I e III em

resposta a infeções virais, e que são particularmente abundantes em estado latente nas amígdalas [33,34].

Existem também células linfoides inatas, como as células *natural killer* (NK) abundantes no sangue e tecidos, bem como as células linfoides inatas de tipo efetor (ILCs) ILC1, ILC2, e ILC3 que são maioritariamente células residentes em tecidos [35,36]. As células NK cells são importantes na resposta imune anti-tumor e anti-viral, enquanto as ILCs atuam sobretudo na promoção da reparação dos tecidos [37].

1.2. Sistema Imune Adaptativo

As funções principais das respostas imunes adaptativas são: o reconhecimento de antigénios "não-self" específicos, distinguindo-os de antigénios do "self"; a geração de vias efetoras específicas para o agente patogénico de modo a eliminá-lo a ele ou às células por ele infetadas; e o desenvolvimento de memória imunológica que possa ser rapidamente mobilizada para eliminar um agente patogénico específico caso ocorra um novo encontro [38]. As respostas imunes adaptativas são a base da imunização eficaz contra doenças infeciosas.

As células da imunidade adaptativa incluem as células T antigénio-específicas, cuja ativação e proliferação é induzida pelas APCs, e as células B, que se diferenciam em plasmócitos para produzir anticorpos [24].

O sistema imunitário adaptativo carateriza-se pelo extenso repertório de recetores para antigénios específicos expressos pelos linfócitos T e B, e pela capacidade de manter a memória imunológica durante décadas [23].

1.2.1. Linfócitos B

Como parte da imunidade adaptativa os linfócitos B constituem um braço crítico do sistema imunitário, sendo responsáveis pela manutenção, a curto e longo prazo, da resposta humoral mediada por anticorpos. Aliado à produção de anticorpos, os linfócitos B desempenham também importantes funções das quais se destacam a apresentação de antígenos, modulação da diferenciação e sobrevivência de células T e produção de citocinas reguladoras e pró-inflamatórias [39,40].

De uma forma geral, o compartimento de linfócitos B maturos pode ser dividido em três populações de acordo com a sua ontogenia e localização anatómica: células B-1, células B da zona marginal (B MZ), e células B foliculares (B FO) ou B convencionais B2 [41,42].

As células B-1 são formadas a partir de progenitores no fígado fetal, persistindo após o período neonatal com capacidade de se auto-renovarem, e com pouca contribuição da medula óssea na idade adulta [43,44]. Estas células encontram-se principalmente nas cavidades pleurais e peritoneais e são classificadas como células *innate-like*, estando, portanto, associadas a respostas T-independentes com produção de anticorpos do tipo IgM, nas respostas mais precoces a antigénios bacterianos [45,46]. Apesar de, em humanos, a existência destas células não ser ainda clara, parecem existir populações homólogas a estas, com um padrão fenotípico distinto mas com semelhantes funções [44].

Como o nome indica, as células B da zona marginal (MZ) localizam-se na zona marginal, que corresponde às regiões mais externas da polpa branca do baço. Nos humanos, as células B MZ caracterizam-se fenotipicamente por elevados níveis de IgM e CD21, níveis moderados de CD19, CD20 e CD1c, e baixos níveis de IgD [47,48], tendo sido também descritas populações com fenótipo e genótipo semelhante no sangue periférico, suportando a capacidade circulatória destas células [49,50]. São igualmente consideradas células *innate-like* com uma rápida capacidade de resposta à presença de agentes na corrente sanguínea, resultando na produção rápida de, sobretudo, IgM e diferenciação em plasmócitos de vida curta [51]. Esta resposta, também de uma forma T-independente, permite o reconhecimento de polissacarídeos presentes nas estruturas capsulares bacterianas [52]. Este reconhecimento está fortemente dependente e associado à sinalização por via da ativação dos recetores *toll-like* (TLR) sendo que doentes com deficiências nestas vias apresentam perda seletiva destas células [53].

No entanto, ao contrário das células B-1, as células B MZ parecem estar também envolvidas em respostas T-dependente em resposta a alguns antigénios proteicos, assim como o transporte de antigénios para os folículos esplénicos onde vão ser capturados pelas células dendríticas foliculares [54,55]. De facto, estudos *in vitro* demonstraram que estas células têm uma capacidade de apresentação antigénica às células T mais elevada do que as próprias células B foliculares, ainda que desconhecida a sua participação na formação de centros germinativos (CG) *in vivo* [56,57]. Apesar de produzirem maioritariamente IgM, estudos mostram, no entanto, que estas células podem também sofrer processos de hipermutação somática e de *class-switch*, com consequente produção de certas subclasses de IgG e IgA, no seguimento de ambos os tipos de resposta [58–60]. No ratinho, a expressão de CD1d (CD1c em humano) é uma molécula semelhante às moléculas de MHC classe 1, e são importantes para a apresentação de antigénios lipídicos às células NKT. [61,62].

As células B MZ representam, assim, uma população bastante versátil pela sua rápida capacidade em produzir anticorpos de maneira T-independente e T-dependente e com possíveis implicações no desenvolvimento de doenças autoimunes. Para alem disso, estas células permitem colmatar a falha temporal desde o início de uma infeção até ao aparecimento de anticorpos IgG de alta afinidade produzidos pelas células B foliculares.

Apesar de terem funções relativamente distintas, as células B MZ partilham o mesmo processo de desenvolvimento na medula óssea das células B foliculares, as quais constituem a maior e mais bem caracterizada população de linfócitos B. Como vamos ver, as células B foliculares circulam entre os vários órgãos linfoides secundários e podem ser encontradas em grande quantidade nos folículos dos gânglios linfáticos e no baço. São precisamente estas células que estão na génese dos CGs, onde vão suceder-se processos-chave da imunidade humoral adaptativa: a hipermutação somática, a maturação da afinidade e a troca do isotipo (class switch), discutido mais à frente.

Precursores e maturação dos linfócitos B

As células da linhagem linfoide que incluem os linfócitos B, T e NK derivam de um progenitor comum, conhecidas como as células progenitoras comuns linfoides (CPLs), as quais, por sua vez, derivam de células estaminais hematopoiéticas multipotentes (HSCs) presentes na medula óssea [63–65].

O desenvolvimento e diferenciação dos linfócitos B ocorre de forma gradual, com a aquisição de propriedades essenciais para a função destas células, e a consequente perda de características imaturas. O microambiente especializado da medula óssea proporciona os sinais necessários para o normal desenvolvimento e diferenciação dos linfócitos B, promovendo a ativação de genes essenciais, e a indução de sinais de sobrevivência estritamente necessários ao longo do desenvolvimento. A produção destes sinais é da responsabilidade das células estromais da medula óssea. Por um lado, estas células formam zonas especificas de contacto direto com os linfócitos B em desenvolvimento através de interações entre moléculas de adesão e respetivos ligandos. Por outro, fornecem citocinas e quimiocinas que vão controlar a diferenciação e a proliferação dos linfócitos B [66–68]. Dos sinais produzidos pelas células estromais destacam-se a IL-7 (papel controverso no desenvolvimento B humano), essencial para as fases iniciais do desenvolvimento B e sobrevivência das CLPs, a TSLP, e a quimiocina CXCL12 [69–72].

O comprometimento das células CLP para a linhagem B ocorre aquando da expressão de fatores de transcrição específicos da linhagem B, nomeadamente E2A, EBF e, principalmente, Pax5. [72–74]. Nesta fase do desenvolvimento as células B designam-se por **células pro-B**, e já não é possível seguirem outra via de diferenciação que não a da linhagem B [75,76]. As diferentes fases e processos do desenvolvimento da célula B estão representadas na **Figura 1.**

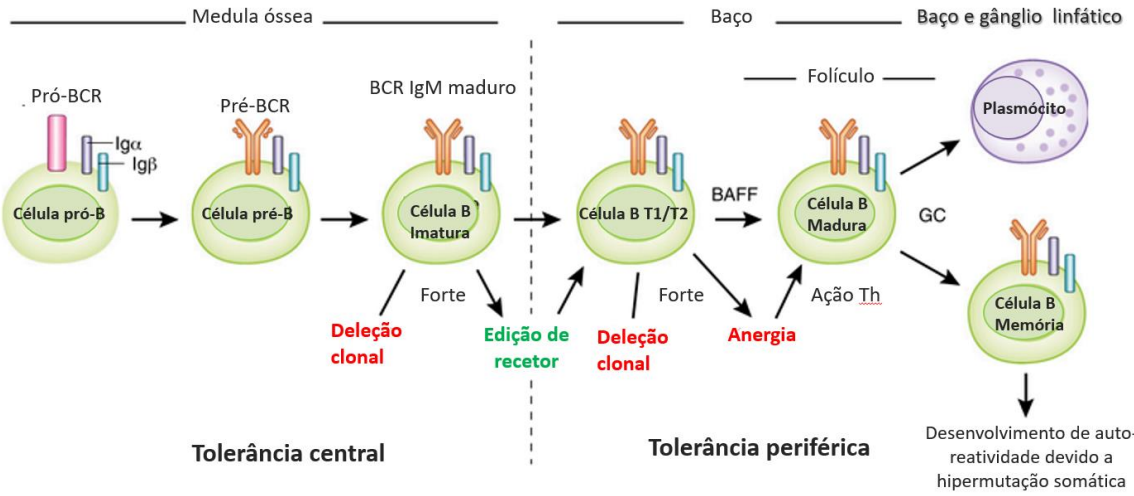

Figura 1. Desenvolvimento dos linfócitos B e mecanismos de tolerância. Adaptado de Hoffman (2016) [77].

O desenvolvimento dos linfócitos B tem início na medula óssea e termina nos tecidos linfoides periféricos, como o baço. Na medula óssea, o desenvolvimento é sequencial desde os linfócitos pro-B, linfócitos pré-B, expressão de IgM, até à expressão de um BCR completo. Os linfócitos B imaturos com alta reatividade para antigénios do próprio sofrem deleção clonal ou promovem o rearranjo dos genes da imunoglobulina. Este processo é conhecido como edição do recetor, que impede a formação de células potencialmente auto-reativas e permite a entrada destas para o *pool* de linfócitos B de transição. Os linfócitos B de transição dependem da sinalização por BAFF para sobreviverem e se diferenciarem em linfócitos B maduros no baço. Os linfócitos B de transição 1 e 2 (T1 e T2) com elevada auto-reatividade morrem por deleção clonal ou permanecem na periferia dos folículos esplénicos e entram num estado de anergia que pode ser revertido após interação com células T e permitir a entrada no *pool* de linfócitos B maduros. Os linfócitos B maduros que são ativados por antigénios estranhos iniciam a formação de centros germinativos (CG) onde se vão originar linfócitos B de memória com troca de isotipo e células plasmáticas. Por fim, durante o processo de hipermutação somática, uma pequena porção de linfócitos B de memoria adquirem auto-reatividade devido aos rearranjos do gene da imunoglobulina, persistindo como clones auto-reativos IgG+ na periferia.

Nesta fase, as células pro-B iniciam os processos de recombinação V(D)J, isto é, ocorre o rearranjo genético do locus da cadeia pesada da imunoglobulina pela ação das enzimas RAG-1 e RAG-2, cuja expressão é induzida pelos fatores de transcrição E2A e EBF. Alem disso, Pax5 induz a síntese de duas importantes proteínas, o CD79a (Igα), componente do recetor da célula B, e o CD19, um co-recetor da célula B e o principal marcador usado na caracterização da linhagem B [74,78,79]. Este rearranjo conta também com a ação da enzima TdT, a qual vai inserir nucleótidos de forma aleatória nas junções dos segmentos rearranjados, aumentando assim a diversidade do reportório de anticorpos produzidos [80,81].

O rearranjo deste locus leva à produção de cadeias pesadas do tipo μ, que vão ser temporariamente colocadas à superfície da célula para serem funcionalmente testadas, ainda na ausência das cadeias leves, que ainda não foram rearranjadas. Neste passo, as células pro-B produzem duas proteínas 'suplentes' com semelhança estrutural às cadeias leves, **λ14.1** e a **VpreB,** que conseguem parear com a cadeia pesada μ, formando, assim, o recetor da células pre-B, ou **pre-BCR** [82].

A montagem de um pre-BCR funcional permite à célula pro-B receber importantes sinais de sobrevivência, promovendo a passagem para a próxima fase do desenvolvimento, as **células pre-B1** [82–84]**.** Desta forma, as células pro-B que falham em produzir uma cadeia μ funcional, são eliminadas, correspondendo a cerca de 45% de células pro-B. No entanto, se um primeiro rearranjo não for bem-sucedido, as células pro-B podem entrar numa segunda fase de rearranjo, ou, caso esta falhe, recorrer ao segundo alelo para efetuar este rearranjo. Assim, a formação de um pre-BCR funcional que consiga sinalizar para o interior da célula, consiste num importante checkpoint na transição da fase de células pro-B para pre-B [85].

Esta sinalização depende das proteínas BLNK e Btk (Bruton's tyrosine kinase) [86,87] e a sua importância reflete-se em imunodeficiências como a agamaglobulinémia de Bruton ligada ao X (XLA), na qual não há produção de células B maduras, devido a mutações no gene da Btk [88,89].

A sinalização através do pré-BCR desencadeia dois importantes eventos. Um deles é a regulação negativa do mecanismo de recombinação na cadeia pesada para garantir a exclusão alélica, de forma a prevenir a expressão duas cadeias pesadas com diferentes especificidades pela mesma célula [90,91]. O outro evento é a indução da proliferação seguida de uma nova expressão das enzimas RAG1 e RAG2, e a abertura do locus da cadeia leve, dando início ao seu rearranjo – **fase pre-B2** [92,93]. Esta proliferação permite que cada célula pre-B1 possa gerar inúmeras células pre-B2 com diferentes especificidades antigénicas. Para alem da exclusão alélica, estas células apresentam ainda exclusão isotópica, isto é, cada célula B expressa um dos tipos de cadeia leve – κ ou λ. Em humanos, o rácio de células B κ:λ é cerca de 65%:35% [94].

Após o correto rearranjo da cadeia leve, esta liga-se à cadeia pesada μ, forma-se um BCR completo na forma de uma molécula IgM, que vai ser translocada para a membrana da célula (IgM de membrana, ou mIgM) e formar um complexo com outras duas proteínas transmembranares, o Igα (CD79a) e o Igβ (CD79b) – **célula B imatura** [95]. Células pre-B2 que falham na produção de IgM sofrem apoptose.

Apesar do processo de rearranjo genético resultar na produção de um vasto leque de BCRs capazes de reconhecer uma enorme diversidade de antígenos, este rearranjo origina, invariavelmente, células B cujo BCR (recetor de célula B) tem a capacidade de reconhecer antigénios do próprio organismo, isto é, auto-antigénios [96]. De fato, estima-se que, nos humanos, cerca de 75% das células B imaturas produzidas sejam auto-reativas [97]. Como tal, a reatividade do BCR para autoantigénios das células B imaturas tem que ser testada e, se necessário, eliminar aquelas que não tolerem a presença destes autoantigénios. Este processo é conhecido como **tolerância central** [98,99].

As células B imaturas cujos BCR não apresentem afinidade para autoantigénios continuam o seu processo de maturação normal (seleção positiva) migrando para a periferia. Se, no entanto, célula B imaturas apresentarem alta afinidade para autoantigénios, a célula entra num processo conhecido como **edição do recetor**, de forma a produzir um novo BCR [100,101]. Caso esta edição não resulte, a célula morre por apoptose num processo denominado por **deleção clonal** ou seleção negativa. Ainda, interações de moderada afinidade com autoantigénios pode resultar num estado de anergia ou ignorância imunológica, com incapacidade de resposta ao antigénio**.** Apesar deste estado de anergia, as células conseguem migrar para a periferia, mas acabam por morrer dentro de poucos dias por falta de sinais de sobrevivência presentes no baço [99,102].

Assim, as células B imaturas tolerantes para autoantigénios vão migrar da medula óssea para a periferia como **células B de transição 1** até chegar ao baço, órgão linfoide secundário onde as células vão dar continuidade ao seu processo de maturação. No entanto, algumas das células B imaturas auto-reativas conseguem sair da medula óssea, em parte devido à impossibilidade de ter todos os autoantigénios do organismo representados num compartimento tão específico e controlado como é a medula óssea. Assim, no baço, estas células vão novamente ser avaliadas quanto à capacidade e intensidade com que reconhecem autoantigénios – **tolerância periférica** [103].

Células B T1 que tenham alta afinidade para autoantigénios membranares (isto é, não solúveis) vão ser eliminadas por apoptose (seleção negativa) devido a uma forte estimulação via BCR e não chegam até aos folículos esplénicos, podendo também entrar num estado de anergia. Por outro lado, células B T1 não auto-reativas entram nos folículos e diferenciam-se em células B T2 [104–106]. As células B T2 vão receber uma estimulação basal por via do BCR num mecanismo ainda pouco esclarecido, levando ao aumento da expressão dos recetores do fator de ativação das células B (BAFF), produzido e secretado principalmente pelas células dendríticas foliculares (FDCs). Assim, a sinalização via BAFF assegura a sobrevivência das células B, sendo reconhecido através de 3 diferentes recetores, nomeadamente BAFF-R, BCMA e TACI [107,108]. Como tal, células que não recebam estes sinais de sobrevivência acabam por morrer [109]. No entanto, estes mecanismos podem ser contornados (p.ex. com níveis elevados de BAFF) permitindo que estas células potencialmente auto-reativas sejam incluídas no *pool* de células B maduras [110]. De facto, desequilíbrios na sinalização por via do BCR e por BAFF-R ocorrem em indivíduos com sobre-expressão de BAFF, estando ligado ao desenvolvimento de doenças autoimunes com destaque para a síndrome de Sjögren, por falha da eliminação das células B auto-reativas [111–113].

De uma forma geral, as células B de transição caracterizam-se por níveis elevados de sIgM, BAFF-R, níveis crescentes de Ig com cadeia pesada do tipo δ (IgD) e do co-recetor CD21, bem como a forte expressão de CD24 e CD38 [114–116]. A partir daqui as células B T2 podem seguir, pelo menos, dois caminhos de diferenciação e maturação. Resumidamente,

nas células B T2 que recebam uma intensa sinalização por via do BCR ocorre uma forte ativação da enzima BTK, que por sua vez ativa a via de sinalização canónica do Fator nuclear-κB (NF-κB), levando à diferenciação em **células B foliculares**, também conhecidas por células B convencionais B2. Nesta fase, a sinalização por via do BAFF-R é necessária para a sobrevivência mas não para a diferenciação em células B FO [115,117]. Por outro lado, a fraca sinalização por via do BCR torna as células permissivas à sinalização por via do recetor NOTCH2 (presente nas células B de transição) com o seu ligando DL-1 expresso nas células endoteliais do baço levando à ativação de fatores de transcrição [118–120]. Aqui, a sinalização via BAFF-R é importante na ativação da via canónica NF-kB, resultando na diferenciação em **células B da zona marginal** [41,115].

Ativação e diferenciação final dos linfócitos B foliculares

As células B foliculares naïve maduras, (doravante designadas por células B naïve) caracterizam-se como sendo células $IgD^{+}IgM^{dim}CD19^{+}CD20^{+}CD21^{+}CD27^{-}$ e são responsáveis pelo reconhecimento e resposta contra antigénios proteicos, a qual requer a participação de células T CD4 (resposta T-dependente) e a consequente diferenciação em células plasmáticas produtoras de anticorpos e células B de memória [41,121].

Após finalizado o seu programa de maturação, as células B naïve migram para os folículos linfoides onde podem ocorrer dois processos distintos. O reconhecimento e interação com antigénios estranhos, resultado na ativação das células B. Ou, na ausência de estimulação, a circulação entre o sangue e os folículos linfoides dos órgãos linfoides secundários (nódulos linfáticos, baço, placas de Peyer, e outros tecidos linfoides), devido à presença do recetor S1PR1 (*Sphingosine-1-Phosphate Receptor* 1) [122]. Nos órgãos linfoides secundários, as células T (discutidas mais à frente) e B naïve ocupam duas zonas distintas, a zona T e os folículos linfoides primários (ou zona B), respetivamente. Estas zonas estabelecem-se devido à presença de diferentes ligandos para os distintos recetores em cada uma das células. As células T naïve possuem o recetor CCR7, e estão presentes nas zonas onde os seus ligandos, CCL19 e CCL21, estão altamente concentrados e a ser secretados por células estromais e células dendríticas [123–125]. Por outro lado, células B naïve em circulação expressam CXCR5 [126], que lhes permite entrar nos folículos primários devido à presença do seu ligando CXCL13, abundantemente produzido pelas células FDCs [127–129]**.**

Quando um determinado antigénio é introduzido no organismo, este vai-se concentrar nos vários órgãos linfoides periféricos já mencionados. Um antigénio transportado pelo sangue vai ser filtrado pelo baço, enquanto que os antigénios dos tecidos e drenados pelo sistema linfático vão ser filtrados pelos gânglios linfáticos mais próximos. A sua entrada pode estar, ou não, associado a células transportadoras de antigénio, como as células dendríticas, macrófagos, ou mesmo as próprias células B [130].

Dentro do folículo, as células B naïve contactam com FDCs e outras células estromais, recebendo sinais de sobrevivência, nomeadamente via BAFF. Se as células B naive estiverem apenas a transportar um antigénio não específico para o seu BCR, por exemplo através de recetores do complemento como o CD21, a presença de níveis mais elevados destes recetores nas FDCs vão remover o antigénio das células B para futura apresentação a outras células B com BCR específico para esse determinado antigénio [131–133]. No entanto, à medida que vão circulando pelo folículo, as células B naive podem reconhecer via BCR a presença de antigénios solúveis ou presentes na superfície de células apresentadoras de antigénios (APCs), nomeadamente DCs [134], FDCs [135] e macrófagos [136], dando início à resposta B. Como vamos ver, este tipo de resposta está altamente dependente das células T, em particular de **células T CD4 foliculares, ou Tfh**.

Durante uma resposta imune, as células T naïve são ativadas por, principalmente, células dendríticas na zona T, mas também pelas células epiteliais das glândulas salivares [137]. Na presença de IL-6 e IL-21 secretadas por estas células, as células T ativadas vão maturar numa população distinta, as células Tfh. Esta diferenciação está associada à expressão do fator de transcrição Bcl-6 e c-Maf e dos recetores ICOS, PD-1 e CXCR5, e à perda de CCR7, com consequente migração para a região interfolicular, onde vão interagir com células B ativadas e finalizar a sua diferenciação em células Tfh [138–145]. As células Tfh ativadas secretam IL-21, que promove a maturação e proliferação de células B [146]. As Tfh auxiliam a ativação de respostas imunes mediadas por linfócitos B, incluindo a recombinação de classe de Ig, a diferenciação de centros germinativos, e a maturação da afinidade [147].

A ativação B tem inicio com o reconhecimento antigénico por células B naïve que possuam BCR específico para esse mesmo antigénio [148,149]. Para além da sinalização via BCR, associam-se nesta fase outros sinais estimuladores como os fornecidos pelo complexo CD21/CD19/CD81, co-recetores do BCR [150]. Este antigénio vai ser de seguida internalizado, processado e colocado em moléculas de MHC-II para ser apresentado às células Tfh (sinal 1). A sinalização via BCR induz ainda expressão de moléculas coestimuladoras nomeadamente CD80, CD86 e CD40 necessárias para a interação com as células Tfh [151].

De seguida, as células B ativadas vão migrar para as zonas interfoliculares, onde vão proliferar e interagir com células Tfh [151]. Isto é conseguido através do aumento da expressão de CCR7 e de EBI2, cujos ligandos (CCL19/21 e 7α, 25-dihydroxycholesterol, respetivamente) são abundantes e produzidos nestas zonas [123–125,152,153]. Nas células B ativadas observa-se ainda a expressão de CD69, um marcador de ativação, e CD27 um marcador de maturação [154]. Nestas zonas, as células Tfh e B ativadas vão entrar em contacto (sinal 2) através de interações via TCR-MHCII, CD40L-CD40, CD28-CD80/CD86, ICOSL-ICOS [139,151,155,156]. Estes sinais co-estimulatórios são cruciais para as células B, mas também para a estabilização e finalização da diferenciação das células Tfh. Por fim, ocorre a libertação de citocinas pelas células Tfh (sinal 3) para a zona de contacto T-B,

nomeadamente IL-21, permitindo a continuação do programa de diferenciação das células B. Outras citocinas secretadas pelas células Tfh incluem a IL-6, TGF-β, IFN-γ, and IL-4, que vão ter, mais à frente, um papel importante no tipo de anticorpos produzidos [157].

Após um período de 4 a 7 dias de intensa comunicação entre células B e T na zona interfolicular, os sinais enviados pelas células Tfh direcionam a maturação e diferenciação B para 3 destinos possíveis: células secretoras de anticorpos (ASCs) de vida curta [59]. células B de memória inicial [158]; e a formação de centros germinativos (CG) [159].

Células secretoras de anticorpos: plasmablastos e células plasmáticas

Após encontro inicial com o antigénio, as células B ativadas migram para a zona interfolicular onde vão proliferar. Após cerca de 4 dias, algumas das B ativadas, aumentam os níveis de expressão de EBI2 e CXCR4 e diminuem níveis de CXCR5 e CCR7 [152,153]. As células B escolhidas para este caminho parecem ser aquelas, dentro do pool total de células B ativadas, com maior afinidade para o antigénio que despoletou a resposta, num mecanismo pouco esclarecido mas, aparentemente independente de IL-21 [160].

Estas células vão migrar para a polpa vermelha do baço ou para as cordas medulares dos gânglios linfáticos, formando um agregado de células B em proliferação conhecido como foco primário, onde se vão formar células com diferenciação intermedia, os plasmablastos. Os plasmablastos são células que começaram já a produzir e secretar anticorpos, possuem ainda uma capacidade proliferativa e BCR à sua superfície e podem ser caracterizadas como células $CD19^{+}CD38^{H1}CD27^{Hi}$MHC-$II^{+}CD269^{+}$. Estas células vão eventualmente originar células plasmáticas ou plasmócitos de curta duração, caracterizadas como $CXCR4^{+}CD27^{+}CD38^{forte}CD138^{+}CD269^{+}$ que possuem uma elevada taxa de secreção de anticorpos mas sem capacidade proliferativa e sem MHC-II à superfície, e secretam predominantemente anticorpos do tipo IgM que constituem a resposta inicial [59,154,161,162]. A expressão dos fatores de transcrição BLIMP-1 e os níveis elevados de IRF-4 favorecem a diferenciação em ASCs [163,164].

Apesar de inicialmente produzirem IgM, a presença de anticorpos do tipo IgG pode ser detetado 5 a 6 dias após imunização, demonstrando que a formação de centros germinativos não é necessária para o processo de troca de isotipo (discutido mais à frente). No entanto, estes anticorpos não apresentam hipermutação somática, processo que ocorre apenas nos centros germinativos [165]. A grande maioria das ASCs de vida-curta do foco primário morrem por apoptose após 7 ou 8 dias do encontro com o antigénio e apesar da baixa afinidade dos anticorpos produzidos, esta reposta inicial é crucial para conter o avançar da infeção até ao aparecimento de anticorpos de alta afinidade [166]. Ainda nesta fase, algumas células de memória inicial expressando principalmente IgM e/ou IgD à superfície são produzidas e enviadas para a circulação (discutido mais à frente) [167].

Segunda fase da resposta imune B – Centros germinativos

Nem todas as células B ativadas vão para fora do folículo formar o foco primário. Algumas vão migrar para o centro do folículo linfoide em conjunto com a sua célula Tfh associada onde vão continuar a proliferar, formando os chamados centros germinativos (CG), ou folículos linfoides secundários [168]. A diminuição da expressão dos recetores EBI2 e CCR7, bem como a expressão dos fatores de transcrição Pax-5, Bcl-6, e os baixos níveis de IRF-4 nas células B ativadas parecem promovem a migração das células e a consequente a formação destes centros germinativos [152,153,163,169,170].

Ao contrário do que acontece na resposta primária, as células B nos centros germinativos sofrem vários processos que vão culminar na produção de anticorpos de alta afinidade. Esses processos incluem a **hipermutação somática, maturação da afinidade**, e **troca de isotipo** ou ***class switch*** [159,171].

Os centros germinativos são compostos principalmente por células B em proliferação, que se dispõem em duas regiões distintas, a **zona escura** e a **zona clara** [171]. A zona escura é caracterizada por uma elevada densidade de células B em proliferação dentro de uma rede de células reticulares que produzem CXCL12 [172]. Por sua vez a zona clara é composta por células B mais dispersas bem como a presença de vários tipos celulares, incluindo células Tfh, FDCs (que produzem CXCL13, ligando do CXCR5) e macrófagos [143,171,173].

As células B em elevada taxa de proliferação na zona escura do centro germinativo denominam-se por centroblastos e expressam CXCR4 (recetor do CXCL12), CXCR5 e baixos níveis de IgD [174]. Os processos de hipermutação somática ocorrem nos centroblastos na zona escura e consiste na introdução de pequenas mutações na região Variável do gene da imunoglobulina, de forma a produzir células B com elevada especificidade e afinidade antigénica [175]. Estas mutações são iniciadas pela enzima AID (*ativation induced cytidine deaminase*), a qual é expressa apenas pelas células B dos centros germinativos [176,177].

À medida que este processo ocorre, os centroblastos reduzem a sua taxa de proliferação, entrando na fase de crescimento com diminuição da expressão de CXCR4 e produção de elevados níveis de imunoglobulina à superfície [178]. Estas células denominam-se agora por **centrócitos**, e vão ser atraídos pela presença de CXCL13 e migrar para a zona clara onde vão reconhecer antigénios presentes nas FDCs para serem internalizados e consequentemente e apresentados às células Tfh via MHC-II. Por sua vez, as células Tfh que reconhecerem estes antigénios apresentados vão ser ativadas e promover a sobrevivência dos centrócitos [179]. Assim, quão melhor for a ligação entre os recetores dos centrócitos e os antigénios, maior vai ser a quantidade de antigénios apresentados em moléculas de MHC-II às células Tfh e consequente produção de sinais de sobrevivência [179–181]. Os

centrócitos cujas mutações resultem numa redução da afinidade de ligação ao antígeno vão reconhecer menos antigénios e, portanto, receber uma quantidade inferior de sobrevivência pelas células Tfh. Se estes sinais de sobrevivência não forem suficientes, os centrócitos vão morrer por apoptose e removidos por macrófagos [181].

As células B com alta afinidade que conseguirem sobreviver, vão voltar a expressar CXCR4 e regressar à zona escura, onde vão entrar em novos ciclos de proliferação (expansão clonal), tornando-se de novamente em centroblastos [181,182]. Este processo de migração dentro dos centros germinativos permite que a afinidade e especificidade das células B seja continuamente refinada durante uma resposta imune – **maturação da afinidade** [171].

Como vimos, os primeiros anticorpos produzidos numa resposta imune são sempre do tipo IgM. Mais tarde, nos centros germinativos, as mesmas regiões variáveis recentemente (hiper)mutadas, vão poder ser associadas a regiões constantes de anticorpos do tipo IgG, IgA, ou IgE [183]. Esta troca é conhecida como troca de isotipo, ou ***class switch*** e envolve recombinações irreversíveis no DNA, pela ação da mesma enzima que atua na hipermutação somática, a AID [176]. As células Tfh são cruciais para que este evento ocorra, através de interações via CD40-CD40L e ICOS-ICOSL [184–186]. De facto, mutações no gene do CD40L reduz drasticamente os eventos de *class switch* e resulta em níveis elevados de IgM – Síndrome de hiper-IgM [187,188]. A seleção da região constante no processo de *class switch* não é ao acaso, com a produção de citocinas pelas células Tfh a modular a classe de imunoglobulina a produzir pela célula B em diferenciação [189]. Por exemplo, a IL-4 induz a transcrição e síntese de anticorpos do tipo IgG1 e IgE. Outras citocinas moduladoras produzidas pelas células Tfh incluem a IL-21 (IgG1 e IgG3), o TGF -β (IgG2 e IgA), a IL-5 (IgA), e o IFN -γ (IgG2a e IgG3) [190–192]. Cada tipo de anticorpo possui funções efetoras distintas e apropriadas para a eliminação de um determinado tipo patógeno. De uma forma geral, os anticorpos IgG promovem a fagocitose de partículas revestidas com anticorpos, os IgE promovem a desgranulação de mastócitos, e os anticorpos IgA conferem proteção contra patogéneos nas superfícies das mucosas [193–195]. Os eventos de *class switch* podem ocorrer mais do que uma vez na mesma célula, e não estão restritos apenas às células B dos centros germinativos [196,197].

Células B do centro germinativo diferenciam-se em células plasmáticas ou células de memória

Após os processos acima descritos e num mecanismo ainda pouco esclarecido, algumas destas células saem da zona clara e diferenciam-se em plasmablastos, com a produção de grandes quantidades de anticorpos. Como já foi referido, para esta diferenciação ocorrer existe uma diminuição da expressão fatores de transcrição Pax5 e Bcl-6, e o aumento de IRF4. O início da expressão de BLIMP-1 induz a diferenciação para células plasmáticas as

quais vão perder capacidade proliferativa, aumentar a produção e secreção de anticorpos, e sofrer alterações na sua superfície. Estas incluem a perda de CXCR5 e o aumento de CXCR4 e de integrinas, permitindo a saída dos centros germinativos dos nódulos linfáticos ou do baço para a medula óssea onde vão receber sinais de sobrevivência e originar células plasmáticas de longa duração [198,199]. A expressão de XBP1 (X-box binding protein 1) por parte das células plasmáticas é também importante para permitir a entrada das células na medula óssea e para regular a sua capacidade secretora [200].

As células plasmáticas que se originaram nos centros germinativos das mucosas, e que produzem principalmente IgA, vão manter-se essencialmente nas mucosas.

A medula óssea fornece às células plasmáticas importantes sinais de sobrevivência, produzidos pelas células estromais, incluindo CXCL12 [68], bem como outras citocinas e quimiocinas secretadas por eosinófilos, megacariócitos e monócitos, incluindo IL-6 e APRIL [201–203]. Assim, as células plasmáticas possuem a capacidade de produzirem anticorpos de elevada afinidade e de sobreviverem durante vários anos.

Células de Memória

No decorrer da resposta imune nos centros germinativos, algumas das células que não originaram células plasmáticas, vão-se diferenciar em **células B de memória** [204]. A ativação e diferenciação dos linfócitos B em células de memória ou em plasmócitos está representada na **Figura 2**.

Apesar do programa de diferenciação das células B de memória estar pouco esclarecido, recentemente, um estudo descreveu a importância da expressão de OBF-1 (*Oct-binding Fator 1*), um regulador da transcrição, juntamente com a expressão do fator de transcrição Spi-B, um inibidor da diferenciação plasmática para a diferenciação em células de memória nos centros germinativos [205,206]. Outros fatores que promovem a formação de células B de memória incluem a sinalização via CD40, a ativação do BCR e a presença de IL-24 [207,208].

As células de memória circulam entre o sangue e os órgãos linfoides secundários e permitem, numa segunda exposição a um determinado antigénio, a montagem de uma resposta mais rápida e eficaz do que na primeira resposta, estando na base dos processos de vacinação [209,210]. Esta diferença na dinâmica das respostas, deve a várias caraterísticas que diferenciam a célula B naïve da célula B de memória, incluindo uma localização mais próxima das zonas de contacto com antigénios, maior afinidade dos BCRs devido a eventos de hipermutação somática, um tempo de vida útil de várias décadas, uma rápida capacidade de proliferação e diferenciação após estimulação devido à presença de um maior numero de moléculas co-estimuladoras (CD27, CD80, CD86, IL21R), entre outros [211].

Estas células podem ser divididas em duas populações principais [204]. Uma delas é composta pelas células de memória geradas na resposta inicial antes da formação dos centros germinativos. Estas células são maioritariamente IgM^+ de baixa afinidade, podendo sobreviver durante várias semanas após contacto inicial com antigénio. Com a formação dos centros germinativos e ocorrência dos eventos que neles acontecem, aparece uma segunda e principal população de células B de memória de alta afinidade do tipo IgG, IgE ou IgA, caracterizando-se como células $IgD^-CD19^+CD20^+CD27^+$ [154,161].

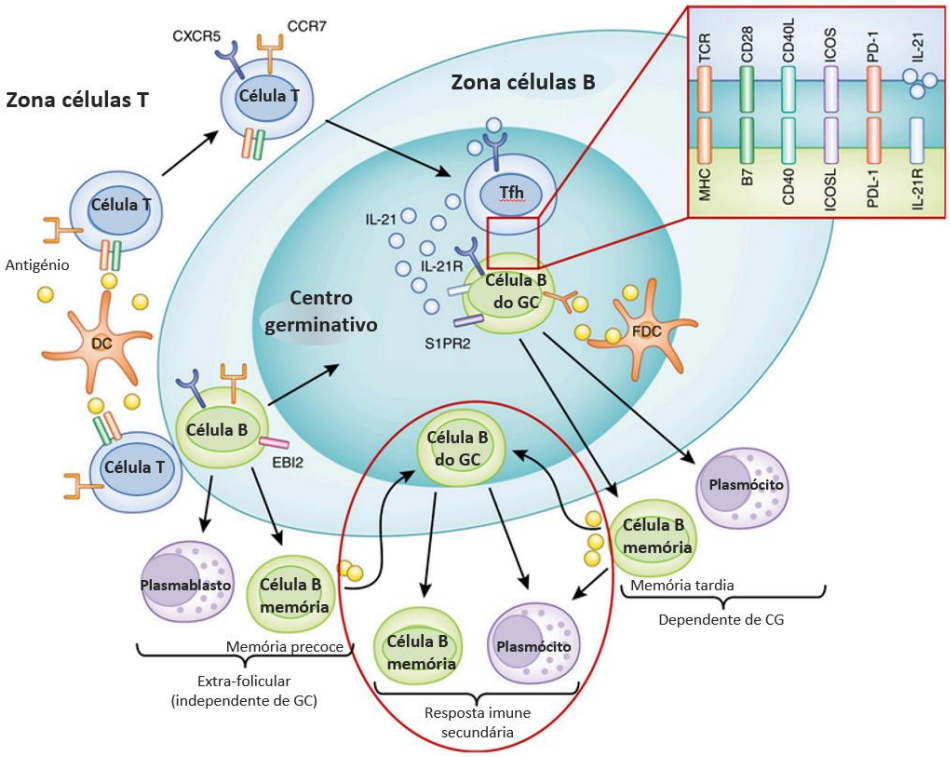

Figura 2. Ativação e diferenciação dos linfócitos B em células de memória ou em plasmócitos. Adaptado de Hoffman (2016) [77].

Os linfócitos B que encontrarem o seu antigénio específico vão migrar para a zona interfolicular através do aumento da expressão de CCR7 e EBI2, onde vão encontrar linfócitos T com especificidade para o mesmo antigénio que despoletou a resposta e promover a sua diferenciação em linfócitos T foliculares (Tfh). Os linfócitos B podem-se diferenciar em plasmablastos ou linfócitos B de memória extrafoliculares, isto é, independentes da formação de centros germinativos (CG). Os linfócitos B que expressam o fator de transcrição Bcl6 voltam para o folículo onde são mantidos através da expressão de S1PR2 para a formação de CGs com Tfhs. Uma vez dentro dos CGs, as interações entre linfócitos B e linfócitos Tfh via MHC2-TCR, B7-CD28, CD40-CD40L, ICOSL-ICOS, PDL1-PD1, e IL-21R-IL-21, facilitam os eventos de hipermutação somática e troca de isotipo, e originam linfócitos B de memória e células plasmáticas de alta afinidade.

No seguimento de um reencontro com o mesmo antigénio, as células B de memória extrafoliculares entram nos CGs e originam linfócitos B de memória e células plasmáticas secundárias, ambas de alta afinidade e com troca de isotipo. Por sua vez, os linfócitos B de memória dependentes de CG conseguem-se diferenciar rapidamente em células plasmáticas secundárias ou reentrar nos CGs para produzir linfócitos B de memória e células plasmáticas secundárias. DC, células dendríticas.

Após novo contacto com o mesmo antigénio, as células B de memória IgM^+ migram para os folículos linfóides para formar novos centros germinativos, iniciando ciclos de proliferação e hipermutação somática antes de se diferenciarem em células plasmáticas. Por sua vez, as células B de memória IgG^+ diferenciam-se rapidamente em plasmablastos que secretam anticorpos IgG de alta afinidade [212,213]. No entanto, as células de memória IgG^+ podem igualmente reentrar nos centros germinativos e sofrer novos ciclos de hipermutação somática e maturação de afinidade e, em certos casos, alterar novamente a sua classe de imunoglobulina [214]. De facto, a geração de células B de memória IgE^+ resulta de células $IgG1^+$ que sofreram novo processo de *class switch* [215,216]. Inicialmente, pensava-se que as células B de memória eram exclusivamente o produto de uma resposta T-dependente. No entanto, dados mais recentes demonstraram que as células B1 podem gerar células B de memória, com características e fenótipo distintos, durante uma resposta T-independente [217–219].

Assim, os anticorpos produzidos numa segunda resposta, e respostas subsequentes, apresentam maior afinidade para o antigénio comparando com os anticorpos produzidos na resposta primária.

1.2.1.1. Linfócitos B reguladores

Os linfócitos B reguladores (Bregs) são células com capacidade supressora que promovem a tolerância imunológica através da produção de IL-10, IL-35 e *transforming growth Fator β* (TGF-β) [220].

A designação "células B reguladoras" foi pela primeira vez empregue por Mizoguchi [221] ao caracterizar uma população de células B $CD1d^{Hi}$ num modelo animal de doença inflamatória intestinal.

A ação supressora das células Bregs deve-se principalmente à produção de IL-10, a qual induz um desvio na diferenciação das células T, inibindo a ação efetora das Th1 e Th17 e promovendo as Tregs, quer em modelos animais [222], quer em humanos [223]. Embora a ação supressora das Bregs sobre as Th1 seja sobretudo dependente da IL-10, a interação com o CD80/CD86 nas Bregs intensifica a supressão das respostas Th1 [223,224]. Indiretamente, as Bregs exercem também uma ação inibitória da diferenciação das Th1 e Th17 através da supressão da produção de citocinas pro-inflamatórias pelas células dendríticas [225,226]. As Bregs expressam também outras citocinas inibitórias, como oTGF-β e a IL-35. Através da produção de TGF-β, as células B podem induzir a apoptose de células efectora $CD4^+$ [227] e anergia das $CD8^+$ [228]. A demonstração recente de que a produção de IL-35 por células B contribui para a supressão da resposta imune [229,230] foi um importante contributo na expansão do conhecimento sobre as funções das Bregs. Adicionalmente, as Bregs contribuem para a manutenção da homeostase das células iNKT [231], elas próprias também de fenótipo regulador. A **Figura 3** representa as várias ações das células Bregs.

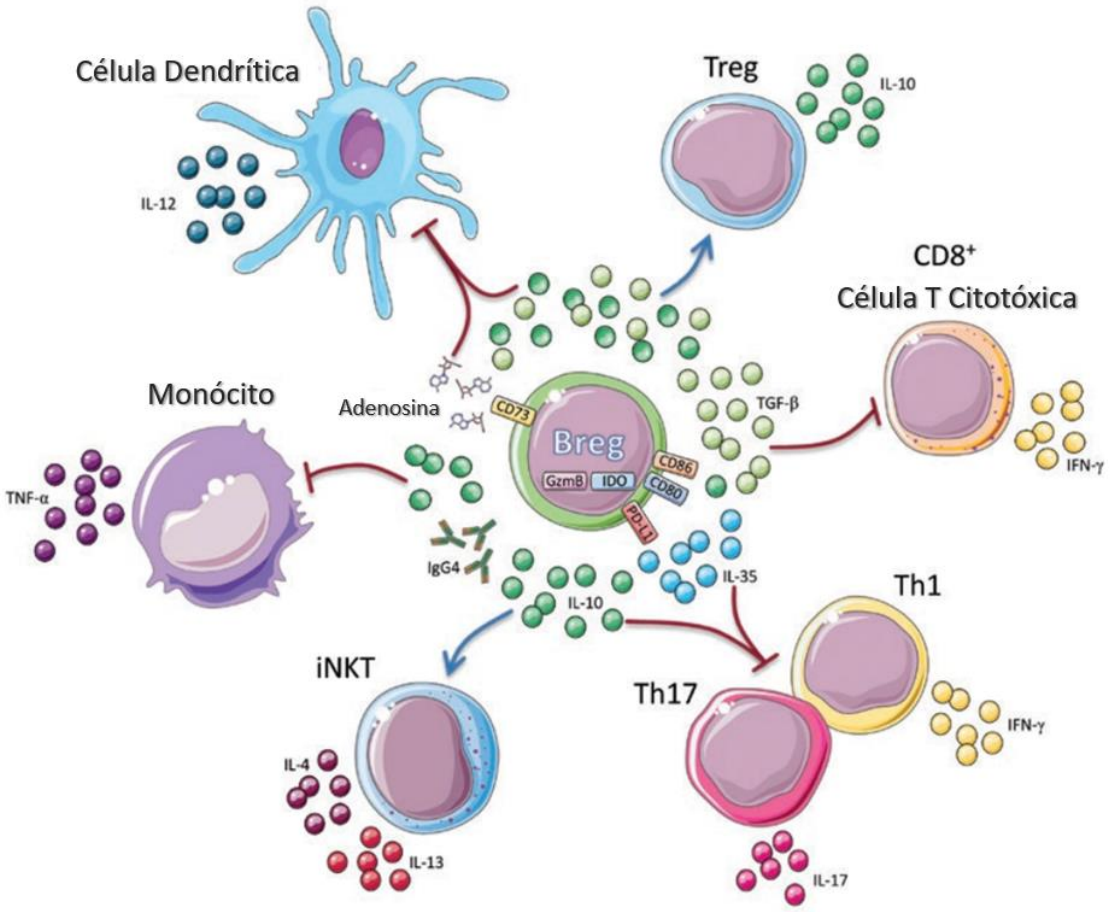

Figura 3. Mecanismos efetores das células B reguladoras (Bregs) humanas. Adaptado de Maravillas-Montero (2017) [232].

Os linfócitos Breg secretam diferentes citocinas anti-inflamatórias, incluindo IL-10, TGF- β e IL-35. A expressão de CD80, CD86, ou PD-1L permite a supressão por interações diretas célula a célula. A utilização de enzimas por parte dos linfócitos Breg incluem a granzima B, IDO, ou o CD73, que promove a geração de adenosina. Por fim, algumas subpopulações de linfócitos Breg conseguem produzir IgG4 após ativação, uma imunoglobulina com algumas propriedades supressoras.

Coletivamente, estes elementos dos linfócitos Breg conseguem controlar as respostas das populações linfoides dos linfócitos (por exemplo: inibição da produção de IL-17 ou INF-γ pelos linfócitos Th17, Th1 ou T CD8), mieloides (suprimem a secreção de TNF-α ou IL-12 por parte de monócitos inflamatórios e células dendríticas, respetivamente). Por último, os linfócitos Breg aumentam ainda as capacidades efetoras dos próprios linfócitos B (promovendo a secreção de IL-10) e dos linfócitos NK invariante (promovem a secreção de IL-4 e IL-13) favorecendo um ambiente anti-inflamatório.

Ao contrário do que sucede com o Foxp3 nas Tregs [233], não foi até à data identificado nenhum fator de transcrição específico das Bregs. As células Bregs não provém de uma única linhagem, constituindo ao invés uma população heterogénea de células B que adquirem capacidade supressora em diversas fases da sua maturação, quando expostas a determinados estímulos [234]. De acordo com a localização, o ambiente inflamatório e a ligação através do BCR, as células B poderão diferenciar-se em Bregs ou em células produtoras de anticorpos [235]. Para a diferenciação e ativação das Bregs é necessária a

interação de várias moléculas, incluindo toll-*like receptors* (TLR), CD40 e BCR, bem como CD80, CD86 e recetores de citocinas [236]. Várias citocinas inflamatórias têm sido associadas à diferenciação das Bregs, como a IL-1 e IL-6 [237], a IL-21 [238] e o *granulocyte-macrophage colony-stimulating Factor* (GM-CSF) [239]. É evidente o aumento do número e da capacidade supressora das células Bregs em resposta à inflamação no contexto de autoimunidade [221,240], alergia [241], ou outras condições [235], e que na ausência de Bregs há exacerbação dessas reações [222,242].

Os métodos de identificação das células Bregs ainda não estão completamente definidos, nomeadamente em relação aos marcadores de superfícies e fatores de transcrição. Uma vez que a característica mais distinta das Bregs é a capacidade de produzir IL-10, os estudos funcionais de produção de IL-10 têm sido utilizados como o principal marcador na identificação destas células [235].

Têm sido descritas várias subpopulações de células B produtoras de IL-10, com variável sobreposição de marcadores de superfície e diversas funções, que são coletivamente designadas Bregs [243]. Estas incluem células B $CD24^{hi}CD38^{hi}$, $CD24^{hi}CD27^{+}$ (B10), $CD38^{+}CD1d^{+}IgM^{+}CD147^{+}GrB^{+}$, plasmablastos $CD27^{int}CD38^{hi}$, e células B $CD19^{+}TIM1^{+}$ [224,225,244–246]. Apesar de todas estas populações terem demonstrado capacidade de supressão de reações pro-inflamatórias, estima-se que em cada um destes subtipos de células a fração capaz de produzir IL-10 e suprimir as respostas imunes não ultrapasse os 20% [235].

A população de células B de transição, identificada pela forte co-expressão de CD24 e CD38, tem uma grande representação de células reguladoras [224,247] e é a população Breg mais estudada. Com frequência, a expressão de CD24 e CD38 é utilizada para quantificação de células Breg em sangue periférico. A frequência das células B $CD24^{hi}CD38^{hi}$ varia em diversas entidades clínicas [248], sendo difícil estabelecer associações entre os números ou frequências destas células e mecanismos fisiopatológicos específicos. Além disso, é admitida a existência de diferentes subtipos de células de transição com perfis reguladores distintos [248].

Na população de células B $CD24^{hi}CD27^{+}$ circulante existe também um subtipo com propriedades reguladoras [244,249], que foram caracterizadas como B10 e B10pro. Estas células representam um pequena fração das células B $CD24^{hi}CD27^{+}$, mas demonstraram uma grande capacidade secretória de IL-10 quando estimuladas [244].

A presença de distúrbios das propriedade reguladores das Bregs foi já descrita em várias doenças autoimunes, incluindo a esclerose múltipla [250], lúpus eritematoso sistémico [224,251,252], SSP [244,253,254], esclerose sistémica [255], artrite reumatóide [223], psoríase e artrite psoriática [256].

1.2.2. Linfócitos T

Percursores e Diferenciação

Os linfócitos T, em conjunto com as células B, constituem o ramo adaptativo e reativo do sistema imunitário. As células T, ao contrário de outras células hematopoiéticas, têm uma longevidade extensa, um grande potencial proliferativo e uma capacidade de diferenciação terminal especializada [257].

Tal como descrito para as células B, também os linfócitos T derivam de células progenitoras comuns linfoides. No entanto, ao contrário do que acontece no desenvolvimento B, estas células migram da medula óssea, através do sangue, para o **timo**, o órgão linfoide primário que fornece um ambiente especializado e organizado para a diferenciação dos precursores de linfócitos T [258]. Este desenvolvimento é semelhante ao das células B, na medida em que inclui também processos de rearranjo genético nos genes que codificam o recetor de antigénios destas células (recetor de célula T ou TCR) e a eventual montagem de um TCR funcional à superfície da célula. No entanto, ao contrário do recetor da célula B, o TCR não é um anticorpo, mas sim um heterodímero de duas cadeias polipeptídicas glicosiladas, codificadas por genes distintos. Assim, o desenvolvimento T permite a produção de duas linhagens T distintas consoante as cadeias que formem o TCR: linhagem α:β ou γ:δ [259]. No âmbito desta tese, irá ser abordado o desenvolvimento das células T α:β.

As células T em desenvolvimento são conhecidas por **timócitos**, e passam também por um controlo rigoroso no que diz respeito ao reportório de células T maduras de forma a assegurar células que reconheçam complexos MHC do próprio, mas que sejam simultaneamente tolerantes à presença de autoantigénios. Os timócitos estão rodeados por uma rede de tecido conjuntivo conhecido por estroma tímico, que fornece um microambiente exclusivo para o desenvolvimento destas células, análogo à importância estroma da medula óssea no desenvolvimento das células B. As células tímicas epiteliais representam grande parte do estroma tímico, e são cruciais para a diferenciação e seleção do reportório T, por apresentarem características fundamentais para este processo, incluindo a expressão antigénica, a produção de fatores tímicos importantes para a maturação dos timócitos, e modulação da resposta linfocitárias [260]. O estroma tímico é também composto por células endoteliais, importantes para a manutenção da vasculatura tímica (e, portanto, para a colonização do timo por progenitores de células T transmitidas pelo sangue) e células mesenquimais, que regulam a proliferação das TECs e progenitores de células T [261]. Por último, este órgão é também ocupado por células dendríticas, células B e macrófagos, todos envolvidos na formação do repertório de células T e na eliminação dos timócitos apoptóticos [261,262].

Os percursores do desenvolvimento T fazem parte de uma populaçao que expressa o marcador CD34 [263], também expresso em células progenitoras hematopoiéticas mas ausente nas populações mais diferenciadas [264–266].

A expressão sequencial dos co-recetores da célula T, nomeadamente CD4, CD3 e CD8 é usada para definir as diferentes fases do desenvolvimento T humano [258]. Assim, os timócitos em desenvolvimento dividem-se de acordo com a expressão destes marcadores em: células triplas negativas (TN); células imaturas com expressao de CD4 (CD4ISP); células duplas positivas para CD4 e CD8 com ou sem expressão de CD3 (DP); e finalmente células T $CD3^+$ com expressao de CD4 ou CD8 [258,267]. Da mesma forma que o CD19 é o marcador principal das células B, o complexo CD3 têm a mesma importancia como marcador de linhagem T. Nas células T αβ maduras, a expressão de CD3 associa-se tambem à expressão do co-recetor CD4 ou CD8, que interagem com moleculas de MHC de classe I ou II, respetivamente, e identificam as duas principais populações de células T no sangue periférico: células T $CD4^+$ ou auxiliares, e células T $CD8^+$ ou citotóxicas.

As células $CD34^+CD1a^-$ TN representam a população mais imatura no timo e não estão, ainda, comprometidas com a linhagem T [268,269]. Este comprometimento está fortemente associado ao início da expressão de $CD1a^+$ [266,270], e com o início do rearranjo dos locus β, λ e δ do gene do TCR [271].

Os vários processos que envolvem a diferenciação e proliferação dos timócitos estão fortemente regulados por uma combinação de diversos fatores envolvidos nas principais vias de sinalização, incluindo interleucina 7 (IL-7), fator de células estaminais, e as vias de sinalização Wnt, Hedgehog e Notch1. [262,272–274].

O Notch1 é um recetor transmembranar altamente conservado que está envolvido na regulação do destino celular em várias linhagens celulares [275], e juntamente com os seus ligandos, Delta-like 1 (DLL1) e DLL4, foi identificado como fator determinante no comprometimento da linhagem de células T e B [276]. A título de exemplo, quando postas em cultura, células $CD34^+$ do sangue do cordão umbilical e da medula óssea na presença de células estromais da medula óssea de ratinho que expressam DLL1, desenvolvem-se em células T maduras. Por contrário, a utilização de inibidores da sinalização Notch afeta substancialmente o desenvolvimento das células T [277,278].

Assim, as células precursoras entram no timo pela junção cortico-medular [279], onde são expostas a níveis elevados de DLL1 expresso pelas células estromais [280]. Esta exposição, resulta na ativação da via de sinalização Notch nas células percursoras, promovendo então a sua diferenciação a favor da linhagem T ou NK, mas não da linhagem B [258].

Um outro fator importante no desenvolvimento de células $CD34^+$ progenitoras de células T é a sinalização por IL-7, produzido pelas células do estroma tímico [281]. Esta citocina atua após ligação ao seu recetor, o IL-7R, composto por duas cadeias alfa (IL-7Rα, ou

CD127), e uma cadeia gama, também conhecida por cadeia gama comum (γc), uma vez que está presente na estrutura de recetores de outras citocinas, incluindo IL-2, IL-4, IL-9, IL-15 e IL-21. Desta forma, mutações nos genes que codificam estas cadeias, ou no genes da Jak3, um outro componente da via de sinalização da IL-7, resulta numa profunda ausência de células T, uma das formas de imunodeficiência combinada severa (SCID) [282–284]. De forma semelhante, a exposição de células CD34+ progenitoras de células T a inibidores da sinalização por via do recetor IL-7R, resulta no bloqueio do seu desenvolvimento, impedindo a transição das células TN para o estadio seguinte [281].

Rearranjo do TCR e seleção β

Nesta fase, em que as células já perderam, na sua maioria, a capacidade de originar células NK ou células dendríticas, os timócitos começam a expressar moléculas de CD4 e a perder completamente a expressão de CD34, designando-se por timócitos CD4ISP (CD4 *Imature Single Positive*) [285].

Durante esta fase, inicia-se o rearranjo dos genes do TCR, de forma a originar células T com TCR (αβ ou γδ) funcionais, por processos de recombinação dos segmentos genéticos V, D e J, de cada locus, por ação das enzimas RAG1 e RAG2 [286].

O comprometimento dos timócitos para com as linhagens αβ ou γδ, é mediado por sinalização via TCR [287]. O rearranjo do locus das cadeias β, γ, e δ inicia-se quase simultaneamente nos timócitos em desenvolvimento, e as duas linhagens celulares divergem de um precursor comum apenas após certos rearranjos genéticos já terem ocorrido [288]. Isto é, a decisão de um timócito em se comprometer com a linhagem Tαβ ou Tγδ, depende do tipo de TCR que é expresso primeiramente durante o seu desenvolvimento. Assim, se um TCRγδ completo se formar antes de um rearranjo bem-sucedido do gene da cadeia β, o timócito vai receber uma sinalização mais forte via TCRγδ, impedindo a continuação do rearranjo da cadeia β. Por contrário, se a formação de uma cadeia β funcional anteceder a conclusão de um TCRγδ, esta cadeia vai-se emparelhar com uma cadeia alfa não-polimórfica, denominada pré-TCRα, formando o chamado pré-TCR. Da mesma forma, a sinalização via pré-TCR interrompe o rearranjo do locus γ e δ, direcionando a célula para a linhagem αβ [289].

A partir desta fase, a continuação do desenvolvimento requer uma sinalização efetiva por via do pré-TCR de cada timócito, de forma a assegurar a sobrevivência, proliferação, e correta seleção para a linhagem T αβ, com interrupção do rearranjo do locus da cadeia β, e inicio do rearranjo do locus da cadeia α [290]. Este processo é conhecido como **seleção β** e representa o primeiro *checkpoint* do desenvolvimento T [258]. Durante este processo, timócitos cujo rearranjo VDJ no locus β origine uma cadeia β não-funcional, não recebem sinais de sobrevivência e/ou proliferação, acabando por morrer. Desta forma, apenas as células que expressem um TCRβ funcional passam à fase seguinte do desenvolvimento, com início da expressão de moléculas de CD8α, seguindo de moléculas de CD8β,

transformando-se primeiro em linfócitos CD8α^{+} e a seguir em CD8αβ^{+}, constituindo uma população de timócitos duplamente positivos para os marcadores CD4 e CD8, ou DP, sem expressão do marcador CD3 (CD3^{-} DP) [291]. É principalmente nesta fase de timócitos DP pequenos, e após vários ciclos de proliferação celular, que se inicia o rearranjo no locus da cadeia α, com consequente eliminação do locus δ (por se encontrar contido no locus α) e formação de círculos de DNA conhecidos como TRECs (*TCR excision circles*) [258,292].

Seleção positiva e negativa das células T em desenvolvimento

À semelhança do que ocorre durante o desenvolvimento linfocitário B, os linfócitos T possuem também a capacidade de discriminar o ''próprio'' (*self*) do ''não-próprio'' (*non-self*). Ou seja, no final do desenvolvimento, os linfócitos T maduros deverão ter a capacidade, por um lado, de reagir contra moléculas de MHC contendo péptidos ''não-próprios'' e, por outro lado, não reagir, ou reagir com reduzida afinidade, contra moléculas de MHC contendo péptidos ''próprios''.

Esta capacidade é avaliada após o rearranjo no locus da cadeia α, através da interação dos dímeros TCRαβ com os complexos MHC:péptido do próprio no timo. Assim, aqueles timócitos cujo TCRαβ interaja com uma baixa afinidade com os complexos MHC:péptido do próprio, expressos pelas células epiteliais tímicas do córtex, recebem sinais de sobrevivência, enquanto que aqueles que não reconhecem estes complexos (constituindo mais de 90% dos timócitos) não recebem sinais de sobrevivência e morrem – **seleção positiva**. Por outro lado, aquelas células cujo TCRαβ apresenta uma afinidade elevada para os complexos MHC:péptido do próprio, expressos por células epiteliais tímicas da zona medular ou por células dendríticas, são eliminados por morte celular programada **– seleção negativa** [293,294]. Este processo é também conhecido por deleção clonal, eliminando células potencialmente auto-reativas [258]. Uma vez que a seleção negativa tem como base a resposta a antigénios do próprio, não é expectável, no entanto, que vários antigénios do próprio sejam expressos no timo (por exemplo, insulina). Assim, a expressão destes antigénios específicos de certos tecidos por parte das células envolvidas neste processo, é controlada pelo gene AIRE (*Autoimune Regulator*), que induz a expressão de inúmeros antigénios [295]

Linhagem CD4 e CD8 dos linfócitos T αβ

A escolha da linhagem CD4 ou CD8 é uma decisão crucial que ocorre nos timócitos DP já com expressão de CD3 (DP CD3^{+}) [296]. Esta decisão está diretamente associada à especificidade do TCR de cada timócito para o MHC durante a seleção positiva que, entre outros fatores, promove a diferenciação seletiva para a linhagem CD8 ou CD4, conforme a especificidade do TCR para moléculas de MHC Classe I ou classe II, respetivamente [297]. A importância das moléculas de MHC reflete-se na síndrome do Linfócito Nu, um tipo de imunodeficiência combinada, causada por mutações no fator de transcrição que regula a

expressão de MHC [298]. A representação sumária do desenvolvimento das células T no timo é apresentada na **Figura 4**.

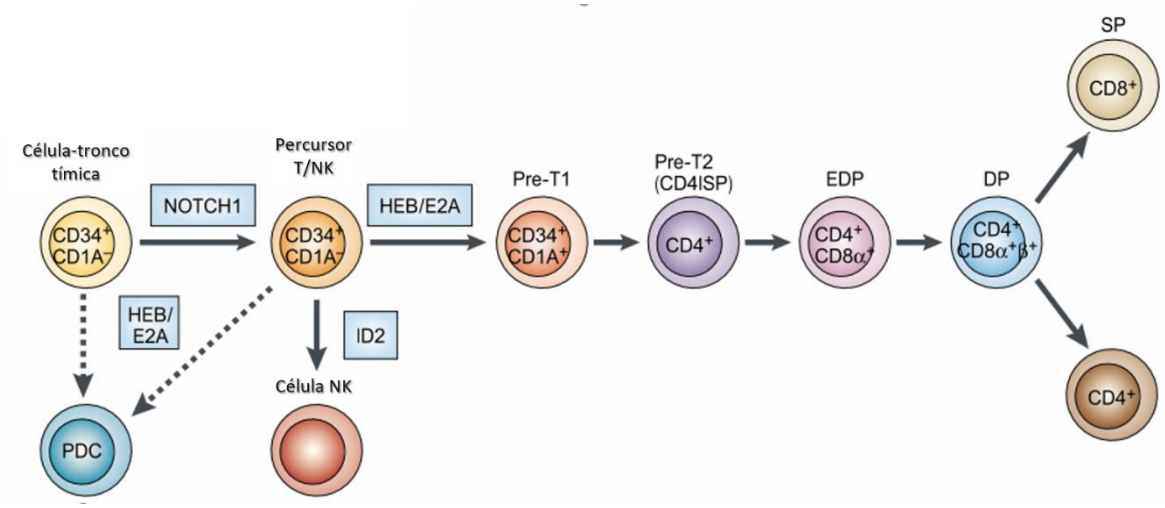

Figura 4. Modelo das fases iniciais do desenvolvimento dos linfócitos T no timo. Adaptado de Spits (2002) [258].

Precursores hematopoiéticos CD34+ colonizam o timo e passam por diferentes etapas, que podem ser distinguidas com base na expressão de marcadores de superfície e pelo estado de rearranjo do recetor dos linfócitos T (TCR). Após comprometimento com a linhagem T, os timócitos CD34+CD1a- começam a expressão de CD1a e iniciam o rearranjo do locus β, γ e δ (timócitos pre-T1). Na próxima fase do desenvolvimento, os timócitos expressam CD4, mas não ainda CD8, e são designadas como timócitos imaturos positivos para CD4 (CD4ISP). Esta população contém os precursores para ambas as linhagens Tαβ e Tγδ. Depois desta fase, os timócitos começam a expressar uma das cadeias da molécula de CD8, CD8α, e são designados por timócitos duplos positivos iniciais (EDP). Por fim, estes timócitos vão expressar as duas cadeias de CD8, tornando-se timócitos DP maduros e originar linfócitos T CD4 e T CD8 maduros. A população de células CD34+CD1a- pode originar precursores da linhagem T e da linhagem NK. A sinalização via Notch1 é crucial para o desenvolvimento da linhagem T no timo.

Atualmente, o modelo mais aceite para explicar o processo de comprometimento para a linhagem CD4 ou CD8 propõe que esta escolha é determinada pela duração da sinalização do TCR, com citocinas, principalmente IL-7, a desempenhar um papel fundamental nesta duração [299].

De acordo com este modelo, os timócitos DP $CD3^+$ baixam a expressão de CD8, tornando-se temporariamente timócitos $CD4^+CD8^{low}$.[300]. A partir daqui, se estas células receberem sinalização via TCR, significa que estão a reconhecer péptidos do próprio em moléculas de MHC II (devido à ausência de CD8). A continuação desta sinalização promove o bloqueio da sinalização por IL-7 e induz a diferenciação em células T CD4 maduras. Por outro lado, se a sinalização pelo TCR for interrompida nos timócitos após a perda de CD8 (por estarem a reconhecer moléculas de MHC I), não ocorre bloqueio da IL-7, promovendo a reversão da expressão dos recetores, com silenciamento da expressão de CD4 e re-expressão de CD8, diferenciando-se em células T CD8 maduras [296,301].

No entanto, é importante referir a importância de fatores de transcrição, nomeadamente Th-POK e RUNX3, na especificação de linhagem, por regularem a transcrição dos genes do CD4 e CD8, respetivamente [302–304]. Outros fatores, como o TOX e o GATA3, parecem estar também envolvidos no comprometimento com a linhagem T CD4 [305306].

Por fim, as células T CD4 e T CD8 com um repertório TCR rigorosamente selecionado e com a aquisição do marcador naïve CD45RA, saem do timo e migram para a periferia, incorporando o *pool* de células T naïve maduras [258].

1.2.2.1. Linfócitos T CD4

Como já foi referido, os linfócitos TCRαβ que sobrevivem aos mecanismos de seleção no timo dividem-se em dois grandes grupos. Os linfócitos T $CD4^+$, conhecidos como linfócitos auxiliares (Th), representando cerca de 40-50% dos linfócitos em circulação, e os linfócitos T $CD8^+$, designados por linfócitos citotóxicos ou Tc, representando 20-25% dos linfócitos no sangue. Para além destes linfócitos ditos convencionais, existem também linfócitos T com propriedades reguladoras/supressoras, descritos mais à frente.

No entanto, os linfócitos T (naïve) que saem do timo, nunca foram ativados por um antigénio, e só após esta interação, é que se poderá originar linfócitos T $CD4^+$ com propriedades auxiliares (ajudam a promover resposta B, T CD8, e na ativação das células do sistema inato) e linfócitos T CD8 com propriedades citotóxicas. Uma característica importante destas células é a expressão do recetor CD28, que irá ser crucial na mediação da resposta imune após contacto com antigénios [307].

A ativação de uma resposta eficaz do sistema imunológico adaptativo contra qualquer agressão, seja ela externa ou interna, envolve uma serie de fases [308], que se encontram intrinsecamente associadas a processos de sobrevivência e de diferenciação, com mudanças fenotípicas e funcionais dos linfócitos T naïve ativados [309].

A ativação T têm início com o reconhecimento por parte de complexos TCR/CD3 presentes nos linfócitos T $CD4^+$ ou $CD8^+$ de péptidos antigénicos expostos na superfície de células dendríticas (DC) em associação com moléculas de MHC classe II e classe I, respetivamente [310]. Esta interação TCR/CD3-MHC:péptido constitui o sinal de ativação primário, e confere especificidade à resposta adaptativa. No entanto, este sinal não é suficiente para induzir uma ativação eficaz e promover a proliferação dos linfócitos T naïve, sendo necessário o envolvimento de outros sinais, nomeadamente pelo recetor CD28, constituindo o sinal 2 [311]. De facto, a existência de um único sinal 1, na ausência de sinais acessórios, pode induzir a célula a entrar num estado de anergia, caracterizado pela incapacidade de responder a estímulos, nomeadamente antigénios [307].

Assim, este duo de sinais mediados pelo complexo TCR/CD3 e do recetor CD28 inicia uma serie de eventos intracelulares que resultam num processo de divisão celular que pode durar vários dias, com o objetivo de formar uma população de linfócitos efetores, juntamente com uma pequena fração de células de memória [308]. Esta fase de proliferação está altamente regulada por recetores sinalizadores expressos unicamente na superfície dos linfócitos T ativados, nomeadamente recetores ativadores da família do CD28 (por exemplo ICOS, expresso maioritariamente por linfócitos T $CD8^+$), da família do TNFR (*Tumor Necrosis Fator Receptor;* por exemplo CD40L, expresso maioritariamente por linfócitos T $CD4^+$) e de recetores de citocinas, bem como pela presença dos seus ligandos no microambiente que os rodeia. De facto, é exatamente este sinal proveniente da interação entre citocinas presentes no microambiente e os recetores que define o sinal 3 da ativação [312]. Dentro dos recetores e suas respetivas citocinas, salienta-se o CD25 (IL-2), CD124 (IL-4), CD132 ou recetor ɣc (IL-2, IL-4, IL-7, IL-9, IL-15 e IL-21), e CD212 (IL-12). As citocinas referidas podem ser produzidas por DCs, por linfócitos T mais diferenciados, assim como por células do microambiente como fibroblastos e células epiteliais [313].

Todos estes processos que ocorrem durante a ativação do linfócito T, resultam no início de um complexo processo diferenciação que irá originar linfócitos T com características efetoras [308]. Os linfócitos T $CD4^+$ diferenciam-se em vários subtipos, como as Th1, Th2, Th17, Treg, Th9, Th22, T reguladoras e T foliculares (Tfh) [314] (**Figura 5**).

1.2.2.1.1. Linfócitos Th1

Estes linfócitos Th1 surgem após ativação de um linfócito $CD4^+$ naïve por uma DC num microambiente rico em IL-12 e INF-ɣ, presumivelmente produzidas pela DC ativada [313315]. A exposição a estas citocinas resulta na ativação dos fatores de transcrição STAT-4, T-bet e Eomes, que vão regular a ativação da transcrição dos genes das citocinas IL-2, INF-ɣ e TNF-β, passando estes linfócitos a produzir e secretar estas citocinas [316317313].

A IL-2 é importante como fator de crescimento para os próprios linfócitos Th1, enquanto que o INF-ɣ atua na amplificação da resposta imunológica, nomeadamente ao nível da ativação de macrófagos, do desenvolvimento de linfócitos Th1, do aumento da atividade citotóxica de linfócitos T CD8 e NK, e do aumento da capacidade apresentadora de antigénios por DCs. No geral, os linfócitos Th1 são responsáveis por potenciar respostas contra bactérias intracelulares e vírus, mediadas por outros linfócitos (T $CD8^+$ e NK), estando assim envolvidos na regulação de respostas celulares [313].

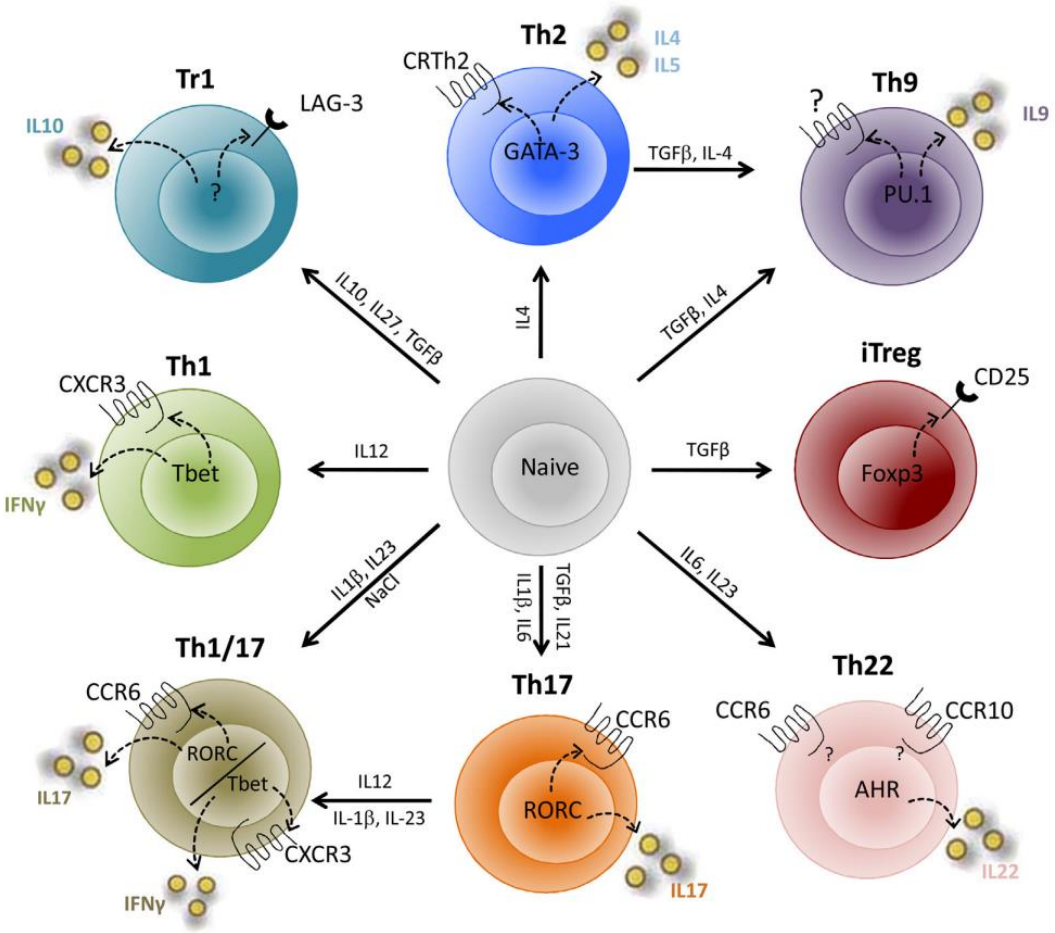

Figura 5. Diferenciação das células T naïve. Adaptado de Geginat. [318]

As células T naïve podem diferenciar-se em diversos subtipos de células T efetoras após sinalização por citocinas específicas. Estes subtipos de células T expressam fatores de transcrição definidores de linhagem que induzem marcadores de superfície específicos, e secretam citocinas efetoras específicas após a estimulação do seu TCR.

1.2.2.1.2. Linfócitos Th2

Os linfócitos Th2 caracterizam-se pela produção de IL-4, IL-5, IL-9, IL-10 e IL-13, e diferenciam-se após ativação por DC num microambiente onde predomina a IL-4, presumivelmente produzida pelo linfócito T $CD4^+$ horas depois da ativação [315,319]. Nesta diferenciação estão envolvidos os fatores de transcrição STAT-6, GATA-3 e C-Maf, que regulam a ativação dos genes das citocinas referidas [313]. De salientar que o GATA-3 não só promove a diferenciação em linfócitos Th2, mas também impede o acesso de STAT-4 às regiões promotores dos genes das citocinas IL-2, INF-ɣ e TNF-β, inibindo a diferenciação em linfócitos Th1. As citocinas produzidas pelas células Th2 atuam ao nível da regulação da produção de Ig's por linfócitos B, ativação de eosinófilos, desgranulação de mastócitos, inibição da formação de linfócitos Th1 e inibição da ativação de macrófagos. Desta forma,

os linfócitos Th2 exercem um papel critico na regulação de respostas humorais e doenças alérgicas, bem como na defensa contra agentes patogénicos extracelulares (helmintas) [315].

1.2.2.1.3. Linfócitos Th17

Como o nome sugere, os linfócitos Th17 caracterizam-se pela produção principal de IL-17, por ativação do fator de transcrição RORɣt [320]. Este programa de diferenciação é induzido após ativação num microambiente onde predominem as citocinas TGF-β, IL-1β, IL-6 e IL-23 [321,322]. Os linfócitos Th17 são células altamente inflamatórias, envolvidas nas respostas contra bactérias extracelulares e fungos, através do recrutamento de monócitos e neutrófilos, estando também envolvidos na patogénese de algumas doenças autoimunes [323324].

1.2.2.1.4. Linfócitos T reguladores

As células T reguladoras (Tregs) são um subtipo de linfócitos T $CD4^+$ que desempenham um papel crucial no equilíbrio do sistema imunitário, ao manterem a tolerância a autoantigénios e prevenirem uma resposta exagerada das células efectoras [325]. As Tregs intervêm na maioria das respostas imunes - incluindo alergia, autoimunidade, inflamação, e reações a micróbios e tumores. Para isso, são capazes de controlar a atividade da maioria dos subtipos celulares do sistema imunitário inato e adaptativo [326].

Sakaguchi, em 1995, identificou pela primeira vez as células $CD4^+CD25^+$ como as responsáveis pela manutenção da tolerância ao self, e provou que a sua depleção se associava ao desenvolvimento de diversas doenças autoimunes em ratinhos [327]. Apesar desta evidência ter aberto as portas ao estudo das Tregs, a comunidade científica na altura encontrava-se focada no estudo de citocinas e outros intervenientes da imunidade humoral, de modo que estas células apenas foram identificadas em humanos passados 6 anos [328]. Apesar da identificação do CD25 como um marcador das Tregs ter permitido estudos funcionais destas células, a sua utilidade é limitada pela sua sobre-expressão em células ativadas, pelo que na altura não era possível distinguir entre células Tregs e células T convencionais ativadas no contexto de inflamação [326]. A identificação do Fator de transcrição *forkhead-box protein* P3 (Foxp3) [329] estabeleceu definitivamente as Tregs como uma linhagem distinta de células T, assistindo-se desde então a um crescimento exponencial do interesse nestas células.

Origem, Classificação e Função das Tregs

De acordo com a sua origem, as células Tregs são classificadas em naturais ou tímicas (nTregs ou tTregs) e induzidas ou adaptativas (iTregs ou pTregs).

As células nTregs $CD4^+$ de origem tímica representam 5 a 10% das células CD4 circulantes [330]. Estas células formam-se durante o período de seleção das células T no timo, sendo demonstrado em ratos a sua migração para o sangue periférico a partir do 3º dia de vida [331]. Os percursores das tTregs são timócitos cuja afinidade do TCR para péptidos do *self* se encontra em níveis intermédios entre os das células que sofrem deleção clonal por apoptose devido a elevada afinidade e os das células que sofrem seleção positiva por baixa afinidade [332,333]. No entanto, admite-se que possa existir já o compromisso com a linhagem Treg ainda antes do processo de seleção pela afinidade do TCR, permitindo a inclusão nesta população de células com recetor de baixa afinidade para o *self* [334]. As Tregs que se desenvolvem no timo migram posteriormente para os órgão linfoides periféricos, onde as suas propriedades são reforçadas pela ativação, ao mesmo tempo que as suas características são perpetuadas por múltiplas divisões celulares [335]. Algumas células Tregs migram para órgãos não-linfoides, como o tecido adiposo visceral, o músculo e o intestino, onde adquirem características adicionais por influência do meio [336]. Esta atividade dinâmica das Tregs é controlada ao nível transcricional e epigenético [335].

O Foxp3 é essencial para a função supressora, potencial proliferativo e metabolismo das células Tregs, e, além disso, impede a diferenciação dos percursores das Tregs em células T efectoras [337]. Recentemente foi descrito que a co-expressão de Foxp3 e Helios (um Fator de transcrição da família Ikarus) representa um estadio crítico na atividade das Tregs [338,339]. Foi também demonstrado que as células Tregs $Helios^+Foxp3^+$ têm propriedades supressoras superiores às Tregs $Helios^-Foxp3^+$ [340]. Devido à sua expressão tímica, o Helios permite descriminar entre tTregs ($Helios^+$) e iTregs ($Helios^-$) em ratos, mas é controverso se em humanos este marcador é igualmente discriminativo [341].

Uma vez adquirida a identidade de Tregs, estas células mantém a sua linhagem estável através de múltiplos mecanismos moleculares, incluindo alguns dos que contribuíram para a sua génese, como o TCR e a IL-2 [342].

As pTregs desenvolvem-se na periferia a partir de células naïve ou, por vezes, a partir de células diferenciadas, após estimulação antigénica em condições propícias [343] (**Figura 6**). Há ainda limitações na compreensão dos processos envolvidos em cada microambiente periférico, mas é segura a necessidade de estimulação do TCR e a presença de TGF-β e IL-2 [344]. Apesar de corresponderam apenas a uma pequena percentagem do total das Tregs, as pTregs são particularmente abundantes em órgãos específicos, como o intestino e a placenta [345]. Deste modo, esta células serão especialmente importantes na manutenção da tolerância a bactérias comensais, alimentos, alergénios, e ao feto [345].

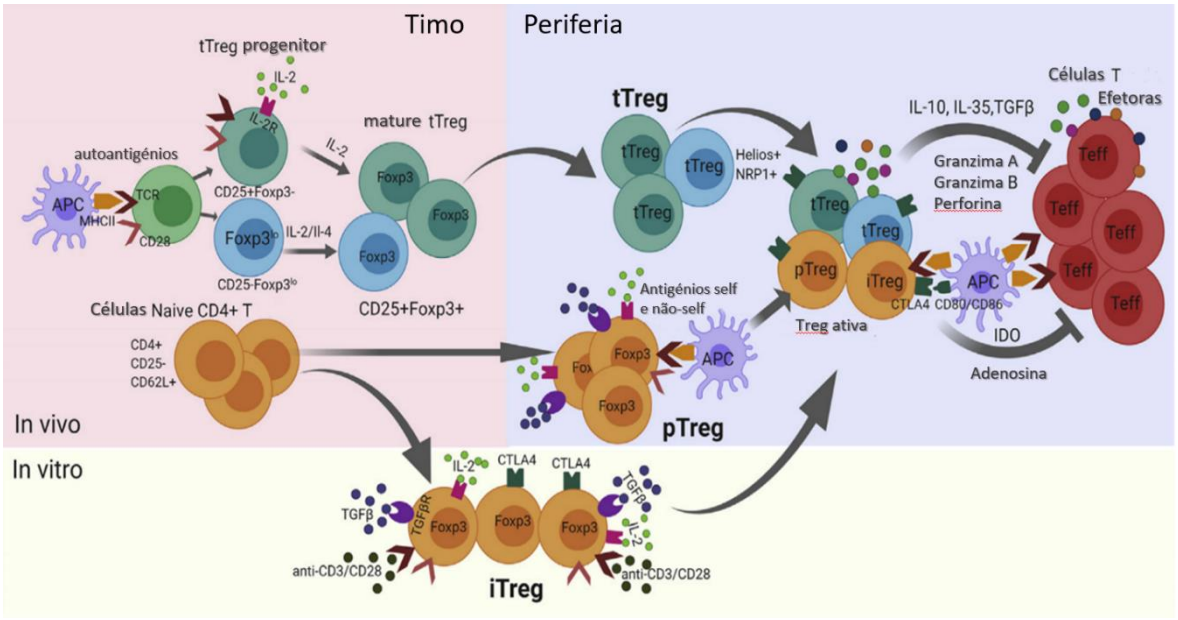

Figura 6. Diferenciação, fenótipos e caraterísticas funcionais das células Treg. Adaptado de Zhang (2020) [346].

As células Treg CD4+ Treg classificam-se em 2 grupos; as tTregs (tímicas), e as Tregs induzidas (pTreg, induzidas in vivo; iTreg, induzidas in vitro). As Treg exercem as suas ações supressoras sobre as células T efetoras por contacto direto através do CTLA-4, ou pela secreção de citocinas imunossupressoras e moléculas pro-apoptóticas.

Ambos os subtipos cooperam na manutenção da homeostase do sistema imunitário [344], sendo as Tregs originadas na periferia tão eficazes na supressão das respostas imunes com as Tregs geradas no timo [347]. As Tregs são capazes de inibir diversos tipos de células, incluindo linfócitos T $CD4^+$ e $CD8^+$, células *natural killer* (NK), linfócitos B, monócitos e células dendríticas [348]. O efeito supressor das Tregs é exercido através de vários mecanismos (Sakaguchi et al. 2009; Schmidt, Oberle, and Krammer 2012; Shevach 2009): produção de citocinas inibidoras, como a Il-10, Il-35 e TGF-β; atividade citotóxica envolvendo a libertação de perforina e granzima; indução da apoptose através da disrupção de vias metabólicas; redução da co-estimulação através da interferência com vários mecanismos funcionais das células dendríticas; inibição da maturação das células B através da redução da ação T-efectora; inibição da síntese de anticorpos e da recombinação da classe de Ig. Os principais mecanismos de ação das Tregs estão representados na **Figura 7.**

Outros subtipos de células T com propriedades reguladoras foram já descritos, para além das Tregs $CD4^+CD25^+Foxp3^+$, como as as células $CD4^+CD25^{low}GITR^+$ [352], as Th3 [353], as Tr1 [354], as Treg $CD8^+CD25^+$ [355], as Treg γδ [356] e as células NKT [357], e as Tregs foliculares (Tfr) [358].

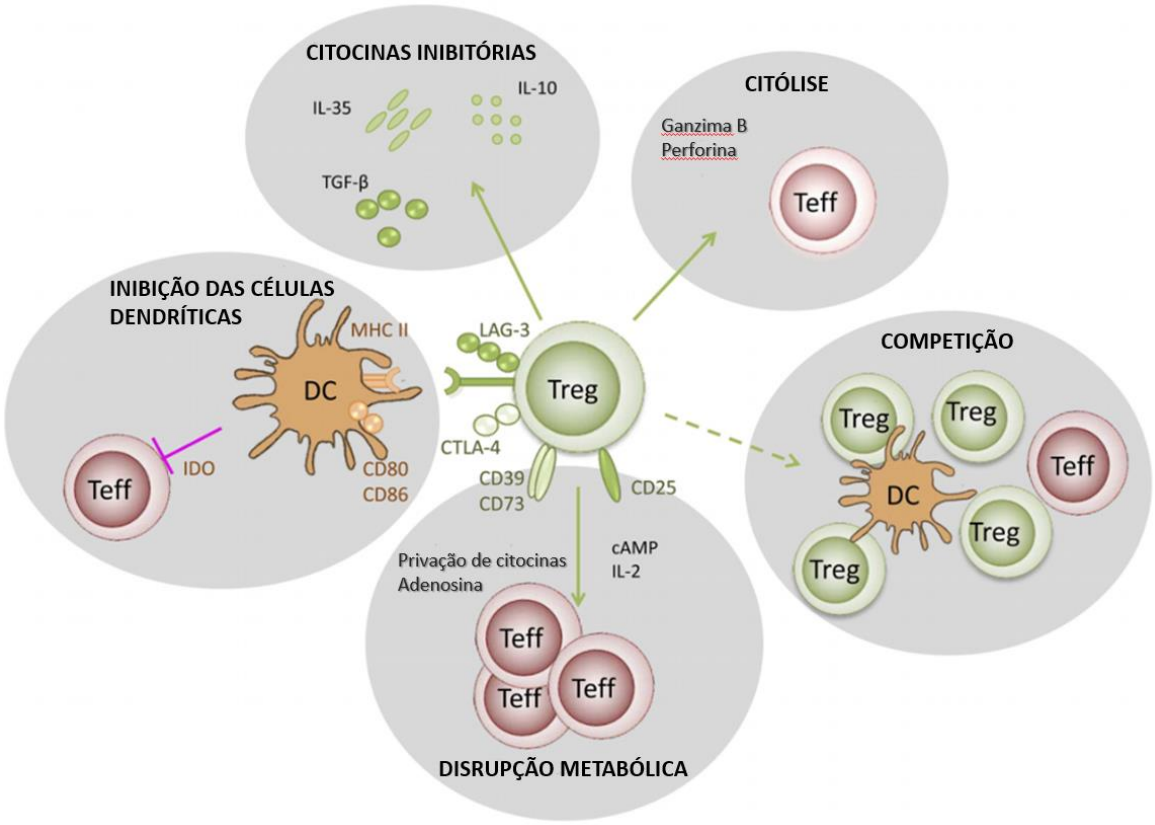

Figura 7. | Mecanismos de ação das células T reguladoras. Adaptado de Caridade (2013) [359].

As células T reguladoras foliculares tem um perfil de marcadores de superfície de célula T folicular (CXCR5hi PD-1hi ICOS+) e localizam-se nos CG, mas também expressam Foxp3 e apresentam um perfil de célula Treg ativada, através da expressão de CTLA-4, GITR, ICOS, e IL-10 [360]. A diferenciação de tTreg e pTreg em Tfr é influenciada por sinais fornecidos por células dendríticas e células B. Estas células apresentam grande heterogeneidade, consoante a sua via de desenvolvimento e progenitor, a especificidade de antigénio, a produção de IL-10 e a expressão de PD1 à superfície cells [361].

Identificação das Tregs

As Tregs são definidas pela expressão de Foxp3, que é essencial ao o seu desenvolvimento e função supressora [362], sendo este marcador considerado o "*gold standard*" para a identificação das Tregs [233]. No entanto, devido à localização intracelular do Foxp3, a identificação das Tregs por citometria de fluxo exige várias etapas, o que a torna morosa e tecnicamente exigente, limitando a sua reprodutibilidade e aplicação clínica [363]. Adicionalmente, a necessidade de fixação das células inviabiliza a realização de estudos funcionais adicionais, pelo que há a necessidade de identificar marcadores de superfície específicos das Tregs.

Antes da emergência do Foxp3 como marcador de eleição para a identificação das Tregs, estas eram definidas apenas pela expressão de CD25, mas a sua utilização isolada foi abandonada após a constatação de que o CD25 é expresso também em células T efectoras ativadas [364]. Vários outros marcadores de superfície têm sido estudados e propostos para identificar as Tregs, mas a demonstração de que a expressão de CD127 se correlacionava inversamente com o CD25 e que as Treg não expressam níveis valorizáveis de CD127, levou à identificação da combinação $CD25^{+}CD127^{low/-}$ como o melhor candidato [365]. Foi demonstrado que as células $CD25^{+}CD127^{low/-}$ não só tinham a maior expressão de Foxp3, como tinham também uma excelente capacidade supressora [365]. Consequentemente, o *Human ImmunoPhenotyping Consortium* (HIPC) propôs um painel padrão para identificação das principais populações linfocitárias, incluindo as Tregs, para as quais apenas seriam utilizados marcadores de superfície, excluindo o Foxp3 [161]. No entanto, uma vez que são conhecidas populações T $CD127^{+}$ que expressam Foxp3, a identificação das Tregs apenas como células $CD25^{+}CD127^{low/-}$, embora interessante na prática clínica, poderá não ser suficiente para fins de investigação [366]. Novas técnicas e protocolos têm sido propostos, como por exemplo a associação de marcadores de superfície e Foxp3 de modo simplificado, com boa reprodutibilidade [363].

1.2.2.2. Linfócitos T foliculares

Os linfócitos T $CD4^{+}$ foliculares providenciam uma ajuda especial aos linfócitos B nos folículos dos órgãos linfoides secundários, sendo por isso essenciais na formação de centro germinativos, maturação da afinidade, e desenvolvimento de linfócitos B de memória e anticorpos de alta afinidade [367].

A diferenciação dos linfócitos Tfh é um processo multifatorial com várias etapas. Não existe, por isso, um único evento que defina a diferenciação dos linfócitos Tfh, ao contrário do que se observa, por exemplo, na diferenciação de linfócitos Th1, que pode ser facilmente induzida por exposição a IL-12. De forma semelhante, a diferenciação dos linfócitos Tfh começa com interações entre DCs e linfócitos T CD4 naïve [368], com consequentes ciclos de proliferação [369,370]. Na presença de IL-6 e IL-21, os linfócitos T CD4 ativados vão iniciar a diferenciação em linfócitos Tfh, com consequente expressão dos fatores de transcrição Bcl-6 e Maf, e dos recetores ICOS, PD-1 e CXCR5 [371]. A expressão de CXCR5 permite a migração dos linfócitos Tfh em diferenciação para a região interfolicular dos folículos dos órgãos linfoides secundários.

A sinalização da IL-6 e IL-21 constituem os primeiros sinais envolvidos na iniciação da diferenciação dos linfócitos Tfh, induzindo a expressão do fator de transcrição Bcl6 e Maf nos linfócitos T CD4 ativados [372]. De facto, vários modelos mostram a necessidade da presença de Bcl6 na expressão inicial de CXCR5 [373,374], enquanto que a ausência de IL-6 resulta em defeitos na diferenciação de linfócitos Tfh [374]. Desconhece-se, no entanto, quais

as células especificas que iniciam a diferenciação dos linfócitos Tfh, sendo provável que não haja nenhum tipo celular específico, uma vez que várias DCs e monócitos conseguem iniciar esta diferenciação em diferentes condições [375].

Uma vez na região interfolicular, os linfócitos Tfh vão interagir com linfócitos B específicos para o antigénio que deu início a esta diferenciação. Esta co-localização é possível porque, como já referido, os linfócitos Tfh possuem CXCR5 (são atraídos para a zona B por CXCL13, abundante nesta região) e perdem a expressão de CCR7, cujos ligandos (CCL19 e CCL21) se encontram na região T. De facto, os linfócitos B são cruciais na continuação do desenvolvimento dos linfócitos Tfh [151], porque para além atuarem como células apresentadoras de antigénio, possuem ICOSL na sua superfície. Esta interação ICOS-ICOSL é fundamental para a estabilização e diferenciação das células Tfh [139,143,369,376]. Por último, a diferenciação final dos linfócitos Tfh envolve a formação dos centros germinativos, onde vão exercer a sua função nos processos acima descritos, através de interações com os linfócitos B e produção das citocinas IL-21, IL-4 e CXCL13 [145,377,378]. Os linfócitos Tfh dos centros germinativos tem uma expressão de proteínas de superfície, fatores de transcrição, e citocinas secretadas bem conservadas entre espécies, sendo caracterizadas como linfócitos T CD4 com forte expressão de CXCR5, PD-1 e Bcl6, e baixa expressão de CCR7 e S1P1R. Estes linfócitos expressam também importantes moléculas de adesão, incluindo SLAMF (*signaling lymphocyte ativation molecule family*)-6 CD84 e SAP (*SLAM-associated protein*) [379].

Uma vez diferenciadas, os linfócitos Tfh não ficam confinados nos centros germinativos. Ao contrário dos linfócitos B, os linfócitos Tfh podem rapidamente sair do centro germinativo e (1) transitar para o centro germinativo de um folículo vizinho [380] ou (2) permanecer temporariamente num folículo adjacente antes de entrar no centro germinativo de origem, ou (3) sair do centro germinativo, baixar a expressão de Bcl6 e desenvolver-se num linfócito Tfh de memória [380,381]. Assim, um linfócito Tfh $CXCR5^{+}$ fora do centro germinativo pode representar diferentes vias de migração, com redução da expressão de Bcl6. Os linfócitos Tfh de memória residem principalmente no baço, nódulos linfáticos e medula óssea, e possuem a capacidade de recirculação no sangue. De facto, cerca de 20% dos linfócitos T CD4 de memória central expressam CXCR5, evidenciando a importância deste compartimento [382,383].

O comportamento do programa de diferenciação dos linfócitos Tfh é semelhante ao referido para as outras subpopulações efetoras, com a participação de principais fatores de transcrição, nomeadamente Bcl6, Maf, STAT3 e STAT4. Estes fatores de transcrição, controlam os aspetos gerais dos linfócitos Tfh, incluindo a localização, função, diferenciação, e repressão de programas de diferenciação alternativos, através da inibição da expressão de recetores necessários para a diferenciação de outros linfócitos T efetores. De facto, Bcl6 tem um papel importante neste aspeto, inibindo a função de Blimp-1, fortemente associado com a diferenciação de outros linfócitos não Tfh [384].

Tal como foi referido para os outras populações efetoras TCD4, também os linfócitos Tfh circulantes podem ser divididos em três principais compartimentos, com base na expressão de CXCR3 e CCR6: linfócitos cTfh1 ($Bcl6^{-}CXCR3^{+}CCR6^{-}$), linfócitos cTfh2 ($Bcl6^{-}CXCR3^{-}CCR6^{-}$), e linfócitos cTfh17 ($Bcl6^{-}CXCR3^{-}CCR6^{+}$) [385]. Para alem destas populações, existem também linfócitos T foliculares com propriedades reguladoras (Tfr), que regulam as respostas adaptativas nos folículos linfoides e centros germinativos. Estes linfócitos expressam marcadores de linfócitos Tfh (CXCR5, Bcl-6, PD-1 e ICOS) e marcadores de linfócitos Treg (FoxP3, IL-10, GITR e CTLA4) [386,387].

Os linfócitos Tfh estão envolvidos em várias doenças autoimunes, sendo esperado que o aumento do conhecimento sobre estas populações origine novas abordagens terapêuticas. De facto, foram observadas frequências aumentadas de linfócitos com perfil Tfh ($CXCR5^{+}$ ou $PD\text{-}1^{Hi}$ ou $ICOS^{Hi}$) em doentes com síndrome de Sjögren [21,388], dermatomiosite juvenil [385], e Lupus Eritematoso Sistémico [216,388] (He et al., 2013; Simpson et al., 2010). Uma vez que estas doenças estão associadas com produção anormal de autoanticorpos, os linfócitos Tfh poderão estar na génese destas doenças autoimunes, não só por facilitarem a produção destes autoanticorpos, mas também por facilitarem a formação e manutenção dos folículos ectópicos, que servem como nichos para outras células potencialmente patogénicas no contexto das doenças autoimunes.

1.2.2.3. Linfócitos T CD8

Ao contrário dos linfócitos T CD4, os linfócitos T CD8 são ativados após reconhecerem antigénios pelas moléculas de MHC classe I nas APCs. Esta população de linfócitos tem um papel muito importante na defesa contra agentes patogénicos intracelulares, incluindo vírus e bactérias, vigilância tumoral, e contribuem na regulação de processos patológicos, tais como doenças autoimunes e alérgicas [389]. De forma semelhante, a ativação dos linfócitos T CD8 envolve a participação de sinais acessórios e citocinas secretadas por APCs e/ou linfócitos T CD4, com influência na diferenciação dos linfócitos T CD8 em diferentes populações efetoras. No entanto, em alguns casos, como DCs infetadas, a infeção cria inflamação suficiente para a produção das citocinas e sinais acessórios necessários para ativar as células T CD8 na ausência de células T CD4 [389].

Após ativação, o linfócito T CD8 tem vários mecanismos de atuação [390,391]. De forma geral, o modelo de atuação dos linfócitos T CD8 na destruição de células alvo assenta na libertação de moléculas citotóxicas, como perforina e granzimas. Enquanto que a perforina forma um poro na membrana da célula-alvo, as granzimas, por sua vez, vão clivar as proteínas dentro da célula alvo, impossibilitando a produção de proteínas virais. Por fim, a célula alvo entra em apoptose. É importante referir que os grânulos citotóxicos são libertados apenas na diretamente contra a célula-alvo, alinhadas ao longo da sinapse imunológica, para evitar danos indiretos nas células adjacentes não infetadas. Os linfócitos

T CD8 secretam ainda citocinas que ajudam na resposta inata e adaptativa contra agentes patogénicos intracelulares, nomeadamente INF-γ (interferão-γ) e TNF-α (Fator de Necrose Tumoral α) [390,391]. Por último, os linfócitos T CD8 exercem a sua função através das interações Fas/FasL. Os linfócitos T $CD8^+$ ativados expressam FasL à superfície celular, ligando do recetor Fas que se encontra à superfície da célula alvo. Esta interação promove a ativação das caspases na célula alvo, resultando na indução da apoptose [392].

Ainda que não estejam tão bem caracterizados, os linfócitos T $CD8^+$ naïve podem também diferenciar-se em vários subtipos com fenótipos efetoras, ainda que nem todos com propriedades citotóxicas, semelhantes aos que foram referidos para os linfócitos T $CD4^+$, após interação com as DCs – Linfócitos Tc1 (produção de INF-γ), Tc2 (produção de IL-4), Tc17 (produção de IL-17), Tc9 (produção de IL-9) e Tc22 (produção de IL-22) [390]. Ainda que os programas de diferenciação destes linfócitos sejam semelhantes aos dos linfócitos $TCD4^+$, nomeadamente o tipo de DC e o ambiente citocínico, existem alguns fatores que parecem ser específicos dos linfócitos T $CD8^+$ [389].

Recentemente, foram descritos linfócitos T CD8 com propriedades reguladoras, ainda que com propriedades fenotípicas e funcionais dos linfócitos T CD4 reguladores .[393]. Linfócitos T CD8 com expressão de CXCR5 e localizados junto dos linfócitos B nos folículos dos órgãos linfoides secundários foram também recentemente descritos em humanos – linfócitos T citotóxicos foliculares (Tfc). Esta população de linfócitos é semelhante aos linfócitos Tfh, com participação dos mesmos fatores de transcrição [394].

2. SÍNDROME DE SJÖGREN PRIMÁRIA

2.1. Definição

A Síndrome de Sjögren (SS) é uma doença inflamatória crónica, sistémica, de origem autoimune, que se caracteriza pela infiltração linfocitária e lesão das glândulas exócrinas, sendo também designada epitelite autoimune [395]. O envolvimento das gândulas salivares e lacrimais, das glândulas exócrinas do epitélio nasal, do aparelho respiratório superior, da vagina e pele origina disfunção glandular e as queixas secas que caracterizam a doença [396]. Podem ocorrer manifestações extra-glandulares ou sistémicas, como artrite, lesões cutâneas, envolvimento pulmonar, neurológico, renal ou vasculite [397].

A SS pode ocorrer isoladamente, sendo designada SS primária (SSP), ou associada a outras doenças sistémicas, em particular à Artrite Reumatóide (AR) [398,399], ao Lupus Eritematoso Sistémico (LES) [400,401] ou à Esclerose Sistémica (ESP) [402,403], sendo nesses casos designado SS secundária ou associada. Apesar da distinção entre SSP e SS secundária estar instituída na prática clínica desde há mais de 50 anos [404], atualmente esta distinção tem sido contestada [405,406]. Pode também ocorrer a presença simultânea de SSP e doenças autoimunes específicas de órgão, como tiroidite autoimune, colangite biliar primária ou hepatite autoimune [407].

2.2. Perspectiva histórica

Henrik Sjögren, um oftalmologista sueco, descreveu em 1933 uma condição que designou queratoconjuntivite sicca [408], e que veio a receber o seu nome - Síndrome de Sjögren. No entanto, já em 1925 Gougerot, um dermatologista francês, havia descrito casos de sindrome sicca e atrofia glandular [409]. A tumefação glandular e as características histológicas tinham sido descritas por Mikulicz, em 1892 [410], embora atualmente se considere que a entidade descrita por este corresponda à Doença Relacionada com a IgG_4 [411,412].

Na década de 1960 a origem autoimune da SSP foi confirmada e importantes avanços no diagnóstico da doença foram introduzidos, como a identificação dos anticorpos anti-Ro/SSA e anti-La/SSB [413] e a elaboração de uma classificação histológica da biópsia de glândula salivar minor por Chisholm e Mason [414]. Foi também descrita a associação entre SSP e linfoma MALT (*mucosa-associated lymphoid tissue*) de células B [415].

O conhecimento sobre a SSP tem progredido continuamente, mas os aspectos fundamentais que caracterizam a doença foram reconhecidos nesta fase inicial: o desenvolvimento de queixas secas, o envolvimento sistémico, a infiltração linfocitária das glândulas exócrinas, a presença de autoanticorpos, e o risco aumentado de linfoma [396].

2.3. Epidemiologia

A SSP afeta predominantemente mulheres, com uma razão feminino-masculino de aproximadamente 10:1 [416]. A doença pode ocorrer em qualquer idade, mas é mais frequente o seu início entre os 40 e os 60 anos [417].

A SSP é uma das doenças autoimunes sistémicas mais frequentes, afetando entre 0,01% e 1% da população [416,418]. Não há dados sobre a prevalência da SSP em Portugal, mas em Espanha a prevelência foi estimada em 0,33% da população [419], quando consideradas as formas primária e secundária. A variabilidade na prevalência verifica-se não só entre diferentes regiões do mundo, mas inclusivé no mesmo país. Entre as razões desta discrepância estarão diferentes metodologias, como os critérios de classificação utilizados e a sua aplicação, bem como a especificidade da recolha da informação, que pode variar desde estudos em contexto comunitário até casuísticas hospitalares. Também é de considerar a influência de fatores genéticos e ambientais na diferença das prevalências regionais [420].

2.4. Fisiopatologia

A imunopatogénese da SSP é complexa e envolve o sistema imune inato e os ramos celular e humoral do sistema imune adaptativo, sob influência de complexas interações genéticas, ambientais e hormonais [421].

Foram identificados diversos processos patogénicos: ativação de vias da imunidade inata e adquiririda, como as relacionadas com os sistemas do Interferão (IFN) tipo I e II [422], ação deficiente das células T reguladoras [423], aumento da atividade das T efectoras [424], linfomagénese com formação de CGE, e ativação anormal das células B e a sua expansão clonal [425]. Os possíveis mecanismos imunopatogénicos subjacentes à SSP estão representados na **Figura 8**.

Crê-se que o fenómeno inicial é a lesão epitelial, provavelmente induzido por um agente patogénico viral, que estará na origem da perda de tolerância [426]. A resposta immune anormal resulta em aumento da apoptose das células acinares e ductais das GS, que leva à ativação de céluals T e NK pela ação de citocinas pró-inflamatórias [427,428]. Esta

libertação maciça de antigénios poderá levar, por um processo de expansão de epitopos, à extensão do processo autoimune a outros locais do corpo [429].

Na fase inicial da SSP, o processo patogénico é dominado pelas células T, cuja progressiva infiltração à volta dos ductos das glândulas salivares e lacrimais compromete a função glandular e leva à secura oral e ocular [430]. Em simultâneo dá-se o recrutamento de linfócitos B e células apresentadoras de antigénio, com formação de agregados linfocitários no tecido glandular, designados "focos" [431]. O aumento da produção de interlucina 21 e da citocina ativadora de linfócitos B (BAFF) promove a ativação dos linfócitos B e a produção de anticorpos [432].

Embora os linfócitos T $CD4^+$ predominem nas lesões epiteliais [433], são os distúrbios das células B que constituem a face mais visível da SSP, quer a nível da expressão e evolução clínica, quer no prognóstico [434–436]. A hiperatividade dos linfócitos B na SSP pode ser reconhecida pela presença de hipergamaglobulinémia, crioglobulinémia, produção de citocinas e de autoanticorpos [437], e resulta num risco aumentado de linfoma [438,439].

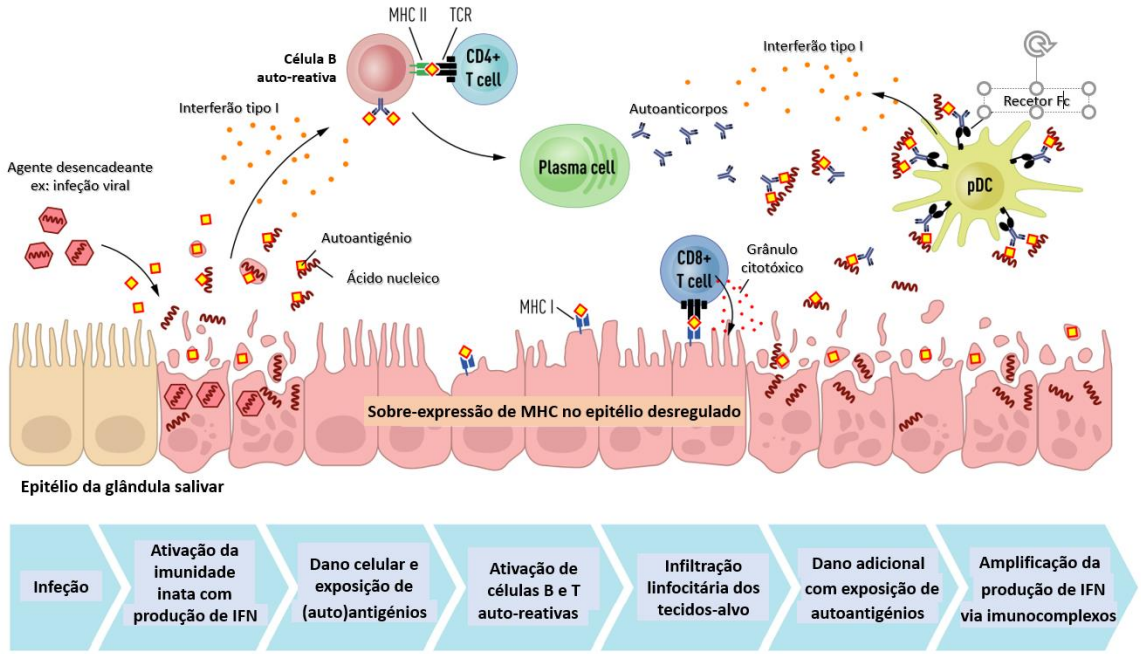

Figura 8 – Possíveis mecanismos imunopatogénicos subjacentes à SSP. Adaptado de Bjork (2020) [440].

Agentes microbianos, como infeções virais, iniciam a disrupção do epitélio das glândulas salivares e induzem a produção de IFN tipo I, criando assim um micro-ambiente inflamatório com autoantigénios libertados e expostos nas células apoptóticas. As células apresentadoras de antigénio processam e apresentam antigénios virais e autoantigénios, levando à ativação de células T e B auto-reativas e subsequente diferenciação e ativação de plasmócitos. As células T auto-reativas agravam a lesão tecidular através da secreção de grânulos citotóxicos, aumentando a exposição de autoantigénios. Num ciclo auto-perpetuante de autoimunidade, os imunocomplexos ligam-se às pDCs, resultando num aumento do IFN tipo I, que novamente induz aumento da produção de autoanticorpos através da amplificação da diferenciação e ativação de células B.

IFN, interferão; pDCs, células dendríticas plasmacitóides; MHC, complexo *major* de histocompatibilidade; TCR, recetor de célula T.

2.4.1 Fatores genéticos

Na SSP, a predisposição genética, mecanismos epigenéticos e Fatores ambientais contribuem para o desenvolvimento da doença [441]. (**Figura 9**)

Os Fatores de risco genéticos mais importantes na SSP estão associados ao complexo major de histocompatibilidade (MHC), nomeadamente os alelos da classe II DRB1*03:01, DQA1*05:01 e DQB1*02:01 [442].

Vários genes não-MHC, relacionados com a imunidade inata e adaptativa, foram também identificados como Fatores de risco [443,444]. Genes envolvidos na via do IFN têm expressão aumentada na SSP, incluindo o STAT4 (*signal transducer and ativator of transcription 4*), IRF5 (IFN *regulatory Fator 5*), IL-12A, e OAS1 (*2-5 oligoadenylate synthetase 1*), e associam-se à presença de anti-SSA e SSB [422]. O aumento da expressão de TLR7 (*toll-like receptor 7*) e TLR9 e da ativação de células dendríticas plasmocitóides são também Fatores genéticos que contribuem para a assinatura do IFN na SSP [445]. Alguns SNP (*single nucleotide polymorphisms*) e alelos de risco relacionados com o IFN, quimiocinas e seu receptores, e com a resposta inflamatória têm também sido identificados [446,447].

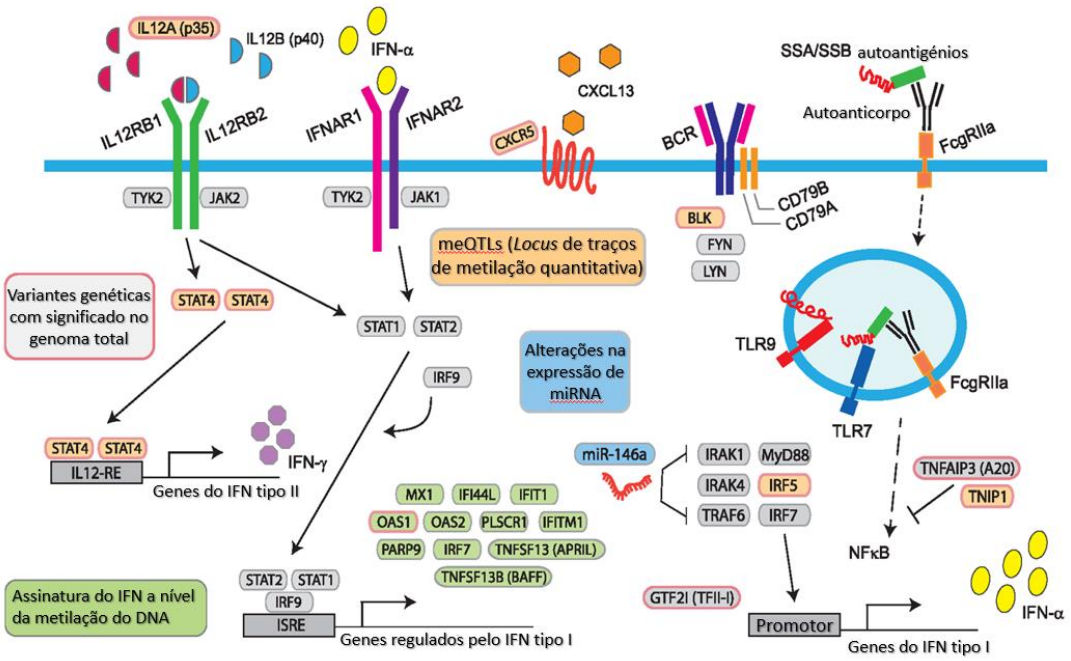

Figura 9. Mecanismos genéticos e epigenéticos associados à susceptibilidade para SSP. Adaptado de Imgenberg-Kreuz (2019) [441].

Caixas Rosa: genes não-HLA com SNP associados à susceptibilidade para SSP com significado no genoma total, incluindo *BLK*, *CXCR5*, *GTF2I*, *IL12A*, *IRF5*, *OAS1*, *STAT4*, *TNFAIP3* and *TNIP1*

Caixas Verdes: genes induzidos pelo IFN, como *MX1*, *IFI44L*, *OAS1*, *OAS2*, *TNFSF13B* e *IRF7,* com regiões CpG hipometiladas na SSP.

Caixas Laranja: meQTLs, referindo-se às associações entre variantes genéticas (SNP) e o nível de metilação de regiões CpG próximas, demonstradas no *BLK*, *CXCR5*, *IL12A*, *IRF5-TNPO3*, *STAT4* and *TNIP1*.

Caixas Azuis: alterações na expressão de miRNA identificadas na SSP, incluindo miRNA-146a.

meQTL: *methylation quantitative trait loci*.

Mecanismos epigenéticos, como a metilação do DNA, miRNAs e lncRNAs, contribuem também para a ativação de genes relacionados com as vias da inflamação [441].

De acordo com o perfil clínico da doença, a expressão génica varia. Doentes com manifestações extra-glandulares têm maior expressão de genes relacionados com a resposta imune inata (apoptose, TLR, IFN) e adaptativa (ativação de células B e T), enquanto que doentes apenas com disfunção glandular ou dor generalizada têm maior expressão de genes relacionados com a percepção sensorial e dor [448].

2.4.2. Fatores desencadeantes e ambientais

2.4.2.1. Agentes Infecciosos

A etiologia da SSP não está ainda esclarecida, mas vários estudos suportam o conceito de desencadeantes virais [449] ou bacterianos [450] que tenham tropismo para as células epiteliais.

Não foram ainda identificados agentes específicos, mas alguns vírus da família Herpesviridae exibem tropismo para os tecidos-alvo da SSP, as glândulas salivares e lacrimais, sendo fortes candidatos a desencadeantes [451]. A agressão viral inicial, combinada com uma predisposiçao genética para a perda de tolerância na SSP, leva a um processo de ativação de células T mediado por antigénios, com subsequente migração para as glândulas exócrinas de células T $CD4^+$ e células B, que interagem promovendo a expansão e formação de plasmócitos e a produção local de auto-anticorpos [452].

Vírus linfotrópicos como o Epstein-Barr (EBV) e o Citomegalovirus (CMV) são especialmente suspeitos de causar a lesão inicial na SSP [453], sendo a implicação do EBV geralmente aceite. Mecanismos como o mimetismo molecular e susceptibilidade genética ao EBV acrescem à excessiva co-estimulação das células T, deficiente resposta T-específica ao EBV [454], reação cruzada de anticorpos anti-EBV ou inibição da apoptose das células B, habitualmente associados à linfoproliferação induzida por antigénio [455]. Foi demonstrado que CGE de glândulas salivares infectadas por EBV promovem a produção de anticorpos anti-EBV, anti-Ro/SSA e anti-La/SSB, e a sobrevivência de células B autoreativas [456].

2.4.2.2. Fatores Endógenos

Stress

Fatores de stress psicológico, agudos ou crónicos, podem induzir perturbações das respostas imunes inatas e adaptativas, mediadas pelo eixo hipotálamo-hipófise-

suprarrenal [457]. De facto, eventos de vida traumáticos antecedendo o início da doença têm sido propostos como um Fator de risco para o desenvolvimento de doenças autoimunes [458]. Na SSP, um estudo concluíu que eventos de vida adversos eram mais frequentes no ano que antecedia o início da doença, e que a percepção de mau suporte social e estratégias de coping deficientes eram mais frequentes, em comparação com indivíduos com linfoma e controlos saudáveis [459].

Hormonas

Vários estudos sugerem também o papel de mecanismos neuroendócrinos na disfunção das glândulas exócrinas, como hormonas e neuropéptidos [460]. Esta pode ser uma das razões para que alguns doentes apresentem sintomas secos muito intensos, na ausência de infiltrados inflamatórios glandulares significativos [461].

O acentuado predomínio do sexo feminino sugere que as hormonas sexuais tenham um papel na etiologia da SSP. OS estrogéneos poderão contribuir para a maior incidência da SSP através do aumento da produção de autoanticorpos, levando à deposição de imunocomplexos, dano nos tecidos, ativação de toll-like receptor (TLR) e do inflamassoma, aumento do IFN, e disfunção glandular [462]. É provável que o contributo hormonal dependa da predisposição genética e de estímulos ambientais, pois alguns estudos associaram maior tempo de exposição hormonal (idade de menarca e de menopausa, história reprodutiva, uso de terapias hormonais, e história de histerectomia) a uma menor prevalência de SSP [463].

Vitamina D

A vitamin D exerce efeitos imunomoduladores, e níveis baixos foram identificados como Fator de risco na Esclerose Múltipla [464]. Na SSP, até à data, não evidência significativa que apoie o envolvimento da carência de vitamina D na patogénese da doença [440].

2.4.2.3. Fatores Exógenos

Tabaco

Parece existir uma tendência para maior risco de SSP em ex-fumadores e um menor risco em fumadores ativos [465], sugerindo uma situação como a que ocorre na Colite Ulcerosa, em que o tabagismo tem um efeito protector [466].

O tabaco também parece reduzir o *focus score* de um modo dose-dependente, e associa-se a menor frequência de presença de anticorpos anti-SSA e SSB [467]. No entanto,

uma explicação alternativa poderá ser que indivíduos com inflamação mais leve e sintomas mais ligeiros tenham uma tendência para manter os hábitos tabágicos, enquanto indvíduos com doença mais grave seriam levados a deixar de fumar devido à irritação ocular, oral e respiratória do fumo [440].

Não se encontraram associações entre o tabagismo e manifestaçõs extra-glandulares [468] ou com atividade sistémica da doença [469].

Álcool

O consumo de álcool pode inibir a inflamação e reduzir a resposta a antigénios [470,471], e deste modo reduzir o risco de autoimunidade. Estudos noutras doenças autoimunes demonstraram uma relação inversa entre o consumo de álcool e o risco de AR [472] e Esclerose múltipla [473].

O papel do consumo de álcool na SSP não é conhecido. No entanto, doentes com SSP apresentaram menor frequência de consumo de álcool em relação a controlos saudáveis [474], embora se possa admitir que a menor ingestão se deva ao desconforto oral provocado pelas bebidas alcoólicas em doentes com xerostomia.

Solventes orgânicos

A associação entre a SSP e a exposição ocupacional a solventes orgânicos foi referida num estudo, mas não foi estabelecida relação causa-efeito uma vez que não foi clara a distinção entre a exposição pré- e pós-início da doença [475].

Sílica

Apesar da exposição à sílica ser reconhecida como um Fator de risco ambiental para o desenvolvimento de AR, LES e ESP [476], os dados disponíveis são insuficientes para estabelecer uma associação da sílica à SSP. Adicionalmente, as mulheres, que são a população predominanatemente afetada pela SSP, têm uma menor probabilidade de ter exposição ocupacional à sílica [440].

Silicone

Vários efeitos imunológicos de silicones têm sido relatados [477]. Embora alguns estudos tenham referido uma relação entre implantes mamários de silicone e o aumento de risco de SSP, a maioria dos estudos não detectou essa associação [440].

2.4.3. Sistema imunitário inato

Após a agressão inicial às células epiteliais das glândulas salivares e/ou lacrimais, a ativação da imunidade inata tem uma papel central na patogénese da SSP [478] (**Figura 10**). A sinalização através de TLR7, presente em monócitos e nas células ductais, leva à ativação da via do IFN tipo I e ao desenvolvimento da sialoadenite [479].

As células dendríticas plasmacitóides (pDC) são as principais produtoras de IFN tipo I, que em condições fisiológicas tem como principal função a defesa contra agentes virais [480]. Para além desse papel, o IFN tipo I tem várias outras funções imunomoduladoras, como a expansão, migração e diferenciação de células B através da indução do *B cell ativating Fator* (BAFF) [481] e da recombinação de classe de imunoglobulina [482](7), aumento da apresentação de antigénio, e citotoxicidade mediada por células T e *Natural Killer* (NK) [483]. Além disso, o IFN-I exerce um efeito indirecto na estimulação da atração e expansão dos linfócitos por via da ativação de células dendríticas convencionais e outras células do sistema imune [428].

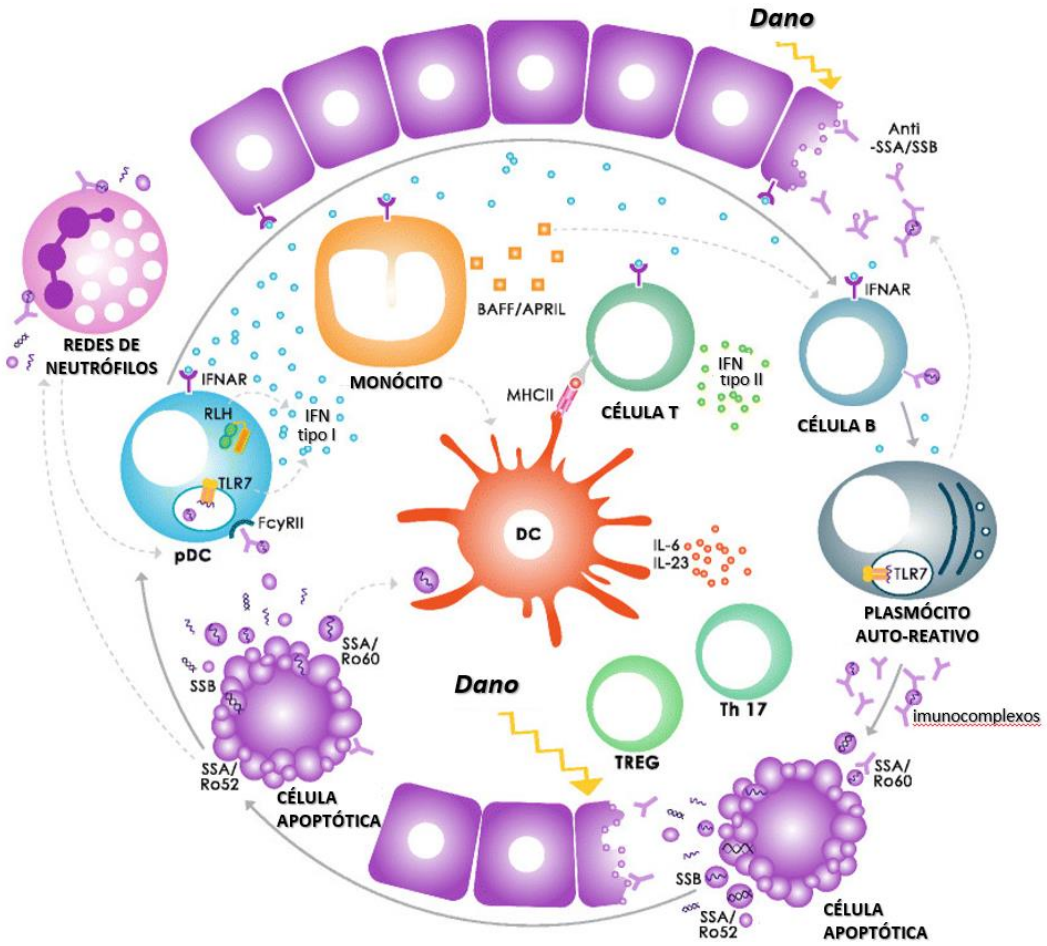

Figura 10. Papel do sistema imune inato no processo de ativação inicial da SSP. Adaptado de Maria (2015) [484].

As células dendríticas e os IFN estão implicados na origem de um processo patogénico que se auto-amplifica. Um dano inicial, como uma infeção ou stress, leva à acumulação de debris apoptóticos, induzindo a produção de IFN tipo I pelas células dendríticas plasmacitóides (pDCs). O IFN tipo I liga-se ao recetor IFNAR (IFNα,β receptor) de células adjacentes, o que induz uma assinatura de IFN e a ativação de células efetoras maduras, pela perpetuação da via TLR7 em plasmócitos auto-reativos e pela auto-amplificação nas pDCs. A sobre-regulação do TLR7 em plasmócitos auto-reativos aumenta a produção de autoanticorpos associados a RNA (ant-SSA/Ro52, SSA/Ro60 e SSB/La). Estes autoanticorpos e debris apoptóticos formam imunocomplexos que aumentam a ativação da via TLR7. Os neutrófilos podem causar dano adicional nos tecidos através da formação de redes de neutrófilos, que são potentes indutoras do IFN tipo I. Por sua vez, os autoanticorpos induzem a amplificação das redes neutrofílicas, perpetuando o ciclo. As pDC ativadas pelo IFN promovem a ativação de células T e NK, que por sua vez produzem grandes quantidades de IFN tipo II (IFNγ). Dados recente sugerem o contributo conjunto de TLRs e RLHs (*cytoplasmic RIG-I-like family of helicases*) na promoção da perda de auto-tolerância e hiper-ativação persistente do IFN.

APRIL, a proliferation inducing ligand; BAFF, B-cell ativating Fator; IL, interleukin; MDA5, melanoma differentiation-associated protein 5; MHC, major histocompatibility complex; RIG-I, retinoic acid-inducible gene 1; Th17, T-helper type 17; TLR, Toll-like receptor; Treg, regulatory T cells.

Na SSP, as pDC apresentam um fenótipo ativo com produção aumentada de IFN e de citocinas pro-inflamatórias, e várias outras populações celulares apresentam sobre-expressão de genes estimulados pelo IFN, como as células dos ductos salivares, monócitos e linfócitos B [485].

As células epiteliais das glândulas salivares, para além de serem o alvo da reação autoimune na SSP, poderão também desempenhar um papel na patogénese. Através da expressão de moléculas co-estimulatórias, com CD80, CD86 e CD40, é possível que actuem como células apresentadoras de antigénio, e através da secreção de mediadores solúveis promovem a ativação e sobrevivência dos linfócitos B [486].

2.4.4 Sistema imunitário adaptativo

Após a ativação do Sistema imunitário inato, a intensa expressão e apresentação antigénica leva ao recrutamento de células T para o epitélio afetado. Aí, ocorre uma ativação e proliferação de células B auto-reativas dependente de antigénio, promovida maioritariamente pelas células T (**Figura 11**). Esta interação B-T é facilitada pela formação de estruturas linfóides terciárias nas GS, designadas centros germinativos, onde pode posteriormente ocorrer linfomagénese [434].

2.4.4.1. Linfócitos T

Nas fases iniciais da SSP, os infiltrados linfocitários nas GS são compostos maioritariamente por linfócitos T (> 75%), sobretudo $CD4^+$ [487], e pequenos números de células B, macrófagos e células dendríticas. Com a progressão da doença aumenta o número de células B e plasmócitos nas lesões autoimunes da SSP [488].

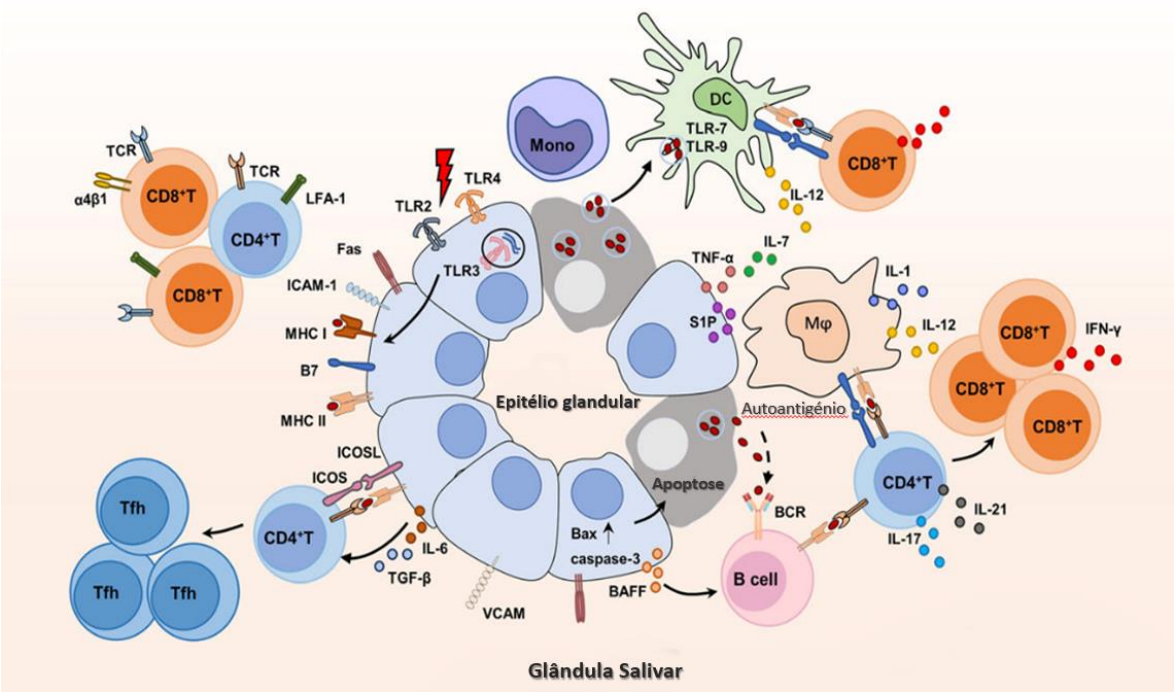

Figura 11. Resposta imune na glândula salivar. Adaptado de Yao (2020) [489].

As respostas imunes nas glândulas salivares na SSP compreendem a ativação do sistema imunitário inato e adaptativo. Na presença de autoantigénios libertados pelas células danificadas e apoptóticas do epitélio glandular, as células apresentadoras de antigénio, como as células dendríticas, macrófagos e células B, facilitam a ativação de células T $CD4^+$ e $CD8^+$ T. As células T ativadas secretam citocinas inflamatórias e amplificam o processo inflamatório.

DC, células dendríticas; Mϕ, macrófago; Tfh, célula T efetora (*helper*) folicular; ICOS, *inducible T cell co-stimulator*; ICOSL, ICOS *ligand*; IFN-γ, interferão gama; IL, interleucina; TNF-α, *tumor necrosis Fator* alfa; MHC, classe do *major histocompatibility complex*; ICAM, *intercellular adhesion molecule*; VCAM, *vascular cell adhesion molecule*; BAFF, *B cell ativating Fator*; TLR, *Toll-like receptor*; TCR, *T cell receptor*; TGF-β, *transforming growth Fator* beta; S1P, *sphingosine 1- phosphate*.

A maioria das células $CD4^+$ que infiltra as glândulas salivares na SSP apresenta um perfil ativado ou de memória [490,491]. Em doentes com SSP, as células T ativadas reagem a um intenso estímulo antigénico, como ocorre com o reconhecimento dos auto-antigénios Ro e La presentes em partículas de células apoptóticas [492], que induzem uma intensa resposta proliferativa [493]. Assim, o reconhecimento de auto-antigénios e a consequente ativação das células T são cruciais na sequência de eventos que levam ao desenvolvimento da SSP. As células T podem proliferar localmente nos tecidos-alvo, ou ser atraídas da circulação para as glândulas por ação de quimiocinas, como o CXCL9 e o CXCL10 [494].

A ativação das células T nas glândulas resulta em lesão estrutural e disfunção secretória [430]. A disrupção da normal arquitectura glandular pode ser mediada pelas células T através de mecanismos apoptóticos induzidos pela via do FasL [495], por ação citotóxica directa envolvendo a libertação de perforina e secreção de citocinas, ou pela ativação das células B [496].

Cedo se assumiu que a patogénese da SSP seria mediada sobretudo por respostas imunes derivadas das Th1 [497,498]. Células CD4+ IFN-γ-positivas estão presentes em grande número nas GS de doentes com SSP, e a análise de citocinas intra-celulares demonstrou a polarização para um fenótipo Th1 [499]. Vários modelos animais de SSP demonstraram também a associação da patogénese da SSP a células produtoras de citocinas Th1 [500,501]. Adicionalmente, IL-2 e IFN-γ são consistentemente detectados nos tecidos-alvo de doentes com SSP [502], enquanto que a IL4 e a IL-5 são detectadas apenas em doentes com grande acumulação de células B nas GS [503]. O papel das células T CD4+ T na patogénese da SSP está representado na **Figura 12**.

As células Th17 são outra subpopulação T que apresenta forte ativação na SSP [504], decorrente certamente do seu papel fisiológico na defesa das mucosas. As células Th17 são encontradas nas lesões autoimunes das glândulas salivares de doentes com SSP [20,505], e promovem a inflamação através da secreção de IL-6, IL-17, IL-21, IL-22 e IL-23 [424]. Efectivamente, níveis aumentados de IL-17, a citocina canónica das Th17, têm sido descritos em doentes com SSP [506,507]. Uma vez que as Th17 também produzem IFN-γ, serão participantes na patogénese da SSP relacionada com a via do IFN. Adicionalmente, a IL-18 e IL-23 produzidas pelas células do epitélio glandular salivar pode contribuir para a ativação das Th17 na SSP [505].

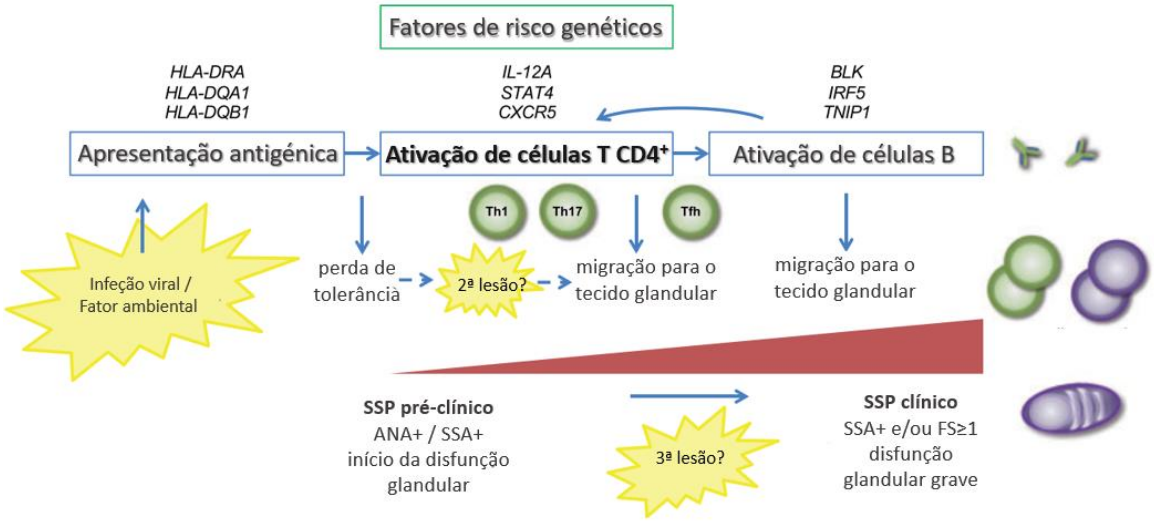

Figura 12. Papel das células T CD4+ T na patogénese da SSP. Adaptado de Verstappen (2019) [452].

Após a agressão inicial, os antigénios são apresentados às células T através de moléculas do MHC classe II, resultando na ativação de células T CD4+. Alelos de risco no HLA-DR e HLA-DQ associadas à SSP podem estar envolvidos na perda de tolerância aos antoantigénios. Variantes de risco da IL-12ª e do STAT4 podem contribuir para diferenciação preferencial em células Th1. Uma segunda agressão, local, poderá induzir a migração de células Th CD4+ para as glândulas salivares ou lacrimais. Este estadio clínico reflete-se em caraterísticas sugestivas de SSP, mas sem infiltrados focais peri-ductais e sem sinais evidentes de hiperatividade das células B. Uma terceira agressão é provavelmente necessária para se estabelecer um ciclo de auto-amplificação entre as células B e T, resultando na hiperatividade B dependente das T. Nesta fase, as caraterísticas típicas da SSP tornam-se evidentes.

As células T, em especial as Th17/Th22, desempenham um importante papel na estruturação dos constituintes das estruturas linfóides terciárias nas glândulas salivares, que acomodam o subsequente recrutamento de células B e a formação de CGE [508].

As células T efetoras presentes nos folículos linfoides, designadas Tfh, desempenham um papel importante na formação e manutenção dos CGE na SSP e na regulação da diferenciação das células B de memória e plasmócitos [367,384]. As Tfh expressam altos níveis de CXCR5 (CXC Chemokine Receptor 5), que é fundamental para o seu acesso aos folículos linfóides [367,382]. O fenótipo das Tfh inclui ainda a expressão dos receptores de superfície ICOS (inducible T cell costimulator) e PD-1 (programmed cell death protein 1), bem como o repressor nuclear da transcrição Bcl-6 (B-cell lymphoma 6) [367,510]. A IL-21 é a citocina-chave produzida pelas Tfh [511]. As células epiteliais das glândulas salivares contribuem para a ativação e diferenciação de células Th em Tfh através da secreção de IL-6 e ICOS [137].

As células Tfh ativadas secretam IL-21, que promove a maturação e proliferação de células B [146]. As Tfh auxiliam a ativação de respostas imunes mediadas por linfócitos B, incluindo a recombinação de classe de Ig, a diferenciação de centros germinativos, e a maturação da afinidade [147] (**Figura 13**).

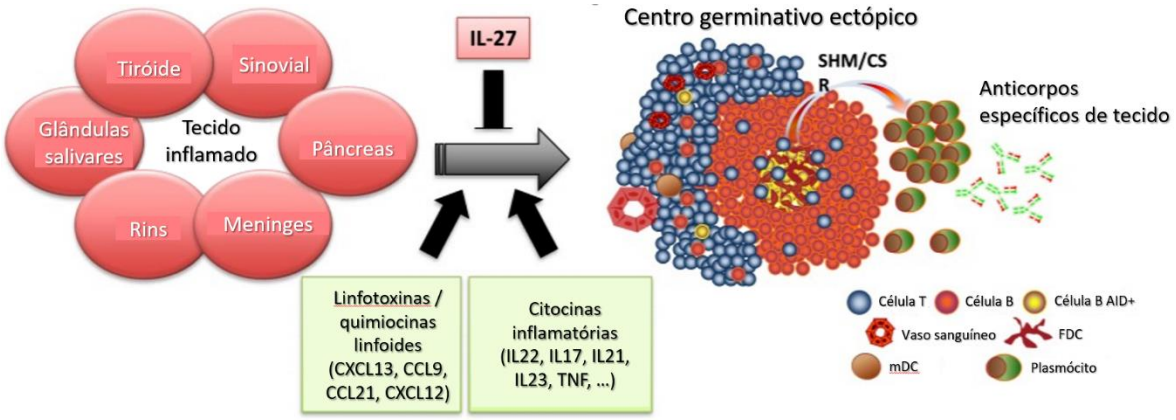

Figura 13. Estruturas linfoides ectópicas em doenças autoimunes. Adaptado de Corsiero (2016) [512].
Várias citocinas inflamatórias e linfotoxinas/quimiocinas linfoides têm importância na formação e manutenção de CGE nos órgão-alvo.

Encontra-se documentado que as células Tfh $CD4^{+}CXCR5^{+}$ estão aumentadas nas glândulas salivares e sangue periférico de doentes com SSP, associadas a plasmócitos e células B aberrantes, o que sugere que as Tfh contribuem para a patogénese da doença pela promoção da maturação de células B [22]. Níveis séricos aumentados de IL-21 associam-se à atividade sistémica da SSP [513]. A expressão de Bcl-6 pelas células T é fundamental para a geração de Tfh e células B dos CG [372,384], e níveis aumentados de mRNA de Bcl-6 foram

encontrados nas GS de doentes com SSP [514]. Doentes com SSP apresentam aumento das células Tfh circulantes e uma expansão da sua diferenciação nas GS [21,22].

Dado que a expressão de CXCR5 é também encontrada em células T $CD8^+$, a existência de células T foliculares citotóxicas (Tfc) é atualmente aceite, contribuindo provavelmente para a regulação das respostas B e produção de anticorpos [515,516].

As células T reguladoras (Tregs) são um subtipo de linfócitos T $CD4^+$ que desempenham um papel crucial no equilíbrio do sistema imunitário, ao manterem a tolerância a autoantigénios e prevenirem uma resposta exagerada das células efectoras [325]. Há um equilíbrio delicado entre as Th17 e as Tregs, pois enquanto o TGF-β é necessário para a génese de ambas as populações, é a presença ou ausência de IL-6 concomitante que leva à génese de células Th17 ou Tregs, respectivamente [517]. Enquanto na AR a maioria dos estudos indica uma depleção das Tregs e aumento das Th17 no sangue periférico e órgãos-alvo, ao qual se juntam defeitos na atividade das Tregs [518], na SSP há uma maior variabilidade dos resultados em relação às frequências e função das Tregs [20].

Num modelo murino de SSP, a razão Treg/Th foi significativamente mais baixa que em controlos, e foi evidente a marcada limitação da indução de iTreg a partir de células T naïve por ação do TGF-β [519]. Adicionalmente, as Tregs do modelo murino de SSP apresentavam produção de IFN, assemelhando-se a um perfil Th1, e a sua transferência para outros ratinhos não foi suficiente para prevenir o desenvolvimento de lesões autoimunes [519].

No modelo murino deficiente em *C-C-chemokine receptor* 7 (CCR7), análogo da SSP, verifica-se um aumento da resposta imune associado à reduzida migração de Tregs para os gânglios linfáticos e consequente disfunção supressora das Tregs [520]. Na mesma linha de investigação, doentes com SSP apresentaram um número significativamente menor de células Treg $CCR7^+Foxp3^+$ nas glândulas salivares quando comparados com controlos [521]. Estes achados sugerem que a anormal expansão, diferenciação e perfil functional das Tregs contribui para a patogénese da SSP.

Em humanos, a maioria dos estudos iniciais descreveu uma redução das Tegs $CD25^{high}$ [522–525] ou percentagens similares mas tendencialmente menores valores absolutos [253,526,527], enquanto outros estudos referem elevação das células $CD4^+CD25^+$ [528], das $CD4^+CD25^+Foxp3^+$ [529], e do subtipo $CD4^+CD25^{hi}Helios^+Foxp3^+$ [530]. Em parte, estas discrepâncias podem ser explicadas pela estratégia usada da identificação das células Tregs, pois nos estudos mais antigos definiam-se as Tregs apenas pela expressão de CD25, enquanto em estudos mais recentes passou a ser utilizada também a expressão de Foxp3 pois o CD25 pode também ser expresso por células T ativadas [20].

Em relação à associação entre as frequências de Tregs e parâmetros fenotípicos e de atividade da doença na SSP, Liu [522] descreveu uma relação inversa entre os valores de Tregs

$CD24^{+}CD25^{+bright}$ e a VS, PCR, FR e IgG. Mais tarde, um estudo do mesmo grupo [530] reportou também uma relação inversa entre os níveis de Tregs $CD4^{+}CD25^{hi}Helios^{+}Foxp3^{+}$ e indicadores como a VS, IgG, IgM e o ESSDAI, bem como menores valores em doentes com anti-SSB positivo. Miao *et al* [527] associaram menores contagens de Tregs a valores de ESSDAI mais elevados.

As células T reguladoras foliculares (Tfr) são um subtipo especializado de Tregs que conseguem aceder aos folículos linfóides e regulam as respostas B após a exposição antigénica. Estas células são definidas como células T CD4+FoxP3+ CD4+ que expressam níveis variados de CXCR5, e têm um programa transcricional consistente com um fenótipo regulador [531484]. As células Tfr contribuem para a regulação das respostas B por vários mecanismos, incluindo a inibição direta das células B através do CTLA-4, e a inibição da produção de citocinas pro-inflamatórias pelas Tfh [532,533]. É provável que as Tfr cells regulem respostas B precoces nos CG, mas não tardias, para limitar as respostas auto-reativas e a produção de autoanticorpos específicos [534].

2.4.4.2 Linfócitos B

A hiperatividade das células B e distúrbios das suas populações circulantes são características da SSP, e desempenham um papel fundamental dos processos autoimunes e linfoproliferativos da doença [434,535]. Estes distúrbios incluem uma alteração do tráfico de células B entre o componente circulatório e as glândulas inflamadas, e polarização da diferenciação de células B com aumento de plasmócitos ativados [434,536]. Além disso, os linfócitos B polarizados são capazes de produzir uma grande variedade de citocinas pro-inflamatórias, como a IL-1, IL-6, IL-7, e TNF-α [537].

A distribuição das populações B tem implicações no desenvolvimento de doenças como a SSP. Em condições normais, as células B auto-reativas naturalmente geradas na medula óssea são removidas por dois principais mecanismos de tolerância (checkpoints). O primeiro ocorre na medula óssea na fase de células B imaturas, enquanto que o segundo ocorre entre a passagem da fase de células transição para a célula B naïve madura. Existe ainda outro checkpoint que assegura a remoção de células B de memória IgM+ antes da formação dos centros germinativos [97,110]. São precisamente estes mecanismos que estão parcial ou totalmente defeituosos e que levam ao desenvolvimento de doenças autoimunes, como o SSP, com perfis de distribuição das populações B característicos [436,538,539].

Para além da presença de auto-anticorpos, doentes com SSP caracterizam-se por profundas alterações na frequência das diferentes subpopulações de células B, quer no sangue periférico quer nas glândulas salivares. Nomeadamente, estes doentes apresentam uma predominância de células de transição e B naïves, e uma diminuição significativa de células B de memória CD27+, particularmente as com fenótipo *unswitch* (CD27+IgD+), sendo esta a alteração mais bem caracterizada nestes doentes [540–543].

Esta alteração pode ser explicada pela migração ou retenção destas populações nas glândulas salivares, onde se observou níveis aumentados desta população [18]. A acumulação está relacionada com a formação de estruturas linfoides ectópicas que se assemelham a centros germinativos e que são visíveis no epitélio das glândulas salivares de 10-30% dos doentes com SSP [544]. Nestas estruturas, a expressão de CXCR5 e de CXCL13, promove o recrutamento de células Tfh e células B.

Parece haver também um aumento nos níveis de plasmablastos $CD27^{hi}CD19^{low}CD20^{-}$ nas glândulas salivares destes doentes com historial de linfoma [18]

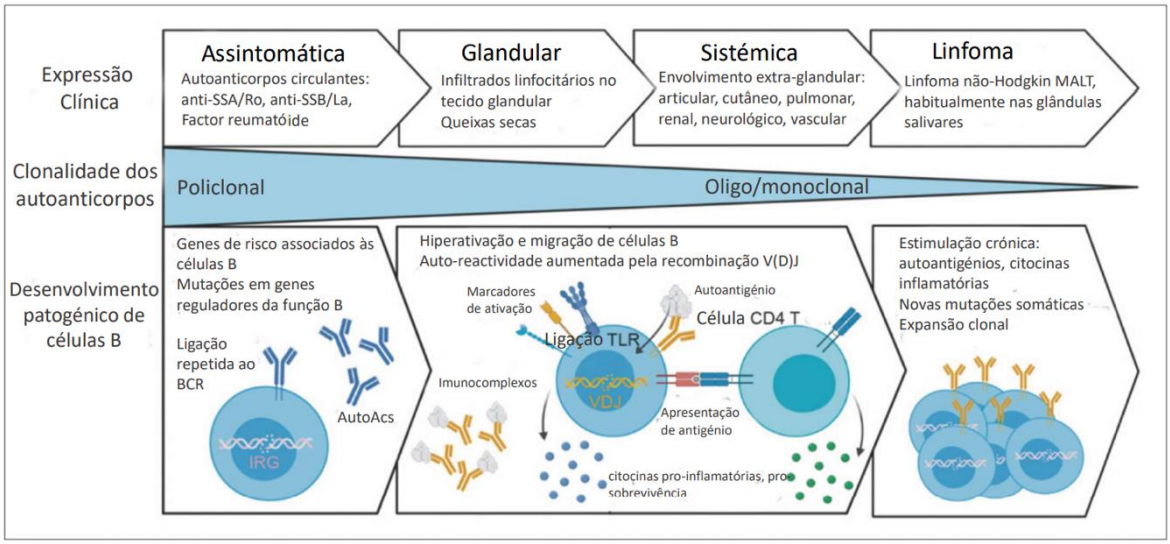

Figura 14. Desenvolvimento e função de células B patogénicas durante a evolução da SSP. Adaptado de Reed (2020) [496].

Múltiplas mutações da linha germinativa B e variantes somáticas de genes da via do IFN permitem que células B portadoras de recetores estereotípicos e auto-reativos se evadam dos mecanismos de tolerância, sobrevivam, proliferem e se diferenciem em células produtoras de anticorpos. A hiperativação de células B auto-reativas, a recombinação da região variável VDJ e a secreação de autoanticorpos pode ocorrer através de vias dependentes ou independentes das células T. Nas vias independents das células T, autoantigénios associados a ácidos nucleicos (ex: ribonucleoproteínas Ro e La) fornecem um duplo sinal, estimulando o BCR (receptor da célula B) e ligando-se a TLR (Toll-like receptors). Nas vias dependentes de céluas T, as células B auto-reativas apresentam antigénio às células T CD4+ através do MHC classe II, resultando na diferenciaçã destas em Th, incluindo Tfh que promovem a hipermutação somática que aumenta a afinidade para autoanticorpos. Os autoanticorpos produzidos formam imunocomplexos patogénicos com autoantigénios, que estimulam sinais inflamatórios adicionais incluindo a ativação de TLR e IFN tipo I. A estimulação crónica por autoantigénios, o microambiente inflamatório e a acumulação de mutações somáticas criam as condições propícias à proliferação descontrolada de células B auto-reativas, levando à expansão clonal e ao linfoma.

O *B-cell ativating Fator* (BAFF), produzido por células inflamatórias, é necessário para a sobrevivência a longo prazo dos plasmócitos e pela sua intensa produção de imunoglobulinas [545]. Na SSP, o microambiente inflamatório do tecido glandular é rico em BAFF, que contribui para a acumulação de células B de memória $CD27^+$ e para o aumento dos plasmócitos nas GS [546,547]. Também o aumento da expressão de CXCR3 e CXCR4 em plasmócitos e plasmablastos em doentes com SSP induz a migração destas células para os locais de inflamação [542]. Os plasmócitos presentes em número aumentado nas GS de doentes com SSP incluem células auto-reativas que produzem autoanticorpos anti-SSA/Ro ou anti-SSB/La [548,549].

Neste perfil molecular pro-inflamatório, destacam-se as quimiocinas atratoras de células B CXCL13 e CXCL12 [550,551]. O CXCL13 é uma citocina homeostática produzida pelas células dendríticas foliculares, células Tfh e células T expostas a antigénio, que regula o movimento das células B através da interação com o receptor correspondente nas células B, o CXCR5 [552]. As células B de memória $CD27^+$ expressam os recetores CXCR5 para o CXCL13 e CXCR4 para o CXCL12 [553,554], pelo que a co-expressão de CXCL12 e CXCL13 poderá atrair esta subpopulação para as glândulas inflamadas [550,554]. Um fator adicional na atração das células B de memória para as GS poderá ser a expressão por estas células de CXCR3, cujos ligandos são extensamente produzidos localmente na SSP [437]. Assim, a atração das células B de memória $CD27^+$ para as GS explicará a redução do número destas células no sangue periférico na SSP. Nas GS, estas células contribuirão para a formação de CGE e sofrem diferenciação em plasmablastos e plasmócitos [555]. A formação de CGE nas GS de doentes com SSP correlaciona-se com maior hiperatividade das células B, presença de autoanticorpos anti-SSA e anti-SSB, hipergamaglobulinémia e crioglobulinémia. Assim, a persistência de CGE contribui para a progressão da doença na SSP [556] (**Figura 15**).

Alguns estudos também implicam distúrbios das células B reguladoras (Bregs) na patogénese da SSP. Foram descritos menores percentagens de células B produtoras de IL-10 $CD19^+CD24^{hi}CD38^{hi}$ em indivíduos com doença ativa, em comparação com doentes com doença inativa e controlos [253]. Noutro estudo, doentes com SSP apresentaram percentagens de células Bregs ($CD19^+CD24^{hi}CD38^{hi}$) superiores aos controlos saudáveis, mas com deficiente capacidade de inibir a produção de citocinas pelas T efectoras [254]. No entanto, um outro estudo que abordou células B produtoras de IL-10 demonstrou incapacidade destas em regular a produção de citocinas pelas células T no LES, enquanto que em doentes com SSP e osteoartrose essa capacidade supressora se mantinha [224].

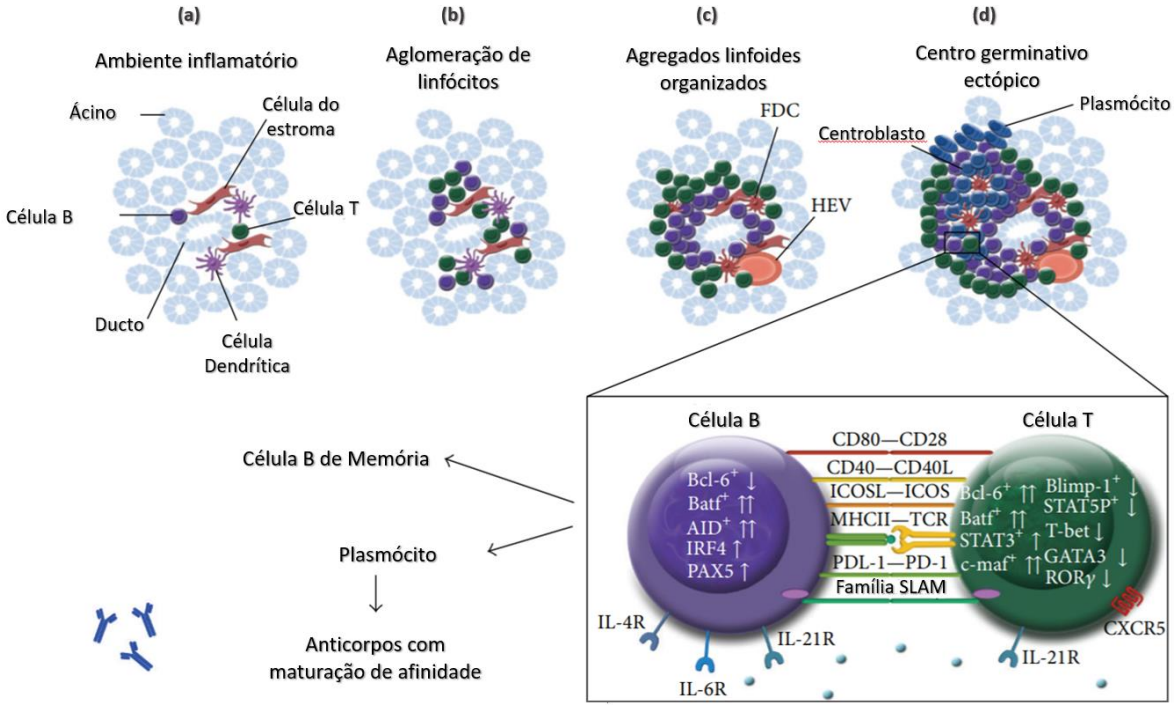

Figura 15. Organização das estruturas linfóides ectópicas nas glândulas exócrinas na SSP. Adaptado de Szabo (2014) [514].

Na fase inicial da inflamação, células T, B e dendríticas atividas acumulam-se em volta dos ductos epiteliais **(a)**. A resposta inflamatória, incluindo citocinas produzidas pelas células do estroma, pode induzir a formação de estruturas linfóides mais organizadas **(b)** e **(c)**. Agregados com um nível superior de organização designam-se centros germinativos (CG) ectópicos, e apresentam zonas distintas ricas em células T e em células B, com uma rede central de células dendríticas foliculares (**d**). As células T foliculares (Tfh) caraterizam-se pela expressão de CXCR5, ICOS, PD-1, CD40L, membros da família SLAM (*signalling lymphocyte ativation molecule*), como o CD84, SAP, e IL-21. A interação entre as Tfh e as células B dos CG é essencial para a formação e manutenção dos CG ectópicos e, consequentemente, para a geração de células B de memória e plasmócitos de vida longa. FDC: *follicular dendritic cells*; HEV: *high endothelial venule*; SAP: SLAM-*associated protein*.

2.5. Expressão Clínica

Os sintomas clássicos da SSP, presentes na grande maioria dos doentes, consistem em secura da boca e olhos, frequentemente acompanhada de secura de outras mucosas ou da pele [395]. Manifestações extra-glandulares ocorrem com frequência e são muito variáveis, podendo consistir em autoimunidade específica de um órgão, envolvimento multi-sistémico, ou inclusivé doença linfoproliferativa [557].

2.5.1. Manifestações Glandulares

A xerostomia corresponde à presença de menor quantidade de saliva, que os doentes referem como sensação de ardor bucal, lábios secos, alterações do paladar, dificuldade em falar ou deglutir, halitose ou aftose oral. A necessidade de utilizar água para auxiliar a deglutição é um dos principais indicadores da redução do fluxo salivar [558].

A xeroftalmia manifesta-se habitualmente por irritação ocular, sensação de ardor, picadas ou corpo estranho, sintomas que se agravam em ambientes secos, ventosos ou com a atenção visual prolongada. Estas manifestações têm grande impacto na função visual e qualidade de vida dos doentes [559].

O complexo sicca pode afetar outras estruturas, devido ao envolvimento inflamatório das glândulas exócrinas da pele e do epitélio do aparelho respiratório, nasal ou vaginal. A nível cutâneo, a secura ou xerose manifesta-se por prurido em mais de 50% dos doentes [560]. A rinite seca é um sintoma presente em até 40% dos doentes, manifestando-se por desconforto, crostas nasas, sinusite, epistáxis ou alterações do olfacto ou paladar [561]. A secura da árvore traqueo-brônquica pode manifestar-se por tosse crónica não-produtiva ou síndrome obstrutivo [562]. A secura vaginal é comum, associando-se a queixas de disparêunia e má qualidade da vida sexual [563].

2.5.2. Sintomas gerais ou constitucionais

Os sintomas constitucionais na SSP incluem febre, emagrecimento e fadiga, e são frequentes no início da doença. Estas manifestações são observadas quer em doentes seropositivos para anti-SSA/SSB, quer em doentes seronegativos. Contudo, o impacto funcional destes sintomas gerais parece ser superior nos doentes seronegativos [564,565].

Pode ocorrer febre baixa em 6-41% dos doentes [566]. No entanto, a presença de febre, sudorese nocturna e emagrecimento (sintomas B) pode indicar a presença de linfoma [567].

Fadiga crónica e má qualidade do sono são referidos por mais de metade dos doentes [568]. Dor musculo-esquelética difusa, semelhante a uma sindrome fibromiálgica, está presente em cerca de 50% dos doentes [569]. Doentes com maior intensidade da secura, fadiga e dor parecem ter menos envolvimento sistémico e menos características imunológicas de SSP [570], tornando muitas vezes difícil a distinção entre alguns casos de SSP e fibromialgia.

2.5.3. Manifestações Sistémicas

Manifestações sistémicas ou extra-glandulares ocorrem em cerca de 50-60% dos doentes [571,572]. Cerca de 15-20% dos doentes apresentam manifestações graves, e necessitam de acompanhamento e terapêutica mais intensivos, devido ao risco de linfoma, estimado em 5% [572,573].

As manifestações extra-glandulares podem ser devidas a diversos mecanismos: epitelite autoimune com infiltração linfocítica peri-epitelial em órgãos-alvo, autoimunidade

específica de órgão com autoanticorpos específicos, manifestações sistémicas associadas à formação de imunocomplexos ou crioglobulinémia, e expansão linfocitária clonal [2].

As manifestações sistémicas podem estar presentes aquando da identificação da doença, ou surgir mais tarde. Nalguns casos, no entanto, o envolvimento sistémico pode anteceder as queixas secas, ou estas serem ligeiras, sendo estes casos de SSP oculto difíceis de diagnosticar [1].

O envolvimento extra-glandular na SSP é diverso, e por vezes multi-sistémico. Sistemas frequentemente envolvidos são a pele, articulações, aparelho respiratório, aparelho urinário, sistema nervoso central e periférico, hematológico e circulatório [574].

Os doentes com doença potencialmente grave podem ser identificados precocemente pela presença de baixos níveis dos Fatores C3 e/ou C4 do complemento, hipergamaglobulinémia, crioglobulinémia, bem como pela positividade para anticorpos anti-SSA e FR [1].

2.5.3.1. Manifestações músculo-esqueléticas

O envolvimento articular é muito frequente na SSP (50% dos doentes), variando de artralgias de ritmo inflamatório a poliartrite não-erosiva [575]. A artrite na SSP tem habitualmente extensão moderada (<5 articulações), carácter intermitente, e envolve preferencialmente as pequenas articulações das mãos e os membros superiores [575,576]. No entanto, sinovite subclínica é frequentemente identificada na ecografia articular [577].

Apesar de queixas de mialgias serem frequentes na SSP, a presença de miosite é rara, com uma prevalência inferior a 3% [578].

2.5.3.2. Manifestações cutâneas

O envolvimento cutâneo é frequente, estando descritas diversas manifestações para além da habitual xerose, como eritema polimorfo, eritema anular (lesões semelhantes a lupus cutâneo subagudo) e púrpura vascular por vasculite linfocítica, vasculite urticariforme, crioglobulinémia ou púrpura hipergamaglobulinémica [575].

Manifestações mais raras incluem úlceras cutâneas, livedo, eritema nodoso, amiloidose ou granuloma anular [579].

A vasculite crioglobulinémica é uma manifestação potencialmente grave, pois para além da habitual púrpura pode ter expressão neurológica (polineuropatia periférica), renal (glomerulonefrite) e linfoproliferativa (risco de linfoma) [580].

2.5.3.3. Manifestações cardiovasculares

O fenómeno de Raynaud é a manifestação vascular mais frequente na SSP, ocorrendo em 10–20% dos doentes [581]. Nestes casos, é frequentemente o primeiro sinal da doença, precedendo as queixas secas em até 75% dos doentes [582]. A expressão clínica e a evolução do fenómeno de Raynaud na SSP é mais favorável que em outras doenças reumáticas como a esclerose sistémica, e a hipertensão arterial pulmonar é muito rara [581].

Outras manifestações cardíacas são raras, como disritmias ou bloqueio cardíaco [583], pericardite ou cardiomiopatia [581].

2.5.3.4. Manifestações respiratórias

Manifestações pulmonares clinicamente significativas ocorrem em 9-20% dos doentes com SSP [584]. No entanto, a avaliação com exames de imagem ou provas de função respiratória revela envolvimento subclínico numa percentagem significativa de doentes (43–75%) [575,585].

Nos doentes com sintomas respiratórios, o envolvimento das pequenas vias aéreas é o achado mais frequente, e pode incluir broquiectasias, bronquilite ou hiperreatividade brônquica [575]. Manifesta-se habitualmente por um padrão funcional obstrutivo e frequência aumentada de infeções respiratórias [586].

Doença pulmonar intersticial pode estar presente em diversos padrões, sendo os mais frequentes a Pneumonite intersticial não-específica (NSIP) (33-45%), Pneumonia intersticial usual (UIP) (16-23,8%), e a Pneumonia intersticial linfocítica (9.5-15%) [587,588]. O envolvimento instersticial está mais frequentemente associado à presença de anti-SSA, FR, baixos nívies de C3, e elevação da proteína C reativa (PCR) [588]. O prognóstico da doença intersticial pulmonar associada à SSP é desfavorável, com melhoria em apenas cerca de 16% dos doentes, estabilização em 47% e deterioração em cerca de 37% dos doentes [587].

2.5.3.5. Manifestações Gastro-Intestinais

Várias manifestações gastrointestinais têm sido descritas na SSP, com cerca de 23% dos doentes referindo queixas de índole functional, como disfagia, dismotilidade esofágica e dispepsia [589], nalgums casos associada a gastroparésia [590]. Outras formas de envolvimento gástrico incluem a gastrite crónica linfocítica e a presença de anticorpos anti-células parietais ou anti-Fator intrínseco, embora a anemia perniciosa seja rara [589].

Outras manifestações tais como dor abdominal, diarreia ou malabsorção podem ocorrer no contexto de enteropatia perdedora de proteínas [591] ou pela associação a doença celíaca [592].

2.5.3.6. Manifestações Hepato-pancreáticas

Alterações das enzimas hepáticas podem estar presentes em 10–50% dos doentes, sendo habitualmente ligeiras e sem significado clínico, e hepatomegália ocorre em 10-20% dos doentes [2]. Hepatite autoimune pode estar presente em até 4% dos doentes [593].

A SSP e a Colangite Biliar Primária (CBP) são ambas epitelites imunomediadas, com mecanismos patogénicos similares e frequentemente encontradas em associação [594]. Até 10% dos doentes com SSP apresentam anticorpos anti-mitocondriais, típicos da CBP, sem manifestações clínicas graves da doença [595]. Em doentes com CBP, coexiste uma SS associada em cerca de 40% dos casos [596].

O envolvimento pancreático, na forma de pancreatite aguda ou crónica e insuficiência pancreática ocorre em até 7% dos doentes com SSP [589]. No entanto, o envolvimento subclínico será muito mais frequente, dada a presença de alterações morfológicas de pancreatite crónica em 25-33% de indivíduos assintomáticos [597].

2.5.3.7. Manifestações Nefro-urológicas

Na SSP os rins podem ser alvo de dois tipos distintos de lesão, a nefrite tubulo-intersticial relacionada com a infiltração linfocitária, e a glomerulonefrite devido à deposição de imunocomplexos [598]. Cerca de 5% dos doentes com SSP tem envolvimento renal, número que é seguramente subestimado pois não inclui casos de envolvimento tubulo-intersticial assintomático [575,599].

O envolvimento dos túbulos renais é o mais frequente, podendo afetar qualquer segmento e manifestar-se por acidose tubular renal, diabetes insípida nefrogénica e disfunção tubular proximal com proteinúria, podendo complicar-se por nefrocalcinose e osteomalácea [599,600].

Na SSP pode também ocorrer cistite intersticial devido à infiltração linfocitária da bexiga, manifestando-se por polaquiúria, disúria, tenesmo vesical e dor hipogástrica [601].

2.5.3.8. Envolvimento neurológico

O envolvimento neurológico na SSP é relativamente frequente (18–45% dos doentes) e afeta quer o sistema nervoso central, quer o periférico (sensitivo-motor e autonómico), com maior prevalência do envolvimento do sistema nervoso periférico [602].

As manifestações neurológicas clinicamente significativas podem ser manifestação inaugural da doença em cerca de 25% dos casos [603].

2.5.3.8.1. Sistema Nervoso Periférico

As manifestações neurológicas periféricas são polimórficas e podem ser classificadas de acordo com a expressão clínica e electrofisiológica em polineuropatia mista, polineuropatia sensitiva axonal, neuropatia sensitiva atáxica, polineuropatia axonal sensitivo-motora, neuropatia sensitiva pura, mononeurite múltipla, e polirradiculoneuropatia crónica desmielinizante (CIDP) [604]. As neuropatias periféricas mais frequentemente detectadas são a polineuropatia sensitiva axonal e polineuropatia sensitivo-motora axonal [605].

Os nervos craneanos podem ser também afetados, em particular o trigémio (por envolvimento do gânglio de Gasser) e o facial (paralisia uni- ou bilateral) [602].

Os mecanismos envolvidos são sobretudo a infiltração linfocítica dos gânglios dorsais, lesões vasculíticas dos *vasa vasorum*, e a eventual presença de anticorpos específicos anti-axónio [606].

A lesão das pequenas fibras não-mielinizadas pode originar uma neuropatia autonómica ou uma neuropatia sensitiva de pequenas fibras [607].

2.5.3.8.2 Sistema Nervoso Central

O envolvimento do Sistema nervoso central pode consistir em mielopatias agudas ou crónicas imitando a esclerose múltipla, ou manifestações mais difusas como meningite asséptica, encefalopatia, sintomas psiquiátricos, coreia ou convulsões [608].

Alguns doentes com SSP apresentam mielite e neurite óptica (Síndrome de Devic), que faz parte do espectro mais amplo da neuromielite óptica [609]. Devido à associação quase universal deste síndrome aos anticorpos anti-aquaporina 4, não é consensual se se tratará de uma manifestação da SSP ou de uma mera associação [610].

Na origem das manifestações do Sistema nervoso central poderá estar a infiltração linfocitária directa, o envolvimento vasculítico de pequenos vasos, e o dano causado por anticorpos anti-neurais e anti-Ro/SSA [611].

As manifestações cognitivas e psiquiátricas são classicamente associadas à SSP, embora não seja claro se são causadas por processos imunológicos, ou se são reativas ao impacto da doença [612].

2.5.3.9. Sistema Hematológico

A presença de anemia está presente em cerca de 20% dos casos, habitualmente normocítica e normocrómica, podendo reflectir a atividade da doença, hemólise, ou ser carencial (ferropénica, défice de B12) [613].

A Leucopénia ocorre em 15-30% dos doentes, e corresponde maioritariamente a linfopénia [613,614]. A linfopénia deve-se sobretudo a uma redução das células CD4+ [615], e é considerada um Fator de risco para o desenvolvimento de linfoma [616]. Neutropénia (<1500/µl) ocorre em cerca de 10% dos doentes, e relaciona-se com a presença de FR e anti-SSA/SSB, sugerindo uma etiologia autoimune [617].

Trombocitopénia é encontrada em 15% dos doentes [613], de causa periférica, sendo que a ocorrência de Púrpura trombocitopénica trombótica é rara [618].

A intensa estimulação de células B reflete-se na presença de hipergamaglobulinémia, crioglobulinémia, gamapatia monoclonal e produção de autoanticorpos [437]. A formação de imuno-complexos leva ao consumo de Fatores de complemento, que se encontram diminuídos em cerca de 25% dos doentes, relacionando-se fortemente com a atividade sistémica e pior prognóstico [619].

2.5.4. Risco de linfoma

Os doentes com SSP têm um risco 16x superior de desenvolver linfoma [620]. Em cerca de 65% dos casos os linfomas associados à SSP são linfomas B não-Hodgkin (LNH) de baixo grau, predominantemente linfomas MALT (*mucosa associated lymphoid tissue*) de tipo histológico da zona marginal [621]. Estes linfomas são maioritariamente extra-nodais, indolentes, e localizam-se habitualmente em órgãos onde a doença é ativa, como as glândulas salivares [622]. Outros dois tipos histológicos, menos frequentes e de pior prognóstico, são os linfomas B difusos de grandes células (15%), que podem ser primários ou evoluir a partir de linfomas MALT pré-existentes, e os linfomas B nodais da zona marginal (10%) [623]. O risco cumulativo de desenvolver linfoma aumenta com a duração da doença, sendo de 3,4% aos 5 anos e de 9,8% aos 15 anos [622].

Vários elementos clínicos, laboratoriais e histológicos têm sido associados ao risco de linfoma (**Figura 16**). Destes, os principais são a tumefação glandular persistente, linfadenopatias, crioglobulinémia, vasculite, baixos níveis de C4, e linfopénia [621]. Durante a evolução da doença, deve investigar-se a eventual presença de linfoma perante febre recurrente, púrpura, neuropatia periférica sensitivo-motora, glomerulonefrite, linfadenopatia, infiltrados pulmonares nodulares, tumefação assimétrica de glândula salivar ou massa intra-parotídea palpável [624].

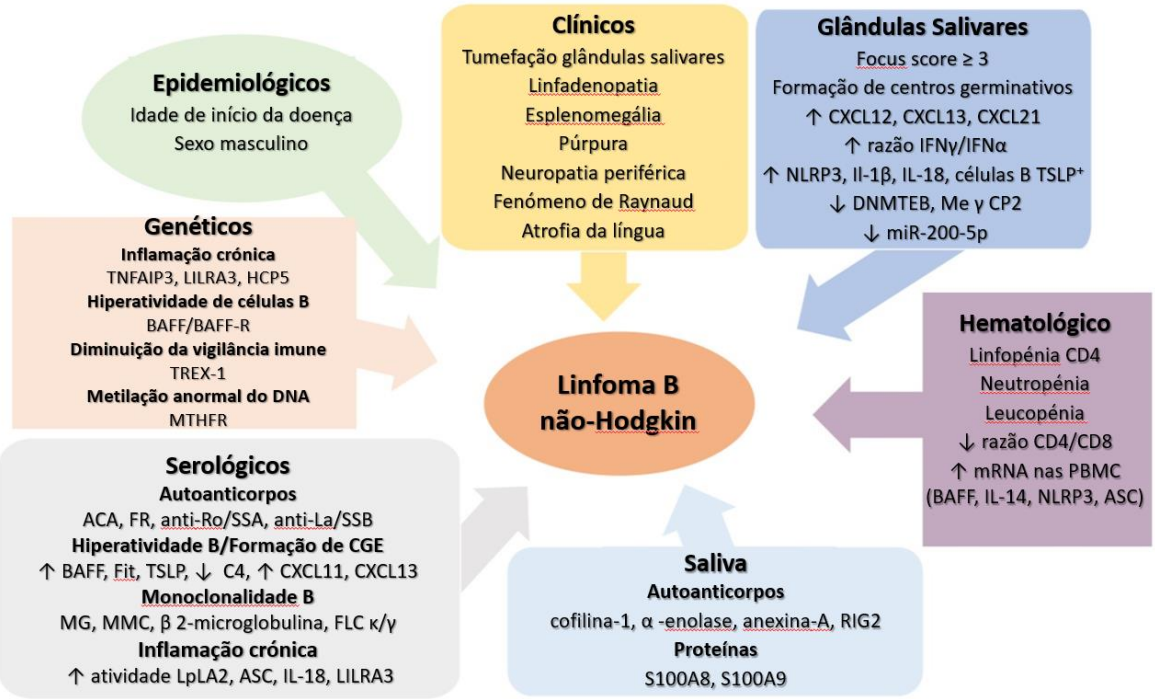

Figura 16. Fatores clínicos e moleculares que contribuem para o desenvolvimento de linfoma na Síndrome de Sjögren. Adaptado de Skarlis (2020) [625].

↑: aumentado; ↓: diminuído; fator reumatóide: FR; *tumor necrosis Fator-alpha induced protein* 3: TNFAIP3; B *cell ativating Fator*: BAFF; B *cell ativating Fator receptor*: BAFF-R; *Interferon* α/γ:IFNα/γ; *three prime repair exonuclease* 1: TREX-1; *leukocyte immunoglobulin- like receptor subfamily A member* 3: LILRA3; *Major histocompatibility complex* P5 *gene*: HCP5; *methylene tetrahydrofolate reductase*: MTHFR; DNA *methyltransferase* 3B: DNMT3B; *methyl CpG binding protein* 2: MeCP2; *peripheral blood mononuclear cells*: PBMCs; *Rho GDP-dissociation inhibitor* 2: RGI2; *Extracellular lipoprotein-associated phospholipase* A2: Lp-PLA2; *free light chains* κ/λ:FLCκ/λ; *Fms-like tyrosine kinase 3 ligand*: Flt-3 L; *chemokine (C-X-C motif) ligand* 11/12/13/21: CXCL11/12/13/21 *NOD-like receptors containing pyrin domain* 3: NLRP3; *apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain [CARD]*:ASC; *Thymic stromal lymphopoietin*: TSLP; *monoclonal gammopathy*: MG; *mixed monoclonal cryoglobulinemia*: MMC; *anti-centromere antibodies*: ACA

2.5.5. Fenótipos clínicos

De acordo com características do doente, como o sexo ou a idade de início, a SSP pode apresentar características fenotípicas distintas [626].

2.5.5.1. Doente Adulto Jovem

Os doentes com início mais jovem (< 35 anos) apresentam maior hiperatividade B em comparação com as faixas estárias mais prevalentes (5ª e 6ª décadas). Esta manifesta-se por maior prevalência de tumefação das gândulas salivares, linfadenopatia, leucopénia, hipergamaglobulinémia e hipocomplementémia C4, resultando em maior risco de linfoma [627,628]. Há também uma maior frequência de anti-SSA, anti-SSB e Fator Reumatóide em doentes com SSP de início jovem [628,629]. Clinicamente, tem sido referido maior frequência de púrpura e envolvimento renal, bem como maior atividade sistémica da doença [628,630]. Apesar disso, quer as queixas, quer algumas manifestações sistémicas como neuropatia periférica e doença intesticial pulmonar são menos prevalentes no indivíduo jovem [631].

2.5.5.2. Doente Idoso

A presença de queixas secas aumenta com a idade e afeta cerca de 30% da população acima dos 65 anos [632]. Há um declínio funcional das glândulas salivares e lacrimais, que se traduz por testes objetivos anormais e maior probabilidade de "falsos positivos", como no teste de Schirmer [633]. Deste modo, no idoso a presença de queixas secas não significa necessariamente a presença de um distúrbio imuno-mediado, e obriga à exclusão de Fatores externos, em especial fármacos xerogénicos [634].

Ao contrário do que se verifica na AR e no LES, a SSP de início no idoso não parece constituir um subtipo distinto de doença [635,636]. No entanto, em comparação com indivíduos cujo início da doença ocorreu na idade habitual (5ª e 6ª décadas), doentes com início mais tardio da doença apresentam uma resposta imune B-mediada menos robusta, com menor frequência de autoanticorpos e de hipergamaglobulinémia [631,637]. Estes doentes também apresentam maior frequência de queixas secas e doença intersticial pulmonar, e menor ocorrência de artrite [627].

2.5.5.3. Sexo masculino

O predomínio do sexo feminino na SSP é superior ao de outras doenças reumáticas,com valores entre 14:1 e 33:1 face ao masculino [638,639].

No sexo masculino a doença é habitualmente mais grave na sua fase inicial, com manifestações extra-glandulares mais frequentes e numerosas, e níveis de anti-SSA mais elevados [640]. No homem são também mais frequentes as manifestações constitucionais, envolvimento intersticial pulmonar, vasculite e linfadenopatia [641]. O risco de linfoma é superior no sexo masculino [642].

Algumas manifestações extraglandulares na SSP seguem a distribuição típica por géneros, com maior frequência de tiroidite, fenómeno de Raynaud, depressão e fibromialgia no sexo feminino [462,636]. No entanto, os homens apresentam menor disfunção glandular, com menos queixas secas e melhores medidas centradas no doente (MCD) [639].

2.6. Diagnóstico

Tal como sucede noutras doenças reumáticas sistémicas, a SSP apresenta uma considerável variabilidade clínica, não existindo nenhuma característica específica que, isoladamente, permita o diagnóstico. Na SSP este é feito considerando o conjunto de manifestações clínicas e alterações laboratoriais no contexto específico de cada indivíduo [1].

O diagnóstico de SSP pode ser considerado num indivíduo que apresente manifestações típicas da doença, nomeadamente secura oral e ocular (síndrome Sicca), tumefação persistente ou intermitente das glândulas salivares, aumento súbito da incidência de cáries, presença de uma doença do tecido conjuntivo acompanhada de secura oral ou ocular, manifestações extra-glandulares típicas (eritema anular, crioglobulinémia, neuropatia periférica, pneumonite intersticial), presença de anticorpos anti-SSA ou anti-SSB, e linfoma MALT numa glândula salivar [643]. A investigação específica inclui parâmetros e testes objetivos para confirmar a hipossecração glandular [644,645], bem como a confirmação da existência de um processo inflamatório autoimune. A marcha diagnóstica exaustiva permite distinguir a SSP de outras patologias ou condições que podem ter uma sobreposição variável com as manifestações da SSP [2] (**Tabela 1).**

Os principais elementos para confirmar a SSP são a presença de anticorpos anti-SSA e anti-SSB [3], e infiltrados linfocitários focais na biópsia de glândula salivar minor (labial) [4,5]. No entanto, diagnóstico clínico nem sempre é fácil, sobretudo nas fases iniciais da doença, em que a disfunção glandular pode ser ainda ligeira [1]. Em alguns casos as manifestações sistémicas podem anteceder o reconhecimento da doença, sendo necessário um elevado grau de suspeição. Apesar dos anticorpos anti-SSA e anti-SSB poderem estar presentes antes do reconhecimento clínico da doença [629], em um terço dos doentes são negativos [646]. A presença de citopénias, hipergamaglobulinémia e elevação da velocidade de sedimentação (VS) pode apoiar a hipótese de um SSP oculto [1].

No entanto, diagnóstico clínico nem sempre é fácil, sobretudo nas fases iniciais da doença, em que a disfunção glandular pode ser ainda ligeira [1]. Noutros casos, as manifestações sistémicas podem anteceder o reconhecimento da doença, sendo necessário um elevado grau de suspeição. Apesar dos anticorpos anti-SSA e anti-SSB poderem estar presentes antes do reconhecimento clínico da doença [629], em um terço dos

doentes são negativos [646]. A presença de citopénias, hipergamaglobulinémia e elevação da velocidade de sedimentação (VS) pode apoiar a hipótese de um SSP oculto [1].

	Síndrome Sicca	Envolvimento Glandular	Envolvimento Articular	Envolvimento Sistémico
Medicação xerogénica	X	-	-	-
Inibidores da Aromatase	X	-	X	(X) SSP-like
Secura relacionada com a idade	X	-	-	-
Sialoadenose metabólica	_	X	-	-
Olho seco não-Sögren	X	-	-	-
Irradiação da cabeça e pescoço	X	-	-	-
Sarcoidose	X	X	X	X
Hiperlipoproteinémia (tipos II, IV, V)	X	X	(X)	-
Doença do Enxerto-versus-hospedeiro	X	X	X	X
Linfoma primário	X	X	-	(X)
Amiloidose	X	X	(X)	(X) Púrpura renal
Sialoadenite viral crónica (HCV, HIV, HTLV-1)	X	(X)	X	X
Outras sialoadenites crónicas inespecíficas	X	X tipicamente unilateral	-	-
Diabetes mellitus	X	(X) Sialoadenose	(X) Queiroartropatia	(X) Neuropatia
Hemocromatose	X	(X)	X CPPD	(X)
Outras doenças do tecido conjuntivo	X	-	X	X
Artrite reumatoide	(X)	-	X	(X)
Granulomatosis com poliangeíte	X	(X)	X	X
Doença relacionada com a IgG4	X	X	(X)	(X)
Ansiedade, fibromialgia	X	-	(X)	-
Inibidores dos Checkpoints	X	(X)	X	X

Tabela 1. Diagnóstico diferencial da Sìndrome de Sjögren Primária.

O critério histológico (*focus score* ≥1) é muito específico, mas tem uma sensibilidade variável [4] e parece ter uma associação à presença de anti-SSA, fator rematóide (FR) e anticorpos anti-nucleares (ANA) [647], de modo que novos biomarcadores são necessários para demonstrar a presença de disfunção autoimune. Como exemplos promissores temos a caracterização do perfil linfocitário por citometria de fluxo [7,8], a identificação de novos autoanticorpos [9] e a ecografia das glândulas salivares [10].

~

2.6.1. Avaliação do componente oral

As queixas de xerostomia habitualmente ocorrem quando a diminuição do fluxo salivar ultrapassa 50% do valor basal, mesmo que o indivíduo ainda possa apresentar fluxos dentro do intervalo de referência[648]. A xerostomia é um sintoma muito prevalente na população mais idosa, sendo a sua causa mais frequente a utilização de fármacos xerogénicos [649]. Além disso, com o envelhecimento ocorre uma redução do fluxo salivar basal, dependente das gândulas submaxilares [650], cuja função é fundamental para manter a hidratação oral e prevenção de cáries. A produção salivar das gândulas parótidas, estimulada durante a ingestão alimentar, é menos afetada com o envelhecimento [651].

Na avaliação objetiva da xerostomia, pode ser medido quer o fluxo não estimulado, quer o estimulado. Considera-se hipossalivação quando o fluxo não estimulado é inferior a 0,1 ml/minuto ou o estimulado inferior a 0,7 ml/minuto [652]. Métodos outrora comuns, como a cintigrafia das gândulas salivares e a sialografia, já não são utilizados de rotina [653], e os peritos responsáveis pela elaboração nos actuais critérios ACR/EULAR apenas consideraram o fluxo salivar não-estimulado como representativo do envolvimento oral [15].

O envolvimento das gândulas salivares na SSP também pode ser avaliado através da ecografia, que revela alterações típicas nas glândulas salivares, como a presença de áreas hipoecogéneas ovóides (as mais específicas), bandas hiperecogénicas lineares, o número de gânglios intra-parotídeos, e a definição do bordo posterior da glândula [10]. As alterações da ecoestrutura das glândulas salivares surgem relativamente cedo na evolução da doença, podendo ajudar no diagnóstico precoce [654]. Estas alterações podem ser quantificadas de acordo coma a sua gravidade/intensidade, estando demonstrada uma correlação positiva entre a pontuação obtida e presença de anticorpos anti-SSA, sialometria anormal, e biópsia de glândula salivar minor positiva [655,656].

2.6.2. Avaliação do componente ocular

A síndrome de olho seco é um distúrbio multifatorial, geralmente causado pela diminuição da produção de lágrima ou pela sua evaporação excessiva [645]. A síndrome de olho seco é um problema comum, afetando até 17% das mulheres e 11% dos homens, de acordo com estudos epidemiológicos dos Estados Unidos[657]. A sua prevalência é especialmente elevada em idosos e em utilizadores de medicamentos xerogénicos.

A avaliação objetiva do olho seco baseia-se no estudo do fluxo lacrimal e na avaliação do dano da superfície da córnea, através do teste de Schirmer-I, teste de quebra do filme lacrimal e observação da córnea marcada com corantes vitais [658–660]. A reprodutibilidade destes testes não é alta, e a variabilidade depende da técnica utilizada, caraterísticas do

ambiente e do próprio doente [6,661,662]. Adicionalmente, a fraca associação entre as queixas subjetivas de olho seco e os testes objetivos dificulta a avaliação do componente ocular em doentes com xeroftalmia [6].

O teste de Schirmer mede a produção lacrimal e é de fácil realização [663]. O fluxo basal é avaliado sem anestesia, sendo valores inferiores a 5mm em 5 minutos considerados anormais e constituindo um dos items nos critérios de classificação da SSP [13,15]. No entanto, o fluxo lacrimal diminui com a idade, pelo que a interpretação do teste no idoso deve ser criteriosa. Adicionalmente, a fraca associação entre as queixas subjetivas de olho seco e os testes objetivos dificulta a avaliação do componente ocular em doentes com xeroftalmia [6].

A avaliação da superfície ocular marcada com corantes vitais, através da lâmpada de fenda, é mais sensível que o teste de Schirmer na deteção de olho seco, existindo várias escalas de quantificação das lesões, como a de Bijsterveld [664], a de Oxford [665] ou a do *Sjögren International Collaborative Clinical Alliance* (SICCA) [660]. Lesões do epitélio da córnea são visíveis com a fluorosceína, e a presença de desvitalização das células conjuntivais é detectada com o verde de lisamina. Para além da sua utilização na deteção e classificação de doentes com SSP, como no caso da de Bijsterveld [13,15] e SICCA [15,666], a aferição e quantificação das lesões da superfície ocular é também utilizada como objetivo em ensaios clínicos [665].

Novos intrumentos de avaliação do olho seco têm vindo a ser estudados, como a *Anterior Segment - Optical Coherence Tomography* (AS-OCT) e a microscopia confocal.

Através da AS-OCT pode medir-se as caraterísticas do menisco lacrimal, sendo assim uma técnica não invasiva para avaliação do filme lacrimal [658,667–672].

A microscopia confocal *in vivo* produz imagens de alta resolução da córnea e da superfície ocular [673], e pode ser utilizada para estudar alterações morfológicas do plexo nervoso subcorneano induzidas pela secura ocular [674,675]. Esta técnica pode vir a ser útil não só na deteção da doença, mas também na monitorização da resposta ao tratamento [676].

2.6.3. Auto-anticorpos

Os anticorpos anti-Ro/SSA e os anti-La/SSB são os mais importantes no diagnóstico da SSP, embora sejam também frequentes noutras doenças autoimunes, como o LES [677]. Na SSP, os anticorpos anti-SSA estão presentes em 33-74% dos doentes e os anti-SSB em 23-52% [626].

Para além dos anticorpos anti-SSA e anti-SSB, outros antoanticorpos tem sido propostos como biomarcadores da SSP, nomeadamente os anti-α-fodrina, anti-receptor

muscarínico tipo-3 da acetilcolina (M3R), anti-proteína da glândula salivar (SP1), anti-anidrase carbónica-6 (CA6), e anti-antigénio secretório da parótida (PSP) [677].

2.6.3.1. Anti-SSA

Os anticorpos anti-SSA reconhecem duas ribonucleoproteínas não relacionadas e com funcões biológicas distintas, denominadas Ro52 e Ro60 [678]. Modelos experimentais sugerem que estes anticorpos poderão ser diretamente patogénicos na SSP.

A proteína Ro52/TRIM21 (tripartite motif 21) é amplamente expressa em células hematopoiéticas e participa na ubiquinação de vários Fatores reguladores da via do IFN, de proteínas da via do Fator nuclear KB, e de proteínas reguladoras do ciclo celular [679]. Ratos com deficiênca de TRIM21 desenvolvem autoimunidade sistémica mediada por células B, com autoanticorpos, hipergamaglobulinémia e glomerulonefrite [680]. O TRIM21 parece ter um papel na diferenciação, compromisso de linhagem e sobrevivência dos linfócitos B, e na sua ausência há um desvio a favor da diferenciação em células B foliculares [681]. Em doentes com LES, a diferenciação de células B em plasmablastos e a produção de anticorpos foi significativamente superior em doentes com anticorpos anti-TRIM21, em comparação com aqueles sem estes anticorpos [682]. Além disso, em ratos as respostas imunes iniciadas pelo Ro52 induziram disfunção das gândulas salivares [683] e olho seco hipossecretor [684], que poderão ser mediadas pelos anticorpos anti-Ro52.

A proteína Ro60, também conhecido por TROVE2 (telomerase-RO-vault-element 2), é uma proteína ligante de RNA, que remove RNAs que falharam a ligação às suas proteínas ligantes específicas, desempenhando assim um papel na prevenção de respostas imunes e na redução do stress celular [685]. Ratos com deficiência de Ro60 desenvolvem uma doença lúpus-*like*, provavelmente causada pela acumulação anormal de RNAs devido à sua deficiente remoção [686].

2.6.3.2. Anti-SSB

O antigénio La/SSB é uma proteína fosforilada de 48 kDa, localizada no núcleo e citoplasma, que desempenha um importante papel no processo de transcrição e maturação pela RNA polimerase III, através da sua ligação às moléculas de RNA durante o ciclo celular [687]. Estudos funcionais demonstraram que os anticorpos anti-SSB/La exercem vários efeitos nas células polimorfonucleadas humanas, incluindo a supressão da fagocitose, a apoptose acelerada e o aumento da produção de IL-8 [688]. A proteína La encontram-se com frequência fisicamente associada ao complexo Ro60-hyRNA [689] (36), o que explica a frequente coexistência de anticorpos anti-La e anti-Ro.

A prevalência de anti-La/SSB na SSP é de 23-52% [626], quase sempre associados a anti-Ro/SSA. A combinação de anti-Ro/SSA e anti-La/SSB é mais específica para a SSP que o anti-Ro isolado, e está associada a doença mais grave [690]. Em doentes com anti-SSA e anti-SSB há maior ocorrência de manifestações sistémicas, como esplenomegália, linfadenopatia, vasculite e fenómeno de Raynaud [691].

A presença isolada de anti-SSB, na ausência de anti-SSA, é rara na SSP e não parece estar associada a características típicas da doença [692], sendo esta uma das razões para a não inclusão deste anticorpo nos critérios de classificação ACR/EULAR de 2016.

2.6.3.3. Anticorpos específicos glandulares

Para além dos anticorpos anti-SSA e anti-SSB, outros antoanticorpos tem sido propostos como biomarcadores da SSP, nomeadamente os anti-α-fodrina, anti-receptor muscarínico tipo-3 da acetilcolina (M3R), anti-proteína da glândula salivar (SP1), anti-anidrase carbónica-6 (CA6), e anti-antigénio secretório da parótida (PSP) [677].

Aquando da sua descrição em 1997, os anticorpos anti-α-fodrina foram considerados biomarcadores muito promissores na SSP, com uma sensibilidade de 90% e uma especificidade de 100% [693]. O interesse nestes anticorpos diminuíu após estudos subsequentes, em diferentes cenários clínicos, terem reportado sensibilidades e especificidades de 59-75% para os IgG, e 55-73% para os IgA, respectivamente [694].

Os anticorpos anti-M3R têm uma sensibilidade e especificidade na SSP de 43% e 95%, respectivamente [695]. Este anticorpos podem ser directamente responsáveis pela hipossecração salivar na SSP [696]. Além disso, têm sido associados a leucopenia devido à aparente subregulação do HLA classe II e do M3R da membrana celular, que é seguida da morte celular mediada por células NK [697].

Os anticorpos anti-SP1, CA6 e PSP, por reconhecerem antigénios específicos das glândulas salivares e lacrimais, têm sido alvo de particular interesse. Em modelos animais, estes anticorpos são detectáveis antes do aparecimento dos anticorpos anti-SSA e SSB [9]. Na SSP, identificam doentes com secura oral e ocular, e baixo focus score, sendo mais frequentes em indivíduos negativos para anti-SSA [698]. Com a excepção dos anticorpos anti-CA6 da classe IgA, presentes sobretudo em doentes com SSP de longa evolução, os anticorpos das classes IgG e IgM raramente foram encontrados na doença evoluída [699].

Estes anticorpos, dos quais o mais promissor é o anti-SP1, poderão vir a ser úteis no diagnóstico precoce [700], bem como na identificação da SS secundária, sobretudo em doentes com AR, Esclerose Sistémica e a Doença Mista do Tecido Conjuntivo [699].

2.6.3.4. Anticorpos gerais – Anti-nucleares e Fatores Reumatóides

Anticorpos anti-nucleares (ANA) estão presentes em 59-85% dos doentes com SSP [636,701–703]. Doentes com ANA positivo têm maior prevalência de hipertrofia das parótidas e maior frequência de manifestações extra-glandulares como fenómeno de Raynaud, vasculite cutânea, envolvimento articular e renal, febre, adenomegálias, citopénias e elevação da VS (>50 mm/h) [636,701,702]. Para além de doentes ANA-positivos apresentarem maior número de órgãos envolvidos, têm maior prevalência de indicadores de atividade imunológica, como hipergamaglobulinémia, fractor reumatóide, anticorpos anti-SSA e SSB e anti-fosfolípidos [701,702].

Não obstante, os ANA são inespecíficos e frequentemente encontrados, em título baixo, em indivíduos saudáveis (sobretudo idosos), doentes com outras doenças autoimunes, e em indivíduos com história familiar de autoimunidade [704].

Fatores reumatóides (FR) são anticorpos dirigidos à porção Fc de imunoglobulinas da classe IgG, podendo ser de qualquer isotipo, mas mais frequentemente da classe IgM, IgG e IgA [705].

Na SSP os FR são encontrados em 36-74% dos doentes [701–703], e associam-se a menor idade de início, sexo feminino e positividade da biópsia de glândula salivar [702,706]. Vários estudos estabeleceram uma correlação entre os FR e a presença de um perfil serológico ativo, com presença de anti-SSA, anti-SSB, crioglobulinas, ANA, hipocomplementémia e hipergamaglobulinémia [636,702,703,707]. Tal como os anti-SSA e SSB, o FR é um marcador de maior disfunção das glândulas salivares e lacrimais [708]. Quer a presença, quer o título de FR, apresentam uma correlação positiva com o número de manifestações extra-glandulares da SSP [702,703,707]. Comparados com doentes FR-negativos, doentes FR-positivos têm com maior frequência manifestações articulares, vasculite cutânea, tumefação das glândulas salivares, citopenias, fenómeno de Raynaud, envolvimento renal e do sistema nervoso central [636,702]. Foi descrita uma associação entre os FR da classe IgA e envolvimento renal, maior focus score na biópsia de glândula salivar, e níveis de outros autoanticorpos [709].

Os FR têm valor prognóstico na SSP devido à sua associação a manifestações sistémicas e marcadores serológicos de doença ativa [710]. Além disso, associam-se ao risco de linfoma, sendo um dos elementos que compõe o modelo preditivo para o desenvolvimento de linfoma não-Hodgkin na SSP [439].

2.6.4. Histopatologia das glândulas salivares

A presença de sialoadenite linfocítica focal na biópsia de gândula salivar minor (labial) é considerada a característica mais típica da SSP [711]. Chisholm e Mason descreveram a técnica

de biópsia labial e elaboraram uma classificação ainda hoje utilizada na SSP [414], utilizando o conceito de focos, definido como a presença de agregados de mais de 50 células (linfócitos) em localização periductal ou perivascular [712].

Apesar de integrar vários critérios de classificação da SSP [13,15,666,713], a análise histopatológica da glândula salivar minor está sujeita a Fatores de confundimento que podem reduzir a sua reprodutibilidade [714,715]. De facto, a sua sensibilidade varia entre 63.9% e 85.7%, e a especificidade entre 61.2% and 100% [4]. Um número significativo de doentes pode não ser diagnosticado devido à baixa sensibilidade do exame, especialmente indivíduos seronegativos para anti-SSA e SSB [716], para o qual podem contribuir Fatores como colheita inadequada e discrepâncias na interpretação [717]. Têm vindo a ser propostos aperfeiçoamentos para melhorar a sensibilidade e o valor preditivo da biópsia de GSM, incluindo o uso rigoroso do focus score [718], a realização de cortes multi-nível [719], e o consenso por peritos para uniformização [720].

Para além do seu clássico papel no diagnóstico e classificação da SSP, o fácil acesso ao tecido glandular através da biópsia de GSM tem sido uma mais-valia no estudo da patogénese da doença, e a relação entre aspectos histológicos e clínicos pode ser preditor da evolução da doença e do prognóstico [721].

O conceito de "*focus score*", introduzido por Greenspan [722], indica o número de agregados linfocitários por área (4 mm^2), com valores mais elevados associados a maior lesão acinar e fibrose [5]. O *focus score* correlaciona-se com importantes parâmetros imunológicos, como os anticorpos anti-SSA e SSB, ANA e FR, e associa-se a menor função glandular e manifestações extra-glandulares como fenómeno de Raynaud, vasculite e leucopenia [5,647].

Outro aspecto histopatológico importante na SSP é a presença de estruturas com características de CGE nas glândulas salivares [723]. Estas estruturas linfóides são relativamente frequentes na SSP, estando presentes em até 40% dos doentes [724], e contribuem para a autoimunidade através da diferenciação local de plasmócitos autorreativos [512]. A presença de CGE está associada a maior prevalência de anticorpos anti-SSA e SSB, maior gravidade da doença, manifestações extra-glandulares, e linfoma MALT de células B [724]. A presença de CGE nas GSM tem um valor preditivo de 16% para linfoma não-Hodgkin, enquanto a sua ausência tem um valor preditivo negativo de 98% [725].

2.7. Critérios de Classificação

Enquanto que o diagnóstico é o corolário de um processo clínico que avalia a probabilidade de um determinado indivíduo ter a doença, no seu contexto específico, a classificação

pretende definir presença ou ausência da doença de acordo com caraterísticas pré-estabelecidas [11]. Os critérios de classificação permitem identificar grupos de doentes bem definidos para estudos clínicos e epidemiológicos, sendo fundamentais na investigação clínica em Reumatologia [726]. A maioria das doenças reumáticas imuno-mediadas são heterogéneas nas suas manifestações clínicas, evolução e prognóstico, e carecem de identificadores únicos, clínicos, laboratoriais, patológicos ou radiológicos que sirvam de "padrão-ouro" no diagnóstico e/ou classificação [727]. Deste modo, a investigação de mecanismos patogénicos e o desenvolvimento de novas terapêuticas nas doenças reumáticas não seria possível sem a utilização de critérios de classificação. Estes têm sido revistos ao longo do tempo com a incorporação de novos items e metodologias [728].

Os fundamentos da classificação são o desempenho dos testes utilizados (sensibilidade, especificidade, *receiver operating characteristics*), enquanto que a base do diagnóstico é o conjunto das variáveis/características relevantes, através das quais se afere a probabilidade da presença da doença [729]. Nos casos em que critérios de classificação apresentassem sensibilidade e especificidade perfeitas (ambas de 100%), então seriam sinónimos de critérios de diagnóstico, pois identificariam corretamente todos os casos individuais [17].

Apesar da maioria dos critérios de classificação das doenças reumáticas sistémicas apresentarem sensibilidade e especificidade moderada a elevada, ainda assim não incluem todas as características clínicas necessárias para o diagnóstico de doenças heterogéneas e de baixa prevalência [726]. No entanto, na prática clínica os critérios de classificação podem ser úteis como uma orientação na investigação e suporte do diagnóstico.

No caso da SSP, entre 1965 e 2016 foram desenvolvidos 13 conjuntos de critérios de classificação [14]. Nos vinte anos que se seguiram à publicação dos primeiros critérios, vários outros foram sendo publicados, por iniciativa individual ou nacional, culminando na publicação de 3 conjuntos distintos de critérios em 1986 [730–732]. Este foi um ponto de viragem, e a partir daí procurou-se a elaboração de critérios de consenso gerados por grupos de peritos, resultando na publicação dos *Preliminary criteria for the classification of Sjögren's syndrome* [713], patrocinados pela Comunidade Europeia.

A versão revista dos critérios de 1993 foi publicada em 2002, elaborada pelo *American-European Consensus Group* (AECG), e inclui 6 domínios, dos quais 2 são subjetivos (secura ocular e oral) e 4 são objetivos (sinais oculares e orais, histopatologia, e auto-anticorpos) [13]. Durante a elaboração dos critérios AECG apurou-se uma sensibilidade de 97,4% e uma especifidade de 89,4% para SSP quando se cumpriam quaisquer 4 dos 6 items. Ao aplicar como critérios obrigatórios na versão final a serologia ou a histopatologia, a sensibilidade desceu para 89,5% e a especifidade aumentou para 95,2%, sendo essa maior especificadade preferível para efeitos de classificação [13]. Desde a sua publicação,

estes critérios de classificação tornaram-se os mais utilizados, com mais de 1500 citações (Rasmussen et al., 2014).

Em 2012, contudo, um sistema de classificação alternativo foi proposto pelo *American College of Rheumatology* (ACR) com o objetivo de aumentar a especificidade perante o crescente número de ensaios clínicos (Shiboski et al., 2012). Estes critérios eram baseados exclusivamente em sinais objetivos (*focus score* positivo, serologia, e *ocular staining score*), mas não demonstraram superioridade quando comparados com os critérios AECG (Rasmussen et al., 2014).

Dada a necessidade de se obterem critérios consensuais, o consórcio *International Sjögren's Syndrome Criteria Working Group* desenvolveu um novo sistema de classificação em 2016, que recebeu o nome das associações de reumatologia que os adoptaram, o ACR e a *European League Against Rheumatism* (EULAR) *Classification Criteria* (ACR-EULAR) (Shiboski et al., 2017). Estes novos critérios incorporam elementos dos critérios AECG e ACR, e atribuem pontos aos 4 items objetivos: *focus score* ≥1 foco/4 mm^2, anti-SSA/Ro, sinais oculares (*ocular staining score* ≥5/Van Bijsterveld *score* ≥4 e teste de Schirmer ≤5 mm/5 min) e fluxo salivar não-estimulado ≤0.1 ml/min, sendo a classificação como SSP estabelecida com 4 ou mais pontos (Shiboski et al., 2017).

A comparação dos critérios AECG e ACR/EULAR não demonstrou superioridade significativa de nenhum deles [14], mas os ACR/EULAR parecem ser ligeiramente menos específicos mas mais sensíveis [146,733,734], permitindo a classificação de alguns doentes que ainda não apresentavam queixas secas [734]. Os elementos constituintes de cada conjunto de critérios e as regras de classificação são apresentadas na **Tabela 2**.

No entanto, apesar da elevada sensibilidade e especificade de ambos os critérios, AECG e ACR/EULAR, nem todos os doentes com um diagnóstico clínico de SSP irão cumprir critérios de classificação [16]. Uma vez que os critérios ACR/EULAR são baseados na pontuação de elementos objetivos, a sua adaptação poderá ser exequível caso novos testes diagnósticos demonstrem mais-valia. Entre possíveis candidatos encontra-se a ecografia das gândulas salivares, cuja valor acrescentado aos critérios foi já estudado [734–737]. Foi inclusivé proposta a atribuição de 1 ponto à avaliação ecográfica caso fosse incluída nos critérios ACR/EULAR [736,737], o que aumentaria o conjunto de testes por onde escolher. Outras técnicas ou biomarcadores que venham a ser validados terão também potencial de aperfeiçoar os critérios de classificação [14].

#	2002 AECG	2016 ACR/EULAR	Pontos
1	Sintomas oculares: resposta positiva a pelo menos uma das questões: Tem diariamente secura ocular persistente e incómoda há > 3 meses? Tem sensação recurrente de corpo estranho ou areia nos olhos? Usa substituto lacrimal mais de 3 vezes por dia?		
2	Sintomas orais: resposta positiva a pelo menos uma das questões: Tem sensação diária de boca seca desde há mais de 3 meses? Teve inchaço recorrente ou persistente das GS em adulto? Tem de beber líquidos para ajudar a deglutição de alimentos secos?		
3	Sinais objetivos oculares: Resultado positivo em pelo menos um dos testes: Teste de Schirmer I, sem anestesia (≤5 mm in 5 min) Marcação ocular com Rosa de Bengala ou outro corante ocular (≥4 de acordo com o esquema de pontuação de van Bijsterveld)	Sinais objetivos oculares: Teste de Schirmer I, sem anestesia (≤5 mm in 5 min) Queratoconjuntivite sicca com: ≥3 pontos no *Ocular staining score* ou ≥4 no esquema de van Bijsterveld	**1** **1**
4	Histopatologia: biópsia de GS minor apresentando sialoadenite linfocítica focal com um *focus score* ≥1 foco / 4 mm^2	Biópsia de GS labial apresentando sialoadenite linfocítica focal com um *focus score* ≥1 foco / 4 mm^2	**3**
5	Evidência objetiva de envolvimento das glândulas salivares: Definido como um resultado positivo em pelo menos um: Fluxo salivar não estimulado (≤1.5 ml em 15 min) Sialografia parotídea mostrando sialectasias difusas, sem evidência de obstrução nos ductos principais Cintigrafia salivar mostrando atraso na captação, baixa acumulação e/ou atraso na secreção do radionuclido	Evidência objetiva de envolvimento das glândulas salivares: Fluxo salivar não estimulado ≤0.1 ml / min	**1**
6	Presença de autoanticorpos: Anticorpos anti-Ro (SSA) e/ou anti-La (SSB)	Presença de autoanticorpos: Anticorpos anti-Ro (SSA)	**3**

(Cont.)

(Cont.)

Regras de Classificação	
A presença de 4 dos 6 items é indicativa de SSP, sendo obrigatório que o item 4 (histopatologia) ou o item 6 (autoanticorpos) sejam positivos	Classifica-se como SSP um indivíduo com ≥4 pontos e sinais /sintomas sugestivos da doença
Critérios de Exclusão	
Critérios AECG: história de radioterapia à cabeça e pescoço; infeção pelo virus da Hepatite C (VHC); infeção pelo Vírus da Imunodeficiência Adquirida (VIH); linfoma pré-existente; sarcoidose; doença do enxerto-versus-hospedeiro (GVHD); uso de medicamentos anticolinérgicos (num período inferior a 4 semi-vidas do fármaco) **ACR/EULAR**: história de radioterapia à cabeça e pescoço; infeção ativa pelo VHC (confirmada por PCR); infeção pelo VIH; linfoma pré-existente; sarcoidose; GVHD; amiloidose; doença relacionada com a IgG4. (O uso de medicamentos anticolinérgicos deve ser suspendo antes da avaliação)	

Tabela 2. Critérios de classificação da SSP. Adaptado de Vitali (2002)[13] e Shiboski (2017) [738].

2.8. Instrumentos Metrológicos na SSP

A avaliação da atividade da doença e das MCD é um desafio na SSP devido à heterogeneidade da doença, mas é fundamental para identificar doentes com elevado impacto funcional, atividade sistémica elevada, risco aumentado de linfoma e maior mortalidade [739], e assim orientar a terapêutica.

Um outro aspeto fundamental da doença é o dano funcional ou estrutural irreversível acumulado, que pode resultar da atividade da doença, de efeitos iatrogénicos das terapêuticas, ou de comorbilidades [740]. Estão atualmente disponíveis 2 escalas para aferição e quantificação do dano na SSA: a *SS Disease Damage Index* (SSDDI) [741] [296] e a *SS Damage Index* (SSDI) [742].

A dor generalizada, frequentemente presente nestes doentes, e as queixas secas orais e oculares, bem como as manifestações secundárias a esses sintomas, são importantes para os doentes com SSP, afetando significativamente os componentes físico, psicológico e social da qualidade de vida relacionada com a saúde [743]. No entanto, é frequente a discordância entre a resposta subjetiva e os testes objetivos, causando desacordo entre o médico e o doente relativamente à relevância da secura, fadiga, mal-estar e outros sintomas [744].

No âmbito da saúde oral, tem sido dada atenção à *Oral Health Related Quality of Life*, que é baixa nos doentes com SSP [745]. O *Oral Health Impact Profile* (OHIP)-14 [746] é uma

versão simplificada do OHIP-49, com 14 items. Este questionário encontra-se validado em português, e demonstrou bom desempenho na avaliação da qualidade de vida relacionada com a saúde oral em doentes com hipossalivação [747].

Também a nível ocular foram desenvolvidas algumas escalas, como o *National Eye Institute Visual Function Questionnaire* [748] e o *Ocular Surface Disease Index* (OSDI) [749]. O OSDI é uma ferramenta extensamente utilizada no âmbito da Oftalmologia, uma vez que avalia quer a frequência das queixas de olho seco, quer o seu impacto na visão [750]. Recentemente, um novo questionário, o *Symptom Assessment in Dry Eye* (SANDE) [751], demonstrou boa correlação com o OSDI, com a vantagem de consistir apenas em 2 questões avaliadas com EVA, ao invés das 12 questões do OSDI. Deste modo, é promissor como um método válido, simples e rápido para avaliação da frequência e gravidade dos sintomas de olho seco.

No entanto, o impacto clínico das queixas secas, bem como as MCD relativas às queixas secas, têm uma fraca correlação com a função secretória glandular, de causa certamente multiFatorial [752]. Além disso, as queixas secas, subjetivas, não se correlacionam com a atividade sistémica da doença, e podem afetar negativamente a qualidade de vida, mesmo na ausência de manifestações de envolvimento extra-glandular [753].

Apesar dos vários instrumentos oriundos de áreas particulares, são necessários instrumentos específicos e abrangentes para a avaliação sistemática da qualidade de vida relacionada com a doença em doentes com SSP [754]. Vários instrumentos metrológicos foram desenvolvidos para utilização em ensaios clínicos, como o *SS disease ativity index* (SSDAI) [741], o *Sjögren's Systemic Clinical Ativity Index* (SCAI) [755], o *Profile of Fatigue and Discomfort* (PROFAD) [756] e o *Sicca Symptoms Inventory* (SSI) [757].

Nos ensaios clínicos iniciais, a eficácia da terapêutica imunossupressora foi avaliada recorrendo sobretudo a escalas visuais analógicas (EVA) para as queixas secas, dor e fadiga. A sensibilidade à variação das EVA é questionável, em particular a relativa à dor, que pode subestimar ou sobrestimar a real variação [758].

Deste modo, a EULAR desenvolveu uma iniciativa com vista à elaboração de instrumentos de avaliação das MCD, da atividade sistémica e do dano.

O *EULAR Sjögren's syndrome patient reported index* (ESSPRI), composto por EVA de queixas secas, dor e fadiga [759], demonstrou um melhor desempenho que outras MCD [760].

O *EULAR Sjögren's syndrome disease ativity index* (ESSDAI) foi desenvolvido para avaliar a atividade sistémica da doença. No ESSDAI, as manifestações sistémicas da SSP são caracterizadas de acordo com a gravidade ou grau de atividade, e alocadas a domínios de acordo com o órgão ou sistema afetado (**Anexo 1**). Considerando o somatório das pontuações dos 12 domínios, a pontuação deste índice de atividade varia entre 0 e 123 [761].

Para a correta utilização do ESSDAI em ensaios clínicos, e também na prática clínica, houve necessidade de clarificar e harmonizar a sua aplicação, com a elaboração de um guia de utilização [762].

O ESSDAI e o ESSPRI não se correlacionam entre si, sugerindo que as manifestações sistémicas e as queixas reportadas pelo doente são componentes distintos da doença, pelo que estes instrumentos complementam-se na avaliação dos doentes, quer em ensaios, quer na clínica [763].

A pontuação do ESSDAI correlaciona-se com marcadores de atividade das células B, como a β2 microglobulina, cadeias leves de imunoglobulina livres [573], a citocina BAFF [764], e associa-se ao risco de linfoma [571,764].

O ESSDAI foi validado prospectivamente e demonstrou ter elevada sensibilidade à variação, ao contrário das MCD [760]. Recentemente, foram definidos níveis de atividade de doença e estabeleceu-se o conceito de melhoria clinicamente importante (MCI): doença moderadamente ativa é definida como um ESSDAI ≥5 e a MCI é considerada como a redução de pelo menos 3 pontos [765]. Com estas definições de níveis de atividade e MCI, os novos ensaios clínicos passaram a incluir apenas doentes com atividade sistémica moderada ou elevada (ESSDAI≥5), e simultaneamente o ESSDAI começou a ser utilizado como medida de eficácia em ensaios clínicos randomizados na SSP, sendo em alguns deles o indicador primário de resposta.

Contudo, os resultados de ensaios clínicos recentes que utilizaram o ESSDAI como medida de resposta foram frequentemente desapontantes. Embora os motivos apontados para a falência sejam múltiplos, foram reconhecidas limitações do ESSDAI como medida principal de resposta, incluindo elevados índices de efeito placebo [766].

Como a má qualidade de vida na SSP é determinada sobretudo por MCD como o ESSPRI ou escalas *Sicca*, em detrimento do ESSDAI [767], estão a ser desenvolvidos índices de resposta combinados. Um dos índices combinados proposto é o *Sjögren's syndrome Responder Index* (SSRI) [768], que combina EVA's de fadiga, secural oral e ocular, com o fluxo salivar não-estimulado e a VS. Uma resposta SSRI-30 foi definida como uma melhoria de 30% em pelo menos 2 dois 5 items. Outro índice combinado é o *Composite of Relevant Endpoints in SS* (CRESS), que foi desenvolvido a partir de um ensaio randomizado com abatacept [769]. O CRESS inclui 5 domínios (ESSDAI, ESSPRI, função glandular salivar e lacrimal, e serologia) e requer resposta em pelo menos 3 destes.

Os resultados dos ensaios TEARS e TRACTISS apoiam a inclusão da ecografia das glândulas salivares num índice de resposta composto [770]. Menores pontuações na EGS inicial associaram-se a uma melhoria dos parâmetros relacionados com a secura, sugerindo que em doentes com elevadas pontuações ecográficas possa já ter ocorrido dano irreversível [771]. Embora seja necessário esclarecer melhor a correlação entre a EGS e a

histologia glandular, devido ao seu fácil acesso a EGS enquadra-se bem no conceito recente de "fenotipagem clínica profunda" [739].

Num estudo com base no registo britânico de SSP, os 4 grupos distintos de doentes definidos de acordo com o conjunto da intensidade da queixas de secura, dor, fadiga, ansiedade e depressão ("baixo impacto dos sintomas", "alto impacto dos sintomas", "predomínio de secura com fadiga" e "predomínio de dor com fadiga") apresentaram diferenças significativas entre si em marcadores laboratoriais e transcriptomas [772].

Face à inconsistência dos instrumentos metrológicos habitualmente utilizados e às novas metodologias descritas, é necessário repensar os instrumentos de medição da resposta primária e considerar a utilização de um conjunto alargado de medidas complementares. Além da fenotipagem clínica, têm sido feitos importantes progressos na fenotipagem imunológica e molecular da SSP (genética, epigenética, transcricional). Por exemplo, um perfil linfocitário caracterizado por diminuição das células T CD4+, das B de memória e das células dendríticas plasmacitóides, e aumento das células T CD4+ e CD8+ ativadas, permitiu estratificar os doentes de acordo com a pontuação do ESSDAI [773]. Noutro estudo, a quantificação de citocinas séricas por um sistema multiplex foi preditora de resposta à terapêutica combinada com hidroxicloroquina e leflunomida [774]. Apesar da sobre-expressão de genes relacionados com o IFN (a "assinatura do IFN") estar presente em cerca de 60% dos doentes com SSP [422], não existe uma relação consistente entre as manifestações clínicas e o perfil transcricional que permita, na atualidade, a utilização de tais "assinaturas" como instrumento de avaliação [775].

2.9. Terapêutica

Na terapêutica da SSP é necessário considerar a abordagem do envolvimento glandular, que inclui as queixas secas e a tumefação glandular, e as manifestações sistémicas, incluindo a fadiga, dor e envolvimento específico de órgão. Foram publicadas recomendações pela *British Society for Rheumatology* e pela EULAR [12,776], e a *Sjögren's Foundation* promoveu a publicação de orientações para a prática clínica elaboradas por peritos [777,778].

Um desafio futuro é a estratificação dos doentes, de acordo com o perfil de atividade sistémica, biomarcadores, queixas secas e e impacto na qualidade de vida, com vista ao desenvolvimento de estratégias terapêuticas personalizadas. Um exemplo, utilizando dados do *United Kingdom SS registry*, demonstrou-se que existiam diferenças nos marcadores laboratoriais e transcriptomas entre 4 grupos distintos de doentes definidos de acordo com a intensidade da queixas de secura, dor, fadiga, ansiedade e depressão [772]: "baixo impacto dos sintomas", "alto impacto dos sintomas", "predomínio de secura com fadiga" e "predomínio de dor com fadiga" [772].

2.9.1. Manifestações glandulares

Em relação às queixas secas é fundamental a eliminação de Fatores de agravamento, como fármacos xerogénicos, irritantes orais e oculares, ambientes secos ou ventosos, entre outras medidas.

A abordagem terapêutica da xeroftalmia segue um esquema por degraus, em que de início se recorre às lágrimas artificiais e lubrificantes, seguida da utilização de fármacos tópicos com ação anti-inflamatória como corticóides e ciclosporina, da oclusão temporária ou definitiva do canal lacrimal, e da aplicação tópica de soro autólogo [779].

A abordagem terapêutica da xerostomia visa aliviar os sintomas e prevenir as cáries, com recurso a pastas fluoradas, anti-sépticos orais, estimulantes salivares de ação local, substitutos salivares e segratogogos como a pilocarpina [778,780]. Infelizmente, as opções terapêuticas para a hipofunção salivar grave são limitadas [781,782].

2.9.2. Manifestações sistémicas

A abordagem terapêutica das manifestações sistémicas requer o uso de terapêuticas imunossupressoras para as quais há vasta experiência em outras doenças reumáticas sistémicas. Assim, é habitual a utilização de metotrexato no tratamento das manifestações articulares, e azatioprina ou o micofenolato mofetil em manifestações específicas de órgão, tendo os corticoesteróides um papel adjuvante [12]. Para manifestações sistémicas com risco de perda de órgão ou de morte, a abordagem não difere da utilizada em manifestações semelhantes noutras doenças autoimunes, com a utilização de pulsos de corticoesteróides, ciclofosfamida, imunoglobulinas endovenosas ou plasmaferese [12].

A hidroxicloroquina é recomendada no envolvimento articular e cutâneo, e pode ter algum efeito na fadiga [776,783]. Um estudo retrospectivo sugeriu um efeito protetor em relação ao dano [784]. Apesar da sua extensa utilização, não foi possível demonstrar a eficácia da hidroxicloroquina na SSP num anterior ensaio clínico randomizado de grande dimensão [785]. É possível que o fracasso deste ensaio se deva à baixa sensibilidade dos instrumentos de avaliação centrados do doente utilizados [786].

Os linfócitos B são desde há muito considerados um alvo lógico na terapêutica da SSP, dado o seu importante papel na patogénese da doença [437]. Pequenos ensaios abertos com Rituximab (anticorpo monoclonal anti-CD20) demonstraram rápida depleção de células B nas glândulas salivares e lacrimais [787,788], sugerindo um eventual papel na terapêutica precoce, reforçada por um possível benefício nas queixas secas e fadiga [789,790]. Contudo, 2 grandes ensaios de fase 3 não cumpriram os objetivos estabelecidos, avaliados através de

instrumentos centrados no doente [791,792]. Não obstante, as recomendações da *British Society for Rheumatology* e da *Sjögren's Syndrome Foundation* admitem o uso *off-label* de Rituximab em doentes com SSP e manifestações graves, como linfoma, trombocitopénia autoimune, neuropatia vasculítica, crioglobulinémia, e manifestações sistémicas refractárias aos agentes imunossupressores convencionais [776,783].

A falência em atingir os objetivos terapêuticos nos ensaios clínicos na SSP pode ser atribuída a vários fatores, como a heterogeneidade da doença, o forte efeito placebo, má sensibilidade dos instrumentos de avaliação, dimensão reduzida da amostra, ou curta duração do ensaio [793]. Esta falência na aprovação de terapêuticas inovadoras estimulou o desenvolvimento de instrumentos metrológicos específicos, abrangentes e sensíveis à mudança, e de biomarcadores para estratificação dos doentes de acordo com a probabilidade de resposta a um determinado agente [794].

Ensaios recentes mostraram resultados promissores recorrendo a novas metodologias de avaliação, nomeadamente com o Iscalimab (anticorpo monoclonal anti-CD40) [795], Ianalumab (anticorpo monoclonal anti-receptor do BAFF) [796,797], RSLV-132 (proteína de fusão RNase-Fc humana) [798], e a combinação de hidroxicloroquina e leflunomida [799].

As terapêuticas atualmente em estudo visam sobretudo as vias imunológicas desreguladas na SSP, como a ativação de células B, a co-estimulação, a sinalização por TLR e a produção de citocinas pró-inflamatórias [800]. Embora a resolução da inflamação nos órgãos afetados seja um importante objetivo da imunoterapia, a recuperação do epitélio das glândulas salivares e lacrimais será também desejável e permanece um desafio. Foi demonstrado que a população de células percursoras epiteliais está reduzida na SSP e apresenta um fenótipo senescente, realçando a necessidade de eliminar estas células senescentes e repor o epitélio saudável, por exemplo através da transferência de células estaminais [801].

Devido à heterogeneidade da SSP, justifica-se uma abordagem médica de precisão, utilizando futuramente características clínicas, imunológicas, genéticas e transcricionais para uma decisão terapêutica personalizada [802].

2.10. Impacto e Prognóstico

A morbilidade na SSP decorre principalmente da deficiente função das glândulas exócrinas, causando diversos sintomas que afetam a vida diária [767].

Estas manifestações, associadas à elevada prevalência de fadiga, dor, alterações do sono e lentificação do pensamento ("brain fog"), contribuem para má qualidade de vida e altos índices de incapacidade para o trabalho [803,804].

Os doentes com SSP apresentaram taxas de incapacidade laboral de 26% aquando do diagnóstico, atingindo 41% 2 anos após o diagnóstico [805].

A mortalidade na SSP, globalmente, não está aumentada em relação à população geral [806]. No entanto, alguns subgrupos de doentes têm de facto aumento da mortalidade, incluindo aqueles com vasculite, crioglobulinémia e linfoma [807–809]. Numa grande coorte de Espanha, o acréscimo de risco de mortalidade na SSP foi de 4.66 (95% CI, 3.85 a 5.60) [571], sendo preditores de maior mortalidade o sexo masculino, a crioglobulinémia e a redução do C4. A presença de vasculite crioglobulinémica no início da doença é um fator independente de maior mortalidade [807]. Elevada atividade sistémica no início da doença também é um fator preditivo de mortalidade, em especial se estiver presente envolvimento pulmonar [571].

CAPÍTULO II. Objetivos

A SSP apresenta uma considerável variabilidade clínica, não existindo nenhuma característica específica que, isoladamente, permita o seu diagnóstico, recorrendo-se a critérios de classificação, como os do ACR/EULAR e os do AECG, para seleção de doentes para fins de investigação.

Na prática clínica, o diagnóstico baseia-se na confirmação objetiva de que um doente com queixas secas apresenta efetivamente redução da capacidade secretória lacrimal ou salivar, e que há um distúrbio imunológico subjacente, sendo este último requisito obrigatório.

São desejáveis novos biomarcadores que traduzam a disfunção imune presente na doença, sendo um dos exemplos promissores a caracterização do perfil linfocitário por citometria de fluxo

O objetivo principal deste projeto consiste na exploração das subpopulações linfocitárias, caraterizadas por citometria de fluxo, como biomarcador auxiliar no diagnóstico e classificação da SSP.

Objetivos gerais

Avaliar se os doentes com SSP apresentam, em relação a controlos saudáveis, doentes com síndrome Sicca não-Sjögren e doentes com Artrite Reumatoide, diferentes perfis de subpopulações de linfócitos B (nomeadamente linfócitos B maduros, B de memória e B reguladores) e de linfócitos T (nomeadamente linfócitos Th1, Th17, T reguladores e T foliculares) no sangue periférico.

Avaliar o impacto da inclusão do estudo das subpopulações linfocitárias na sensibilidade e especificidade dos critérios de classificação ACR/EULAR e AECG.

Objetivos específicos

Determinar o perfil imunológico de doentes com SSP através da caraterização de linfócitos B e T no sangue, por citometria de fluxo e estabelecer valores de cut-off (em comparação com grupos saudáveis) das subpopulações linfocitárias que maximizem a especificidade e sensibilidade para aplicação na prática clínica.

Averiguar da existência de alterações dos linfócitos B em doentes com SSP, nomeadamente de uma razão (Bm2+Bm2')/(eBm5+Bm5) aumentada, de uma diminuição dos linfócitos B CD27+ de memória, e de uma diminuição nos linfócitos B reguladores CD19+ $CD24^{hi}CD38^{hi}$, conforme descrito na literatura.

Averiguar da existência de alterações dos linfócitos T em doentes com SSP, nomeadamente de uma elevação do número de células Th17 CD4+, de um aumento da razão Th17/T reg e de um aumento do número de linfócitos T foliculares, conforme descrito na literatura.

Avaliar as caraterísticas clínicas e distribuição das populações linfocitárias, de acordo com o perfil serológico (Acs anti- SSA) de doentes com SSP.

Avaliar a concordância dos critérios de classificação AECG e ACR/EULAR, a sua comparação com o padrão-ouro (o diagnóstico clínico) e o possível valor adicional da avaliação de subpopulações linfocitárias específicas para estes critérios de classificação.

Objetivos complementares

Avaliar a distribuição das subpopulações linfocitárias consoante o perfil serológico do EBV.

Avaliar aplicação de um novo biomarcador ocular, a morfologia do plexo nervoso corneano sub-basal, e comparar as caraterísticas de doentes com SSP, Sicca, AR e controlos saudáveis

Avaliar a relação entre a morfologia do plexo nervoso corneano sub-basal e caraterísticas fenotípicas da SSP, incluindo atividade da doença e a distribuição das subpopulações linfocitárias.

CAPÍTULO III. Association between memory B-cells and clinical and immunological features of primary Sjögren's syndrome and *Sicca* patients | *Associação entre as células B de memória e as caraterísticas clínicas e imunológicas de doentes com Síndrome de Sjögren e síndrome* Sicca.

Association between memory B-cells and clinical and immunological features of primary Sjögren's syndrome and *Sicca* patients

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Abstract

B-cells play a pivotal role in primary Sjögren's syndrome (pSS) pathogenesis. We aim to (1) evaluate the distribution of B-lymphocyte subpopulations in pSS and *Sicca* patients, (2) establish cut-off points that discriminate pSS from controls, (3) evaluate the association between memory B-cells and phenotypic features in pSS. We included 57 pSS patients, 68 *Sicca* and 24 healthy controls. Circulating B-cells were characterized by flow cytometry as naïve and memory subsets and classified from Bm1 to Bm5. Compared to controls, pSS patients had lower percentages (29.5 vs 44.4%) and absolute numbers (47 vs 106 cells/μl) of memory B-cells. Through ROC curves, a cut-off of ≤ 58 total memory B-cells/μl yielded a specificity of 0.88 and a sensitivity of 0.60 for pSS, and was met by 59.6% of pSS patients, 38.8% of *Sicca* and 12.5% of controls. A cut-off of < 23.5 Switched-memory B-cells/μl yielded a specificity of 0.88 and a sensitivity of 0.54 and was met by 54.4% of pSS patients, 37.3% of *Sicca* and 12.5% of controls. In pSS, lower total memory B-cells count was associated with longer disease duration (14.3 vs 8.1 years, $p = 0.006$) and more active disease profile, as evaluated by the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) (3.1 vs 1.4, $p = 0.043$). Decreased numbers of memory B-cells clearly discriminated pSS from controls and can also have prognostic value. It remains to be clarified whether *Sicca* patients with decreased memory B-cells represent pSS and if B-cell profiling could help in the diagnosis of pSS.

Keywords Sjögren's syndrome · Flow cytometry · Memory B cells · Diagnosis · Autoimmunity

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Introduction

Primary Sjögren's syndrome (pSS) is characterized by lymphocytic infiltration and damage of the exocrine glands, resulting in glandular dysfunction with xerostomia and xerophthalmia [1]. Systemic manifestations include arthritis, vasculitis, lung, neurological and renal involvement [2]. The main elements for pSS diagnosis are the presence of antiSSA antibodies [3] and focal lymphocytic infiltrates in the minor salivary gland (MSG) biopsy [4, 5]. These have been included as mandatory items used in the classification criteria [6–8], from which the American-European Consensus Group (AECG) [6] have been the most used in the clinical practice for the last 15 years. Recently, a new set of criteria was proposed by the ACR/EULAR joint initiative [8], based on a weighted sum of objective items, which could potentially be adapted, if new diagnostic tests arise.

Clinical diagnosis remains difficult, especially in the early stages of the disease [9], since many patients with pSS may not meet the immunological criteria. Although anti-SSA antibodies can be detected before the recognition of clinical disease [10], they are negative in one-third of patients [11]. The histological criterion (focus score ≥ 1) has also variable sensitivity [4] and seems to be associated with the occurrence of anti-SSA, rheumatoid factor (RF) and antinuclear antibodies (ANA) [12]. Therefore, it is of great interest to identify and validate other instruments to support the diagnosis, especially in patients that although not fulfilling the classification criteria, may have the disease, as judged by the experts. Examples of potential new instruments are the study of lymphocytic subpopulations by flow cytometry [13, 14], the identification of new autoantibodies [15] and salivary gland sonography [16].

The immunopathogenesis of pSS is complex and involves the innate immune system and both arms of the adaptive immune system, cellular and humoral [17]. B-cell disturbances are the hallmark of pSS and play a pivotal role in the disease pathogenesis and clinical evolution [18–20]. The hyperactivity of B-lymphocytes in pSS is recognized by hypergammaglobulinemia, cryoglobulinemia, cytokine and antibody production [21], and results in an increased risk of lymphoma [22].

B-lymphocytes leave the bone marrow as transitional B-cell and proceed to the secondary lymphoid organs, where their maturation continues under modulation of T-lymphocytes [23]. B cells in different stages of differentiation may be identified in peripheral blood based on their expression of distinct surface markers patterns. There is a great interest in identifying distinct B-cell subset profiles that may have a potential role for the diagnosis of autoimmune diseases [14]. In pSS, the distribution of peripheral B-cell subpopulations is altered, with an increase of the naive subset and the decrease of circulating memory cells [24–26]. A decreased frequency of memory cells has also been identified in patients with *Sicca* syndrome without criteria for pSS [25].

CD27 is a marker of somatically mutated B cells and memory B-cells. The expression of CD27, IgD and IgM allows the identification of naïve B-cells ($CD27{-}IgM^{+}IgD^{+}$), unswitched

($CD27^+IgM^+$ IgD^+) and switched ($CD27^+IgM-IgD-$) memory B-cells in the peripheral blood [27].

The Bm1–Bm5 classification of the $CD19^+$ B-cell compartment has also been used in the context of autoimmune diseases. According to the expression of CD38 and IgD, it allows the identification of several development stages of B cells which may leave the germinative centres and be detected in circulation [28], classified from Bm1 to Bm5 [29]. This classification partially overlaps with the IgD/ CD27 classification. Bm1 includes transitional and naïve B-cells (IgD^+CD38-), which become Bm2 (IgD^+CD38^+) upon activation and progress to germinal centre founder (IgD^+CD38^{++}). Bm3 centrocytes and Bm4 centrocytes ($IgD-CD38^+$) are seldom identified in circulation and will differentiate inside the germinal centre into either plasma cells, or early and late memory cells (eBm5: $IgD-CD38^+$ and Bm5: $IgD-CD38-$, respectively).

In pSS, specific changes in the distribution of these populations have been described, namely the increase of the Bm2 and Bm2' subsets with a decrease of the eBm5 and Bm5 subpopulations [24]. In recent papers, these changes have been suggested to be useful as diagnostic tools [30, 31].

Our study aims to evaluate the distribution of B-lymphocyte subpopulations in patients with pSS and *Sicca* syndrome through flow cytometry and to establish cut-off points for pSS classification in relation to healthy controls. Moreover, we aim to evaluate the relation between lymphocyte subpopulations and phenotypic features in pSS patients.

Materials and Methods

Population

We have included adult patients followed at the Rheumatology department of Instituto Português de Reumatologia and Hospital Cuf Descobertas, with confirmed or suspected pSS. We have consecutively recruited patients, which were classified as pSS if they fulfilled the AECG classification criteria [6], or as non-Sjögren *Sicca* syndrome (designated as "*Sicca* group") if they did not fulfil pSS criteria. The exclusion criteria from the AECG criteria were applied to all patients, and additional exclusion factors were considered for both groups: IgG4-related disease, history of other corneal diseases and refractive surgery. Recruitment and evaluation have been performed between September 2014 and March 2017. The control group consisted of healthy women without *Sicca* symptoms, selected from the Ophthalmology outpatient clinic.

Disease activity in pSS patients was determined with the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) [32]. pSS patients were stratified according to the disease activity in two subgroups: low disease activity (ESSDAI < 5) and moderate or high disease activity (ESSDAI ≥ 5) [33].

Informed consent has been obtained from all patients and controls.

This study was approved by the Ethics committee of Hospital Cuf Descobertas, Ethics committee of Instituto Português de Reumatologia and NOVA Medical School Ethics (no. 17/2016/CEFCM).

Flow cytometry measurements

A total of 6 ml of whole peripheral blood was collected into EDTA containing tubes. All samples were processed and analyzed within 24 h after collection.
To characterize B-lymphocyte subpopulations, a pre-validated panel of membrane markers was used, with the following monoclonal antibodies: CD19, CD24, CD27, CD38, Anti-IgD and Anti-IgM.

The acquisition of samples was performed using a 4-color BD FACS Calibur™ cytometer (BD Biosciences). Cell Quest Pro™ software (BD Biosciences) was used for both acquisition and analysis of samples.

In each tube, at least 5×10^3 $CD19^+$ events (B-lymphocytes) were acquired.

Each studied B-cell subset was evaluated in percentages and absolute counts. For the CD27/IgD classification (Figure 1), naive, memory, unswitched memory and switched memory B-cells were evaluated (Figure 1). As for the Bm1-5 classification, six other subsets were considered: Bm1, Bm2, Bm2', Bm3 + Bm4, eBm5 and Bm5 B-cells. For absolute counts, a single-platform strategy was used, with BD Trucount tubes™.

Complete gating strategies are described in supplementary data and illustrated in Supplementary Figure 1.

Statistical analysis

An exploratory analysis was carried out for all variables. Quantitative variables were described with mean and standard deviation (SD) or median and inter-quartile range (IQR: 25th percentile–75th percentile), as appropriate. Categorical data were presented as frequencies and percentages. To compare the distribution of the B-cell subsets between pSS, *Sicca* and control groups, Kruskal–Wallis test was applied, and whenever differences between at least two of these groups were identified, multiple comparisons were used to overcome the multiple testing problem. The Receiver Operating Characteristic (ROC) curves were used to establish cut-off points in the B-cells subset levels and to estimate corresponding sensitivity and specificity. Data analysis was performed using R (R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, yea*r* = 2017, http://www.R-project.org).

Results

Patients' characteristics

Fifty-seven patients were included in the pSS group, 68 in the *Sicca* group and 24 in the control group.

pSS mean age was 58.3 years with standard deviation (SD) of 11.9 years, and *Sicca* patients' mean age and SD were 60.5 and 10.7 years, respectively. In the control group, the mean age was 51.1 years with SD of 6.6 years. The average disease duration in pSS patients was 11.8 years with SD of 7.8 years, and in *Sicca* patients was 9.7 and 5.0 years, respectively. The clinical and immunological characteristics of both patient groups are presented in Table 1.

Table 1 pSS and *Sicca* patient's characteristics

	pSS (N=57)	*Sicca* (N=68)
Ocular symptoms, *n* (%)	54 (94.7)	66 (95.6)
Oral symptoms, *n* (%)	55 (96.5	65 (95.6)
Ocular signs, *n* (%)	34/56 (62.5)	35/66 (53.8)
Oral signs, *n* (%)	34/50 (70.8)	42/62 (67.7)
Parotid enlargement, *n* (%)	8 (14.0)	1 (1.5)
Extraglandular disease (ever), *n* (%)	23 (40.4)	24 (35.3)
Joint symptoms, *n* (%)	24 (42.1)	31 (47.7)
Skin involvement, *n* (%)	18 (31.6)	16 (24.6)
Other Extraglandular involvement, *n* (%)	5 (8.8)	0 (0)
Raynaud's phenomenon, *n* (%)	8 (14.0)	17 (26.2)
Focus score ≥1, *n* (%)	43/56 (79.6)	0 (0)
SSA, *n* (%)	38 (66.7)	1 (1.5)
SSB, *n* (%)	18/50 (36.0)	1/61 (1.6)
ANA ≥ 1/320, *n* (%)	45 (78.9)	37 (54.4)
ANA ≥ 1/640, *n* (%)	32 (56.1)	16 (23.9)
Rheumatoid factor, *n* (%)	24/50 (48.0)	18/66 (27.3)
Gammaglobulin ≥ 1.6 g/dl, *n* (%)	14 (24.6)	3/66 (4.5)
Therapy (any), *n* (%)	32 (56.1)	30 (46.2)
Glucocorticoids, *n* (%)	19 (33.3)	18 (26.5)
Hydroxychloroquine, *n* (%)	20 (35.1)	20 (29.4)
Immunossupressants, *n* (%)	10 (17.5)	8 (11.8)

Patient's characteristics are represented as number of occurrences (n) and percentages (%). Whenever there were missing values, percentages reflect the number of occurrences over the number of patients tested for the item. Joint symptoms include arthritis and joint pain of inflammatory origin, but only cases that would score in the articular domain of ESSDAI were considered as extra-glandular disease. Likewise, in some patients skin involvement (which not included xerosis) was not considered as extra-glandular disease if it would not score in the cutaneous domain of ESSDAI

pSS, primary Sjögren's syndrome; SF, salivary flow; SSA/SSB, Sjögren's syndrome A/B antibody; ANA, antinuclear antibody; RF, rheumatoid factor; ESSDAI, European Sjögren's syndrome disease activity index

B-cell subsets according to IgD and CD27 classification

pSS and *Sicca* patients had lower absolute number of lymphocytes in comparison to controls ($p = 0.001$ and $p = 0.053$, respectively), as well as lower B-cell numbers, with statistical significance when comparing pSS with controls ($p = 0.031$). There were no

significant differences between both patient groups regarding total lymphocyte and B-cell numbers (p = 0.490 and p = 0.165, respectively) (Table 2). Regarding naïve B-cells, there were no significant differences between patient groups and controls (Table 2; Supplementary Table 1). Significant differences were found between pSS and controls in absolute counts of all memory populations: total memory (TMem) ($CD19^+CD27^+$), switched memory (SwM) ($CD19^+IgD-CD27^+$) and unswitched memory (UnSwM) ($p < 0.001$ for all) (Table 2). Comparing pSS patients with controls, we found a weak evidence of lower percentages of TMem B-cells (p = 0.078) in patients, and more significant differences in the UnSwM subset (p = 0.043) (Supplementary Table 1). Percentages of memory B-cells in *Sicca* patients were similar to pSS patients (30.8 and 29.5%, respectively), and lower than controls (30.8 and 44.4%, respectively), although the difference was not statistically significant (p = 0.300) (Supplementary Table 1). Absolute memory B-cells numbers in *Sicca* were intermediate between those of pSS (66 and 47 cells/µl, respectively, p = 0.103) and controls (66 and 106 cells/ µl, respectively, p = 0.071) (Figure 1; Table 2), being more significant when considering the memory subsets SwM cells (p = 0.053 when comparing with controls) and UnSwM cells (p = 0.058 when comparing with pSS).

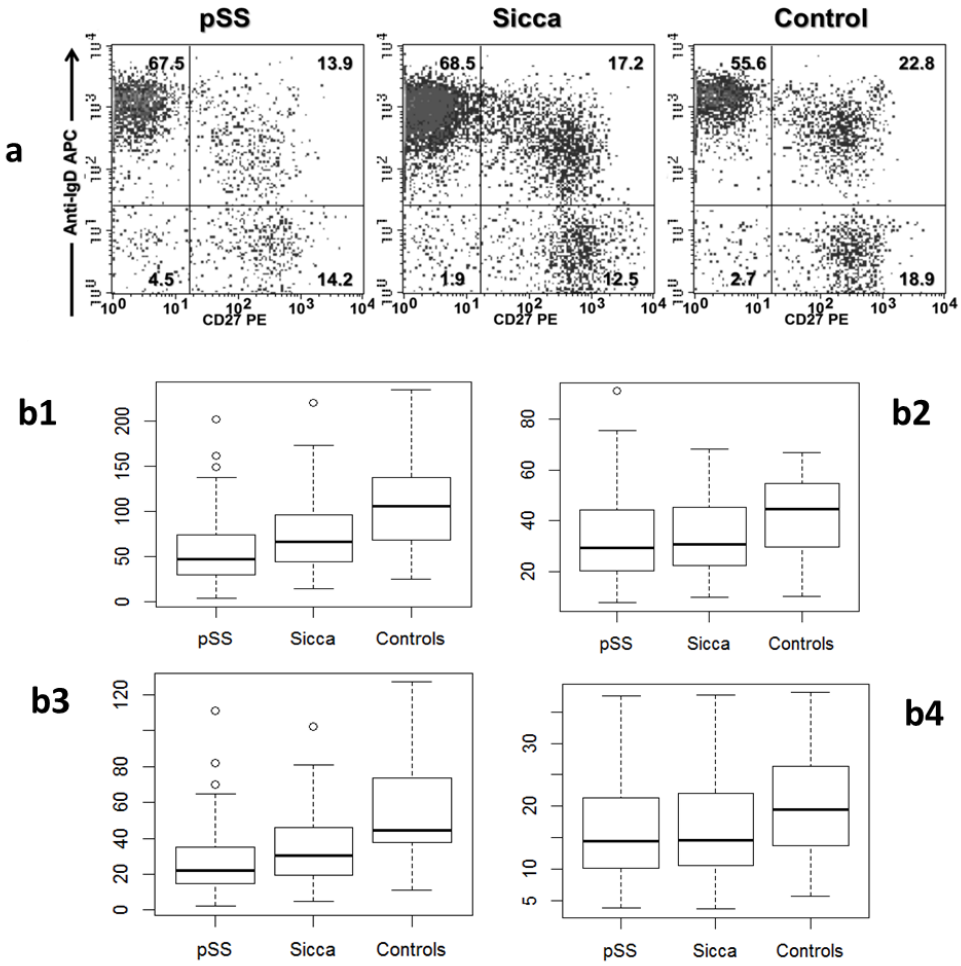

Figure 1 **a** Examples of typical blood memory B-cell subset profiling by flow cytometry in pSS patients, *Sicca* patients and controls. Annotations of the figure: analyses are gated on IgD and CD27. **b** Box-plots with the

distribution of absolute numbers and percentages of total Memory B-cells (b1 and b2, respectively) and of Switched memory B-cells (b3 and b4, respectively). pSS, primary Sjögren's syndrome.

Bm1–Bm5 classification of mature B-cells

In pSS patients, the percentage of Bm1 cells was lower than in controls and *Sicca* ($p = 0.067$ and $p = 0.064$, respectively) (Supplementary Table 1). The difference was even more significant for absolute counts (Table 2) ($p < 0.001$ versus controls, and $p = 0.002$ versus *Sicca*).

Regarding Bm2 and Bm2' cells, no significant differences were found between the three groups, although in the pSS group the Bm2 count was lower than in the other two groups. The percentages of eBm5 and Bm5 cells did not differ between groups, but patients with pSS presented significantly lower numbers compared to controls ($p < 0.001$ for eBm5 and $p = 0.002$ for Bm5). *Sicca* patients presented values between those of pSS and controls, without statistical significance. Even though patients with pSS presented higher Bm2 + Bm2'/eBm5 + Bm5 ratios than controls, the difference was not statistically significant ($p = 0.613$).

Cut-off points for optimal diagnosis and best specificity

pSS patients and controls were compared and ROC curves were used to identify cut-off points with optimal sensitivity and specificity concerning naive and memory populations, as well as Bm1–Bm5 subsets. In most cases, the absolute values produced a better area under the ROC curve (AUC) than percentages (Table 3; Supplementary Table 2).

The best discriminators were the TMem cells count (in which a cut-off equal to 72 cell/µl had a specificity of 0.75 and a sensitivity of 0.74 for pSS) and the SwM cells subset count (in which a cut-off equal to 35.5 cells/µl had a specificity of 0.79 and a sensitivity of 0.75 for pSS) (Table 3). The former cut-off was fulfilled by 75.4% of pSS patients and 20.8% of controls, whereas 61.2% of *Sicca* patients also presented SwM counts lower than the cut-off. Regarding the TMem cut-off of 72 cells/µl, it was fulfilled by 73.7% of pSS patients, 25.0% of controls, and 53.7% of *Sicca* patients.

In the Bm1–Bm5 classification, the item with the best performance was the sum of eBm5 with Bm5 populations, in which, using a cut-off value of 38 cells/µl, a specificity of 0.75 and a sensitivity of 0.72 for pSS was obtained (Table 3). That cut-off was fulfilled by 71.9% of pSS patients, 25.0% of controls, and 55.2% of *Sicca* patients.

The ratio Bm2 + Bm2'/ eBm5 + Bm5 presented an optimal cut-off value of 3.83, however, with a sensitivity of only 0.53 and specificity of 0.75.

To reduce the chance of false positives, we have tested several specificity values between 0.80 and 0.95 and determined that a 0.88 specificity yielded the cut-offs that best distinguished pSS patients from controls, without severely compromising sensitivity. Overall, slightly better results were obtained with absolute values than with percentages (Table 3; Supplementary Table 2). A cut-off of equal to 58 TMem cells/µl was met by 59.6% of pSS patients, 12.5% of controls and 38.8% of *Sicca* patients, and a cut-off of equal to 23.5

SwM cells/µl was met by 54.4% of pSS patients, 12.5% of controls and 37.3% of *Sicca* patients. In the Bm1–Bm5 classification, the eBm5 + Bm5 population was a good discriminator (AUC = 0.77), with a cut-off of 23.33 cells/µl corresponding to 0.88 specificity, and 0.47 sensitivity. Values lower than this cut-off were obtained in 47.4% of pSS patients, 8.3% of controls and 31.3% of *Sicca* patients.

Table 2 Comparison of B-cell subsets absolute counts in pSS, *Sicca* syndrome and healthy controls

B-cell types	pSS	*Sicca* Syndrome	Controls	Groups' comparisons (p-value*)		
	n = 57	*n* = 68	n = 24	pSS vs Controls	*Sicca* Syndrome vs Controls	pSS vs *Sicca* Syndrome
Cells/µL, median (25^{th}-75^{th} percentile)						
Lymphocytes	1615 (1143-2312)	2005 (1509-2297)	2228 (1998-2287)	0.001	0.053	0.490
B cells	177 (109-261)	229 (143-308)	252 (173-354)	0.031	1	0.165
IgD/CD27						
Naive	108 (61-186)	138 (76-208)	137 (89-223)	0.647	1	0.478
Total Memory	47 (29-74)	66 (44-96)	106 (71-134)	<0.001	0.071	0.103
Unswitched	22 (12-37)	35 (21-53)	57 (35-80)	<0.001	0.190	0.058
Switched	22 (15-35)	30 (20-46)	45 (38-71)	<0.001	0.053	0.182
Double-Negative	3 (2-8)	4 (2-9)	4 (2-8)	1	1	1
Bm1-5						
Bm1	18 (10-27)	28 (18-43)	38 (22-61)	<0.001	1	0.002
Bm2	101 (57-162)	138 (76-186)	130 (104-199)	0.245	1	0.389
Bm2'	11 (6-29)	11 (6-19)	12 (7-29)	1	1	1
$Bm3^{+}Bm4$	3 (1-5)	3 (2-4)	3 (2-4)	1	1	1
eBm5	15 (9-23)	18 (11-29)	27 (20-38)	<0.001	0.062	0.267
Bm5	11 (8-19)	16 (10-22)	24 (15-34)	0.002	0.143	0.331
$Bm2^{+}Bm2'/Bm5^{+}eBm5$	3.9 (2.2-6.9)	4.1 (2.1-5.9)	2.9 (1.9-4.1)	0.613	0.726	1

pSS, primary Sjögren's syndrome
*Obtained by Kruskal–Wallis test multiple comparisons

Table 3 Levels of B-cell subsets (absolute values) for optimal and high specificities for the classification of pSS

B-cell subsets or combinations of subsets	AUC (95% CI)	Optimal[a]			Specificity = 0.88	
		Cut-off point	Sensitivity	Specificity	Cut-off point	Sensitivity
IgD/CD27						
Naive	0.61 (0.48, 0.74)	107.00	0.49	0.71	63.00	0.28
Memory Total	0.78 (0.67, 0.89)	72.00	0.74	0.75	58.00	0.60
Unswitched	0.76 (0.65, 0.88)	36.50	0.74	0.75	17.50	0.37
Switched	0.78 (0.67, 0,89)	35.50	0.75	0.79	23.50	0.54
Ratio Naive/Memory T	0.67 (0.55, 0.80)	1.52	0.65	0.71	3.38	0.33
Ratio Naive/Switched M	0.63 (0.50, 0.76)	3.17	0.63	0.62	8.00	0.23
Double-Negative	0.52 (0.39, 0.66)	3.50	0.54	0.50	0.50	0.09
Bm1-Bm5						
Bm1	0.76 (0.63, 0.88)	27.50	0.77	0.71	13.63	0.42
Bm2	0.64 (0.52, 0.77)	104.50	0.53	0.75	77.50	0.39
Bm2'	0.53 (0.40, 0.66)	19.50	0.65	0.46	4.50	0.19
eBm5	0.77 (0.66, 0.89)	24.50	0.79	0.71	14.50	0.49
Bm5	0.74 (0.62, 0.86)	12.99	0.61	0.83	10.00	0.42
$Bm2^{+}Bm2'$	0.63 (0.50, 0.76)	132.50	0.60	0.62	82.50	0.37
$eBm5^{+}Bm5$	0.77 (0.65, 0.88)	38.00	0.72	0.75	23.33	0.47
$Bm2^{+}Bm2'/Bm5^{+}eBm5$	0.62 (0.49, 0.75)	3.83	0.53	0.75	6.77	0.28

AUC, Area Under Curve; CI, Confidence Interval; pSS, primary Sjögren's syndrome a Cut-off points obtained by maximizing both sensitivity and specificity

Comparison with phenotypic features of pSS

Considering the cut-off values for 0.88 specificity established in TMem and SwM B-cell counts, we compared the immunological profile and phenotypic features of pSS patients bellow and above the established cut-off (Table 4).

For both cell populations, patients with cell counts bellow the cut-off were older and had longer disease duration than patients with cell counts above the cut-off, however only the disease duration reached statistical significance (14.3 versus 8.1 years, $p = 0.006$ for TMem, and 13.8 versus 9.4 years, $p = 0.042$ for SwM) (Supplementary Table 3).

Regarding the presence of a focus score ≥ 1 in MSG biopsy, it was more frequent in patients with cell counts below the established cut-off, with an odds ratio of 1.4 for TMem and 1.7 for SwM, although the differences were not statistically significant ($p = 0.618$ and $p = 0.452$, respectively).

Autoantibody occurrence (anti-SSA, SSB, ANA and RF) was higher in the pSS patients' group with TMem ≤ 58 or SwM < 23.5 cell/µl, but not reaching statistical significance.

Patients with positive anti-SSA were more likely to present TMem or SwM counts below the cut-off, compared to SSA-negative patients, with an odds ratio estimate of 2.1 for TMem and 1.5 for SwM. Similarly, a greater odds ratio estimate was determined for anti-SSB positivity (3.5 for TMem and 2.9 for SwM) and for ANA ≥ 1/320 (2.5 for TMem and 1.9 for SwM).

Although the presence of extra-glandular manifestations did not differ between patients below or above the B-cell cut-offs, patients below the cut-off did had significantly higher ESSDAI score than patients above the cut-off (3.1 versus 1.4, $p = 0.043$ for TMem, and 3.3 versus 1.3, $p = 0.022$ for SwM—Supplementary Table 3). Among the 9 patients with at least moderate disease activity (ESSDAI ≥ 5), 8 presented TMem and SwM cell-counts below the cut-off (with $p = 0.082$ and $p = 0.049$, respectively, compared to patients above the cut-off). By univariate analysis, we determined that patients with ESSDAI ≥ 5 were 6.8 and 8.7 times more likely to have TMem ≤ 58 cells/μl and SwM < 23.5 cells/ μl, respectively. Also, patients with gammaglobulin levels above 1.6 g/dl were 3.2 and 2.6 times more likely to have TMem ≤ 58 cells/μl and SwM < 23.5 cells/μl, respectively.

Discussion

B-cell subpopulations defined by the Bm1–Bm5 classification have been previously evaluated as putative diagnostic elements for pSS [30, 31]. To our knowledge, this is the first study that attempts to establish cut-offs for the naïve and memory B-cell subpopulations defined by the IgD/CD27 expression. Therefore, we have assessed the distribution of B-lymphocyte subsets in two groups of patients (pSS and *Sicca*) and in healthy controls, to determine the cut-off values that better discriminate between pSS and healthy individuals using both gating strategies (IgD/CD27 and Bm1–Bm5 classification with IgD/CD38).

In our study, we have found a significant reduction in the absolute numbers and percentage of UnSwM B-cells in pSS patients compared to controls (in line with the lower number of lymphocytes). These results are in line with those obtained in other studies [20, 24–26, 34], and are likely to represent the impairment of B cell tolerance checkpoints. The mobilization of self-reactive naïve B cells from the bone marrow to the periphery is increased in pSS [20], with migration of peripheral memory B-cells to the affected salivary glands [19, 26] where they account for the majority of infiltrating B-cells. Probably there is also a skew of B-cell differentiation towards plasma cells [24, 35]. Age can be associated with a decrease in naïve B-cells and an accumulation of mature B-cells [36]. However, we have found that patients with lower TMem and SwM B-cell counts were older and had longer disease evolution. The impairment in these particular B-cell subsets may be related to either an increased tissue migration pattern, or a failure in the differentiation of such post-germinal centre B cells.

Table 4 Immunological profile and phenotype of pSS patients according to total memory and switched memory cell count cut-off status

	Total Memory B-cells				Switched Memory B-cells			
	≤58 cells/µl (n=34) (%)	>58 cells/µl (n=23)	*p*-value	OR 95% CI	<23.5 cells/µl (n=31)	>23.5 cells/µl (n=26)	*p*-value	OR 95% CI
Focus score ≥1								
Positive	27/33 (81.8)	16/21 (76.2)	0.618	1.40 (0.35, 5.42)	25/30 (83.3)	18/24 (75.0)	0.452	1.67 (0.44, 6.61)
Negative	6/33 (18.2)	5/21 (23.8)			5/30 (16.7)	6/24 (25.0)		
Anti-SSA								
Positive	25/34 (73.5)	13/23 (56.5)	0.185	2.18 (0.70, 6.71)	22/31 (71.0)	16/26 (61.5)	0.453	1.53 (0.50, 4.71)
Negative	9/34 (26.5)	10/23 (43.5)			9/31 (29.0)	10/26 (38.5)		
Anti-SSB								
Positive	14/30 (46.7)	4/20 (20.0)	0.061	3.50 (1.01, 14.49)	13/28 (46.4)	5/22 (22.7)	0.088	2.95 (0.89, 11.02)
Negative	16/30 (53.3)	16/20 (80.0)			15/28 (53.6)	17/22 (77.3)		
ANA ≥ 1/320								
Positive	29/34 (85.3)	16/23 (69.6)	0.160	2.54 (0.70, 9.85)	26/31 (83.9)	19/26 (73.1)	0.324	1.92 (0.53, 7.37)
Negative	5/34 (14.7)	7/23 (30.4)			5/31 (16.1)	7/26 (26.9)		
RF positive								
Positive	17/31 (54.8)	7/19 (36.8)	0.219	2.08 (0.66, 6.97)	14/28 (50.0)	10/22 (45.5)	0.750	1.20 (0.39, 3.73)
Negative	14/31 (45.2)	12/19 (63.2)			14/28 (50.0)	12/22 (54.5)		
Gamma ≥1.6g/dl								
Positive	11/34 (32.4)	3/23 (13.0)	0.107	3.19 (0.85, 5.58)	10/31 (32.3)	4/26 (15.4)	0.148	2.62 (0.75, 10.75)
Negative	23/34 (67.6)	20/23 (87.0)			21/31 (67.7)	22/26 (84.6)		
EGM								
Positive	15/34 (44.1)	8/23 (34.8)	0.482	1.48 (0.50, 4.55)	15/31 (48.4)	8/26 (30.8)	0.180	2.11 (0.72, 6.50)
Negative	19/34 (55.9)	15/23 (65.2)			16/31 (51.6)	18/26 (69.2)		
ESSDAI ≥ 5								
Positive	8/34 (23.5)	1/23 (4.3)	0.082	6.77 (1.12, 130.65)	8/31 (25.8)	1/26 (4.3)	0.049	8.70 (1.44, 167.82)
Negative	26/34 (76.5)	22/23 (95.7)			23/31 (74.2)	25/26 (95.7)		
Objective ocular signs								
Positive	17/33 (51.5)	18/23 (78.3)	0.047	0.30 (0.08, 0.94)	14/30 (46.7)	21/26 (80.8)	0.011	0.21 (0.06, 0.67)
Negative	16/33 (48.5)	5/23 (21.7)			16/30 (53.3)	5/26 (19.2)		
Objective oral signs								
Positive	18/29 (62.1)	16/19 (84.2)	0.109	0.31 (0.06, 1.19)	17/26 (65.4)	17/22 (77.3)	0.369	0.56 (0.15, 1.96)
Negative	11/29 (37.9)	3/19 (15.8)			9/26 (34.6)	5/22 (22.7)		
Dry eye symptoms								
Positive	32/34 (94.1)	22/23 (95.7)	0.800	0.73 (0.03, 0.73)	29/31 (93.5)	25/26 (96.1)	0.664	0.58 (0.03, 6.41)
Negative	2/34 (5.9)	1/23 (4.3)			2/31 (6.5)	1/26 (3.9)		
Dry mouth symptoms								
Positive	32/34 (94.1)	23/23 (100.0)	0.992	-	29/31 (93.5)	26/26 (100.0)	0.992	-
Negative	2/34 (5.9)	0/23 (0.0)			2/31 (6.5)	0/26 (0.0)		

Table 4 (*Legend*) Odds ratios estimates obtained after discretizing Total Memory and Switched Memory in the two categories: above and below the established cut-offs

Each phenotypic feature is represented as positive and negative cases and corresponding percentages. pSS patients were divided in two groups according to the numbers of total memory (TMem) B-cells (≤ 58 and > 58 cells/μl) and phenotypic features in both groups were compared. The same comparison was performed according to the numbers of switched memory (SwM) B-cells (< 23.5 and > 23.5 cells/μl). A TMem cell count cut-off of 58 cells/μl has a specificity and a sensitivity of 0.88 and 0.60 for pSS, respectively. A SwM cell count cut-off of 23.5 cells/μl has a specificity of 0.88 and a sensitivity of 0.54 for pSS, respectively

CI, confidence intervals; SSA/SSB, Sjögren's syndrome A/B antibody; ANA, antinuclear antibody; RF, rheumatoid factor; ESSDAI, European Sjögren's syndrome disease activity index

Although the percentages of SwM and UnSwM cells in each group were equivalent, the pSS group presented significantly lower percentages of UnSwM B-cells compared to the control group. The influence of the evolution time in the distribution of the B cell subpopulations should be approached in future prospective studies.

Cut-off values for two scenarios were obtained, and we have chosen greater specificity over optimal sensitivity and specificity, because our objective was to minimize the chance of false positives, comparing pSS and controls. Better discrimination was obtained with absolute counts rather than percentages. Patients fulfilling the established cut-off for either TMem (≤ 58 cells/μl) or SwM (< 23.5 cells/μl) were more likely to present other phenotypic features of pSS, such as FS ≥ 1, anti-SSA and anti-SSB antibodies, ANA, RF and increased IgG. Although statistical significance was not achieved, probably due to the small size of our population, these results support a strong association between lower memory B-cells and immunologic features of pSS. Whether such B-cell cut-offs could be used in substitution of any of the immunological criteria remains to be clarified. The association of higher disease activity, as determined by the ESSDAI score, with lower total memory and switched memory B-cells may be indicative of the role of these immunological disturbances in disease severity. Adding to its hypothetical diagnostic utility, the assessment of memory B-cells could have prognostic value.

Our results share similarities with those of Roberts et al. [25], which described significantly lower frequencies of SwM and UnSwM B-cells in pSS. However, their work corresponded UnSwM B-cell with phenotypic features of pSS, whereas in our study we have focused in TMem and SwM. Differences in phenotypic associations between SwM and UnSwM B-cell subsets is yet to be clarified.

The distribution of Bm1–Bm5 cells in our study also confirmed the increase in the Bm2 and Bm2' populations, and the decrease in the eBm5 and Bm5, although less marked than described by other investigators [30, 31]. The optimal Bm2 + Bm2'/eBm5 + Bm5 ratio cut-offs of ≥ 3,8 in absolute counts and ≥ 3,2 in percentages that we obtained were lower than the cut-off of ≥ 5 obtained by Binard et al. [30] (considering only percentages), and were associated with lower sensitivity and specificity. However, populations' characteristics may contribute to these differences, namely the older age of our control group (51.1 versus 36.8 years) and the lower occurrence of anti-SSA in our pSS group, which at 66.7% positivity, is lower than the southern Europe prevalence of 71.8% reported in a large data collection [37]. Although the independent association of the

Bm2 + Bm2'/eBm5 + Bm5 ratio with a pSS diagnosis was confirmed in another study, the integration of this test in the AECG criteria did not improve the diagnostic performance [31]. Additionally, due to current therapy, our patients could have less active disease, possibly contributing to a lower Bm2 + Bm2'/eBm5 + Bm5 ratio. In fact, a significant percentage of patients was on low-dose steroid therapy (less than 10 mg/day), and although circulating B-cells are less affected by glucocorticoids than T-cells [38], the long-term influence or the cumulative-dose influence on B-cells has not been properly evaluated. Comparing patients with or without therapy or excluding medicated patients would introduce a bias because no medicated patients would tend to have mild or inactive disease. We must also stress out that we aimed to have a study that could represent the daily clinical basis.

In our study, *Sicca* patients presented lymphocyte populations' counts with values in between those obtained for pSS and controls. Such patients constitute a diagnostic challenge, because many of their clinical characteristics overlap with pSS but they lack the specific immunologic markers for pSS, namely the anti-SSA antibodies and the focal lymphocytic infiltrates in the salivary glands. Considering the IgD/CD27 classification, it is interesting to note that the percentage of naive and memory B-cells in *Sicca* patients was similar to pSS and different from controls, while their absolute numbers were superior to pSS, but notably lower than controls. This intermediate lymphocyte profile probably indicates the presence of disturbed immunological mechanisms in these individuals, which may have milder forms of the disease or be in an earlier stage. In our *Sicca* group, about 40% of patients had either TMem or SwM cell count below the established threshold, and we can therefore admit that some of these individuals may effectively have pSS. Roberts et al. [25] have also evaluated *Sicca* patients and described lower memory B-cells percentages compared to controls, and a subset of *Sicca* patients with a pSS-like transcription profile. In our study, however, more significant differences were obtained in absolute counts of memory B-cells than in their percentages.

We acknowledge some limitations of our study, related to the composition of the patient groups, since B-cell populations' distribution may be influenced by age, duration of disease and past or current therapy. Our pSS and *Sicca* patient's groups were constituted of individuals recruited in a clinical setting, therefore including patients with a broad range of age and disease duration, as well as variable disease severity.

Although we tried to minimize the age difference between healthy controls and patients, their difference was still significant. Major age differences between groups could affect classification cut-offs. Nevertheless, the age difference between our groups did not prove to be relevant for the interpretation of our results.

Regarding the effect of therapies in the B-cell subset distribution, in a clinical setting it could only be ascertained in prospective studies or clinical trials. The study of B-cell subsets in non-medicated patients in a clinical setting like ours would probably have a trend towards the selection of mild cases not needing therapy, which would limit the validity of our results. It would be feasible in a triage setting, although there would probably exist an over-representation of earlydisease patients, whose B-cell populations' distribution may not be similar to those of more evolved patients.

The criteria used for patient inclusion may pose an additional dilemma, since the use of the classification criteria could fail to identify some patients. However, the use of the "gold standard" clinical diagnosis may increase subjectivity and decrease the reproducibility of the results. Nevertheless, the evaluation of the discriminatory role of B-cell subpopulations in the classification of pSS should be ascertained by comparison with the "gold standard" that is the clinical diagnosis. For that purpose, pSS and *Sicca* patients not fulfilling pSS classification criteria should be subjected to an expert-driven diagnostic re-evaluation, to identify true pSS based on clinical opinion, and B-cell subsets must be compared in both "clinical" and "classification"-determined pSS patient groups.

B-cell subpopulations assessment by flow cytometry is a non-invasive procedure that is reproducible and easy to perform.

We have demonstrated that in pSS, the presence of lower memory B-cells counts was associated with a longer disease duration and a more active disease, which could represent a possible role as prognostic markers. Therefore, we aim to continue the present study in the future to include more patients, allowing us to clearly prove that these measurements may be used as clinical biomarkers for the follow-up of these patients. In fact, we have clearly demonstrated the chance to identify a cut-off point in these cell populations that can be used to clearly distinguish healthy controls from patients. Additionally, they might also be useful for the early diagnosis of pSS in *Sicca* patients. Considering the association of lower memory B-cell counts with typical pSS phenotypic features such as hypergammaglobulinemia and antibody occurrence, B-cell subpopulations assessment in *Sicca* patients could potentially identify individuals in higher risk of progression to pSS.

It remains to be clarified if decreased memory B-cells could complement the immunological items currently used for pSS diagnosis. If so, future classification criteria integrating such items could identify patients presently unclassifiable as pSS and allow their access to novel therapies and clinical trials.

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Author contributions FB conceived the original research idea, while all of the authors designed the study and created the study protocol. FB and JVP recruited the patients and collected the data. JC and NA recruited the healthy controls and collected the data. CM, GN and TL analyzed the blood samples using flow cytometry. CG and ALP performed the statistical analysis. JCB and LMB supervised all the work and the research protocol. All of the authors contributed to data analysis and interpretation. FB drafted the manuscript, and all of the authors revised it and contributed to it intellectually. All of the authors have approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics

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References

1. Kassan SS, Moutsopoulos HM (2004) Clinical manifestations and early diagnosis of Sjögren syndrome. *Arch Intern Med* **164**:1275– 1284. https://doi.org/10.1001/archinte.164.12.1275
2. Mavragani CP, Moutsopoulos HM (2014) Sjögren's syndrome. *Annu Rev Pathol Mech Dis* **9**:273–285. https://doi.org/10.1146/annurev-pathol-012513-104728
3. Kyriakidis NC, Kapsogeorgou EK, Tzioufas AG (2014) A comprehensive review of autoantibodies in primary Sjögren's syndrome: clinical phenotypes and regulatory mechanisms. *J Autoimmun* **51**:67–74
4. Guellec D, Cornec D, Jousse-Joulin S et al (2013) Diagnostic value of labial minor salivary gland biopsy for Sjögren's syndrome: a systematic review. *Autoimmun Rev* **12**:416–420
5. Fisher BA, Brown RM, Bowman SJ, Barone F (2015) A review of salivary gland histopathology in primary Sjögren's syndrome with a focus on its potential as a clinical trials biomarker. *Ann Rheum Dis* **74**:1–6. https://doi.org/10.1136/annrheumdis-2015-207499
6. Vitali C, Bombardieri S, Jonsson R et al (2002) Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* **61**:554–558. https://doi.org/10.1136/ard.61.6.554
7. Shiboski SC, Shiboski CH, Criswell LA et al (2012) American College of Rheumatology classification criteria for Sjögren syndrome: a data-driven, expert consensus approach in the *SICCA* cohort. *Arthritis Care Res* **64**:475–487
8. Shiboski CH, Shiboski SC, Seror R et al (2017) 2016 American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjögren's syndrome. *Ann Rheum Dis* **76**:9–16. https://doi.org/10.1136/annrheumdis-2016-210571
9. Brito-Zerón P, Theander E, Baldini C et al (2016) Early of primary Sjögren's syndrome: EULAR-SS task force clinical recommendations. *Expert Rev Clin Immunol* **8409**:1–20. https://doi.org/10.1586/1744666X.2016.1109449
10. Theander E, Jonsson R, Sjöström B et al (2015) Prediction of Sjögren's syndrome years before diagnosis and identification of patients with early onset and severe disease course by autoantibody profiling. *Arthritis Rheumatol* **67**:2427–2436. https://doi.org/10.1002/art.39214
11. Trier NH, Nielsen I, Friis T et al (2016) Comparison of antibody assays for detection of autoantibodies to Ro 52, Ro 60 and La associated with primary Sjögren's syndrome. *J Immunol Methods* **433**:44–50. https://doi.org/10.1016/j.jim.2016.03.001
12. Daniels TE, Cox D, Shiboski CH et al (2011) Associations between salivary gland histopathologic diagnoses and phenotypic features of Sjögren's syndrome among 1726 registry participants. *Arthritis Rheum* **63**:2021–2030. https://doi.org/10.1002/art.30381
13. Sack U, Boldt A, Mallouk *n* et al (2016) Cellular analyses in the monitoring of autoimmune diseases. *Autoimmun Rev* **15**:883–889
14. Carvajal Alegria G, Gazeau P, Hillion S et al (2017) Could lymphocyte profiling be useful to diagnose systemic autoimmune diseases? *Clin Rev Allergy Immunol* **53**:219–236. https://doi.org/10.1007/s12016-017-8608-5
15. Shen L, Suresh L, Lindemann M et al (2012) Novel autoantibodies in Sjogren's syndrome. *Clin Immunol* **145**:251–255. https://doi.org/10.1016/j.clim.2012.09.013
16. Jousse-Joulin S, Milic V, Jonsson MV et al (2016) Is salivary gland ultrasonography a useful tool in Sjögren's syndrome? A systematic review. *Rheumatology* **55**:789–800. https://doi.org/10.1093/rheum antology/kev385
17. Nocturne G, Mariette X (2013) Advances in understanding the pathogenesis of primary Sjögren's syndrome. *Nat Rev Rheumatol* **9**:544–556

18. Hansen A, Lipsky PE, Dörner T (2007) B cells in Sjögren's syndrome: indications for disturbed selection and differentiation in ectopic lymphoid tissue. *Arthritis Res Ther* **9**:218. https://doi.org/10.1186/ar2210
19. Hansen A, Daridon C, Dörner T (2010) What do we know about memory B cells in primary Sjögren's syndrome? *Autoimmun Rev* **9**:600–603
20. Corsiero E, Sutcliffe N, Pitzalis C et al (2014) Accumulation of self-reactive naïve and memory B cell reveals sequential defects in B cell tolerance checkpoints in Sjögren's Syndrome. *PLoS One*. https://doi.org/10.1371/journal.pone.0114575
21. Kroese FGM, Abdulahad WH, Haacke E et al (2014) B-cell hyperactivity in primary Sjögren's syndrome. *Expert Rev Clin Immunol* **10**:483–499. https://doi.org/10.1586/17446 6X.2014.891439
22. Fragkioudaki S, Mavragani CP, Moutsopoulos HM (2016) Predicting the risk for lymphoma development in Sjogren syndrome: an easy tool for clinical use. *Medicine (Baltimore)* **95**:e3766. https://doi.org/10.1097/MD.000000000000376
23. LeBien T, Tedder T (2008) B lymphocytes: how they develop and function. *Blood* **112**:1570–1580. https://doi.org/10.1182/blood
24. Bohnhorst J, Thoen JE, Natvig JB, Thompson KM (2001) Significantly depressed percentage of $CD27^+$ (memory) B cells among peripheral blood B cells in patients with primary Sjogren's syndrome. Scand *J Immunol* **54**:421–427. https://doi.org/10.1046/j.13653083.2001.00989.x
25. Roberts MEP, Kaminski D, Jenks SA et al (2014) Primary Sjögren's syndrome is characterized by distinct phenotypic and transcriptional profiles of IgD^+ unswitched memory B cells. Arthritis *Rheumatol* **66**:2558–2569. https://doi.org/10.1002/art.38734
26. Hansen A, Odendahl M, Reiter K et al (2002) Diminished peripheral blood memory b cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome. *Arthritis Rheum* **46**:2160–2171. https://doi.org/10.1002/art.10445
27. Klein U, Rajewsky K, Küppers R (1998) Human immunoglobulin (Ig)M^+ IgD^+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med* **188**:1679–1689. https://doi.org/10.1084/jem.188.9.1679
28. Bohnhorst JO, Bjorgan MB, Thoen JE et al (2001) Bm1–Bm5 classification of peripheral blood b cells reveals circulating Germinal Center Founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjogren's syndrome. *J Immunol* **167**:3610–3618. https://doi.org/10.4049/jimmunol.167.7.3610
29. Pascual V (1994) Analysis of somatic mutation in five B cell subsets of human tonsil. *J Exp Med* **180**:329–339. https://doi.org/10.1084/jem.180.1.329
30. Binard A, Le Pottier L, Devauchelle-Pensec V et al (2009) Is the blood B-cell subset profile diagnostic for Sjögren's syndrome? *Ann Rheum Dis* **68**:1447–1452. https://doi.org/10.1136/ard.2008.096172
31. Cornec D, Saraux A, Pers J-O et al (2014) Diagnostic accuracy of blood B-cell subset profiling and autoimmunity markers in Sjögren's syndrome. *Arthritis Res Ther* **16**:R15. https://doi.org/10.1186/ar4442
32. Seror R, Ravaud P, Bowman SJ et al (2010) EULAR Sjogren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjogren's syndrome. *Ann Rheum Dis* **69**:1103–1109. https://doi.org/10.1136/ard.2009.110619
33. Seror R, Bootsma H, Saraux A et al (2016) Defining disease activity states and clinically meaningful improvement in primary Sjögren's syndrome with EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient-reported indexes (ESSPRI). *Ann Rheum Dis* **75**:382–389. https://doi.org/10.1136/annrheumdis-2014-206008
34. Szabó K, Papp G, Szántó A et al (2016) A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjögren's syndrome and systemic lupus erythematosus. *Clin Exp Immunol* **183**:76–89. https://doi.org/10.1111/cei.12703
35. Hamza N, Bos N, Kallenberg CGM (2012) B-cell populations and sub-populations in Sjögren's syndrome. *Presse Med* **41**:e475-83. https://doi.org/10.1016/j.lpm.2012.05.021
36. Weiskopf D, Weinberger B, Grubeck-Loebenstein B (2009) The aging of the immune system. *Transpl Int* **22**:1041–1050

37. Brito-Zerón P, Acar-Denizli N, Zeher M et al (2017) Influence of geolocation and ethnicity on the phenotypic expression of primary Sjögren's syndrome at diagnosis in 8310 patients: a cross-sectional study from the Big Data Sjögren Project Consortium. *Ann Rheum Dis* **76**:1042–1050. http://doi.org/10.1136/annrheumdis-2016-209952
38. Slade JDHB (1983) Prednisone-induced alterations of circulating human lymphocyte subsets. *J Lab Clin Med* **101**:479–487. https://doi.org/10.5555/URI:PII:0022214383901580

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Supplementary Material

Detailed Methods

Flow cytometry evaluation

After collection, peripheral blood samples were processed for further analysis by flow cytometry.

To obtain absolute cell counts from a single platform strategy, a lyse-no wash technique was used. For each patient, a volume of 50uL of peripheral blood was incubated in Trucount tubes™ (BD Biosciences, San Jose, CA, USA) with FITC Anti-CD19 and PerCP Cy5.5 Anti-CD45 monoclonal antibodies (Biolegend, San Diego, CA, USA). Cells were then lysed for 10 minutes with FACS Lysing solution (BD Biosciences) and directly acquired in the flow cytometer.

In parallel, the protocol for specific B-cell phenotyping was performed using a pre-validated panel of monoclonal antibodies with a combination of PerCP Cy5.5 Anti-CD19, APC Anti-CD24, PE Anti-CD27, FITC Anti-CD38, APC Anti-IgD and APC Anti-IgM (Biolegend). This lyse-wash technique included a 15 minutes incubation with the monoclonal antibodies, at room temperature in the dark. Afterwards, samples were lysed with BD FACS Lysing solution (BD Biosciences), washed with PBS and then acquired in the flow cytometer.

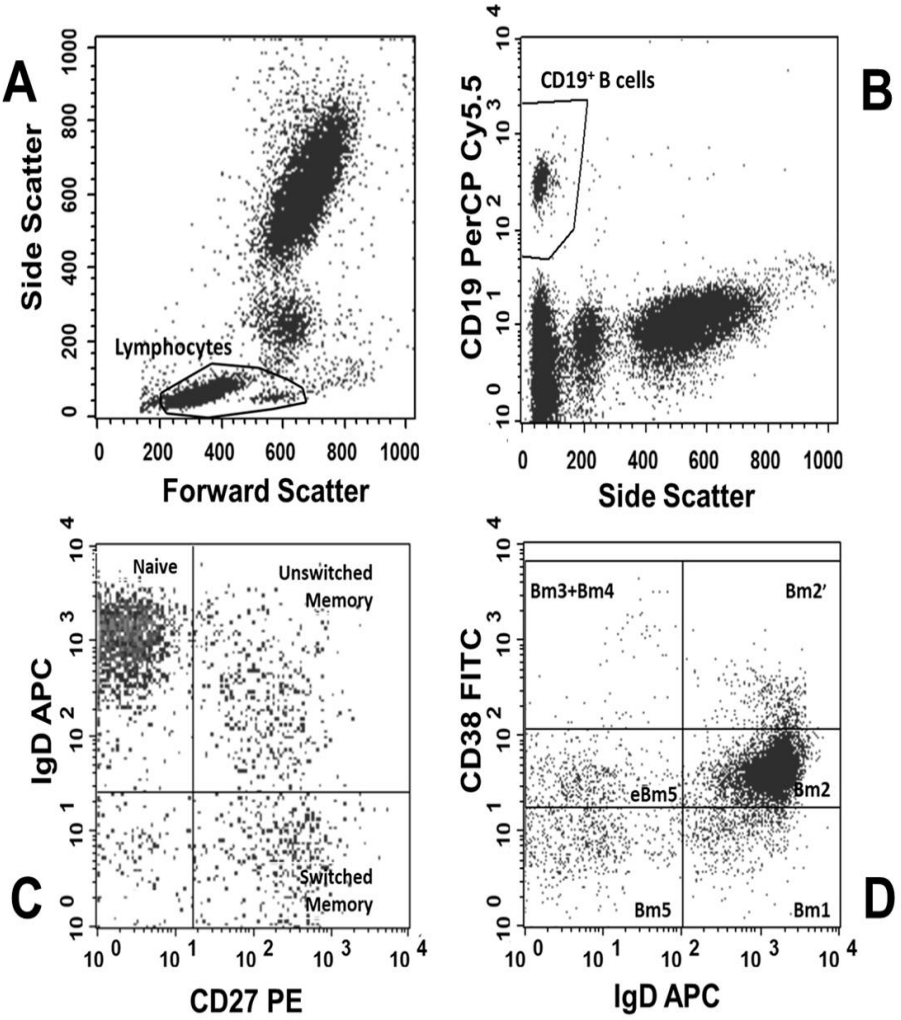
Flow cytometry data analysis

In the single platform evaluation, lymphocytes were gated in a CD45/SSC dot plot ($CD45^{hi}$ SSC^{low}). Then, within lymphocytes, B cells were identified as the $CD19^{+}$ events. Further analysis included the classification of B cells ($CD19^{+}$) according to the expression of IgD and CD27 (Naïve: $IgD^{+}CD27^{-}$, total memory: $CD27^{+}$, unswitched memory: $IgD^{+}CD27^{+}$, and switched memory: $IgD^{-}CD27^{+}$), and the expression of IgD and CD38 (Bm1-5 classification, Bm1: $CD38^{-}IgD^{+}$, Bm2: $CD38^{+}IgD^{+}$, Bm2´: $CD38^{Hi}IgD^{+}$, $Bm3^{+}Bm4$: $CD38^{Hi}IgD^{-}$, eBm5: $CD38^{+}$IgD-, and Bm5: $CD38^{-}IgD^{-}$). Gating strategy is displayed in supplementary Figure 1.

Absolute counts of total lymphocytes were calculated from the number of counting beads present Trucount tubes™ (lot dependent), the number of bead events acquired per tube and the number of lymphocyte events acquired per tube. Absolute counts of B cells were then obtained according to the respective percentage of B cells in total lymphocytes.

B cell subsets percentages were calculated from total B cells (for both the IgD/CD27 and the Bm1-5 classifications), and the respective absolute counts were obtained as well.

Supplementary Figure 1 Gating strategy for the characterization of the circulating B cell compartment



Schematic dot-plots representative of the gating strategy used for identifying circulating B-cells and their respective subsets, according to the IgD/CD27 and Bm1-5 classifications. **A:** Lymphocyte gate was made in the $CD45^{hi}$ SSC^{low} events. **B:** B cells were identified as the $CD19^{+}$ events within the lymphocyte gate. **C:** Identification of Naïve: $IgD^{+}CD27^{-}$, total memory: $CD27^{+}$, unswitched memory: $IgD^{+}CD27^{+}$, and switched memory: $IgD^{-}CD27^{+}$ B cells. **D:** Identification Bm1: $CD38^{-}IgD^{+}$, Bm2: $CD38^{+}IgD^{+}$, Bm2´: $CD38^{Hi}IgD^{+}$, $Bm3^{+}Bm4$: $CD38^{Hi}IgD$, eBm5: $CD38^{+}IgD$-, and Bm5: $CD38^{-}IgD^{-}$.

Supplementary Table 1 Comparison of B-cell subsets (percentages) in pSS, *Sicca* syndrome and Healthy Controls

B-cell types	*pSS*	*Sicca* Syndrome	Healthy Controls	Groups' comparisons (p-value*)		
	n = 57	n = 68	n = 24	pSS *versus* Controls	*Sicca versus* Controls	pSS *versus Sicca*
Cells/µL, median (25^{th}-75^{th} percentile)						
Lymphocytes	31.5 (24.6-37.2)	32.7 (25.7-37.0)	36.7 (29.9-39.7)	0.095	0.299	1
B cells	9.7 (7.0-13.3)	11.7 (8.9-15.0)	10.4 (8.6-13.3)	1	1	0.149
IgD/CD27						
Naive	66.6 (52.0-77.2)	67.3 (50.1-75.3)	53.0 (43.5-65.7)	0.213	0.453	1
Total Memory	29.5 (20.4-44.2)	30.8 (22.6-45.0)	44.4 (32.1-54.6)	0.078	0.300	1
Unswitched	13.6 (9.0-22.0)	15.9 (11.1-24.3)	21.6 (15.9-30.4)	0.043	0.426	1
Switched	14.5 (10.1-21.3)	14.6 (10.6-21.9)	19.5 (13.9-26.4)	0.410	0.453	1
Double-Negative	2.2 (1.6-4.1)	2.0 (1.1-3.2)	2.1 (1.5-2.7)	1	1	1
Bm1-5						
Bm1	9.6 (5.8-15.3)	13.4 (7.5-18.7)	13.9 (10.5-22.2)	0.067	1	0.064
Bm2	60.3 (48.8-67.0)	60.7 (48.0-68.2)	53.6 (48.9-61.2)	1	0.991	1
Bm2'	8.1 (4.2-13.3)	5.2 (3.2-8.2)	5.0 (3.5-7.9)	0.553	1	0.092
$Bm3^{+}Bm4$	1.4 (1.0-3.5)	1.2 (0.9-1.8)	1.2 (1.0-1.8)	1	1	1
eBm5	8.9 (6.3-12.7)	8.5 (6.2-12.6)	11.8 (8.9-15.6)	0.243	0.241	1
Bm5	6.9 (4.5-12.8)	7.8 (4.6-10.7)	8.9 (7.4-11.8)	1	1	1
$Bm2^{+}Bm2'$/ $Bm5^{+}eBm5$	3.8 (2.1-6.9)	4.1 (2.1-6.0)	2.9 (1.9-4.1)	0.718	0.852	1

*Obtained by Kruskal-Wallis test multiple comparisons.
pSS, primary Sjögren's syndrome

Supplementary Table 2 Levels of B-cell subsets (percentages) for optimal and high specificities for the classification of pSS.

B-cell subsets or combinations of subsets	AUC (95% CI)	Optimal*			Specificity = 0.88	
		Cut-off point	Sensitivity	Specificity	Cut-off point	Sensitivity
IgD/CD27						
Naive	0.65 (0.53, 0.78)	58.02	0.63	0.71	75.80	0.30
Memory Total	0.68 (0.55, 0.81)	38.37	0.67	0.71	22.38	0.33
Unswitched	0.68 (0.56, 0.81)	17.71	0.68	0.71	12.46	0.42
Switched	0.62 (0.49, 0.76)	17.77	0.63	0.58	9.54	0.19
Double-Negative	0.57 (0.44, 0.70)	2.41	0.46	0.67	3.70	0.28
Ratio Naive/Memory T	0.67 (0.55, 0.80)	1.52	0.65	0.71	3.38	0.33
Ratio Naive/Switched M	0.63 (0.50, 0.76)	3.17	0.63	0.62	8.00	0.23
Bm1-Bm5						
Bm1	0.69 (0.56, 0.81)	12.58	0.68	0.67	8.96	0.49
Bm2	0.60 (0.46, 0.73)	56.71	0.58	0.62	65.62	0.32
Bm2'	0.62 (0.50, 0.74)	6.38	0.60	0.67	8.68	0.47
eBm5	0.65 (0.52, 0.78)	9.39	0.56	0.71	6.66	0.30
Bm5	0.59 (0.45, 0.72)	7.95	0.60	0.71	4.68	0.28
$Bm2^{+}Bm2'$	0.64 (0.51, 0.77)	68.92	0.54	0.75	75.41	0.37
$eBm5^{+}Bm5$	0.62 (0.49, 0.75)	19.67	0.65	0.62	10.91	0.25
$Bm2^{+}Bm2'/Bm5^{+}eBm5$	0.62 (0.49, 0.74)	3.20	0.60	0.62	6.78	0.26

*Cut-off points obtained by maximizing both sensitivity and specificity.
AUC, Area Under Curve; CI, Confidence Interval; pSS, primary Sjögren's syndrome.

Supplementary Table 3 Comparison of age, disease duration and ESSDAI between patients with Total Memory and Switched Memory absolute values lower and higher than the established cut-offs.

	Total pSS (n=57)	Total Memory				Switched Memory			
		≤58 cells/µl (*n*=34)	>58 cells/µl (*n*=23)	p-value	$\widehat{OR}$ (95% CI)	<23.5 cells/µl (*n*=31)	>23.5 cells/µl (*n*=26)	p-value	$\widehat{OR}$ (95% CI)
Age (years), mean (SD)	58.3 (11.9)	60.7 (9.5)	54.7 (14.2)	0.065	1.00 (1.05, 1.10)	61.0 (9.3)	55.0 (13.9)	0.064	1.05 (1.00, 1.10)
Disease duration (years), mean (SD)	11.8 (7.8)	14.3 (8.2)	8.1 (5.5)	0.006	1.14 (1.05, 1.26)	13.8 (8.4)	9.4 (6.5)	0.042	1.08 (1.01, 1.17)
ESSDAI , mean (SD), [min-máx])	2.4 (2.9) [0-14]	3.1 (3.4) [0-14]	1.4 (1.6) [0-6]	0.043	1.35 (1.05, 1.90)	3.3 (3.5) [0-14]	1.3 (1.5) [0-6]	0.022	1.42 (1.10, 2.00)

Odds ratios estimates obtained after discretizing Total Memory and Switched Memory in the two categories: above and below the established cut-offs.

*Abb*CI, confidence intervals; pSS, primary Sjögren's syndrome; ESSDAI, European Sjögren's syndrome disease activity index

CAPÍTULO IV. Association between EBV serological patterns and lymphocytic profile of SjS patients support a virally triggered autoimmune epithelitis | *A associação entre os padrões serológicos do vírus Epstein-Barr e o perfil linfocitário na Síndrome de Sjögren apoia a influência viral na epitelite autoimmune.*

Association between EBV serological patterns and lymphocytic profile of SjS patients support a virally triggered autoimmune epithelitis

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Abstract

Sjögren's syndrome (SjS) is characterized by lymphocytic infiltration of exocrine glands, i.e. autoimmune epithelitis. Lymphocytes are central in SjS pathogenesis, with B-cell hyperactivity mediated by T-cells. B-cells are main targets of Epstein-Barr virus (EBV) infection, a frequently suggested trigger for SjS. We aimed to evaluate how the EBV infection modulates B and T-cell subsets in SjS, including as controls Rheumatoid arthritis patients (RA) and healthy participants (HC). SjS patients presented decreased $CXCR5^+$ T-cells, although IL21-secreting Tfh and Tfc cells were increased. Tfc were positively correlated with ESSDAI scores, suggesting their relevant role in SjS pathogenesis. As previously described, SjS patients showed expanded circulating naïve B-cell compartments. SjS patients had a higher incidence of EBV-EA-D-IgG^+ antibodies, characteristic of recent EBV-infection/reactivation. SjS patients with past infection or recent infection/reactivation showed increased $CXCR3^+$ Th1 and $CXCR3^+$ Tfh1 cells compared to those without active infection. SjS patients with a recent infection/reactivation profile presented increased transitional B-cells compared to patients with past infection and increased plasmablasts, compared to those without infection. Our results suggest EBV-infection contributes to B and T-cell differentiation towards the effector phenotypes typical of SjS. Local lymphocyte

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activation at ectopic germinal centres, mediated by Tfh and Tfc, can be EBV-driven, perpetuating autoimmune epithelitis, which leads to gland destruction in SjS.

Introduction

Sjögren's syndrome (SjS) is a chronic systemic autoimmune disease, with an estimated prevalence between 0.2–0.5%[1], affecting predominantly middle-aged women. It is characterized by lymphocytic infiltration of the exocrine glands, referred to as autoimmune epithelitis[2]. Lachrymal and salivary glands (SG) are the most affected glands, originating the hallmark features of xerostomia and xerophthalmia. Extraglandular manifestations are common and can be caused by either lymphocytic infiltration of epithelial tissues, or immune complex disease[3].

Lymphocytes are central in the pathogenesis of SjS[4], and a lymphocyte profile with increased naïve B-cells and decrease memory B-cells is typical[5], reflecting the increased migratory pathway of differentiated B-cells into affected organs[6]. A deviation of B-cell differentiation towards plasma cells has also been described in SjS[7].

In SjS, T-cells infiltrate affected organs, like the SG, and support hyperactivity of B-cells[8]. In fact, interactions between T-cells and activated B-cells occur in GC-like structures developed in target tissues, such as the SG[9]. Recently, follicular helper T-cells (Tfh) have been addressed as players in SjS pathogenesis. Tfh cells are a major source of interleukin (IL)-21, which mediates B-cell survival and promotes ectopic formation of germinal-centre (GC)-like structures[10]. SjS patients present increased circulating Tf cells and expanded Tfh differentiation in the SG[11,12]. Tfh cells also express the chemokine receptor X5 (CXCR5), which induces their homing towards lymph nodes, particularly to B-cell sites. Since the expression of CXCR5 has been encountered in $CD8^+$ T-cells, the existence of a follicular cytotoxic ($CD8^+$) T-cell subset (Tfc) is now accepted, as well as their possible role in the regulation of GC B-cell responses and autoantibody production[13,14].

The aetiology of SjS is still poorly understood, but the concept of an infectious trigger is widely spread. Virallytriggered autoimmunity in SjS possibly results from an antigen-driven $CD4^+$ T-cell activation. Combined with a genetic predisposition to loss of tolerance, this activation process elicits a migration of both $CD4^+$ T-cells and B-cells towards exocrine glands, where the expansion and formation of plasma cells occurs[8]. Lymphotrophic viruses, namely Cytomegalovirus (CMV) and Epstein-Barr virus (EBV), are strong candidates for triggering the disease[15]. EBV primary infection occurs in B lymphocytes of the oropharyngeal mucosa, where lytic and latent phases of the viral cycle take place. In the active lytic phase, EBV replicates and propagates, while in the latent phase it remains inactive in B-cells[16]. Viral agents also interfere with T-cell mediated responses[17]. In chronic viral infections, helper T-cells sustain cytotoxic T-cell responses as long as viral antigens persist[18]. Moreover, the proinflammatory Th1 profile, usually present in acute viral infections, is somehow replaced by Tf in response to viral persistence and prolonged T-cell receptor stimulation[19].

The implication of EBV in SjS is widely accepted. Mechanisms such as molecular mimicry and genetic susceptibility to EBV infection can overlap with T-cell costimulatory overactivity, impaired EBV-specific T-cell response, cross-reactivity of anti-EBV antibodies or inhibition of B-cell apoptosis, often associated with stimulation-driven polyclonal and monoclonal lymphoproliferation (recently reviewed by Máslínska[20]). Nonetheless, the impact of viral infection on the typical B-cell profile and hypergammaglobulinemia observed in SjS patients needs further clarification. Thus, we aimed to evaluate circulating B and T-cell subsets of SjS patients and to assess their relation to the EBV background of patients.

Results

Population. Fifty-seven SjS patients were recruited along with 20 Rheumatoid Arthritis (RA) patients and 24 healthy controls (HC). From our cohort, we assessed EBV serology in 34 SjS patients, 20 RA patients, and 20 HC. Participants' characteristics are presented in Supplementary Table 1.

T-cell subsets. SjS patients presented lower $CD4^+$ T-cell percentages and absolute counts than HC ($p = 0.002$ and $p < 0.0001$, respectively). Accordingly, the absolute counts of $CXCR5^+$ Tf cells were also lower in the SjS group compared to both HC ($p < 0.0001$) and RA patients ($p = 0.038$). However, the percentages of IL21-secreting $CD4^+$ T-cells were increased in SjS patients when compared to both RA patients and HC ($p < 0.0001$). Nonetheless, a positive correlation between the percentages of $IL21^+$ $CD4^+$ T-cells and the percentages of $CXCR5^+$ Tfh cells ($r = 0.281$, $p = 0.034$) was observed.

$CD8^+$ T-cells percentages were higher in SjS patients compared to HC ($p = 0.001$), but not absolute counts. Additionally, IL21-secreting $CD8^+$ T-cells' were increased in SjS patients when compared to both HC ($p = 0.029$) and RA patients ($p < 0.001$). $CXCR5^+CD8^+$ T-cells were positively correlated with ESSDAI scores ($p = 0.029$, $r = 0.430$) of SjS patients. Results are summarized in Table 1 and Supplementary Table 2.

B-cell subsets. Considering the IgD/CD27 classification, the percentages of IgD^+CD27- B-cells (naïve) were higher in SjS patients when compared to HC ($p = 0.028$) and RA patients ($p = 0.043$), and SjS patients also presented higher absolute counts of this subset compared to RA ($p = 0.015$). Total memory B-cells ($CD27^+IgD^{+/-}$) and unswitched memory B-cells ($CD27^+IgD^+$) were lower in SjS patients compared to HC ($p = 0.001$). However, only absolute counts of switched memory B-cells ($CD27^+IgD^-$) were lower in SjS compared to HC ($p < 0.001$), and no differences were observed towards RA patients.

Percentages	SjS	RA	HC	p-value
T-cell subsets				
T-cells	74.97 [69.14-78.43]	80.25 [71.59-82.54]	74.71 [70.11-79.57]	0.151
CD4 T-cells	**61.23 [53.09-67.65]**	66.26 [52.80-71.62]	**69.09 [60.83-75.89]**	**0.006**
CXCR5$^+$ Tfh	18.44 [14.69-23.56]	20.00 [15.48-25.47]	20.80 [17.38-22.93]	0.502
Tfh1$^{\#}$	**36.23 [30.18-41.25]**	**28.64 [23.87-38.20]**	31.65 [27.30-35.47]	**0.014**
Tfh17$^{\#}$	21.15 [16.63-26.72]	24.92 [17.08-29.57]	20.88 [17.57-29.11]	0.405
IL-21$^+$	12.41 [8.25-14.92]	8.98 [7.32-11.39]	9.69 [6.32-11.94]	**0.031**
IL-17$^+$	2.20 [1.46-3.17]	2.43 [1.20-3.90]	2.40 [1.72-6.74]	0.750
IL-21$^+$ IL-17$^+$	0.67 [0.50-0.94]	0.71 [0.34-0.94]	0.68 [0.28-1.09]	0.829
CD8 T-cells	**38.40 [31.88-46.92]**	33.74 [28.38-47.21]	**30.90 [23.44-39.17]**	**0.012**
CXCR5$^+$ Tfc	2.53 [1.99-3.60]	1.98 [1.42-3.69]	3.44 [1.98-3.80]	0.456
IL-21$^+$	4.05 [2.31-5.65]	**2.32 [1.15-3.03]**	2.79 [0.97-4.40]	**0.001**
IL-17$^+$	0.89 [0.56-1.40]	1.06 [0.74-1.80]	1.14 [0.75-2.08]	0.217
IL-21$^+$ IL-17$^+$	0.27 [0.14-0.45]	0.18 [0.09-0.59]	0.33 [0.16-0.75]	0.350
B-cell subsets				
B-cells	**9.73 [6.87-13.34]**	**6.38 [4.40-8.60]**	**10.40 [8.57-13.65]**	**< 0.001**
Naïve	66.58 [51.84-77.34]	55.80 [30.28-69.42]	53.01 [43.22-69.21]	**0.030**
Memory	**29.46 [20.23-44.44]**	32.17 [25.38-56.76]	**44.45 [27.37-54.71]**	**0.032**
Unswitched Memory	**13.64 [8.91-22.57]**	15.99 [10.80-27.53]	**21.61 [14.57-32.20]**	**0.029**
Switched Memory	14.50 [10.00-21.85]	17.41 [13.59-30.69]	19.52 [13.72-26.48]	0.107
Double negative	2.21 [1.51-4.10]	**5.59 [2.33-7.74]**	2.07 [1.44-2.93]	**0.006**
Bm1	**9.60 [5.62-15.63]**	14.27 [11.19-21.66]	13.86 [10.12-22.68]	**0.003**
Bm2	**60.32 [48.75-67.10]**	**48.81 [31.48-63.13]**	53.57 [47.42-61.74]	**0.036**
Bm2’	8.08 [3.72-13.44]	3.97 [1.87-9.91]	5.03 [3.46-8.20]	0.053
Bm3+4	1.40 [0.93-3.57]	1.61 [0.82-3.52]	1.19 [0.94-1.97]	0.476
eBm5	8.85 [6.07-12.79]	10.90 [8.28-14.26]	11.76 [8.26-15.69]	0.082
Bm5	**6.87 [4.40-13.07]**	**17.14 [7.42-26.14]**	8.86 [6.81-11.98]	**0.006**

Table 1. Percentages of T and B-cell subsets in all groups. T and B cells subsets’ percentages presented in mean ± standard deviation. Bold numbers highlight the populations that were significantly different. Kruskal-Wallis test was applied for statistical significance. $^{\#}$ Tf1 and Tfh17 are represented as percentages among CXCR5$^+$ Tfh cells. *SjS* Sjögren’s syndrome, *RA* rheumatoid arthritis, *HC* healthy controls.

Using the Bm1-5 classification, B-cells were classified as Bm1, Bm2, Bm2', Bm3+4, eBm5, and Bm5 subsets21. The percentages of Bm1 cells were significantly lower in SjS compared to RA ($p = 0.005$) and HC ($p = 0.008$), and the absolute counts were also significantly lower in SjS ($p = 0.002$) compared to HC. Bm2 (naïve) and Bm2' (transitional) percentages were significantly higher in SjS compared to RA patients ($p = 0.015$ for Bm2 and $p = 0.041$ for Bm2'), and Bm2' absolute counts followed the same trend (SjS vs RA; $p = 0.003$). Lower percentages ($p = 0.037$) and absolute values ($p < 0.001$) of eBm5 cells were found in SjS when compared to HC. As for Bm5 cells, SjS patients presented lower percentages than RA ($p = 0.011$), and lower absolute counts than HC ($p = 0.001$). The results are summarized in Table 1 and Supplementary Table 2.

EBV serological markers. All patients and controls were negative for anti-VCA IgM and anti-EA IgA, except for 1 HC that presented borderline levels for anti-EA IgA. All samples were positive for anti-VCA IgG, except for 2 SjS patients, who showed negative values for these antibodies. Most patients and HC showed positive values for anti-EBNA IgG (76.5% of SjS; 80.0% of RA and 85.0% of HC), and negative values for anti-VCA IgA (79.4% of SjS; 75.0% of RA and 80.0% of HC), without significant differences between groups. Interestingly, for anti-EA IgG, significant differences were observed between SjS patients and HC (32.4% in SjS; 20.0% in RA; 5.0% in HC). The results are presented in Table 2.

Group	**SjS (n=34)**	**RA (n=20)**	**HC (n=20)**	***p* value**
IgG antibodies – quantitative assays (positive / negative)				
EBV CA IgG +	32/2	20/0	20/0	n.s.
EBV EA IgG +	11/23	4/16	1/19	0.022*
EBV EBNA IgG +	26/8	16/4	17/3	n.s.
IgA/IgM antibodies – semiquantitative assays (positive / borderline / negative)				
EBV CA IgA	1/6/27	2/3/15	3/1/16	n.s.
EBV CA IgM	0/0/34	0/0/20	0/0 /20	n.s.
EBV EA IgA	0/0/34	0/0/20	0/1/19	n.s.

Table 2 – EBV serological evaluation in SjS, RA and HC. Results for the different anti-EBV antibodies in the different patient groups (positive/negative for quantitative assays; positive/borderline/negative for semi-quantitative assays). *SjS* Sjögren's syndrome, *RA* rheumatoid arthritis, *HC* healthy controls. *$p < 0.05$, for SjS versus HC (Fisher's exact test); n.s., non-significant.

EBV serological patterns in SjS patients. Recognizing that SjS patients presented an increased prevalence of Anti-EBV EA-D IgG and also an important presence of anti-EBNA IgG, we further divided these patients into 3 subgroups according to the serological EBV profile observed: G1 (n = 18), previous infection (EA-IgG-, EBNA IgG+); G2 (n = 11), recent infection/reactivation (EA IgG+, EBNA IgG+/-), and G3 (n = 5), no serological evidence of

active infection (EA IgG-, EBNA IgG-)[20]. Demographic and clinical data are presented in Table 3.

	Sjögren's syndrome n = 34	Distinct EBV serology Sjögren's subgroups		
		G1 EA IgG- EBNA IgG+ n = 18	**G2 EA IgG+ EBNA IgG+/-** n = 11	**G3 EA IgG– EBNA IgG–** n = 5
Age (years, median, Min-Max)	57.1 (28.6-74.8)	57.1 (28.6-71.4)	49.1 (29.9-74.8)	63.8 (49.2-67.4)
Age of onset (years, median, Min-Max)	43.7 (24.5-68.3)	43.8 (24.5-58.7)	39.3 (25.3-68.3)	50.9 (36.0-61.2)
Age at diagnosis (years, median, Min-Max)	48.9 (26.7-71.7)	49.3 (26.7-62.0)	48.5 (29.7-71.7)	53.8 (41.6-65.2)
Symptom duration (years, median, Min-Max)	11.9 (1.0-29.5)	13.1 (1.3-29.5)	8.1 (1.0-26.1)	12.8 (5.4-17.8)
Ocular symptoms, n (%)	31 (91.2)	17 (94.4)	10 (90.9)	4 (80.0)
Oral symptoms, n (%)	33 (97.1)	18 (100)	11 (100)	4 (80.0)
Ocular signs, n (%)	22 (64.7)	11 (61.1)	8 (72.7)	3 (60.0)
Oral signs, n (%)	23 (67.6)	15 (83.3)	5 (45.5)	3 (60.0)
Parotid enlargement, n (%)	6 (17.6)	5 (28.8)	1 (9.1)	0 (0.0)
Focus Score ≥1, n (%)	23/32 (71.9)	15/17 (88.2)	3/10 (30.0)	5 (100)
Active disease, n (%)	17 (50.0)	9 (50.0)	7 (63.6)	1 (20.0)
ESSDAI (median, Min-Max)	2 (0-14)	2 (0-14)	2 (0-6)	2 (0-4)
ESSDAI ≥5, n (%)	5 (14.7)	4 (22.2)	1 (9.1)	0 (0.0)
Extra-glandular disease (ever), *n* (%)	16 (47.1)	8 (44.4)	5 (45.5)	3 (60.0)
Joint symptoms (ever), *n* (%)	13 (38.2)	5 (28.8)	5 (45.5)	3 (60.0)
Skin involvement (ever), *n* (%)	10 (29.4)	4 (22.2)	5 (45.5)	1 (20.0)
Other Extraglandular involvment	2 (5.9)	2 (11.1)	0 (0.0)	0 (0.0)
Raynaud's phenomenon	5 (14.7)	3 (16.7)	2 (18.2)	0 (0.0)
SSA (%)	27 (79.4)	13 (72.2)	10 (90.9)	4 (80.0)
SSB (%)	13/30 (43.3)	7/17 (41.2)	3/8 (37.5)	3 (60.0)
ANA ≥ 1/320, n (%)	28 (82.4)	15 (83.3)	8 (72.7)	5 (100)
ANA ≥ 1/640, n (%)	21 (61.8)	12 (66.7)	6 (54.5)	3 (60.0)
Rheumatoid Factor, *n* (%)	16/29 (55.2)	9/16 (56.3)	4/9 (44.4)	3/4 (75.0)
Gammaglobulin ≥ 1,6 g/dl, n (%)	11 (32.4)	5 (28.8)	5 (45.5)	1 (20.0)
Therapy (any), n (%)	21 (61.8)	13 (72.2)	5 (45.5)	3 (60.0)
Glucocorticoids, n (%)	12 (35,3)	6 (33.3)	3 (27.3)	3 (60.0)
Hydroxychloroquine, n (%)	12 (35,3)	8 (44.4)	2 (18.2)	2 (40.0)
Imunossupressants, n (%)	6 (17,6)	5 (28.8)	0 (0.0)	1 (20.0)

Table 3. Characterization of SjS patients and of SjS subgroups with distinct EBV serology. Patient's characteristics are represented as number of occurrences (n) and percentages (%). Whenever there were missing values, percentages reflect the number of occurrences over the number of patients tested for the item. Ocular evaluation included Schirmer's test and corneal staining score. The oral signs item consisted of a

(Cont.) decreased unstimulated salivary flow. Focus score was defined as the number of lymphocyte aggregates (≥50 cells) per 4 mm^2 of glandular area of the biopsy sample. Joint symptoms include arthritis and joint pain of inflammatory origin, but only cases that would score in the articular domain of ESSDAI were considered as extra-glandular disease. Likewise, in some patients skin involvement (which not included xerosis) was not considered as extra-glandular disease if it would not score in the cutaneous domain of ESSDAI. Clinically active disease was defined as activity in any ESSDAI domain, except the hematologic and biologic. *SjS* primary Sjögren's syndrome, *F* female, *M* male, *y* years, *SSA/SSB* Sjögren's syndrome A/B antibody, *ANA* antinuclear antibody, *RF* rheumatoid factor, *ESSDAI*, EULAR Sjögren's syndrome disease activity index.

SjS patients with recent infection/reactivation markers (G2) had earlier disease manifestations and shorter disease duration.

Half of the patients from group G1 and 2/3 of those in group G2 had active disease at the time of recruitment, with G1 patients showing higher ESSDAI scores than G2 patients. The lowest ESSDAI scores were observed in G3 patients. Skin involvement was more frequent in G2 patients. Parotid enlargement and Raynaud's phenomenon were not documented in G3 patients. A higher proportion of G2 patients presented increased gammaglobulin, with higher mean IgG compared to G1 and G3 (1.52 g/dl vs 1.42 g/dl vs 1.29 g/dl, respectively). None of the abovementioned differences reached statistical significance, though.

Regarding T-cells, G1 and G2 patients presented increased $CXCR3^+$ $CD4^+$ T cells (Th1) cells and $CXCR3^+$ $CXCR5^+$ $CD4^+$ T cells (Tfh1) compared to G3 (Th1: G1vsG3, $p = 0.121$, and G2vsG3, $p = 0.009$; Tfh1: G1vsG3, $p = 0.003$, and G2vsG3, $p = 0.066$).

As for B-cells, transitional Bm2' cells were augmented in G2 patients compared to G1 ($p = 0.024$). Moreover, plasmablasts (Bm3 + Bm4) were increased in G1 and G2 patients compared to G3 (%, G1vsG3, $p = 0.088$ and G2vsG3, $p = 0.003$; absolute counts, G1vsG3, $p = 0.020$ and G2vsG3, $p = 0.003$).

These data are presented in Table 4 and Supplementary Table 3.

Discussion

Our study aimed to explore the relation between the EBV serological profile of SjS patients and the distribution of circulating B and T-lymphocyte subsets. First, we report interesting differences in follicular T-cell subsets between SjS patients and both HC and RA patients. Despite circulating $CXCR5^+$ T cell subsets were decreased in SjS patients, functionally IL21-secreting $CD4^+$ (Tfh) and $CD8^+$ (Tfc) T cells seem to be more pronounced in these patients. IL21-secreting $CD8^+$ T cells (Tfc) were even positively correlated with ESSDAI scores, suggesting their relevant role in SjS pathogenesis. Moreover, we confirmed the enriched circulating naïve B-cell compartment of SjS patients (compared to both control groups, healthy and autoimmune), previously reported in the literature[5].

The major observation of our study, however, comes from the EBV profile, with SjS patients presenting a greater incidence of EBV-EA-D-IgG positivity, a profile characteristic of recent infection/reactivation of EBV infection. Furthermore, SjS patients with either

serological evidence of past EBV infection or recent infection/reactivation presented higher values of CXCR3$^+$ CD4$^+$ T cells (Th1) and CXCR3$^+$ CXCR5$^+$ CD4$^+$ T cells (Tfh1) compared to those without serological evidence of active infection. Also, the B-cell compartment was distinctive in SjS patients with signs of recent EBV infection/reactivation: showing higher levels of transitional Bm2' cells compared to patients with past infection and increased plasmablasts, compared to patients without serological evidence of infection.

Percentages	**G1** **EA IgG$^-$ EBNA IgG$^+$** **(n=18)**	**G2** **EA IgG$^+$ EBNA IgG$^{+/-}$** **(n=11)**	**G3** **EA IgG$^-$ EBNA IgG$^-$** **(n=5)**	***p*-value**
T-cell subsets				
T-cells	77.0 [68.7-83.9]	75.6 [71.9-78.4]	74.8 [63.2-76.2]	0.395
CD4 T-cells	59.9 [50.2-63.6]	62.8 [53.0-67.3]	59.7 [47.4-66.7]	0.727
CXCR5$^+$ Tfh	17.1 [13.8-24.1]	19.7 [14.2-23.7]	17.4 [13.6-27.7]	0.851
Tfh1$^{\#}$	37.3 [34.1-41.3]	37.4 [31.3-43.1]	28.2 [19.9-32.7]	**0.025***
Tfh17$^{\#}$	21.6 [16.1-27.8]	18.7 [13.9-26.2]	23.3 [20.2-37.5]	0.168
IL-21$^+$	12.4 [8.1-14.5]	13.27 [9.4-15.0]	13.4 [6.9-24.7]	0.857
IL-17$^+$	2.42 [1.65-3.38]	2.76 [1.63-3.21]	2.20 [1.52-3.62]	0.882
IL-21$^+$ IL-17$^+$	0.68 [0.53-1.20]	0.73 [0.64-0.99]	0.58 [0.47-1.12]	0.666
CD8 T-cells	40.1 [36.4-49.8]	37.2 [32.7-47.0]	40.4 [33.4-52.7]	0.716
CXCR5$^+$ Tfc	2.40 [2.25-3.38]	2.80 [2.10-3.60]	1.90 [1.50-2.40]	0.152
IL-21$^+$	3.94 [2.52-5.30]	4.42 [2.53-8.47]	3.59 [2.04-33.68]	0.698
IL-17$^+$	1.04 [0.57-1.38]	1.21 [0.90-1.61]	0.64 [0.42-2.13]	0.467
IL-21$^+$ IL-17$^+$	0.28 [0.14-0.50]	0.37 [0.23-0.48]	0.34 [0.15-1.14]	0.833
B cell subsets				
B cells	9.8 [6.6-11.3]	11.0 [8.3-18.4]	7.7 [5.3-9.5]	0.130
Naïve	66.7 [48.7-74.4]	71.9 [49.4-77.5]	66.6 [50.4-77.1]	0.751
Memory	31.4 [23.3-49.0]	26.5 [19.6-49.7]	29.3 [22.4-47.1]	0.589
Unswitched Memory	15.0 [13.0-24.8]	12.0 [7.9-20.0]	15.0 [6.1-28.5]	0.145
Switched Memory	15.3 [10.0-26.8]	14.2 [10.3-22.5]	16.3 [10.9-18.4]	0.978
Double negative	2.11 [1.52-3.06]	2.30 [1.59-3.32]	4.07 [1.04-6.91]	0.803
Bm1	10.0 [7.2-15.7]	5.5 [4.12-10.0]	12.5 [4.5-24.9]	0.203
Bm2	60.5 [45.5-67.1]	60.0 [47.4-64.0]	51.5 [47.7-64.2]	0.831
Bm2'	6.9 [2.3-11.9]	13.6 [6.4-17.4]	5.9 [3.8-18.6]	**0.087***
Bm3+4	2.21 [1.00-4.24]	2.83 [1.31-4.48]	1.10 [0.74-1.26]	**0.043***
eBm5	9.7 [6.0-13.9]	9.2 [7.6-12.8]	11.5 [7.0-12.1]	0.997
Bm5	6.45 [4.67-14.81]	6.24 [4.05-11.45]	8.11 [6.07-14.83]	0.647

Table 4. Immune profile of SjS patients with distinct EBV serology patterns (percentages). Percentage values for all T and B cells subsets in median [minimum–maximum] in SjS patients evaluated for EBV serology. *SjS* Sjögren's syndrome, *EBV* Epstein-Barr virus. $^{\#}$Tfh1 and Tfh17 are represented as percentages among CXCR5$^+$ Tfh cells. *Bold numbers highlight the populations that were significantly different. Kruskal-Wallis test was applied for statistical significance.

The factors underlying the onset and development of SjS are still uncertain. Nevertheless, typical immune profiles have been characterized in these patients, which can

be relevant to unveil important links to other triggering players in this autoimmune disease. Despite B-cells are the main target for EBV latent infection, T-cells have also a role and have been studied in autoimmune diseases for which EBV is considered a potential trigger. For instance, EBV-specific $CD8^+$ T-cells are increased during B-cell transformation and in the productive viral replication phases of EBV in infected RA[22] and SLE patients[23]. Additionally, the EBV-specific $CD8^+$ T-cell pool is reduced by immunosuppressive therapy[24].

Our study supports the presence of a promoted follicular T-cell environment in SjS patients, traduced by the increased secretion of IL21 by both $CD4^+$ and $CD8^+$ T-cells. We found no differences in the percentages of circulating $CXCR5^+$ follicular T-cells between groups, and absolute counts for this subset were even decreased in SjS patients, possibly due to the decreased absolute counts of $CD4^+$ T-cells observed in these patients. Similar data had been described in the work of Brokstad[25], which reported no differences for total $CXCR5^+$ CD4 T-cell percentages, but only changes in particular subsets of these cells in SjS patients, such as the increase in Tfh-like $ICOS^+PD\text{-}1^+$ cells. Interestingly, in our study, another Tfh-like subset was increased in SjS patients, the $IL21^+$ Tfh cells. Indeed, both $CD4^+$ and $CD8^+$ T-cells were more prone to produce IL21, the Tfh modulating cytokine. If the lower absolute numbers may indicate retention of $CXCR5^+$ follicular T-cells at the exocrine glands, as supported by previous studies showing a T-cell predominance in lymphocytic infiltrates of these organs[26], SjS patients seem to be predisposed to promote Tfh differentiation. Also, when naïve T-cells and salivary gland epithelial cells are co-cultured, Tfh differentiation is observed, i.e. T-cells acquire a classical Tfh phenotype and are able to secrete IL21[27]. Thus, this systemic follicular function may be overexpressed in SjS patients, also as an effect of the local altered interplay. We have previously reported that the ESSDAI score, which is a measure of disease activity in SjS, seemed to be correlated with $IL21^+$ $CD8^+$ T cells (Tfc) levels[28]. In fact, patients with more active disease present increased circulating Tfc cells, though the causative link between these observations is still to be clarified (i.e. whether higher levels of Tfc cells lead to increased disease severity or, on the contrary, happen in response to disease aggravation).

In addition, the increase in IL21-expressing T-cells resembles the profile of a chronic active viral infection, as proposed by Fahey and collaborators, who showed that viral persistence redirects T-cell differentiation towards the Tfh profile in animal models[19]. Moreover, patients with infectious mononucleosis show an increase in a particular subset of Tfh cells in peripheral blood[29], which supports our hypothesis that viral triggers may take part in the modulation of the immune responses also in SjS patients[29].

Interestingly, Fahey and colleagues[19] proposed that viral-induced Tfh cells deviate from an original Th1 profile. In line with this, we observed that SjS patients with serological evidence for recent infection/reactivation presented increased Th1 and Tfh1 subsets. Thus, the autoimmune background of SjS patients could provide T-cells with alternate activation signals leading them to assume both Th1 and Tfh1 profiles under viral persistence, since it is accepted that the pathogenesis of SjS is mediated by T1-derived responses[27].

Strikingly, the implication of Tfc cells in SjS pathogenesis is supported by their increase in patients with higher disease activity. In line with our results, serum levels of IL21 had already been associated with systemic disease activity in SjS[30], but our results seem to highlight a role for CD8 T-cells in this scenario. Initially, CXCR5$^+$ CD8 T-cells were described as early effector memory CD8 T-cells present in B-cell follicles of human tonsils[13]. Recently these cells were implicated in the control of chronic viral infections[29,31,32]. Also, associations between humoral responses and CXCR5$^+$ CD8 T-cells[32,33] were identified, as these cells express co-stimulatory molecules. In fact, increased immunoglobulin production by B-cells occurs when they are co-cultured with CXCR5$^+$ CD8 T-cells, suggesting these cells have other immune functions besides cytotoxic activities[33]. Considering that dysregulated humoral responses are present in SjS, Tfc cells, along with Tfh cells, may induce the atypical antibody production of SjS patients. Nevertheless, the major function of the follicular CD8$^+$ T-cells may still be limiting the replication of viral agents in B-cell follicles, as these cells show increased cytotoxic capacities[34].

As for B-cells, it is accepted they are EBV's main target[35]. Several studies tried to relate SjS pathogenesis with a specific clonality of B-cells. One of the hallmarks of Sjögren's syndrome is, in fact, the formation of ectopic lymphoid structures (ELS) in the SG. ELS are composed of B-cell/T-cell follicles, supported by networks of stromal follicular dendritic cells, which support ectopic GC reactions[36]. Active EBV infection has been associated with ELS in the SG of SjS patients and appears to contribute to local growth and differentiation of disease specific autoreactive B-cells[37]. Despite the possibility of an EBV-triggered B-cell proliferation in SjS, EBV-infected memory B-cells were found to express lower levels of self- and poly-reactive antibodies than their uninfected counterparts[38].

As corroborated by our data, SjS patients present a typical circulating B-cell compartment, enriched in transitional/naïve subsets, in opposition to memory subsets. If we consider the observations from Coleman and colleagues on the effect of EBV in B-cells[39], we may also suggest a possible role for EBV in the alterations observed in the B-cell compartment of SjS patients. In fact, these authors have recognized that murine transitional B-cells from the spleen can be reservoirs for gammaherpesvirus like EBV, which can remain latent in these cells, prolonging their life span indefinitely. Our results are in line with this hypothesis, as SjS patients with serological evidence of recent infection/reinfection presented higher percentages of transitional Bm2'. Whether this is an effect of EBV or other concomitant viral infection, remains to be elucidated. However, the viral input for this feature of SjS patients can be also supported by our observation that transitional B cells were particularly increased in patients with recent infection/reactivation. We acknowledge that the assessment of the viral genome in different B-cell subsets could clarify this idea.

Regarding the serological EBV markers, we found an increased prevalence of Anti-EBV EA-D IgG in SjS patients, compared to both RA and HC, as described in previous works[37,40]. The anti-EBV EA-D IgG prevalence in our SjS patients (about 33% vs 5% in HC) was very close to the one reported by Pasoto and colleagues[41] (36% vs 4.5% in HC), which

strengths our data, and led us to further assess the immune compartments according to the EBV serological profile of SjS patients. Interestingly, patients with evidence of EBV infection, and particularly those with recent infection/reactivation (EA-D IgG positive) had earlier disease manifestations, but also a distinct immune profile, with a shift towards pro-inflammatory Th1/Tfh1 subsets in the T-cell compartment. Furthermore, SjS patients with evidence of recent infection/reactivation exhibited higher levels of transitional B-cells and plasmablasts, which may traduce the importance of EBV in the modulation of the immune responses in SjS patients, with possible clinical impact, as suggested by an earlier onset of clinical manifestations. The effect of cytotoxic T-cell (CTL) responses, with both $CD8^+$ and $CD4^+$ T cells, or even other unconventional T cell subsets, may restrict the expansion of latently infected B-cells in long-term carriers or patients with past infection[42,43]. This later immune balance may be the cause for the differences observed in transitional B-cells between SjS patients with recent infection/reinfection and patients with previous infection (G1). Usually increased in SjS, these cells represent a potential EBV reservoir, and are markedly increased in G2 patients, showing however a relapse to lower values in past infections as an effect of an effective immune control happening in these patients (G1).

To better comprehend how EBV profiles modulate immune populations, or whether the changes are SjS-driven, a comparison between SjS patients and HC with similar EBV serological patterns would be very helpful. However, considering the reduced number of HC that could be included in the EBV subgroups G1 (3 HC) and G2 (1 HC), such analysis was not possible. In the future, we aim to better address this question, extending the study to a larger cohort, with more SjS patients, but also healthy controls.

Clinical differences between groups were also difficult to assess due to the small size of our patients' groups. The ESSDAI score in both groups of patients with positive EBV serology (G1 and G2) was higher than in EBV-negative patients (G3). G1 patients had a non-significantly higher ESSDAI compared to G2 patients, in line with a recent report by Sanosian[44], who didn't find distinct ESSDAI scores in anti-EBV EA-positive compared to anti-EBV EA-negative patients. No differences were found in specific clinical ESSDAI domains between G1 and G2 groups, contrasting to the report of Pasoto[41], who found higher articular activity in anti-EBV EA-positive patients. Nevertheless, the greater frequency of active disease in our G2 patients may suggest an influence of recent EBV infection/reactivation in disease activity status, which may be further supported by the higher levels of IgG observed in this group, despite no statistical significance was achieved. Furthermore, our observations on the B-cell compartment are in line with this, as the increased transitional Bm2' and plasmablasts observed in the EBV infection/reactivation group also suggests a higher B-production and differentiation on these patients, possibly contributing in parallel for the increased gammaglobulin levels and greater disease activity.

Interestingly, in our study, IgA and IgM EBV-antibodies behaved similarly in SjS, RA and HC, with a predominance of negative samples for these biomarkers in all groups. As for VCA IgM, an acute-phase marker, despite it may be present in different viral scenarios, no differences were expected in SjS according to previous reports[45].

In other systemic autoimmune diseases, particularly SLE, increased levels of IgA antibodies against the two lytic antigens studied (VCA and EA) have been reported[45,46]. Literature is scarce for this evaluation in SjS patients, nonetheless, both VCA-IgA and EA-IgA seem to be less present in SjS that in other autoimmune conditions such as SLE. Along with a few previous studies[45,46], our data suggest a lower mucosal immune response against EBV in SjS, compared to other autoimmune conditions, with anti-EBV IgA antibodies prevalence similar to HC. Whether this corresponds to a better or worst control of latent infections in SjS remains to be elucidated.

To our knowledge, our study is the first to report an association between EBV serological patterns and the immune profile of SjS patients, despite EBV EA (early antigen) had already been correlated with autoantibodies production[40]. Although we observed no further differences in the clinical manifestations of SjS patients according to their EBV serology, we were able to identify distinct immune profiles according to the EBV serological pattern. Still, we must recognize the low number of patients considered and the absence of other confirmatory methodologies for the effective viral infection. Nonetheless, our data support the idea that different EBV serological profiles affect circulating B and T-cells in SjS patients. In fact, a more active serological background, as the ones observed in groups G1 and G2, may suggest a viral influence in the immune system driving it to the more pro-inflammatory scenario observed in SjS patients when compared to both other autoimmune conditions or healthy controls.

Other authors have supported the hypothesis that reactivation in the lytic phase of EBV infection promotes immunological dysfunction in SjS[37]. Considering our results, we also believe special attention should be given to the group of SjS patients with serological evidence for recent infection/reactivation, which present a proinflammatory profile, with increased Th1/Tfh1 ratio cells along with elevated transitional B-cells and increased plasmablast differentiation.

We acknowledge limitations in our study, such as the absence of standard molecular biology assays to confirm EBV infection. In future studies, it would be relevant to assess not only serology, but also EBV viral load, and eventually other viruses with potential impact in SjS development, such as CMV. Also, our study was performed exclusively in peripheral blood, and we realize it may not properly reflect the numbers and interactions of immune cells at exocrine glands. For instance, SG biopsies would not only clarify the hypotheses on cell traffic between affected organs and the circulating lymphocyte pool but would also allow us to prove the presence of EBV in such organs.

Nevertheless, from our results, it is possible to suggest that EBV plays a role in inducing B and T-cells towards an effector phenotype. EBV enters the replicating phase in the exocrine glands, where this facilitated interaction between EBV antigens and effector T-cells might lead to a breakdown of tolerance. The ensuing autoimmune response mediated by effector B and T-cells might lead to a localized lymphocyte activation with the formation of ectopic GC or GC-like structures. This process, mediated by Tfh and Tfc, can thus perpetuate the autoimmune epithelitis and result in gland destruction.

Our work provides a new perspective on how EBV might be involved in lymphocytic alterations known to be a feature in SjS. Clarifying the role of follicular CD4 and CD8 T-cells in the context of viral infection can be of great value in confirming a viral-triggered autoimmune response in SjS, but a specific strategy for the characterization of these cells in peripheral blood and target organs is still needed.

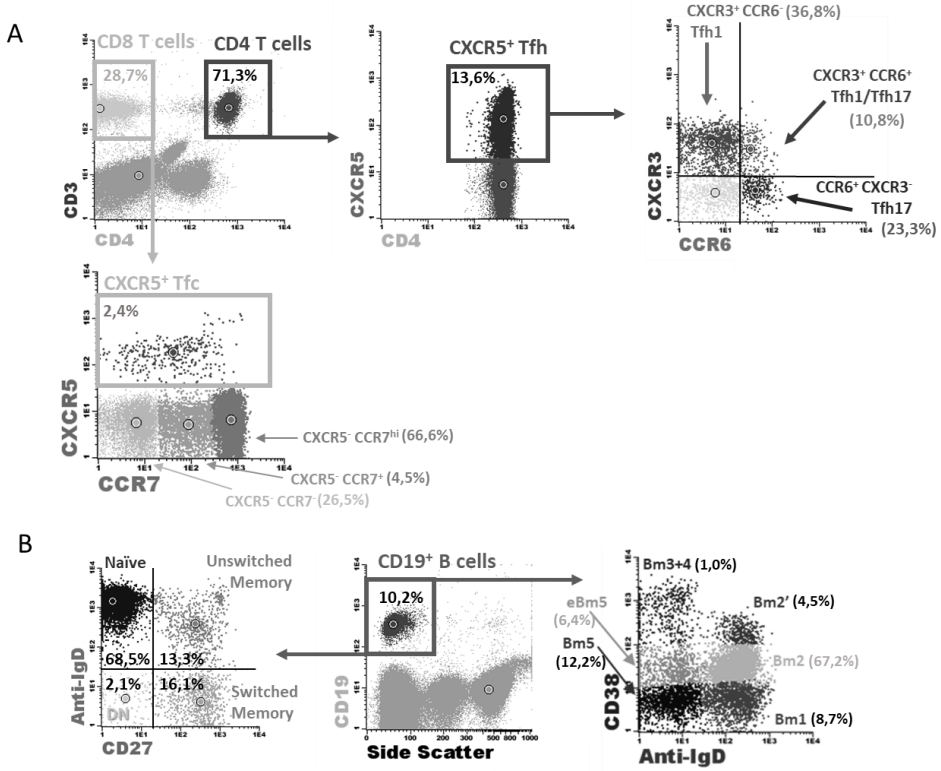

Figure 1. Gating strategy for the identification of circulating T and B-cell subsets. (**A**) Gating strategy for the identification of CD4 T-cells ($CD3^+$ $CD4^+$ lymphocytes): $CXCR5^+$ CD4 T-cells were identified (Tfh), and within this subset, according to the expression of CXCR3 and CCR6, Tfh1 ($CXCR3^+$ $CCR6^-$), Tfh17 ($CXCR3^-$ $CCR6^+$) and Tfh1/Tfh17 ($CXCR3^+$ $CCR6^+$) cells were identified. CD8 T-cells were identified as the $CD3^+$ $CD4^-$ population of lymphocytes. Within CD8 T-cells, $CXCR5^+$ cells were identified (Tfc), but also $CXCR5^-$ cells were characterized according to the expression of CCR7 (negative, positive and high positive). (**B**) Gating strategy for the identification of B-cells ($CD19^+$ lymphocytes). Using the expression of IgD and CD27 cells were divided in naïve (IgD^+ $CD27^-$), unswitched memory (IgD^+ $CD27^+$), switched memory (IgD^- $CD27^+$) and double negative (IgD^- $CD27^-$). Using the Bm1-5 classification, considering IgD and CD38, B-cells were divided in Bm1 ($CD38^-$ IgD^+), Bm2 ($CD38^+$ IgD^+), Bm2' ($CD38^{hi}$ IgD^+), Bm3+4 ($CD38^{hi}$ IgD^-), eBm5 ($CD38^+$ IgD^-) and Bm5 ($CD38^-$ IgD^-). +: positive; - : negative; hi: high; Tfh—Follicular helper T cells; Tfc—Follicular cytotoxic T cells; DN—Double negative.

Our study can also constitute a starting point for approaching the role of $CXCR5^+$ and $IL21^+$ CD8 T-cells (Tfc) in the context of autoimmunity. The association between $CXCR5^+$ CD8 T-cells and disease activity in SjS observed in our study may be an indicator of their involvement in the pathophysiology of autoimmune epithelitis. In the current scenario where Tfc cells involvement in autoimmune pathologies is yet to be elucidated, our study pioneers the association of Tfc cells with human autoimmunity and paves the way for

further studies regarding Tfc cells in autoimmune diseases. Indeed, considering the possible pathogenic role of EBV in the pathogenesis of SjS, therapies directed towards the interaction between EBV and activated effector T-cells and B-cells could halt the EBV-triggered lymphocytic activation, and have a relevant clinical applicability.

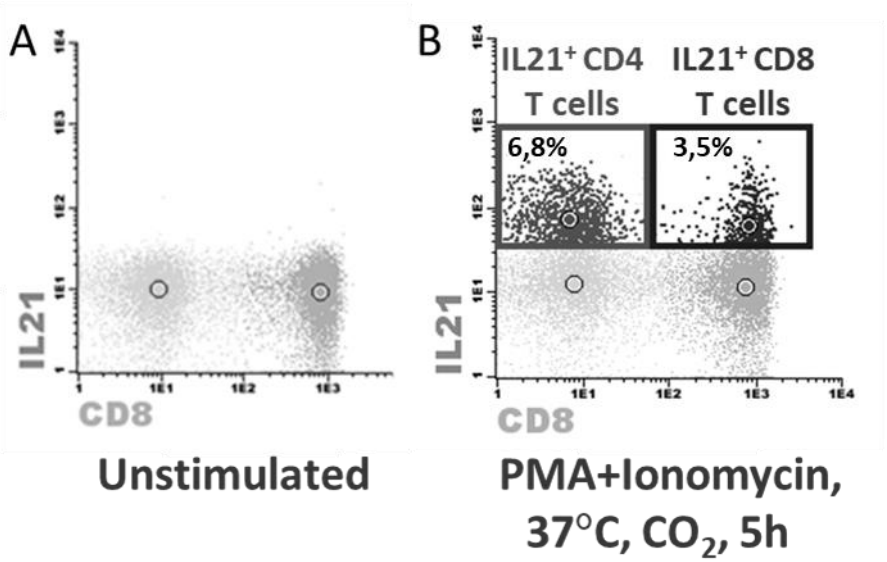

Figure 2. Gating Strategy for the identification of circulating CD4 and CD8 T-cells secreting IL-21 after stimulation. After identifying T-cells according to the expression of CD3 in the lymphocyte gate, CD4 T-cells were identified as the $CD3^+$ $CD8^-$ subset and CD8 T-cells as the $CD3^+$ $CD8^+$. (**A**) Expression of IL-21 and IL-17 in CD4 and CD8 T-cells after a 5-h incubation period with no stimulation. (**B, C**) Expression of IL-21 and IL(-)17 in CD4 and CD8 T-cells after a 5-h stimulation with PMA and ionomycin, in the presence of brefeldin A. Unstimulated controls were used for each sample, to assess the levels of positivity for IL-21 and IL-17.

Methods

Population. In this study, we included SjS patients classified according to the 2016 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria[47], and Rheumatoid Arthritis (RA) patients classified according to the 2010 ACR/EULAR criteria[48]. Patients were consecutively recruited, considering as additional exclusion criteria for SjS the use of B-cell-depleting therapies, and for RA patients the presence of xerostomia or xerophthalmia, as well as the use of any biologic disease-modifying anti-rheumatic drug. Disease activity in SjS was evaluated with the EULAR SjS disease activity index (ESSDAI)[49]. Clinically active disease was defined as activity in any ESSDAI domain, except the hematologic and biologic.

The healthy control group (HC) consisted of women without symptoms or signs of xerostomia or xerophthalmia, or any history of autoimmune rheumatic diseases, selected from the Ophthalmology outpatient clinic of Hospital CUF Descobertas.

Informed consent was obtained from all participants. The study was approved by the Ethics committees of both recruiting institutions, and NOVA Medical School Ethics Committee (no. 17/2016/CEFCM).

Flow cytometry procedures. For the immunophenotyping protocols, peripheral blood samples collected in EDTA-coated tubes were processed and analyzed within 24 h of collection. A pre-validated panel of monoclonal antibodies (mAbs) was used for the characterization of T and B-cell subsets, including CD3, CD4, CD8, CD19, CD24, CD27, CD38, CCR6, CCR7, CXCR3, CXCR5, Anti-IgD, and Anti-IgM. A lyse-wash protocol was performed for both T and B-cell characterization. A lyse-no wash single platform strategy was used to obtain absolute counts of all cell subsets (BD Trucount tubes BD Biosciences, San Diego CA, USA). All samples were acquired in a 4-color cytometer (BD FACS-Calibur, BD Biosciences). CellQuest Pro (BD Biosciences) software was used for acquisition and analysis purposes and Infinicyt 2.0 (Cytognos S.L., Salamanca, Spain) software was also used for more differentiated subset analysis.

Whenever appropriate, fluorescence-minus-one control tubes were prepared to assess the positivity of dimer expressions. The subsets analyzed, and the respective gating strategies, are displayed in Fig. 1. Within T-cells, we characterized $CD4^+$ and $CD8^+$ (CD4-) subsets, including $CXCR5^+$ Tfh and Tfc cells, and the Tfh1 and Tf17 profiles, according to the expression of CXCR3 and CCR6, respectively. B-cells' subsets were addressed according to the classical IgD/CD27 classification, and the Bm1-5 classification, often used in autoimmunity settings[21].

Functional assays for the evaluation of IL21 production by T-cells. Heparinized peripheral blood samples were used to assess IL21 and IL-17 production by $CD4^+$ and $CD8^+$ T-cells.
In brief, cells were stimulated with PMA and ionomycin, for 5 h at 37°C in a 5% CO_2 atmosphere in the presence of brefeldin-A. After stimulation, cells were lysed, washed and incubated with anti-CD3 and anti-CD8 mAbs for surface staining. For intracellular stain, cells were treated according to the protocol defined by the manufacturer for the BD Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (*BD Biosciences*) and then marked with anti-IL21 and anti-IL-17 mAbs, after cell fixation and permeabilization. For each patient, stimulated and unstimulated tubes were run in parallel to assure proper stimulation and staining controls. Gating strategy is presented in Fig. 2, with $IL21^+$ ($IL17^-$) cells being identified within $CD8^+$ and $CD8^-$ T cells, respectively considered as Tfc and Tfh cells.

EBV serological markers. Enzyme-linked immunosorbent assays (ELISA) were used for the assessment of IgG, IgA and IgM antibodies (Abs) against EBV antigens (Ags). All ELISA kits were obtained from Euroimmun (*Euroimmun, Luebeck, Germany*) and used according to the manufacturers' instructions. The following Abs for EBV Ags were determined: IgG for diffuse early Ag (EA-D), IgG for viral capsid Ag (VCA), IgG for nuclear Ag-1 (EBNA1), IgA for

EA-D, IgA for VCA and IgM for VCA. All tests for IgG Abs were quantitative, while IgA and IgM were semiquantitative. In quantitative assays, sample concentration was determined using 3-point calibration curves constructed with ELISA-Logit software, available at https://ednieuw.home.xs4all.nl/Calibration/Logit/Logit.htm (V24May2017). The cut-off level for all IgG antibodies assayed was 20 RU/ml. For semiquantitative assessments, a single calibrator was determined in triplicate per assay. The ratio sample/calibrator was used to assess positivity levels (Negative: ratio < 0.8; Borderline: ratio ≥ 0.8 to < 1.1; Positive: ratio ≥ 1.1). Patients were randomly assigned to undergo EBV serology evaluation.

Statistics. Graph Pad Prism™ 6.0 (*Graph Pad Sofware, San Diego, CA, USA*) was used for statistical analysis. The normality of data sets was assessed using D'Agostino & Pearson omnibus and Shapiro–Wilk normality tests. ANOVA and Kruskal–Wallis tests were made for multiple analyses among groups, followed by Dunn's multiple comparisons test. When a significant difference was found, comparisons were done using Unpaired Student's t-test with Welch's correction or Mann–Whitney test, for every two groups. For categorical variables, Fischer's or Chi-square tests were applied to assess differences between groups. Statistical significance was considered for p values < 0.05.

Ethics approval. This study was approved by the Ethics committee of *Hospital Cuf Descobertas*, 8/09/2014, Ethics committee of *Instituto Português de Reumatologia*, 3/07/2015 and NOVA Medical School Ethics (No. 17/2016/CEFCM).

Informed consent. All patients have signed informed consent to participate according to the Declaration of Helsinki.

References

1. Pontarini, E., Lucchesi, D. & Bombardieri, M. Current views on the pathogenesis of Sjögren's syndrome. *Curr. Opin. Rheumatol*. **30**, 215–221 (2018).
2. Mitsias, D. I., Kapsogeorgou, E. K. & Moutsopoulos, H. M. Sjögren's syndrome: why autoimmune epithelitis?. *Oral Dis.* **12**, 523–532 (2006).
3. Abrol, E., González-Pulido, C., Praena-Fernández, J. M. & Isenberg, D. A. A retrospective study of long-ter m outcomes in 152 patients with primary Sjögren's syndrome: 25-year experience. *Clin. Med*. *(Northfeld. Il.)* **14**, 157–164 (2014).
4. Nocturne, G. & Mariette, X. B cells in the pathogenesis of primary Sjögren syndrome. *Nat. Rev. Rheumatol.* **14**, 133–145 (2018).
5. Psianou, K. *et al.* Clinical and immunological parameters of Sjögren's syndrome. *Autoimmun. Rev.* **17**, 1053–1064 (2018).
6. Hansen, A. *et al.* Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome. *Arthritis Rheum*. **46**, 2160–2171 (2002).
7. Szyszko, E. A. *et al.* Salivary glands of primary Sjögren's syndrome patients express factors vital for plasma cell survival. *Arthritis Res. Ther.* **13**, R2 (2011).
8. Verstappen, G. M., Kroese, F. G. M. & Bootsma, H. T cells in primary Sjögren's syndrome: targets for early intervention. *Rheumatology* https://doi.org/10.1093/rheumatology/kez004 (2019).

9. Salomonsson, S. *et al.* Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjögren's syndrome. *Arthritis Rheum.* **48**, 3187–3201 (2003).
10. Zotos, D. *et al.* IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. *J. Exp. Med.* **207**, 365–378 (2010).
11. Szabo, K. *et al.* Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. *Clin. Immunol*. **147**, 95–104 (2013).
12. Li, X. *et al.* Role of the frequency of blood $CD4^+CXCR5^+CCR6^+$T cells in autoimmunity in patients with Sjögren's syndrome. *Biochem. Biophys. Res. Commun*. **422**, 238–244 (2012).
13. Quigley, M. F., Gonzalez, V. D., Granath, A., Andersson, J. & Sandberg, J. K. CXCR5+ CCR7- CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles*. Eur. J. Immunol*. **37**, 3352–3362 (2007).
14. Chen, Y. *et al.* CXCR5+PD-1+ follicular helper CD8 T cells control B cell tolerance. *Nat. Commun.* **10**, 1–15 (2019).
15. Igoe, A. & Scofeld, R. H. Autoimmunity and infection in Sjögren's syndrome. *Curr. Opin. Rheumatol*. **25**, 480–487 (2013).
16. Faulkner, G. C., Krajewski, A. S. & Crawford, D. H. The ins and outs of EBV infection. Trends Microbiol. 8, 185–189 (2000).
17. Khanolkar, A., Fuller, M. J. & Zajac, A. J. T cell responses to viral infections: lessons from lymphocytic choriomeningitis virus. *Immunol. Res***. 26**, 309–321 (2002).
18. Matloubian, M., Concepcion, R. J. & Ahmed, R. $CD4^+$ T cells are required to sustain $CD8^+$ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* **68**, 8056–8063 (1994).
19. Fahey, L. M. *et al.* Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J. Exp. Med.* **208**, 987–999 (2011).
20. Maślińska, M. The role of Epstein-Barr virus infection in primary Sjögren's syndrome. *Curr. Opin. Rheumatol.* **31**, 475–483 (2019).
21. Bohnhorst, J. Ø., Bjørgan, M. B., Toen, J. E., Natvig, J. B. & Tompson, K. M. Bm1–Bm5 classification of peripheral blood b cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjögren's syndrome. *J. Immunol.* **167**, 3610–3618 (2001).
22. Lünemann, J. D. *et al.* Increased frequency of EBV-specific effector memory $CD8^+$ T cells correlates with higher viral load in rheumatoid arthritis. *J. Immunol.* **181**, 991–1000 (2008).
23. Berner, B. R. *et al.* Phenotypic and functional analysis of EBV-specific memory CD8 cells in SLE. *Cell. Immunol.* **235**, 29–38 (2005).
24. Cassaniti, I. *et al.* Evaluation of EBV- and HCMV-specific T cell responses in Systemic Lupus Erythematosus (SLE) patients using a normalized Enzyme-Linked Immunospot (ELISPOT) assay. *J. Immunol. Res.* 2019, Article ID 4236503 (2019).
25. Brokstad, K. A. *et al.* T follicular-like helper cells in the peripheral blood of patients with primary Sjögren's syndrome. *Scand. J. Immunol.* **88**, e12679 (2018).
26. Singh, N. & Cohen, P. L. The T cell in Sjogren's syndrome: force majeure, not spectateur. *J. Autoimmun*. **39**, 229–233 (2012).
27. Saito, M. *et al.* Unique phenotypes and functions of follicular helper T cells and regulatory T cells in Sjögren's syndrome. *Curr. Rheumatol. Rev.* **14**, 239–245 (2018).
28. Monteiro, R. *et al.* Follicular helper and follicular cytotoxic T cells in primary Sjögren's Syndrome: clues for an abnormal antiviral response as a pathogenic mechanism. *Ann. Med.* **51**, 42–42 (2019).
29. Perdomo-Celis, F., Taborda, N. A. & Rugeles, M. T. Circulating CXCR5-expressing $CD8^+$ T-cells are major producers of IL-21 and associate with limited HIV replication. J. Acquir. Immune Defic. Syndr. 78, 473–482 (2018).
30. Gong, Y. Z. *et al.* Differentiation of follicular helper T cells by salivary gland epithelial cells in primary Sjögren's syndrome. *J. Autoimmun*. **51**, 57–66 (2014).

31. Mylvaganam, G. H. *et al.* Dynamics of SIV-specific CXCR5$^+$ CD8 T cells during chronic SIV infection. *Proc. Natl. Acad. Sci.* **114**, 1976–1981 (2017).
32. Jiang, H. *et al.* CXCR5$^+$CD8$^+$T cells indirectly offer B cell help and are inversely correlated with viral load in chronic hepatitis B infection. *DNA Cell Biol.* **36**, 321–327 (2017).
33. Shen, J. *et al.* A subset of CXCR5$^+$CD8$^+$ T cells in the germinal centers from human tonsils and lymph nodes help B cells produce immunoglobulins. *Front. Immunol.* **9**, 2287 (2018).
34. Leong, Y. A. *et al.* CXCR5+ follicular cytotoxic T cells control viral infection in B cell follicles. *Nat. Immunol.* **17**, 1187–1196 (2016).
35. Ehlin-Henriksson, B., Gordon, J. & Klein, G. B-lymphocyte subpopulations are equally susceptible to Epstein-Barr virus infection, irrespective of immunoglobulin isotype expression. *Immunology* **108**, 427–430 (2003).
36. Jonsson, M. V., Skarstein, K., Jonsson, R. & Brun, J. G. Serological implications of germinal center-like structures in primary Sjögren's syndrome. *J. Rheumatol*. **34**, 2044–2049 (2007).
37. Croia, C. *et al.* Implication of Epstein-Barr virus infection in disease-specific autoreactive B cell activation in ectopic lymphoid structures of Sjögren's syndrome. *Arthritis Rheumatol*. **66**, 2545–2557 (2014).
38. Lossius, A., Johansen, J., Torkildsen, Ø., Vartdal, F. & Holmøy, T. Epstein-Barr virus in systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis—association and causation. *Viruses* **4**, 3701–3730 (2012).
39. Coleman, C. B., Nealy, M. S. & Tibbetts, S. A. Immature and transitional B cells are latency reservoirs for a gammaherpesvirus. *J. Virol*. **84**, 13045–13052 (2010).
40. Kivity, S. *et al.* Infection and autoimmunity in Sjogren's syndrome: a clinical study and comprehensive review. *J. Autoimmun.* **51**, 17–22 (2014).
41. Pasoto, S. G. *et al.* EBV reactivation serological profile in primary Sjögren's syndrome: an underlying trigger of active articular involvement? *Rheumatol. Int.* **33**, 1149–1157 (2013).
42. Long, H. M., Meckif, B. J. & Taylor, G. S. The T-cell response to Epstein-Barr virus-new tricks from an old dog. *Front. Immunol.* **10**, 1–11 (2019).
43. Moss, D. J., Burrows, S. R. & Khanna, R. EBV: Immunobiology and host response. in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis* 904–914 (2007). https://doi.org/10.1017/CBO9780511545313.052
44. Sanosyan, A. *et al.* Discrepancy of serological and molecular patterns of circulating Epstein-Barr virus reactivation in primary Sjögren's syndrome. *Front. Immunol*. https://doi.org/10.3389/fimmu.2019.01153 (2019).
45. Sternbæk, L. *et al.* Increased antibody levels to stage-specifc Epstein-Barr virus antigens in systemic autoimmune diseases reveal a common pathology. *Scand. J. Clin. Lab. Investig.* **79**, 7–16 (2019).
46. Draborg, A. H. *et al.* Epstein-Barr virus early antigen diffuse (EBV-EA/D)-directed immunoglobulinA antibodies in systemic lupus erythematosus patients. *Scand. J. Rheumato*l. **41**, 280–289 (2012).
47. Shiboski, C. H. *et al.* 2016 American College of Rheumatology/European League against rheumatism classification criteria for primary Sjögren's syndrome: a consensus and data-driven methodology involving three international patient cohorts. *Arthritis Rheumatol*. **69**, 35–45 (2017).
48. Aletaha, D. *et al.* 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League against rheumatism collaborative initiative. *Arthritis Rheum.* **62**, 2569–2581 (2010).
49. Seror, R. *et al.* EULAR Sjögren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjögren's syndrome. *Ann. Rheum. Dis*. **69**, 1103–1109 (2010).

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Author contributions
F.B., C.M. and R.M. conceived the original research idea, while all of the authors designed the study and created the study protocol. F.B. and J.V.P. recruited the patients and collected the data. J.C. and N.A. recruited healthy controls and collected the data. C.M. and R.M. performed the flow cytometry analysis and the EBV serology testing, and collected the data. C.M. performed the statistical analysis. J.C.B. and L.M.B. supervised all the work and the research protocol. All of the authors contributed to data analysis and interpretation. F.B. and R.M. drafted the manuscript, and all of the authors revised it and contributed to it intellectually. All of the authors have approved the final version of the manuscript.

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Competing interests
The authors declare no competing interests.

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Supplementary Tables

	SjS (*N* = 57)	RA (*N* = 20)	HC (*N* = 24)
Female gender, n (%)	56 (98.2)	16 (80.0)	24 (100)
Age, years median (Min-Máx)	60.6 (28.6 – 78.3)	56.7 (30.30-78.0)	51.3 (38.9 – 63.2)
Age of onset, years	47.1 (24.5-68.3)	38.6 (22.2-61.9)	-
Age at diagnosis, years	53.8 (26.7-77.2)	42.5 (22.9-64.0)	-
Duration of disease, years	11.3 (1.0-29.5)	10.2 (1.5-38.2)	-
ESSDAI, median (Min-Máx)	2 (0-14)		
Ocular symptoms, *n* (%)	54 (94.7)		
Oral symptoms, *n* (%)	55 (96.5)		
Ocular signs, *n* (%)	36 (63.2)		
Oral signs, *n* (%)	42 (73.7)		
Focus Score ≥1[a]	43/55 (78.2)		
Parotid enlargement (ever), *n* (%)	8 (14.0)		
Extraglandular disease (ever)[b], *n* (%)	23 (40.4)		
Joint symptoms (ever)[b], *n* (%)	24 (42.1)		
Skin involvement (ever)[c], *n* (%)	18 (31.6)		
Other extraglandular involvement (ever), *n* (%)	5 (8.8)		
Raynaud's phenomenon (ever), *n* (%)	8 (14.0)		
SSA, *n* (%)	38 (66.7)		
SSB, *n* (%)	18/50 (36.0)		
ANA ≥1/320	45 (78.9)		
Rheumatoid Factor, *n* (%)	24/50 (48.0)		
Gammaglobulin ≥1.6 g/dL, *n* (%)	14 (24.6)		
Therapy (any), *n* (%)	32 (56.1)		
Glucocorticoids, *n* (%)	19 (33.3)		
Hydroxychloroquine, *n* (%)	20 (35.1)		
Immunosuppressants, *n* (%) [d]	10 (17.5)		

Supplementary Table 1. SjS, RA patients and HC characteristics. The patient's characteristics are represented as number of occurrences (n) and percentages (%). [a] Defined as the presence of 1 or more dense aggregates of 50 or more lymphocytes, per 4 mm^2 of glandular area. [b] Joint symptoms include arthritis and joint pain of inflammatory origin, but only cases that would score in the articular domain of ESSDAI were considered as extraglandular disease. [c] Skin involvement considered was polymorphous exanthema, purpura or vasculitis, and subacute cutaneous lupus lesions. [d] Any of the following: Methotrexate, Azathioprine, Leflunomide, or Cyclosporine. * SjS vs RA, Fisher's exact test. [#] Brown-Forsythe ANOVA test, with Dunnett's T3 multiple comparisons test showing significant difference between SjS and HC SjS, Sjögren's syndrome; RA, Rheumatoid arthritis; HC – Healthy controls; ESSDAI, European Sjögren's syndrome disease activity index; SSA/SSB, Sjögren's syndrome A/B antibody; ANA, antinuclear antibody.

Absolute Counts	SjS	RA	HC	p-value
T-cell subsets				
T-cells	1180 [778 - 1778]	1369 [1229 - 1742]	1777 [1426 - 2155]	0.001
CD4 T-cells	746 [431 – 1073]	908 [691 – 1122]	1108 [1010 – 1408]	< 0.001
CXCR5^{+} Tfh	134 [82 – 203]	181 [137 – 219]	241 [221 – 284]	< 0.001
Tfh1	51 [25 - 72]	51 [39 - 73]	80 [48 - 102]	0.002
Tfh17	28 [16 - 46]	41 [27 - 58]	53 [37 - 71]	<0.001
IL-21^{+}	84 [52 – 122]	91 [63 – 105]	113 [71 – 167]	0.157
IL-17^{+}	13 [8 -26]	22 [12 – 34]	30 [21 – 46]	0.001
IL-21^{+} IL-17^{+}	5 [3 – 8]	6 [3 – 8]	7 [5 – 13]	0.143
CD8 T-cells	434 [321 – 643]	528 [336 – 667]	533 [394 – 753]	0.225
CXCR5^{+} Tfc	3 [2 – 5]	3 [1 – 7]	5 [3 – 6]	0.222
IL-21^{+}	18 [10 – 27]	9 [5 – 15]	13 [6 – 26]	0.041
IL-17^{+}	3 [2 – 7]	5 [3 – 9]	6 [4 – 12]	0.037
IL-21^{+} IL-17^{+}	1 [1 – 2]	2 [1 – 2]	2 [1 – 4]	0.164
B-cell subsets				
B-cells	177 [97 - 261]	133 [83 – 153]	252 [173 - 393]	<0.001
Naïve	108 [61 – 191]	68 [31 – 97]	108 [61 - 191]	0.002
Memory	47 [29 - 75]	46 [24 - 57]	106 [65 – 141]	<0.001
Unswitched Memory	22 [12 - 38]	18 [13 - 30]	57 [32 - 81]	<0.001
Switched Memory	22 [15 - 37]	20 [13 - 36]	45 [37 - 76]	<0.001
Double negative	3 [2 - 9]	7 [2 - 11]	4 [2 - 9]	0.318
Bm1	18 [10 – 27]	19 [11 - 25]	38 [19 - 62]	0.001
Bm2	101 [57 - 164]	59 [22 - 84]	130 [97 - 214]	<0.001
Bm2’	11 [6 – 29]	4 [2 – 10]	12 [7 - 30]	0.005
Bm3+4	3 [1 – 5]	2 [1 – 3]	4 [2 - 5]	0.089
eBm5	15 [9 – 23]	11 [7 – 17]	27 [19 - 45]	<0.001
Bm5	11 [8 – 19]	14 [9 - 28]	24 [14 - 35]	0.002

Supplementary table 2. Absolute counts of T and B-cell subsets in all groups. T and B cells subsets absolute counts presented in median [25th – 75th percentile]. ***** Bold numbers highlight the populations that were significantly different. Kruskal-Wallis test was applied for statistical significance. **#** Tfh1 and Tfh17 are represented as percentages among CXCR5^{+} Tfh cells. SjS, Sjögren's Syndrome. RA, Rheumatoid Arthritis. HC, Healthy Controls.

Absolute Counts	G1 EA IgG$^-$ EBNA IgG$^+$ (n=18)	G2 EA IgG$^+$ EBNA IgG$^{+/-}$ (n=11)	G3 EA IgG$^-$ EBNA IgG$^-$ (n=5)	p - value
T-cell subsets				
T-cells	1126 [780 - 2145]	1263 [755 - 1772]	930 [814 - 1638]	0.925
CD4 T-cells	755 [395 - 1227]	823 [395 - 1159]	607 [423 - 903]	0.892
CXCR5$^+$ Tfh	134 [88 - 189]	140 [82 - 207]	119 [68 - 209]	0.774
Tfh1	49 [35 - 67]	60 [35 - 72]	34 [17 - 52]	0.283
Tfh17	27 [15 - 43]	28 [14 - 44]	28 [19 - 64]	0.949
IL-21$^+$	90 [45 - 132]	93 [51 - 122]	64 [45 - 159]	0.943
IL-17$^+$	14 [10 - 32]	20 [10 - 29]	9 [8 - 35]	0.764
IL-21$^+$ IL-17$^+$	7 [2 - 13]	7 [4 - 8]	3 [3 - 9]	0.662
CD8 T-cells	501 [319 - 1072]	597 [333 - 636]	346 [305 - 811]	0.783
CXCR5$^+$ Tfc	15 [9 - 27]	15 [5 - 22]	8 [5 - 17]	0.237
IL-21$^+$	20 [12 - 47]	18 [10 - 37]	23 [7 - 121]	0.967
IL-17$^+$	5 [2 - 11]	4 [3 - 8]	3 [2 - 10]	0.681
IL-21$^+$ IL-17$^+$	2 [1 - 3]	1 [1 - 3]	2 [1 - 4]	0.809
B-cell subsets				
B-cells	169 [103 - 259]	187 [168 - 261]	112 [62 - 209]	0.334
Naïve	97 [55 - 188]	126 [73 - 186]	61 [42 - 145]	0.355
Memory	50 [36 - 112]	64 [33 - 92]	40 [19 - 52]	0.306
Unswitched Memory	26 [19 - 61]	24 [12 - 38]	15 [8 - 26]	0.156
Switched Memory	23 [15 - 37]	32 [21 - 52]	19 [8 - 33]	0.250
Double negative	3 [2 - 8]	3 [2 - 9]	3 [2 - 7]	0.769
Bm				
Bm1	19 [10 - 29]	16 [9 - 19]	13 [8 - 22]	0.370
Bm2	91 [49 - 178]	101 [74 - 139]	57 [36 - 115]	0.326
Bm2’	11 [5 - 22]	24 [11 - 29]	8 [4 - 33]	0.132
Bm3+4	3 [2 - 5]	7 [2 - 8]	1 [1 - 2]	0.009*
eBm5	15 [9 - 26]	18 [13 - 24]	13 [4 - 26]	0.434
Bm5	10 [8 - 20]	18 [8 - 27]	9 [6 - 23]	0.608

Supplementary Table 3. Immune profile of SjS patients with distinct EBV serology patterns (absolute counts). Absolute values for all T and B cells subsets presented in median [minimum – maximum] in SjS patients evaluated for EBV serology. ***** Bold numbers highlight the populations that were significantly different. Kruskal-Wallis test was applied for statistical significance. *SjS*, Sjögren's syndrome. *EBV*, Epstein-Barr virus.

CAPÍTULO V. Lymphocyte subpopulations in Sjögren's syndrome are distinct in anti-SSA positive patients and related to disease activity | As subpopulações linfocitárias na Síndrome de Sjögren SSA-positiva são distintas e relacionam-se com a atividade da doença.

Lymphocyte subpopulations in Sjögren's syndrome are distinct in anti-SSA-positive patients and related to disease activity

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Abstract

Objectives Sjögren's syndrome (SjS) patients exhibit great phenotypical heterogeneity, reinforced by the positiveness of antiSSA antibody. We aimed to evaluate lymphocyte subpopulations in SSA-positive (SSA+SjS) and SSA-negative (SSA−SjS) SjS patients, *Sicca* patients, and healthy controls (HC), and to investigate associations between lymphocyte subpopulations and disease activity in SjS.

Methods According to the fulfilment of the ACR/EULAR 2016 classification criteria, patients were included as SjS or as *Sicca*. HC were selected from the Ophthalmology outpatient clinic. Lymphocyte subpopulations were characterized by flow cytometry. Statistical

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analysis was performed with GraphPad PrismTM, with statistical significance concluded if $p < 0.05$.

Results We included 53 SjS patients (38 SSA^+ and 15 SSA−), 72 *Sicca*, and 24 HC. SSA+SjS patients presented increased $IL21^+CD4^+$ and $CD8^+$ T cells compared to *Sicca* and HC, whereas compared to SSA−SjS patients, only IL-21^+CD4^+ T cell percentages were increased and Tfh17 percentages and numbers were decreased. Compared to *Sicca* and HC, SSA+SjS patients had higher levels of $CD24^{Hi}CD38^{Hi}$ B cells, naïve B cells, and $IgM^-/^+CD38^{++}$ plasmablasts, and lower levels of memory B cells, including $CD24^{Hi}CD27^+$ B cells. SSA+SjS patients with clinically active disease had positive correlations between ESSDAI and IL-21^+CD4^+ ($p = 0.038$, $r = 0.456$) and IL-21^+CD8^+ T cells ($p = 0.046$, $r = 0.451$).

Conclusions In SjS, a distinct lymphocyte subset distribution profile seems to be associated with positive anti-SSA. Moreover, the association between ESSDAI and IL-21^+CD4^+ and IL-21^+CD8^+ (follicular) T cells in SSA+SjS patients suggests the involvement of these cells in disease pathogenesis and activity, and possibly their utility for the prognosis and assessment of response to therapy.

Key Points

- SSA+SjS *patients have a pronounced naïve/memory B cell imbalance.*
- SSA+SjS *patients have more active disease associated with IL-21^+ $CD4^+$ and IL-21^+ $CD8^+$ follicular T cell expansion.*
- *IL-21^+CD4^+ and IL-21^+CD8^+ T cell quantification may be useful for the prognosis and assessment of response to therapy*

Keywords B lymphocytes · Disease activity · Sjögren's syndrome · SSA · T lymphocytes

Background

Sjögren's syndrome (SjS) is a chronic immune-mediated disease characterized by lymphocytic infiltration and lesion of exocrine glands, with a variable clinical spectrum, from isolated mucosal dryness to systemic involvement with extraglandular manifestations [1].

The 2016 American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) classification criteria [2] demand either anti-SSA antibodies or a positive minor salivary gland (SG) biopsy (highly specific but invasive and dependent on standardization).

SjS's immunopathogenesis is complex and involves the innate and adaptive immune system. B cell hyperactivity is the hallmark of SjS, playing a major role in pathogenesis and clinical evolution [3]. Interactions between T cells and activated B cells occur in germinal center (GC)–like structures within target organs, such as SG and lachrymal glands, which become dysfunctional [4]. Additionally, T cells infiltrate target organs and produce pro-inflammatory cytokines resulting in B cell activation [5].

Follicular helper T cells (Tfh) have recently emerged as a key player in SjS pathogenesis. Tfh cells are a major source of IL-21, a cytokine that mediates B cell differentiation and proliferation and promotes the ectopic formation of GC-like structures

[6]. The chemokine receptor X5 (CXCR5) induces the homing of follicular T cells to lymph nodes, particularly to B cell sites, and is a surface marker useful in the identification of follicular-related T cells. CXCR5 is also expressed in $CD8^+$ T cells, suggesting the existence of a follicular-related cytotoxic ($CD8^+$) T cell subset (Tfc) [7]. Tfh differentiation is promoted in the SG of SjS patients, who seem to present increased circulating Tfh cells [8].

Although the frequency of regulatory T cells (Tregs) in SjS is variable in the literature, SjS patients seem to present Tregs deficiencies [9], contributing to disease pathogenesis.

Regarding B cells, increased naïve B cells and decreased memory B cells, particularly unswitched memory, are typical in SjS patients [10]. Some B cell subsets, known as regulatory B cells (Bregs), contribute to the regulation of immune responses, mainly through the secretion of interleukin-10 (IL10). Bregs suppress effector T cells, inhibit Tfh differentiation, and induce Tregs [11]. Recently, $CD19^+CD24^+CD38^{hi}$ Bregs showed reduced IL-10 production and defective Tfh inhibitory capacity in SjS patients [12].

The presence of anti-SSA antibodies is associated to distinct phenotypic features of SjS [13]. We sought to investigate whether lymphocyte subpopulations were distinctive in SSA^+ SjS patients versus SSA−SjS, *Sicca* patients, and healthy controls (HC), and their impact in disease activity.

Methods

Population

We enrolled patients with confirmed or suspected SjS, who were consecutively recruited and classified as SjS if ACR/ EULAR 2016 classification criteria were fulfilled, or otherwise included in the *Sicca* group. All patients had been subjected to a multidisciplinary evaluation which included ocular evaluation (Schirmer's test and corneal staining score), and unstimulated salivary flow assessment (Table 1). Secondary SjS patients were excluded. The HC group consisted of women without *Sicca* symptoms, selected from the Ophthalmology outpatient clinic.

Disease activity in SjS was assessed with the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [14]. Patients were considered having clinically active disease if they scored in any ESSDAI domain except for the hematologic and biologic ones. Patients were stratified according to disease activity as low (ESSDAI < 5) or moderate/high (ESSDAI ≥ 5) [15].

Informed consent was obtained from all participants. This study was approved by the Ethics committees of Hospital *CUF Descobertas*, *Instituto Português de Reumatologia* and NOVA Medical School (no 17/2016/CEFCM).

Flow cytometry measurements

Blood samples were collected into EDTA-containing tubes, processed, and analyzed within 24 h after collection. Prevalidated panels of monoclonal antibodies were used to characterize lymphocyte subpopulations, including anti-$CD3^A$, anti$CD4^A$, anti-$CD19^B$, anti-

CD24[B], anti-CD25[B], anti-CD27[B], anti-CD38[B], anti-CD127[B], anti-CCR6[A], anti-CCR7[B], antiCXCR3[A], anti-CXCR5[A], anti-IgD[B], and anti-IgMA ([A]BD Biosciences; [B]Biolegend), as described elsewhere [16].

Table 1 Patients' characteristics

	Sjögren's Syndrome (SjS)				Group's comparison (*p* value)		
	Total SjS *n* = 53	SSA+SjS *n* = 38	SSA-SjS *n* = 15	*Sicca* *n* = 72	Total SjS vs *Sicca*	SSA+SjS vs SSA-SjS	SSA+SjS vs *Sicca*
Sex, F/M	53/0	38/0	15/0	71/1	1,000	1,000	1,000
Age at onset (y), mean (SD)	46,2 (11,6)	44,4 (11,1)	50,7 (11,8)	50,8 (10,0)	*0,022*#	0,092#	*0,004*#
Age (y), mean (SD)	57,5 (11,9)	56,8 (12,4)	60,0 (9,0)	60,8 (11,0)	0,127#	0,538#	0,109#
Age at diagnosis (y), mean (SD)	51,7 (11,9)	49,6 (12,0)	57,0 (10,2)	57,4 (10,4)	*0,006*#	*0,032*#	*0,001*#
Symptom duration (y), mean (SD)	11,5 (7,6)	12,3 (7,6)	9,3 (7,3)	10,0 (5,4)	0,509#	0,183#	0,174#
Ocular symptoms, n (%)	50 (94,3)	36 (94,7)	14 (93,3)	69 (95,8)	0,650	1,000	0,610
Oral symptoms, n (%)	51 (96,2)	36 (94,7)	15 (100,0)	69 (95,8)	1,000	1,000	0,610
Low Schirmer's / keratitis *Sicca*, n (%)	33 (62,3)	22 (57,9)	11 (73,3)	39 (54,2)	0,464	0,359	0,840
Decreased Salivary Flow, n (%)	41 (77,4)	30 (78,9)	11 (73,3)	50 (69,4)	0,417	0,722	0,370
Clinically active disease, n (%)	26 (49,1)	21 (55,3)	5 (33,3)	10 (13,9)	*<0,001*	0,224	*<0,001*
ESSDAI, mean (SD) [min-Máx]	2,5 (3,0)[0-14]	3,0 (3,2)[0-14]	1,3 (1,9) [0-7]	NA	-	*0,025#*	-
ESSDAI ≥5, n (%)	9 (17,0)	8 (21,1)	1 (6,7)	NA	-	0,418#	-
Salivary gland swelling, n (%)	8 (15,1)	8 (21,1)	0 (0)	2 (2,8)	*0,018*	0,088	*0,003*
Joint symptoms, n (%)	23 (43,4)	18 (47,4)	5 (33,3)	32 (50,8)	1,000	0,539	0,842
Skin symptoms, n (%)	18 (34,0)	13 (34,2)	5 (33,3)	16 (22,2)	0,160	1,000	0,182
Other extraglandular involvment, n (%)	9 (17,0)	8 (21,1)	1 (6,7)	1 (1,4)	*0,002*	0,418	*0,001*
Raynaud's phenomenon, n (%)	7 (13,2)	7 (18,4)	0 (0,0)	18 (25,0)	0,118	0,172	0,483
Focus score ≥1, n (%)	39 (76,5)[a]	24 (66,7)[c]	15 (100)	3 (4,2)	*<0,001*	*0,011*	*<0,001*
SSA, n (%)	38 (71,7)	38 (100)	0 (0,0)	1 (1,4)	*<0,001*	*<0,001*	*<0,001*
SSB, n (%)	19 (39,6)[b]	19 (57,6)[d]	0 (0,0)	1 (1,4)	*<0,001*	*<0,001*	*<0,001*
ANA ≥ 1/320, n (%)	43 (81,1)	33 (86,8)	10 (66,7)	39 (54,2)	*0,002*	0,124	*0,001*
ANA ≥ 1/640, n (%)	31 (58,5)	28 (73,7)	3 (20,0)	26 (36,1)	*0,019*	*0,001*	*<0,001*
RF positive, n (%)	31 (58,5)	26 (68,4)	5 (33,3)	19 (26,4)[e]	*0,001*	*0,030*	*<0,001*
Gammaglobulin ≥ 1.6 g/dl, n (%)	14 (26,4)	13 (34,2)	1 (6,7)	3 (4,2)[e]	*0,001*	0,080	*<0,001*
Therapy (any), n (%)	30 (56,6)	21 (55,3)	9 (60,0)	32 (44,4)	0,207	1,000	0,319
Glucocorticoids, n (%)	18 (34,0)	14 (36,8)	4 (26,7)	18 (25,0)	0,320	0,539	0,270
Hydroxychloroquine, n (%)	19 (35,8)	13 (34,2)	6 (40,0)	21 (29,2)	0,445	0,756	0,666
Immunossupressants, n (%)	9 (17,0)	6 (15,8)	3 (20,0)	9 (12,5)	0,608	0,701	0,771

Patient's characteristics are represented as number of occurrences (*n*) and percentages (%). Whenever there were missing values, percentages reflect the number of occurrences over the number of patients tested for the item ([a] *n* = 51; [b] *n* = 48; [c] *n* = 36; [d] *n* = 33; [e] *n* = 71). Joint symptoms include arthritis and joint pain of inflammatory origin, but only cases that would score in the articular domain of ESSDAI were considered extra-glandular disease. Likewise, in some patients, skin involvement (which not included xerosis) was not considered extra-glandular disease if it would not score in the cutaneous domain of ESSDAI. Statistically

(Cont.) significant results are indicated in italic.
SjS, primary Sjögren's syndrome; *F*, female; *M*, male; *y*, years; *SSA/SSB*, Sjögren's syndrome A/B antibody; *ANA*, antinuclear antibody; *RF*, rheumatoid factor; *ESSDAI*, European Sjögren's syndrome disease activity index
*Fisher's exact test # Mann-Whitney U test # Mann-Whitney *U* test.

Samples were acquired in a 4-color BD FACS Calibur™ cytometer (BD Biosciences) and analyzed with Cell Quest Pro™ software (BD Biosciences). This study addressed several subsets of T cells, including Tregs and CXCR5$^+$ follicular-related T cells, as well as naïve and memory B cell subsets.

Each subset was evaluated in percentages and absolute counts, using BD Trucount tubes™ (BD Biosciences).

Complete gating strategies are described in Figure 1.

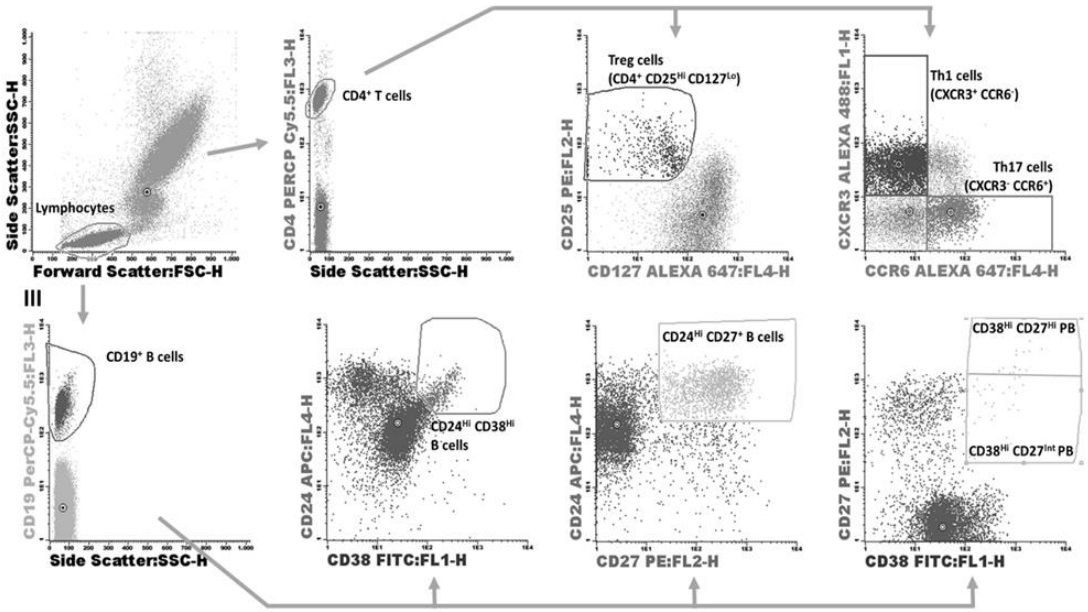

Fig. 1 Gating strategy for the identification of distinct T and B cell subsets. (a) Lymphocytes were identified according to forward and side scatter characteristics. (b and e) CD4$^+$ T and B cells were identified as CD4$^+$ (b) and CD19$^+$ (e) cells within the lymphocyte gate, respectively. (c) Treg subset was identified according to the expression of CD25 and CD127 within the CD4$^+$ T cells gate. (d) T-helper (Th) subsets were identified according to the expression of CXCR3 and CCR6 in Th1 (CXCR3$^+$CCR6$^-$) and Th17 (CXCR3$^-$ CCR6$^+$) cells. (f) B cells with a regulatory phenotype were identified as CD24^{Hi}CD38^{Hi} cells within the B lymphocyte gate. (g) B cell subsets were identified according to the expression of IgD and CD27 in naive, IgD$^+$CD27$^-$; unswitched memory/marginal zone like (MZ), IgD$^+$CD27$^+$; switched memory, IgD$^-$CD27$^+$; and double-negative (DN) memory B cells, IgD− CD27.

Functional assays for the evaluation of IL-21- producing CD4 and CD8 T cells

To characterize IL-21 and IL-17-producing T cells, heparinized blood was collected and stimulated with PMA (phorbol myristate acetate; 50 ng/mL) and ionomycin (1 µg/mL), for 5 h at 37 °C in a 5% CO_2 atmosphere, in the presence of brefeldin A. After stimulation, cells

were lysed, washed, and then incubated with anti-CD3 and anti-CD8 for surface staining. Afterwards, cells were treated according to the manufacturer's instructions for the BD Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (BD Biosciences) and marked with anti-IL-21 and anti-IL17. Stimulated and unstimulated tubes were run in parallel to assure proper stimulation and staining controls. Gating strategies are presented in Figure 2.

Statistical analysis

GraphPad Prism™ software 8.3.0 for Windows was used for statistical analysis. Categorical variables were expressed as numbers and percentages and analyzed using Fisher's exact test. Normality was assessed using the D'Agostino and Pearson test, and continuous variables were presented as means (standard deviation) or medians (25th–75th percentile), as applicable. The Mann-Whitney U test was used to compare every 2-independent groups. Spearman correlation coefficients were calculated to assess correlations. Statistical significance was concluded when $p < 0.05$.

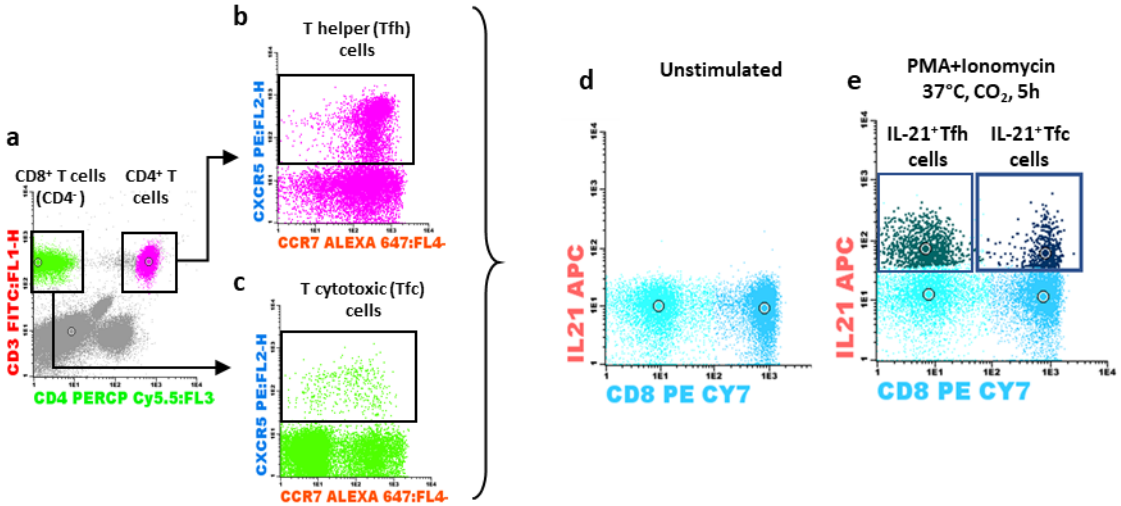

Fig. 2 Identification of follicular T cell subsets. (a–c) Gating strategy for the identification of follicular-related ($CXCR5^+CCD7^{-/+}$) cytotoxic (b) and helper (c) T cells. (d and e) IL-21-producing $CXCR5^+$ $CD4^+$ and $CD8^+$ T cells were analyzed for the expression of IL-21 after a 5-h incubation period without stimulation (d) and after stimulation (e) with PMA (phorbol myristate acetate) and ionomycin, for 5 h at 37 °C in a 5% CO_2 atmosphere in the presence of brefeldin A. Unstimulated tubes were used to assess IL-21 positivity

Results

Patient's characteristics

Fifty-three SjS and 72 *Sicca* patients were included. Thirtyeight SjS patients (71.7%) presented anti-SSA antibodies (SSA+SjS) while 15 (28.3%) were anti-SSA-negative (SSA–SjS). The HC group included 24 women. Table 1 describes the demographic, clinical, and immunological characteristics of patients.

SSA+SjS had a younger age of onset (44.4 years vs 50.7 years; $p = 0.092$) and were diagnosed earlier (49.7 years vs 57.0 years; $p = 0.032$) than SSA−SjS. None of the SSA−SjS patients presented parotid enlargement or Raynaud's phenomenon, although significance was not reached compared to SSA+SjS. Clinically active disease was present in 55.3% of SSA+SjS patients, with a mean ESSDAI of 3.0 (3.2), and in 33.3% of SSA−SjS with a mean ESSDAI of 1.3 (1.9) ($p = 0.025$). Rheumatoid factor (RF) was positive in 68.4% of SSA+SjS, significantly more frequent than in SSA−SjS (33.3%, $p = 0.030$) and *Sicca* (26.4%, $p < 0.001$). Hypergammaglobulinemia was also more frequent in SSA+ SjS (34.2%) compared to SSA−SjS (6.7%, $p = 0.080$) and *Sicca* (4.2%, $p < 0.001$).

Lymphocyte characterization

Complete results are presented in Table 2 and Supplementary Table 1.

$CD4^+$ and $CD8^+$ T cell subsets

Compared to both *Sicca* and HC, SjS patients presented lower $CD4^+$ T cell counts, especially when considering the SSA+ SjS subgroup (percentages, $p \leq 0.032$; absolute counts, $p \leq 0.046$). $CD4^+$ T cell counts were also significantly lower in *Sicca* compared to HC ($p < 0.001$). Both SjS groups presented higher $CD8^+$ T cell percentages compared to *Sicca* and HC, with no differences in counts.

Treg counts were lower in all patient groups when compared to HC ($p \leq 0.042$), although with higher percentages in SSA+SjS compared to *Sicca* ($p = 0.007$) (Figure 3). When considering only SSA+SjS patients with moderate/high disease activity (ESSDAI ≥ 5, $n = 8$), a negative correlation was found between Treg counts and the ESSDAI ($p = 0.011$, $r = -0.854$) (Figure 4).

Percentages of $CXCR5^+$ $CD4^+$ or $CD8^+$ T cells were similar in all patient groups. Nevertheless, lower levels of $CXCR5^+$ T-helper cell counts were observed in SSA+SjS compared to *Sicca* ($p = 0.026$), and in all patient groups compared to HC ($p \leq 0.022$).

Table 2 Comparison of T and B cell subsets' percentages in SjS, *Sicca*, and healty control groups

	Sjögren's Syndrome			*Sicca*	HC	Group's comparisons (*p*-values)					
	Total SjS *n* = 53	SSA+SjS *n* = 38	SSA-SjS *n* = 15	*n* = 72	*n* = 24	SSA+ vs SSA-	SSA+ vs *Sicca*	SSA+ vs HC	SSA- vs *Sicca*	SSA- vs HC	*Sicca* vs HC
Lymphocytes (within leukocytes)	31,5 (23,4-38,1)	31,7 (23,9-36,4)	31,5 (22,3-40,2)	32,2 (25,7-37,1)	36,7 (29,3-39,8)	0,499	0,531	*0,034*	0,900	0,242	*0,027*
B cells	9,7 (6,9-13,3)	9,8 (5,9-16,0)	9,7 (7,0-11,0)	11,7 (8,7-14,8)	10,4 (8,6-13,7)	0,636	0,319	0,723	*0,023*	0,231	0,392
T cells	74,8 (67,3-78,5)	74,9 (67,3-78,5)	74,6 (70,5-78,8)	73,1 (67,6-77,3)	74,7 (70,1-79,6)	0,721	0,409	0,391	0,266	0,836	0,059
T-cell Subsets											
$CD4^+$	61,2 (53,1-66,6)	61,2 (51,2-66,9)	59,7 (55,1-65,1)	66,0 (60,0-72,6)	69,1 (60,8-75,9)	0,897	*0,004*	*0,001*	*0,032*	*0,006*	0,227
Th1	40,3 (28,6-46,0)	38,1 (27,2-45,9)	41,2 (32,1-46,3)	38,6 (30,2-47,4)	35,7 (31,7-45,9)	0,383	0,535	0,878	0,620	0,464	0,625
Th17	19,5 (13,9-28,6)	18,0 (13,8-27,2)	26,6 (19,2-31,6)	22,7 (16,4-28,7)	23,1 (17,0-31,7)	0,154	0,085	0,089	0,603	0,916	0,630
Tregs	8,2 (6,5-10,4)	8,6 (7,1-10,6)	7,1 (6,0-8,5)	7,6 (6,7-8,9)	7,4 (6,7-8,8)	0,085	*0,007*	0,067	0,464	0,535	0,948
CD8+	38,8 (33,0-46,9)	38,6 (32,5-48,9)	40,4 (34,9-44,9)	34,1 (27,4-39,7)	30,9 (23,4-39,2)	0,911	*0,010*	*0,004*	*0,025*	*0,007*	0,231
$CXCR5^+CD4^+$ T-cells (within CD4+ T-cells)	18,4 (14,7-23,6)	18,5 (14,2-23,0)	16,9 (15,3-24,7)	18,3 (14,9-21,5)	20,8 (17,4-22,9)	0,615	0,907	0,214	0,580	0,892	0,121
Surface expression of CCR7*											
Naïve ($CCR7^+$)	18,0 (14,5-23,2)	18,0 (13,9-22,0)	16,7 (14,9-23,7)	18,1 (14,7-21,1)	20,4 (17,2-22,2)	0,594	0,665	0,182	0,611	1.000	0,137
Differentiated ($CCR7^-$)	0,4 (0,3-0,9)	0,5 (0,3-0,9)	0,4 (0,3-0,6)	0,3 (0,2-0,5)	0,4 (0,3-0,7)	0,528	*0,003*	0,411	0,094	0,915	0,060
Surface expression of CCR3 and CCR6											
$CXCR5^+CD4^+$ Th1 ($CCR3^+CCR6^-$)	36,2 (30,7-42,4)	37,6 (30,9-44,0)	34,1 (30,3-37,8)	31,4 (28,8-36,8)	31,7 (27,3-35,5)	0,204	*0,003*	*0,012*	0,339	0,280	0,538
$CXCR5^+CD4^+$ Th17 ($CCR3^-CCR6^+$)	20,5 (16,6-26,7)	19,4 (15,8-24,7)	25,6 (20,4-28,6)	22,9 (18,9-27,3)	20,9 (17,6-29,1)	*0,036*	*0,024*	0,240	0,465	0,351	0,547

Table 2 *(Cont.)*

	Sjögren's Syndrome			*Sicca*	HC	Group's comparisons (*p*-values)					
	Total SjS *n* = 53	SSA+SjS *n* = 38	SSA-SjS *n* = 15	*n* = 72	*n* = 24	SSA+ vs SSA-	SSA+ vs *Sicca*	SSA+ vs HC	SSA- vs *Sicca*	SSA- vs HC	*Sicca* vs HC
	9,9 (7,0-12,8)	9,3 (6,8-12,7)	10,0 (7,7-14,0)	8,8 (6,5-10,7)	8,2 (5,8-10,1)	0,537	0,106	0,078	0,064	0,051	0,563
$CXCR5^{+}CD4^{+}$ $CCR3^{-}CCR6^{-}$	31,9 (26,8-38,1)	32,3 (26,2-38,4)	31,7 (27,2-33,2)	35,0 (31,2-41,9)	37,0 (31,5-43,3)	0,515	0,054	*0,026*	*0,019*	*0,009*	0,418
$CXCR5^{+}CD8^{+}$ T^{-}cells (within $CD8^{+}$ T^{-}cells)	2,9 (1,9-3,7)	2,9 (1,8-3,6)	2,6 (2,0-4,2)	2,8 (2,1-4,2)	3,4 (2,0-3,8)	0,949	0,534	0,360	0,650	0,716	0,755
Surface expression of CCR7											
Naïve ($CCR7^{+}$)	1,9 (1,2-2,9)	2,0 (1,2-3,0)	1,8 (1,2-2,8)	1,9 (1,4-3,2)	2,1 (1,5-3,2)	0,707	0,757	0,608	0,578	0,419	0,713
Differentiated ($CCR7^{-}$)	0,6 (0,4-1,0)	0,6 (0,3-0,9)	0,6 (0,5-1,5)	0,8 (0,5-1,1)	0,8 (0,3-1,4)	0,249	0,106	0,535	0,850	0,516	0,745
$IL^{-}21$ production											
$CD4^{+}$											
$IL21^{+}$	12,5 (8,5-14,9)	13,3 (10,1-16,3)	8,5 (6,9-13,4)	9,6 (7,5-12,6)	9,7 (6,3-11,9)	*0,015*	0,000	*<0,001*	0,707	1.000	0,664
$IL21^{+}IL17^{+}$	0,7 (0,6-0,9)	0,7 (0,6-0,9)	0,7 (0,4-1,2)	0,7 (0,4-1,0)	0,7 (0,3-1,1)	0,721	0,549	0,603	0,971	0,695	0,875
$CD8^{+}$											
$IL21^{+}$	4,3 (2,3-5,8)	4,6 (2,5-7,4)	3,5 (1,3-4,8)	3,1 (2,1-4,4)	2,8 (1,0-4,4)	0,071	*0,009*	*0,008*	0,787	0,537	0,317
$IL21^{+}IL17^{+}$	0,3 (0,2-0,5)	0,4 (0,2-0,5)	0,2 (0,1-0,4)	0,2 (0,2-0,5)	0,3 (0,2-0,8)	*0,033*	0,101	0,900	0,287	0,059	0,176
B^{-}cell Subsets											
IgD/CD27 (within B^{-}cells)											
Naïve	66,6 (51,6-77,4)	70,3 (52,7-78,5)	62,7 (46,9-74,8)	67,3 (51,0-75,7)	53,0 (43,2-69,2)	0,281	0,429	*0,032*	0,546	0,282	0,066

Table 2 *(Cont.)*

	Sjögren's Syndrome			*Sicca*	HC	Group's comparisons (*p*-values)					
	Total SjS *n* = 53	SSA+SjS *n* = 38	SSA-SjS *n* = 15	*n* = 72	*n* = 24	SSA+ vs SSA-	SSA+ vs *Sicca*	SSA+ vs HC	SSA- vs *Sicca*	SSA- vs HC	*Sicca* vs HC
Memory	29,5 (20,2-45,2)	27,8 (19,5-44,3)	33,4 (23,1-48,8)	30,8 (22,1-44,9)	44,5 (27,4-54,7)	0,212	0,292	*0,011*	0,670	0,199	*0,042*
Unswitched memory	13,5 (8,7-21,6)	13,1 (8,4-20,6)	14,8 (10,2-25,5)	15,9 (11,0-24,9)	21,6 (14,6-32,2)	0,342	0,074	*0,004*	0,796	0,144	0,078
Switched memory	14,5 (10,0-22,5)	13,3 (9,8-21,6)	18,6 (12,2-24,1)	14,5 (10,2-21,6)	19,5 (13,7-26,5)	0,243	0,741	0,055	0,217	0,733	*0,049*
$CD24^{Hi}CD38^{Hi}$	6,3 (2,8-9,6)	6,3 (2,2-11,0)	4,2 (3,2-7,2)	4 (2,2-5,5)	3,2 (2,4-5,8)	0,518	*0,006*	0,058	0,146	0,194	0,819
$CD24^{Hi}CD27^{+}$	16,9 (10,1-27,6)	15,0 (9,0-24,7)	19,6 (12,7-38,4)	21,3 (14,0-34,1)	32,0 (21,7-42,5)	0,173	*0,014*	*<0,001*	0,813	0,122	*0,031*
Plasmablasts $IgM^{-/+}CD38^{++}$	2,0 (0,9-3,5)	2,5 (1,2-4,5)	1,9 (0,7-2,7)	1,2 (0,7-1,9)	1,4 (1,0-1,9)	0,114	**0,001**	**0,032**	0,576	0,739	0,500

Mann-Whitney nonparametric *U* test was used for group's comparison. Results are presented as medians and interquartile range, median (IQR). Statistically significant results are indicated in italic.

SjS, Siögren 's syndrome: *HC*, healthy controls; *IQR*, interquartile range.

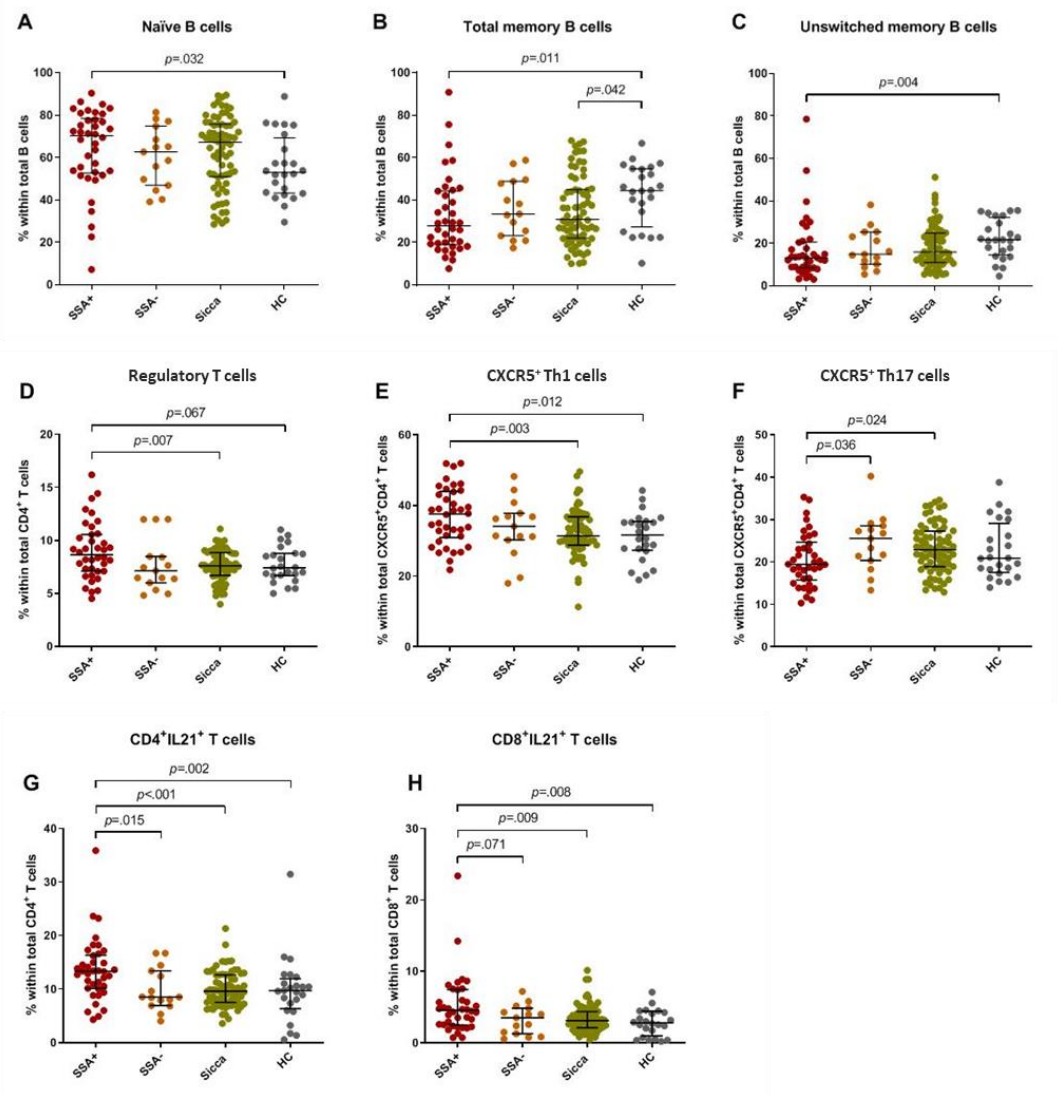

Figure 3 Distributions of T and B-cell subsets' percentages in all groups.

Regarding CXCR5 $^{+}$Th subsets, CXCR5^{+}Th1 (CCR3CCR6−) percentages were higher in SSA+SjS than in *Sicca* ($p = 0.003$) and HC ($p = 0.012$), while CXCR5^{+}Th17 (CCR3− CCR6^{+}) percentages were lower in SSA+SjS compared to SSA−SjS ($p = 0.036$) and *Sicca* ($p = 0.024$). However, CXCR5^{+}Th1 counts were lower in SSA+SjS and *Sicca* compared to HC ($p \leq 0.001$), whereas SSA+SjS presented lower CXCR5^{+}Th17 counts compared to all groups ($p \leq 0.015$) (Fig. 3). To investigate possible relations with disease activity, we further explored the association between Tfh-related subsets and the ESSDAI (Fig. 4). SSA+SjS patients showed a trend for a positive correlation between ESSDAI and CXCR5^{+}Th1 percentages ($r = 0.294$, $p = 0.073$), which was statistically significant in patients with clinically active disease ($r = 0.616$, $p = 0.003$), and among those, even stronger when considering patients with moderate/high disease activity ($r = 0.927$, $p = 0.002$). Contrarily, ESSDAI and CXCR5^{+}Th17 percentages ($r = -0.341$, $p = 0.036$) showed a negative correlation, also slightly stronger in patients with active disease ($r = -0.456$, $p = 0.038$). Despite the low number of patients with moderate/high disease activity, a similar trend was also observed for ESSDAI and

CXCR5^{+}Th17 percentages (r = − 0.659, p = 0.088) in this group. No correlations were found between Tfh-related subsets and ESSDAI in SSA−SjS patients.

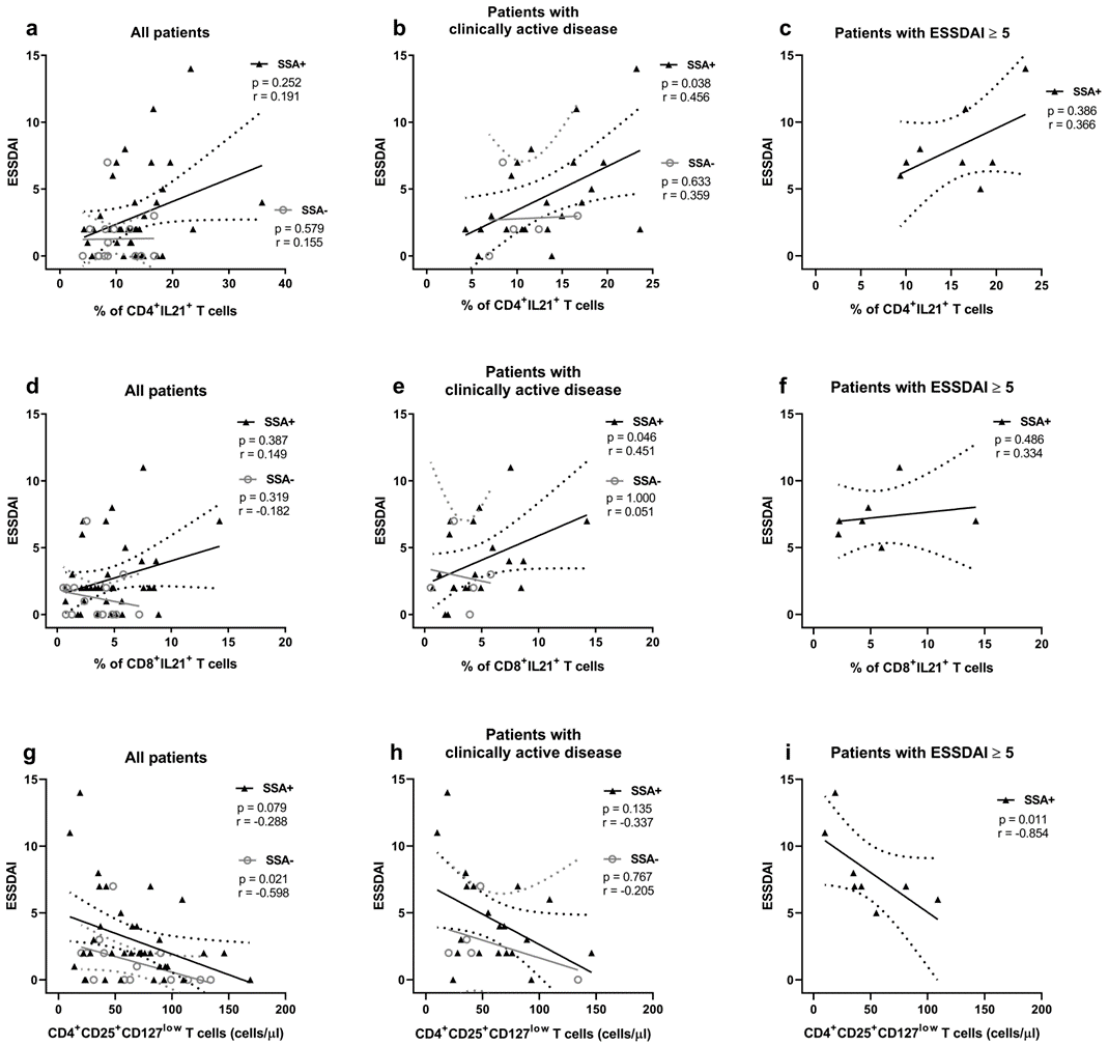

Figure 4 Correlation between Treg counts and the ESSDAI.

IL-21-secreting CD4 and CD8 T cells

We found increased percentages of IL-21^{+}CD4^{+} T cells (13.3%) in SSA+SjS compared to SSA −SjS (8.5%, p = 0.015), *Sicca* (9.6%, p < 0.001), and HC (9.7%, p = 0.002), without differences in absolute counts. Similarly, IL-21^{+}CD8^{+} T cells percentages were significantly increased in SSA+SjS (4.6%) compared to *Sicca* (3.1%, p = 0.009) and HC (2.8%, p = 0.008), and tendentially significant compared to SSA −SjS (3.12%, p = 0.071) (Fig. 3).

Contrary to SSA −SjS, SSA+SjS patients showed higher IL-21^{+}CD8^{+} T cell counts compared to *Sicca* (p = 0.031). Furthermore, ESSDAI positively correlated with both

IL21^{+}CD4^{+} (r = 0.456, p = 0.038) and IL-21^{+}CD8^{+} (r = 0.451, p = 0.046) T cells in SSA+SjS patients with clinically active disease, but not in SSA−SjS (Fig. 4).

B cell subsets

The levels of total B cells were similar between groups, though increased percentages of B cells were observed in *Sicca* compared to SSA −SjS (p = 0.023), and decreased counts were present in SSA+SjS compared to HC (p = 0.033). We found no differences in the distinct IgD/CD27 B cell subsets between SSA+SjS and SSA −SjS. Interestingly, SSA+SjS patients showed increased naïve (p = 0.032) and decreased unswitched memory B cell percentages (p = 0.004) compared to HC. No differences were observed between SSA−SjS and the remaining groups, though these patients presented lower unswitched and switched memory B cell counts than HC ($p \leq 0.025$). *Sicca* patients also presented decreased percentages and absolute counts of total memory ($p \leq 0.042$) and switched memory ($p \leq 0.049$) B cells compared to HC.

When analyzing subsets related to regulatory B cells, memory CD24^{Hi}CD27^{+} B cells showed lower counts and percentages in SSA+SjS patients than in *Sicca* ($p \leq 0.014$) and HC (< 0.001). CD24^{Hi}CD27^{+} B cell percentages were similar in SSA−SjS and HC, although SSA−SjS presented decreased counts (p = 0.006). CD24^{Hi}CD38^{Hi} B cell percentages were higher in SSA+SjS compared to *Sicca* (p = 0.006).

Concerning IgM^{-}/$^{+}$CD38^{Hi} plasmablasts, SSA+SjS showed increased percentages compared to *Sicca* (p = 0.001) and HC (p = 0.032).

Discussion

SjS patients display great clinical and biological heterogeneity [17].

We explored the clinical and immunological features of SSA^{+}/SSA − patients focusing on the lymphocyte subpopulations distribution and its association with disease activity.

In our study, SSA+SjS patients presented higher proportions of IL-21-producing CD4^{+} and CD8^{+} T cells, a pronounced decrease in memory B cells, and increased naïve B cells, compared to HC. Moreover, SSA+SjS patients presented higher disease activity, and associations between ESSDAI and several lymphocyte subsets, including IL-21^{+}-producing CD4^{+} and CD8^{+} T cells, and Tregs. To our knowledge, this is the first study to approach CXCR5^{+}Tc cells in SjS patients according to anti-SSA positivity.

In SjS, the presence of anti-Ro/SSA and anti-La/SSB is a dominant feature, reflecting the hyperactivity of autoreactive B cells/plasma cells [18, 19]. Anti-SSA antibodies are associated with younger age of disease onset, worst exocrine gland function, and parotid gland enlargement [20], as well as several extra-glandular manifestations [13]. An increased risk of lymphoma is predicted by clinical adverse prognostic factors, along with the disease activity (ESSDAI scores) [21].

Our SSA+SjS patients had a younger age of onset and, conversely, a longer disease duration compared to SSA−SjS. ESSDAI was higher in SSA+SjS, but due to the small sample, clinical differences were not statistically significant, although SG swelling and Raynaud's phenomenon only occurred in SSA+SjS. RF and hypergammaglobulinemia were more prevalent in SSA+SjS. All these differences are in accordance with published data [13, 20].

In SjS patients, we found not only T cell lymphopenia but also decreased B cell counts, which also occurred to a lesser degree in *Sicca* patients.

Mandl [22] found significantly lower $CD4^+$ T cell counts and percentages in SjS, and lower absolute $CD4^+$ T cell counts in SSA+SjS, compared to *Sicca* patients. Another study showed that SSA^+/SSB^+SjS patients had decreased circulating $CD4^+$ T cells compared to SSA−/SSB−SjS and HC [23]. Also, $CD4^+$ T cell numbers were lower in both SjS groups compared to HC and in SSA^+/SSB^+SjS compared to SSA−/SSB−SjS [23]. Our findings are partially in line with these studies, as both SSA+SjS and SSA−SjS groups had lower $CD4^+$ T cells compared to *Sicca* and HC groups. Interestingly, $CD4^+$ T cell counts were also lower in *Sicca* compared to HC.

Lymphopenia in SjS has been attributed to the migration of peripheral blood $CD4^+$ T cells to the exocrine glands [24] and therefore may traduce a more active disease profile. However, it has been recently proposed that decreased T cells in SjS represent a senescent phenotype due to earlier increased proliferation on naïve $CD4^+$ T cells contributing to their later exhaustion [25]. Epigenetic cell counting, a promising novel tool to quantify immune cells in the salivary glands, may contribute to a better understanding of cell distribution and immunopathology [26].

The higher percentages of $CD8^+$ T cells found in both SjS groups compared to *Sicca* and HC differ from Mandl et al. [22] study, which found no differences in $CD8^+$ T cells percentages while describing, as we did, lower $CD8^+$ T cell counts in both SSA^+ and SSA−SjS [22].

Follicular T cells participate in T cell–dependent B cell responses in ectopic lymphoid structures. Typically, they express the follicular homing chemokine receptor CXCR5, which guides their migration into B cell follicles, and secrete the B cell helper cytokine IL-21 [27].

Increased Tfh frequencies have been described in the blood and SG of SjS (recently reviewed by Verstappen [5]), possibly related to extra-glandular manifestations [5, 28]. Our results showed no differences concerning $CXCR5^+CD4^+$ T cell frequencies between SjS (SSA^+ and SSA−) and the other groups, while lower numbers were found in SSA+SjS compared to *Sicca* and HC. When addressing $CXCR5^+$Th1 and Th17 profiles, according to CXCR3 and CCR6 expression, we found higher frequencies of $CXCR5^+$Th1 and lower frequencies of $CXCR5^+$Th17 in SSA+SjS patients, especially when comparing SSA+SjS with SSA−SjS patients. Moreover, SSA+SjS had lower $CXCR5^+$Th17 counts compared to the other groups, and Th17 percentages were lower in SSA+SjS compared to SSA− and *Sicca*.

Despite some reports describe decreased circulating Tfh1 in SjS patients compared to HC, and higher or similar Tfh17 levels [29, 30], Aqrawy [31] observed a significant decrease of $CXCR5^+$Th17 frequencies in SjS. Our data are consistent with the later study,

supporting possible retention of CXCR5^{+} Th17 cells in the SG at the site of inflammation, thus decreasing their circulating levels. The lower frequencies found in SSA+SjS patients might traduce a more intense SG accumulation in these patients, although a more specific phenotypic characterization of Tfh cells (PD1 marker) was not performed.

A positive correlation between ESSDAI and CXCR5^{+Th}1 percentages was observed in SSA+SjS patients, stronger in patients with moderate/high disease activity. By contrast, a negative correlation between ESSDAI and CXCR5^{+}Th17 percentages was present. Previous reports correlated Tfh17- like cells with disease activity and antibody production, suggesting also increased expression of effector cell markers such as PD1, CD40L, and IL-21 in these cells compared to Tfh1 cells [30]. Since the associations between ESSDAI score and CXCR5^{+}Th subsets only occurred in SSA+SjS patients, we admit that the immune mechanisms underlying T cell dynamics and disease manifestations may differ according to the presence of anti-SSA antibodies.

Follicular T cells are major sources of IL-21, an autocrine cytokine that regulates GC responses, B cell activation, and immunoglobulin production [32]. Thus, we characterized IL21 production by CD4^{+} and CD8^{+} T cells to identify T cells with a follicular phenotype. SSA+SjS patients presented higher percentages of IL-21-secreting CD4^{+} T cells compared to all other groups, and higher percentages of IL-21-secreting CD8^{+} T cells compared to *Sicca* and HC. Our results suggest that, among SjS patients, those SSA^{+} may have a different biological and clinical profile, with more intense GC-like reactions. This is in line with a recent study by Pontarini [33], which showed that circulating CXCR5^{+}ICOS^{+}PD-1^{+} Tfh subsets producing high levels of both IL-21 and IFN-γ were enriched in SjS patients with anti-Ro/SSA and anti-La/SSB autoantibodies, especially those with ectopic lymphoid structures, and positively correlated with SG focus score.

Follicular CD8^{+} T cells have been identified in the synovial ectopic follicles of patients with rheumatoid arthritis [34], and recent data confirmed an important role of CD8^{+} T cells in the development and progression of the SG lesions in SjS [35]. Our data, showing an increased follicular function in SSA+SjS patients, assessed by IL-21 production by CD4^{+} and CD8^{+} T cells, support an important role of both subsets for antibody production by the infiltrating B cells.

Noteworthy, we have also proven a strong positive correlation between ESSDAI and both IL-21^{+}CD4^{+} and IL21^{+}CD8^{+} T cells in SSA+SjS patients with clinically active disease, a feature not found in SSA−SjS patients. This is in accordance with Mingueneau's study [24], where the frequencies of circulating HLA-DR-expressing activated CD8^{+} and CD4^{+} T cells correlated with the ESSDAI. Our observation that IL-21^{+}CD8^{+} T cells presented the same association with disease activity as IL-21^{+}CD4^{+} T cells suggest a role of these cells in B cell responses. Therefore, the increase in circulating follicular-related CD8^{+} T cells in SSA+SjS probably indicates their role in the active clinical profile of these patients. Considering the fundamental role of IL-21 in GC dynamics, inducing B cell generation and differentiation into plasma cells, and that increased serum levels of IL-21 have been detected in patients

with immune-mediated diseases, including SjS[36], our findings of increased percentages of IL-21- producing $CD4^+$ and $CD8^+$ T cells in SSA+SjS patients are not surprising.

For Treg characterization, we used the phenotype $CD4^+CD25^+CD127^{low}$ recommended by the Human Phenotyping Project [37], as these cells significantly express Foxp3 and correlate positively with the classic regulatory T cell $CD4^+CD25^+Foxp3^+$ [8].

Although both groups of SjS, as well as *Sicca* patients, had lower Treg counts compared to HC, SSA+SjS patients presented higher percentages compared to the other groups. Gottenberg [38] had previously described a higher proportion of functional $CD4^+CD25^{high}$ regulatory T cells in SjS, especially in patients with extra-glandular manifestations or antiSSA/SSB antibodies, suggesting reactive feedback. Recently, higher levels of Tregs have also been reported by Maria [39] (in SjS patients with positive Interferon gene expression signature) and Verstappen [40]. However, Tregs are controversial in SjS [9]. Sudzius [23] did not find significant differences in circulating $CD4^+CD25^+Foxp3^+$ T cells in SjS patients with and without anti-SSA antibodies. Nevertheless, in their study, both patients and controls presented low levels of $CD4^+CD25^+Foxp3^+$ T cells (< 1.5% of total CD4 T cells) [41].

A negative correlation between the frequency of circulating $CD4^+CD25^+Foxp3^+$ cells and the proportion of Tregs infiltrating the SG was reported in SjS [42]. Moreover, patients with advanced pathologic lesions had higher circulating levels of $Foxp3^+$ cells and tissue infiltration by Tregs was reduced. Additionally, this study showed that $CD4^+CD25^+CD127^{low}$ T cell frequencies paralleled $Foxp3^+$ T cells in SjS and controls, supporting our choice for the $CD4^+CD25^+CD127^{low}$ phenotype.

Interestingly, in SSA+SjS with moderate/high disease activity, we found a strong negative correlation between ESSDAI and circulating Treg counts. Considering our data and the previous observations from Christodoulou [42], activity scores seem to proportionally increase to the level of infiltrating Tregs, relating to a decrease in circulating levels. Nevertheless, it cannot be excluded that the association between decreased Treg counts and ESSDAI may be simply a reflex of the lymphopenia in patients with more active disease. However, other authors failed to encounter associations between disease activity and circulating Tregs [9].

SSA+SjS patients presented several features of B cell activation, in accordance with our previous data [16], with higher proportions of naïve mature B cells and lower memory B cells compared to HC.

The increase in naïve mature B cells is widely described for SjS and has been attributed to impairment of early B cell tolerance checkpoints [3]. Higher proportions of naïve B cells in SSA+SjS support a more severe immune dysregulation. The mobilization of self-reactive naïve B cells from the bone marrow to the periphery is increased in SjS [3], and recently Glauzy [43] found that among the expanded naïve B cells in SjS, most clones were polyreactive, pointing to their emergence from defective central and peripheral B cell tolerance checkpoints in SjS patients.

Also, increased migration of memory B cells to the affected SG occurs [44], accompanied by a shift in B cell differentiation towards plasma cells [45]. The consequent increased production of autoantibodies in the SG appears to strongly reflect the serologic humoral response [46]. Therefore, we admit that our SSA+SjS patients have a greater reduction in circulating memory B cells due to their migration to the SG, reinforced by the more frequent occurrence of SG enlargement and hypergammaglobulinemia, both of which are markers of B cell activation in SjS [47].

Additionally, $IgM^{-/+}CD38^{Hi}$ plasmablast percentages were increased in SSA+SjS compared to both *Sicca* and HC, whereas no differences were found comparing SSA−SjS. This is in line with the proposed identification of this population as plasma cell precursors, responsible for antibody production in SjS.

SSA−SjS also presented increased naïve and decreased memory B cell percentages and numbers compared to HC, although the small number of patients did not allow for further clarification.

The frequencies of new emigrant/transitional B cells expressing polyreactive B cell receptors were shown to be increased in SjS patients, a fact attributed to defective central B cell tolerance in SjS [43]. Considering $CD24^{Hi}CD38^{Hi}$ B cells as transitional cells, their significant increase in SSA+SjS may suggest a greater defect in central B cell tolerance allowing for their increased release into the circulation.

$CD24^{Hi}CD38^{Hi}$ B cells, as well as $CD24^{Hi}CD27^{+}$ B cells, are populations known to be enriched in regulatory cells [48]. In our study, decreased numbers and percentages of $CD24^{Hi}CD27^{+}$ B cells and increased $CD24^{Hi}CD38^{Hi}$ B cells occurred in SSA+SjS, whereas SSA−SjS only presented decreased numbers of $CD24^{Hi}CD27^{+}$ B cells. Our results on these populations are difficult to interpret as functional assays for IL-10 production were not performed. However, decreased $CD24^{hi}CD27^{+}$ B cells and a concomitantly reduced frequency of $IL10^{+}CD24^{hi}CD27^{+}$ B cells were reported in systemic lupus erythematosus (SLE) patients compared to HC [49]. The authors also reported decreased $CD24^{hi}CD38^{hi}$B cell frequency in SLE patients, but no differences in $IL10^{+}CD24^{hi}CD38^{hi}$ B cells. Despite the changes found for $CD24^{Hi}CD38^{Hi}$ and $CD24^{Hi}CD27^{+}$ B cells in our study, the frequencies and function of IL-10-producing cells among those subsets remain undetermined.

We acknowledge limitations in our study, such as the inclusion of patients with a broad range of age and disease duration, variable disease activity and severity, and exclusive peripheral blood evaluation. Additionally, a significant number of patients in both groups were under glucocorticoid and/ or hydroxychloroquine therapy, which could potentially influence lymphocyte subset distribution, although we consider our population to be representative of the daily clinical practice. We also recognize the potential risk of bias due to multiple paired testing in our results. Finally, the use of different markers and phenotypic approaches can explain inconsistencies with the literature, and the absence of functional assays for regulatory T and B cells may overlook potential functional deficiencies in our study.

Our data support that SSA+SjS patients have more active disease, including markers of B cell activity such as hypergammaglobulinemia, RF, and SG enlargement. Overall, it seems that the underlying immune mechanisms differ according to the presence of anti-SSA antibodies, with a distinct lymphocyte subset profile observed in SSA+SjS patients. The more pronounced naïve/memory B cell imbalance in SSA+SjS patients reinforces the relevance of the expanded follicular T cell compartment and B cell activity. Furthermore, the association between ESSDAI and $IL21^{+}CD4^{+}$ and $IL\text{-}21^{+}CD8^{+}$ follicular-related T cells suggests the possible involvement of these cells in the pathogenesis of the disease and their possible application for prognosis and monitoring of disease activity, particularly in SSA+SjS patients.

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Authors' contribution F Barcelos and C Martins conceived the original research idea, while all of the authors designed the study and created the study protocol. F Barcelos, J Vaz-Patto, and N Madeira recruited the patients and collected the data. J Cardigos and N Alves recruited the healthy controls and collected the data. C Martins analyzed the blood samples using flow cytometry. C Martins and M Ângelo-Dias performed the statistical analysis. JC Branco and L-M Borrego supervised all the work and the research protocol. All of the authors contributed to data analysis and interpretation. F Barcelos and N Madeira drafted the manuscript, and all of the authors revised it and contributed to it intellectually. All of the authors have approved the final version of the manuscript.

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Compliance with ethical standards

This study was approved by the Ethics committee of *Hospital CUF Descobertas*, 8/09/2014, Ethics committee of *Instituto Português de Reumatologia*, 3/07/2015 and NOVA Medical School Ethics (no 17/ 2016/CEFCM).

Disclosures None.

Consent to participate All patients have signed an informed consent to participate, according to the Declaration of Helsinki.

Consent to publication All authors have revised the final version of the manuscript and gave the consent to publication.

References

1. Ramos-Casals M, Brito-Zeron P, Siso-Almirall A, Bosch X (2012) Primary Sjogren syndrome. *Bmj* **344**:e3821–e3821. https://doi.org/10.1136/bmj.e3821
2. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, Rasmussen A, Scofield H, Vitali C, Bowman SJ, Mariette X, the International Sjögren's Syndrome Criteria Working Group, Heidenreich AM, Lanfranchi H, Vollenweider C, Schiødt M, Devauchelle V, Gottenberg JE, Saraux A, Pincemin M, Dörner T, Tzoufias A, Baldini C, Bombardieri S, de Vita S, Kitagawa K, Sumida T, Umehara H, Bootsma H, Kruize AA, Radstake TR, Vissink A, Jonsson R, Ramos-Casals M, Theander E, Challacombe S, Fisher B, Kirkham B, Larkin G, Ng F, Rauz S, Akpek E, Atkinson J, Baer AN, Carsons S, Carteron N, Daniels T, Fox B, Greenspan J, Illei G, Nelson D, Parke A, Pillemer S, Segal B, Sivils K, St.Clair EW, Stone D, Vivino F, Wu A (2017) 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for primary Sjögren's syndrome: a consensus and data-driven methodology involving three international patient cohorts. *Arthritis Rheumatol* **69**:35–45. https://doi.org/10.1002/art.39859
3. Corsiero E, Sutcliffe N, Pitzalis C, Bombardieri M (2014) Accumulation of self-reactive naïve and memory B cell reveals sequential defects in B cell tolerance checkpoints in Sjögren's Syndrome. *PLoS One* **9**:e114575. https://doi.org/10.1371/journal. pone.0114575
4. Salomonsson S, Jonsson MV, Skarstein K, Brokstad KA, Hjelmström P, Wahren-Herlenius M, Jonsson R (2003) Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjögren's syndrome. *Arthritis Rheum* **48**:3187–3201. https://doi.org/10.1002/art.11311
5. Verstappen GM, Kroese FGM, Bootsma H (2019) T cells in primary Sjögren's syndrome: targets for early intervention. *Rheumatology* **1**–11 https://doi.org/10.1093/rheumatology/kez004
6. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, Corcoran LM, Godfrey DI, Toellner KM, Smyth MJ, Nutt SL, Tarlinton DM (2010) IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. *J Exp Med* **207**:365–378
7. Quigley MF, Gonzalez VD, Granath A et al (2007) CXCR5^{+} CCR7- CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. *Eur J Immunol* **37**:3352–3362. https://doi.org/10.1002/eji.200636746
8. Yu N, Li XX, Song W, Li D, Yu D, Zeng X, Li M, Leng X, Li X (2012) CD4^{+}CD25^{+}CD127low/− T cells: a more specific Treg population in human peripheral blood. *Inflammation* **35**:1773–1780. https://doi.org/10.1007/s10753-012-9496-8
9. Alunno A, Carubbi F, Bistoni O et al (2015) T regulatory and T helper 17 cells in primary Sjögren's syndrome: facts and perspectives. *Mediators Inflamm*. 2015:**1**–10
10. Bohnhorst JØ, Thoen JE, Natvig JB, Thompson KM (2001) Significantly depressed percentage of CD27^{+} (memory) B cells among peripheral blood B cells in patients with primary Sjogren's syndrome. *Scand J Immunol* **54**:421–427. https://doi.org/10.1046/j.1365-3083.2001.00989.x
11. Achour A, Simon Q, Mohr A, Séité JF, Youinou P, Bendaoud B, Ghedira I, Pers JO, Jamin C (2017) Human regulatory B cells control the TFH cell response. *J Allergy Clin Immunol* **140**:215–222. https://doi.org/10.1016/j.jaci.2016.09.042
12. Lin X, Wang X, Xiao F, Ma K, Liu L, Wang X, Xu D, Wang F, Shi X, Liu D, Zhao Y, Lu L (2019) IL-10-producing regulatory B cells restrain the T follicular helper cell response in primary Sjögren's syndrome. *Cell Mol Immunol* **16**:921–931. https://doi.org/10.1038/s41423-019-0227-z
13. Ramos-Casals M, Solans R, Rosas J, Camps MT, Gil A, del PinoMontes J, Calvo-Alen J, Jiménez-Alonso J, Micó ML, Beltrán J, Belenguer R, Pallarés L, GEMESS Study Group (2008) Primary Sjögren syndrome in Spain: clinical and immunologic expression in 1010 patients. *Medicine (Baltimore)* **87**:210–219. https://doi.org/10.1097/MD.0b013e318181e6af

14. Seror R, Ravaud P, Bowman SJ, Baron G, Tzioufas A, Theander E, Gottenberg JE, Bootsma H, Mariette X, Vitali C, EULAR Sjögren's Task Force (2010) EULAR Sjogren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjogren's syndrome. *Ann Rheum Dis* **69**:1103–1109. https://doi.org/10.1136/ard.2009.110619
15. Seror R, Bootsma H, Saraux A, Bowman SJ, Theander E, Brun JG, Baron G, le Guern V, Devauchelle-Pensec V, Ramos-Casals M, Valim V, Dörner T, Tzioufas A, Gottenberg JE, Solans Laqué R, Mandl T, Hachulla E, Sivils KL, Ng WF, Fauchais AL, Bombardieri S, Priori R, Bartoloni E, Goeb V, Praprotnik S, Sumida T, Nishiyama S, Caporali R, Kruize AA, Vollenweider C, Ravaud P, Meiners P, Brito-Zerón P, Vitali C, Mariette X, EULAR Sjögren's Task Force (2016) Defining disease activity states and clinically meaningful improvement in primary Sjögren's syndrome with EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient-reported indexes (ESSPRI). *Ann Rheum Dis* **75**:382–389. https://doi.org/10.1136/annrheumdis-2014- 206008
16. Barcelos F, Martins C, Papoila A, Geraldes C, Cardigos J, Nunes G, Lopes T, Alves N, Vaz-Patto J, Branco J, Borrego LM (2018) Association between memory B-cells and clinical and immunological features of primary Sjögren's syndrome and *Sicca* patients. *Rheumatol Int* **38**:1063–1073. https://doi.org/10.1007/s00296- 018-4018-0
17. Quartuccio L, Baldini C, Bartoloni E, Priori R, Carubbi F, Corazza L, Alunno A, Colafrancesco S, Luciano N, Giacomelli R, Gerli R, Valesini G, Bombardieri S, de Vita S (2015) Anti-SSA/SSB-negative Sjögren's syndrome shows a lower prevalence of lymphoproliferative manifestations, and a lower risk of lymphoma evolution. *Autoimmun Rev* **14**:1019–1022. https://doi.org/10.1016/j.autrev. 2015.07.002
18. Tengnér P, Halse AK, Haga HJ, Jonsson R, Wahren-Herlenius M (1998) Detection of anti-Ro/SSA and anti-La/SSB autoantibodyproducing cells in salivary glands from patients with Sjogren's syndrome. *Arthritis Rheum* **41**:2238–2248 https://doi.org/10.1002/1529-0131(199812)41:12<2238::AID-ART20>3.3.CO;2-M
19. Stott DI, Hiepe F, Hummel M, Steinhauser G, Berek C (1998) Antigen-driven clonal proliferation of B cells within the target tissue of an autoimmune disease. The salivary glands of patients with Sjogren's syndrome. *J Clin Invest* **102**:938–946. https://doi.org/10. 1172/JCI3234
20. Tzioufas AG, Tatouli IP, Moutsopoulos HM (2012) Autoantibodies in Sjögren's syndrome: clinical presentation and regulatory mechanisms. *Press Méd* (Paris, Fr 1983) **41**:e451–e460. https://doi.org/10.1016/j.lpm.2012.05.022
21. Nocturne G, Virone A, Ng WF, le Guern V, Hachulla E, Cornec D, Daien C, Vittecoq O, Bienvenu B, Marcelli C, Wendling D, Amoura Z, Dhote R, Lavigne C, Fior R, Gottenberg JE, Seror R, Mariette X (2016) Rheumatoid factor and disease activity are independent predictors of lymphoma in primary Sjögren's syndrome. *Arthritis Rheumatol* **68**:977–985. https://doi.org/10.1002/art.39518
22. Mandl T, Bredberg A, Jacobsson LTH, Manthorpe R, Henriksson G (2004) $CD4^+$ T-lymphocytopenia–a frequent finding in anti-SSA antibody seropositive patients with primary Sjögren's syndrome. *J Rheumatol* **31**:726–728
23. Sudzius G, Mieliauskaite D, Siaurys A et al (2015) Distribution of peripheral lymphocyte populations in primary Sjögren's syndrome patients. *J Immunol Res* 2015:854706. https://doi.org/10.1155/2015/854706
24. Mingueneau M, Boudaoud S, Haskett S, Reynolds TL, Nocturne G, Norton E, Zhang X, Constant M, Park D, Wang W, Lazure T, le Pajolec C, Ergun A, Mariette X (2016) Cytometry by time-of-flight immunophenotyping identifies a blood Sjögren's signature correlating with disease activity and glandular inflammation. *J Allergy Clin Immunol* **137**:1809–1821. https://doi.org/10.1016/j.jaci.2016.01.024
25. Fessler J, Fasching P, Raicht A, et al. (2020) Lymphopenia in primary Sjögren's syndrome is associated with premature aging of naïve $CD4^+$ T cells. *Rheumatology* **1**–10. https://doi.org/10.1093/rheumatology/keaa105
26. Blokland SLM, van Vliet-Moret FM, Hillen MR, Pandit A, Goldschmeding R, Kruize AA, Bouma G, van Maurik A, Olek S, Hoffmueller U, van Roon JAG, Radstake TRDJ (2020) Epigenetically quantified

immune cells in salivary glands of Sjögren's syndrome patients: a novel tool that detects robust correlations of T follicular helper cells with immunopathology. *Rheumatology* (Oxford) **59**:335–343. https://doi.org/10.1093/rheumatology/kez268

27. Yu D, Vinuesa CG (2010) The elusive identity of T follicular helper cells. *Trends Immunol* **31**:377–383. https://doi.org/10.1016/j.it.2010.07.001
28. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M (2013) Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. *Clin Immunol* **147**:95–104. https://doi.org/10.1016/j.clim.2013.02.024
29. Kurata I, Matsumoto I, Sumida T (2020) T follicular helper cell subsets: a potential key player in autoimmunity. *Immunol. Med*
30. Li X-y, Wu Z-b, Ding J et al (2012) Role of the frequency of blood $CD4^+CXCR5^+CCR6^+$T cells in autoimmunity in patients with Sjögren's syndrome. *Biochem Biophys Res Commun* **422**:238–244. https://doi.org/10.1016/j.bbrc.2012.04.133
31. Aqrawi LA, Ivanchenko M, Björk A, Ramírez Sepúlveda JI, Imgenberg-Kreuz J, Kvarnström M, Haselmayer P, Jensen JL, Nordmark G, Chemin K, Skarstein K, Wahren-Herlenius M (2018) Diminished CXCR5 expression in peripheral blood of patients with Sjögren's syndrome may relate to both genotype and salivary gland homing. *Clin Exp Immunol* **192**:259–270. https://doi.org/10.1111/cei.13118
32. Long D, Chen Y, Wu H, Zhao M, Lu Q (2019) Clinical significance and immunobiology of IL-21 in autoimmunity. *J. Autoimmun*. **99**: 1–14
33. Pontarini E, Murray-Brown WJ, Croia C, Lucchesi D, Conway J, Rivellese F, Fossati-Jimack L, Astorri E, Prediletto E, Corsiero E, Romana Delvecchio F, Coleby R, Gelbhardt E, Bono A, Baldini C, Puxeddu I, Ruscitti P, Giacomelli R, Barone F, Fisher B, Bowman SJ, Colafrancesco S, Priori R, Sutcliffe N, Challacombe S, Carlesso G, Tappuni A, Pitzalis C, Bombardieri M (2020) Unique expansion of $IL\text{-}21^+$ Tfh and Tph cells under control of ICOS identifies Sjögren's syndrome with ectopic germinal centres and MALT lymphoma. *Ann Rheum Dis* **79**:1588–1599. https://doi.org/10.1136/annrheumdis-2020-217646
34. Kang YM, Zhang X, Wagner UG, Yang H, Beckenbaugh RD, Kurtin PJ, Goronzy J J, Weyand CM (2002) CD8T cells are required for the formation of ectopic germinal centers in rheumatoid synovitis. *J Exp Med* **195**:1325–1336. https://doi.org/10.1084/jem. 20011565
35. Gao CY, Yao Y, Li L, Yang SH, Chu H, Tsuneyama K, Li XM, Gershwin ME, Lian ZX (2019) Tissue-resident memory $CD8^+$ T cells acting as mediators of salivary gland damage in a murine Clin Rheumatol model of Sjögren's syndrome. *Arthritis Rheumatol* **71**:121–132. https://doi.org/10.1002/art.40676
36. Kang KY, Kim HO, Kwok SK, et al. (2011) Impact of interleukin21 in the pathogenesis of primary Sjögren's syndrome: increased serum levels of interleukin-21 and its expression in the labial salivary glands. *Arthritis Res Ther* **13**. https://doi.org/10.1186/ar3504
37. Finak G, Langweiler M, Jaimes M, Malek M, Taghiyar J, Korin Y, Raddassi K, Devine L, Obermoser G, Pekalski ML, Pontikos N, Diaz A, Heck S, Villanova F, Terrazzini N, Kern F, Qian Y, Stanton R, Wang K, Brandes A, Ramey J, Aghaeepour N, Mosmann T, Scheuermann RH, Reed E, Palucka K, Pascual V, Blomberg BB, Nestle F, Nussenblatt RB, Brinkman RR, Gottardo R, Maecker H, McCoy JP (2016) Standardizing flow cytometry immunophenotyping analysis from the Human ImmunoPhenotyping Consortium. *Sci Rep* **6**. https://doi.org/10. 1038/srep20686
38. Gottenberg J-E, Lavie F, Abbed K et al (2005) CD4 CD25high regulatory T cells are not impaired in patients with primary Sjögren's syndrome. *J Autoimmun* **24**:235–242. https://doi.org/10.1016/j.jaut.2005.01.015
39. Maria NI, van Helden-Meeuwsen CG, Brkic Z, Paulissen SMJ, Steenwijk EC, Dalm VA, van Daele PL, Martin van Hagen P, Kroese FGM, van Roon JAG, Harkin A, Dik WA, Drexhage HA, Lubberts E, Versnel MA (2016) Association of increased Treg cell levels with elevated indoleamine 2,3-dioxygenase activity and an imbalanced kynurenine pathway in interferon-positive primary Sjögren's syndrome. *Arthritis Rheumatol* **68**:1688–1699. https://doi.org/10.1002/art.39629

40. Verstappen GM, Meiners PM, Corneth OBJ, Visser A, Arends S, Abdulahad WH, Hendriks RW, Vissink A, Kroese FGM, Bootsma H (2017) Attenuation of follicular helper T cell–dependent B cell hyperactivity by abatacept treatment in primary Sjögren's syndrome. *Arthritis Rheumatol* **69**:1850–1861. https://doi.org/10. 1002/art.40165
41. Yi JS, Rosa-Bray M, Staats J, Zakroysky P, Chan C, Russo MA, Dumbauld C, White S, Gierman T, Weinhold KJ, Guptill JT (2019) Establishment of normative ranges of the healthy human immune system with comprehensive polychromatic flow cytometry profiling. *PLoS One* **14**:e0225512. https://doi.org/10.1371/journal.pone. 0225512
42. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos NM, Moutsopoulos HM (2008) Foxp3^{+} T-regulatory cells in Sjögren's syndrome. *Am J Pat*hol **173**:1389–1396. https://doi.org/10.2353/ajpath.2008.080246
43. Glauzy S, Sng J, Bannock JM, Gottenberg JE, Korganow AS, Cacoub P, Saadoun D, Meffre E (2017) Defective early B cell tolerance checkpoints in Sjögren's syndrome patients. Arthritis *Rheumatol* **69**:2203–2208. https://doi.org/10.1002/art.40215
44. Hansen A, Odendahl M, Reiter K, Jacobi AM, Feist E, Scholze J, Burmester GR, Lipsky PE, Dörner T (2002) Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome. *Arthritis Rheum* **46**:2160–2171. https://doi.org/10.1002/art.10445
45. Szyszko EA, Brokstad KA, Øijordsbakken G et al (2011) Salivary glands of primary Sjögren's syndrome patients express factors vital for plasma cell survival. *Arthritis Res Ther* **13**:R2. https://doi.org/10.1186/ar3220
46. Maier-Moore JS, Koelsch KA, Smith K, Lessard CJ, Radfar L, Lewis D, Kurien BT, Wolska N, Deshmukh U, Rasmussen A, Sivils KL, James JA, Farris AD, Scofield RH (2014) Antibodysecreting cell specificity in labial salivary glands reflects the clinical presentation and serology in patients with sjögren's syndrome. *Arthritis Rheumatol* **66**:3445–3456. https://doi.org/10.1002/art. 38872
47. Kroese FGM, Abdulahad WH, Haacke E, Bos NA, Vissink A, Bootsma H (2014) B-cell hyperactivity in primary Sjögren's syndrome. *Expert Rev Clin Immunol* **10**:483–499. https://doi.org/10. 1586/1744666X.2014.891439
48. Mauri C, Menon M (2017) Human regulatory B cells in health and disease: therapeutic potential. *J. Clin. Invest*. **127**:772–779
49. Jin L, Weiqian C, Lihuan Y (2013) Peripheral CD24^{hi} CD27^{+} CD19^{+} B cells subset as a potential biomarker in naive systemic lupus erythematosus. *Int J Rheum Dis* **16**:698–708. https://doi.org/10.1111/1756-185X.12229 .

Supplementary Table

Supplementary Table 1. Comparison of T and B-cell subsets' numbers in SjS, *Sicca*, and Healthy Control groups

Cells/µl, median (IQR)	Sjogren's Syndrome (SjS)			*Sicca*	HC	Group's comparisons (*P*-values)					
	Total SjS *n* = 53	SSA+ SjS *n* = 38	SSA- SjS *n* = 15	*n* = 72	*n* = 24	SSA+ vs SSA-	SSA+ vs *Sicca*	SSA+ vs HC	SSA- vs *Sicca*	SSA- vs HC	*Sicca* vs HC
Lymphocytes	1615 (1131-2475)	1475 (1115-2387)	1891 (1119-2587)	1939 (1506-2275)	2228 (1987-3040)	0,345	0,093	*<0,001*	0,829	0,122	*0,003*
B cells	186 (97-263)	187 (81-263)	161 (109-265)	228 (141-297)	252 (173-393)	0,988	0,140	*0,033*	0,214	0,065	0,147
T cells	1180 (756-1180)	1112 (750-1717)	1263 (882-1924)	1403 (1038-1656)	1777 (1426-2155)	0,320	0,152	*0,001*	0,841	0,108	*0,001*
T-cell Subsets											
CD4+	735 (431-1083)	694 (433-1051)	823 (424-1388)	889 (694-1157)	1108 (1010-1408)	0,259	*0,016*	*<0,001*	0,841	*0,046*	*<0,001*
Th1	259 (176-431)	236 (163-3479	398 (183-554)	347 (211-467)	473 (360-555)	0,062	*0,008*	*<0,001*	0,611	0,237	*0,003*
Th17	125 (82-224)	121 (72-206)	185 (104-271)	196 (123-264)	275 (203-395)	0,190	*0,007*	*<0,001*	0,818	*0,028*	*0,002*
Tregs	65 (36-92)	67 (36-89)	63 (36-99)	68 (45-83)	92 (67-112)	0,685	0,585	*0,001*	0,989	*0,042*	*<0,001*
CD8+	472 (320-662)	401 (298-623)	523 (324-723)	439 (319-574)	533 (391-753)	0,261	0,999	0,086	0,177	0,695	*0,049*
CXCR5+CD4+ T-cells	134 (84-203)	126 (78-189)	170 (118-261)	169 (119-201)	241 (221-295)	0,103	*0,026*	*< 0,001*	0,721	*0,022*	*< 0,001*
Surface expression of CCR7											
Naïve (CCR7+)	131 (80-196)	123 (74-185)	166 (117-254)	167 (116-196)	234 (216-283)	0,103	*0,031*	*<0,001*	0,749	*0,022*	*<0,001*
Differentiated (CCR7-)	3 (2-5)	3 (2-5)	4 (3-5)	3 (2-4)	5 (3-9)	0,511	0,226	*0,019*	0,094	*0,084*	*<0,001*
Surface expression of CCR3 and CCR6											
CXCR5+CD4+ Th1 (CCR3+CCR6-)	51 (26-72)	46 (26-63)	61 (23-94)	54 (33-72)	80 (48-102)	0,166	0,240	*<0,001*	0,484	0,136	*0,001*

Supplementary Table 1. *(Cont.)*

Cells/µl, median (IQR)	Sjogren's Syndrome (SjS)			*Sicca*	HC	Group's comparisons (*P*-values)					
	Total SjS *n* = 53	SSA+ SjS *n* = 38	SSA- SjS *n* = 15	*n* = 72	*n* = 24	SSA+ vs SSA-	SSA+ vs *Sicca*	SSA+ vs HC	SSA- vs *Sicca*	SSA- vs HC	*Sicca* vs HC
CXCR5+CD4+ Th17 (CCR3-CCR6+)	28 (15-43)	23 (14-33)	41 (22-58)	35 (23-54)	53 (37-71)	*0,015*	*0,001*	*<0,001*	0,588	0,090	*0,002*
CXCR5+CD4+ CCR3+CCR6+	12 (7-19)	10 (7-17)	19 (9-26)	14 (8-20)	18 (14-259	0,073	0,168	*0,001*	0,325	0,633	*0,012*
CXCR5+CD4+ CCR3-CCR6-	44 (25-64)	40 (24-59)	54 (24-70)	55 (42-71)	90 (75-110)	0,191	*0,002*	*<0,001*	0,712	*0,001*	*<0,001*
CXCR5+CD8+ T-cells (within CD8+ T-cells)	12 (8-18)	12 (7-18)	13 (10-19)	13 (7-19)	17 (18-23)	0,300	0,755	0,059	0,421	0,506	0,082
Surface expression of CCR7											
Naïve (CCR7+)	9 (5-14)	9 (5-14)	10 (7-13)	9 (5-12)	11 (7-20)	0,628	0,850	0,152	0,366	0,524	0,072
Differentiated (CCR7-)	2 (2-5)	2 (1-5)	4 (3-7)	3 (2-5)	4 (2-9)	*0,022*	0,194	0,078	0,123	0,578	0,399
IL-21 production											
CD4+											
IL21+	86 (51-123)	88 (52-123)	82 (41-110)	85 (60-108)	113 (71-167)	0,522	0,896	0,168	0,479	0,137	0,060
IL21+IL17+	5 (3-8)	5 (3-8)	5 (3-10)	5 (4-8)	7 (5-13)	0,384	0,191	0,081	0,836	0,377	0,140
CD8+											
IL21+	18 (11-28)	20 (11-29)	17 (8-23)	14 (7-22)	13 (6-26)	0,408	*0,031*	0,154	0,646	0,715	0,985
IL21+IL17+	1 (1-2)	2 (1-2)	1 (1-2)	1 (1-2)	2 (1-4)	0,459	0,195	0,273	0,896	0,108	*0,017*

Supplementary Table 1. *(Cont.)*

Cells/µl, median (IQR)	Sjogren's Syndrome (SjS)			*Sicca*	HC	Group's comparisons (*P*-values)					
	Total SjS *n* = 53	SSA+ SjS *n* = 38	SSA- SjS *n* = 15	*n* = 72	*n* = 24	SSA+ vs SSA-	SSA+ vs *Sicca*	SSA+ vs HC	SSA- vs *Sicca*	SSA- vs HC	*Sicca* vs HC
B-cell Subsets											
IgD/CD27											
Naïve	106 (61-196)	113 (44-200)	90 (69-168)	136 (75-204)	137 (86-228)	0,864	0,234	0,209	0,208	0,161	0,694
Memory	50 (27-76)	47 (25-71)	69 (30-92)	65 (37-95)	106 (65-141)	0,222	*0,021*	*<0,001*	0,666	*0,022*	*0,002*
Unswitched memory	22 (11-37)	22 (10-35)	32 (15-55)	35 (20-54)	57 (32-81)	0,425	*0,007*	*<0,001*	0,365	*0,017*	*0,008*
Switched memory	23 (15-41)	22 (15-39)	32 (18-45)	30 (18-45)	45 (37-76)	0,233	0,065	*<0,001*	0,962	*0,025*	*0,001*
$CD24^{Hi}CD38^{Hi}$	9 (4-19)	13 (3-24)	6 (5-11)	8 (4-13)	8 (5-18)	0,521	0,221	0,821	0,850	0,361	0,380
$CD24^{Hi}CD27^{+}$	30 (15-49)	27 (12-43)	31 (20-53)	46 (27-75)	81 (51-103)	0,214	*0,001*	*<0,001*	0,257	*0,006*	*0,003*
Plasmablasts $IgM^{-/+}CD38^{++}$	3 (1-6)	4 (1-6)	2 (1-5)	2 (1-4)	4 (2-5)	0,245	0,122	0,786	0,718	0,119	*0,035*

Statistically significant results are indicated in italic.

CAPÍTULO VI. (A). Tear Meniscus and Corneal Sub-basal Nerve Plexus Assessment in Primary Sjögren's Syndrome and *Sicca* Patients | *Avaliação do menisco lacrimal e do plexo nervoso corneano sub-basal em doentes com Síndrome de Sjögren e síndrome* Sicca.

Tear Meniscus and Corneal Sub-basal Nerve Plexus Assessment in Primary Sjögren Syndrome and *Sicca* Syndrome Patients

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[Abstract]

Purpose: To evaluate lower tear meniscus and corneal sub-basal nerve plexus in primary Sjögren's syndrome (pSS) and *Sicca* syndrome patients.

Methods: Cross-sectional study of 116 patients with *Sicca* syndrome associated with pSS and not associated with Sjögren's syndrome (nonSS *Sicca*) and 20 normal control subjects. Tear meniscus height and area were measured using anterior segment optical coherence tomography; corneal sub-basal nerve plexus density, length, and tortuosity were evaluated using in vivo confocal microscopy. Data analysis was performed using IBM-SPSS Statistics 24.0.

Results: Corneal sub-basal nerve plexus density and length were significantly lower, and tortuosity was significantly higher in pSS and non-SS *Sicca* groups than in normal control subjects ($P < 0.001$; $P = 0.018$, respectively). Corneal sub-basal nerve plexus presented a strong association with Schirmer test I and tear breakup time. Cut-off values of sub-basal nerve plexus density (36.5 nerve/mm^2) and length (12.5 mm/ mm^2) presented 80.2% to

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81.9% sensitivity and 85% specificity for detecting *Sicca* syndrome patients. No significant differences were found between the 3 groups regarding tear meniscus height and area.

Conclusions: Corneal sub-basal nerve plexus in vivo confocal microscopy may be a useful tool in the assessment of dry eye disease in *Sicca* syndrome, complementing the information provided by the conventional modalities used in dry eye disease evaluation.

Key Words: dry eye disease, primary Sjögren's syndrome, tear meniscus, anterior segment, optical coherence tomography, corneal in vivo confocal microscopy.

Introduction

Dry eye is a highly prevalent ocular finding, especially in the elderly, users of xerogenic drugs, and patients with chronic rheumatologic diseases. It is a multifactorial disease, potentially associated with relevant clinical consequences in the ocular surface, with great impact on the visual function and quality of life of the affected individuals.[1]

Despite being frequently subject to external factors and patients' individual variations, which may be difficult to predict or control, dry eye evaluation is still mainly based on the assessment of aqueous tear flow and ocular surface damage, using Schirmer test I, tear breakup time (TBUT), and vital staining scores of the ocular surface.[2–5]

Novel biomarkers to complement the information provided by these conventional dry eye tests contributing to the diagnosis, severity assessment, and outcome of *Sicca* patients are needed.[6] With this purpose, the development and use of novel objective, noninvasive, reproducible, and easy to apply tools have been gaining great focus on the evaluation of dry eye disease (DED), bringing invaluable information to clinical practice.[4,7]

In fact, DED being a disease that directly affects the tear film and ocular surface, an objective evaluation of these 2 main interveners may change the paradigm of dry eye evaluation.[8] In this sense, anterior segment optical coherence tomography (AS-OCT) provides high-resolution cross-sectional images of the cornea and anterior segment. The measurement of tear meniscus using AS-OCT enables a noninvasive and practical approach to assess the overall tear film.[4,9–14] Another promising instrument is the in vivo confocal microscope, which produces high-resolution images of the living cornea and ocular surface.[15] Damage and consequent stimulation of corneal sensory nerves have been described in DED.[16,17] Indeed, evidence of morphological changes in the corneal sub-basal nerve plexus induced by DED may be useful not only in detecting the disease but also in monitoring patient response to treatment.[18]

As a part of a project aiming to search for new objective and serologic biomarkers for the diagnosis of primary Sjögren's syndrome (pSS), we designed a study to evaluate novel diagnostic tools for DED, namely, tear meniscus ASOCT and corneal sub-basal nerve plexus in vivo confocal microscopy and to compare those tests with conventional objective dry-

eye tests in a population with *Sicca* syndrome. We also expect to clarify if the former are sensitive tools to detect DED in patients with *Sicca* syndrome.

Materials and Methods

Clinical Study Design and Population

A cross-sectional study of 116 patients with *Sicca* syndrome and 20 healthy women was conducted. All subjects signed a written informed consent before their participation in the study. Approval from the Ethics Committees of Hospital Cuf Descobertas and of *Instituto Português de Reumatologia* was obtained, and the tenets of the Declaration of Helsinki were followed.

Patients with *Sicca* manifestations were consecutively recruited from the Rheumatology Department of the *Instituto Português de Reumatologia* and *Hospital Cuf Descobertas* between September 2014 and March 2017. All patients had ocular or oral *Sicca* symptoms or signs and had previously undergone a complete evaluation to diagnose pSS, which included minor salivary gland biopsy and autoantibody profiling. Patients who fulfilled the American-European Consensus Group criteria[3] were diagnosed with pSS, whereas those who did not fulfill those criteria were classified as the non-Sjögren's syndrome *Sicca* group (non-SS *Sicca*). Secondary Sjögren's syndrome (sSS) patients were not enrolled in the study. All healthy volunteers who came to the Ophthalmology outpatient clinic during the same period of recruitment, without any *Sicca* symptoms or signs, fulfilling all eligibility criteria composed the normal control group. The exclusion criteria proposed by the American-European Consensus Group were applied to all subjects. Additional exclusion criteria were the history of IgG4-related disease; other systemic or organ-specific autoimmune disease; diabetes mellitus and potential neurodegenerative diseases; treatment with xerogenic drugs or drugs of known corneal toxicity; eyelid malposition or movement disorders; lacrimal drainage pathway obstruction, including the presence of tear duct plugs; ocular surface diseases other than DED, including corneal dystrophy or degeneration, active ocular infection, or allergy; contact lens wear within 2 weeks from the ophthalmic evaluation; or history of corneal laser procedures and refractive, glaucoma or retinal surgeries. Patients were instructed not to use artificial tears within 8 hours before their ophthalmic evaluation.

Demographic and clinical data were collected, and a complete ophthalmologic examination was performed. Additionally, the latest laboratory evaluation was considered.

Ocular Examinations

Dry eye assessment was performed according to the following sequence: tear meniscus measurement using ASOCT, followed by Schirmer test I without anesthesia, TBUT and

corneal fluorescein staining score assessment, and finally, corneal sub-basal nerve plexus evaluation by in vivo confocal microscopy. All subjects were examined by the same examiner, who was unaware of subject condition/group, under standardized conditions of room illumination (low illumination), temperature (20–25°C), and relative air humidity (40%–60%).[2] Both eyes were examined in the majority of the patients. However, for statistical analysis, the eye recording the highest corneal staining score (CSS) or, in case of equal CSS, the lower score on Schirmer test I, was selected for analysis.

Anterior Segment Optical Coherence Tomography: Tear Meniscus Imaging

Tear meniscus was assessed using Visante OCT (Carl Zeiss Meditec, Dublin, CA), as previously described elsewhere.[19] Tear meniscus scans were captured with a vertical beam, set at a 90-degree angle, in the protocol "AC Biometry PreOp." Cross-sectional tomographs of both upper and lower tear meniscus with an axial resolution of 18 μm and a transverse resolution of 60 μm were obtained simultaneously. However, for the purpose of this study, only the lower tear meniscus was subsequently analyzed.

Subjects were instructed to look at a fixation point located in the device, while maintaining spontaneous blinking. The light beam was focused on the ocular surface centered at the pupil area and the patient directed to blink. Immediately after blinking, the scan was performed. Lower tear meniscus height was manually measured using the software callipers of the device, corresponding to the vertical line formed by the union of the touch points between tear meniscus and the corneal surface and the lid margin.[19] Tear meniscus area was measured using Image J software (Java software program developed by the National Institutes of Health, Bethesda, MD). Three images were analyzed, and the mean value was obtained.

Conventional DED Tests

Conventional ocular tests for DED evaluation were performed following the previously defined sequence.[20] First, Schirmer test I was performed without anesthesia, before any drops were instilled in the eye. Sterile Schirmer strips, bended at the notch, were placed on the temporal lower lid margin. All patients were instructed to keep eyelids closed during the test. The strip was removed after 5 minutes or earlier when the strip became totally wet by the tear fluid, and the length of moisture was measured in millimeters. Immediately after completing Schirmer test I, a drop of 0.5% fluorescein was applied to the conjunctival fornix, and the participant instructed to blink. The excess of dye was removed. Two minutes later, TBUT, defined as the time in seconds between the patient's last blink and the first appearance of dry spots on the corneal surface, was measured using the cobalt blue filter with high illumination and a ×10 magnification. A TBUT of less than 10 seconds was considered abnormal and consistent with dry eye.[20] Finally, CSS was assessed using

fluorescein staining and classified into 6 groups according to severity, based on corneal surface staining of the Oxford grading scale: 0—absent, I—minimal, II—mild, III—moderate, IV—marked, and V—severe.[21]

In Vivo Confocal Microscopy: Corneal Sub-basal Nerve Plexus Assessment

Corneal sub-basal nerve plexus in vivo confocal microscopy (Heidelberg Retina Tomograph III, Rostock Cornea Module) was performed, according to the protocol established by Tavakoli and Malik.[22] Patients were instructed to look straight, and the objective lens was brought toward the center of the cornea. The entire central corneal thickness was scanned, while the confocal imaging plane was adjusted manually, from superficial to deeper planes, with the acquisition modality set at "Section Mode." Two-dimensional digital images with 400 $\times$ 400 μm, a lateral digital resolution of 1 μm/ pixel and a depth resolution of 2 μm/pixel, were obtained. Three to 5 best-focused, nonoverlapping, and most representative images were analyzed using Image J software, plugin Neuron J. Central corneal images were selected based on the live imaging given by the camera attached to the microscope and the nerves' vertical orientation.[23,24] As previously reported, sub-basal nerve plexus was characterized in terms of nerve density, length, and tortuosity. Nerve density was defined as the total number of major nerves per square millimeter of corneal tissue; nerve length, was defined as the total length of all nerve fibers and branches, in millimeter per square millimeter of corneal tissue[25]; and nerve tortuosity was classified as grade 0 = almost straight; grade 1 = slightly tortuous; grade 2 = moderately tortuous; grade 3 = quite tortuous; grade 4 = very tortuous.[26]

Statistical Analysis

A descriptive analysis of demographic and clinical variables was performed. Quantitative variables were described with mean (M) and SD. Categorical data were presented as frequencies and percentages, and ordinal variables by the mean rank. Comparisons between the 2 diagnostic subgroups of patients and the control group were performed using χ^2 tests with Monte Carlo estimation for categorical data and Kruskall–Wallis tests for ordinal data. A one-way analysis of variance test was used for continuous variables, and a Scheffe post hoc test was conducted to test for differences between the 3 groups. Despite the size of the control group, the analysis of variance assumptions was checked and guaranteed. The Pearson correlation coefficient was used to examine relationships among continuous variables. Spearman rank correlation was applied for detecting correlations with ordinal variables. The Cramer V coefficient was used to analyze associations between categorical and ordinal variables, and eta coefficient to associate categorical and continuous variables. A receiver operating characteristic (ROC) curve analysis for corneal sub-basal nerve plexus density and length was performed. In this study, $P < 0.05$ was

considered significant. Data analysis was performed using IBM-SPSS Statistics for Windows, Version 24.0 (IBM Corp, Armonk, NY).

Results

Patients' Characteristics

Fifty-four patients with a mean age of 57.8 years (SD = 11.9 years) were included in the pSS group. Sixty-two patients with a mean age of 60.7 years (SD = 11.0 years) were included in the non-SS *Sicca* group. Finally, 20 subjects with a mean age of 50.9 years (SD = 6.5 years) were included in the normal control group. All patients were female. Mean disease duration was 11.6 years (SD = 7.7 years) in pSS patients and 9.9 years (SD = 5.0 years) in non-SS *Sicca* patients. Disease onset, defined by the first ocular dryness complaints reported, and age at diagnosis were significantly lower in pSS group compared with non-SS *Sicca* group ($P = 0.02$, $P = 0.007$, respectively). Demographic, clinical, and immunological characteristics of the subjects in study are presented in Table 1.

Ocular Examinations

The results found in the ophthalmologic evaluation are reported in Table 2. Schirmer test I found that means were significantly lower in pSS and non-SS *Sicca* groups, in contrast to the normal control group ($P < 0.001$, Table 3). Subjects in the pSS and non-SS *Sicca* groups presented a significantly higher frequency of lower TBUT in contrast to those in the normal control group ($P < 0.001$), and the difference was greater for patients with pSS. Concerning CSS, pSS showed a score significantly higher in contrast to non-SS *Sicca* and normal control groups ($P < 0.001$). No significant differences were found between the 3 groups regarding tear meniscus height and area (Figure 1). Corneal sub-basal nerve plexus density and length were significantly lower in pSS and non-SS *Sicca* groups compared with normal control group ($P < 0.001$ for both comparisons); tortuosity was significantly higher in the pSS group in contrast to the normal control group ($P = 0.0.013$; Table 3; Figure 2). Regarding the Schirmer test I and TBUT, no significant differences were found between pSS and non-SS *Sicca* patients. Additionally, nerve density, length, and tortuosity did not present significant differences between pSS and nonSS *Sicca* groups (Table 3).

Associations between the ophthalmic variables studied are reported in Table 4. A positive association was found between corneal sub-basal nerve plexus density, and Schirmer test I and TBUT ($r = 0.364$, $P < 0.001$; $\eta = 0.247$, $P = 0.004$, respectively). Corneal sub-basal nerve plexus length was positively associated with Schirmer test I ($r = 0.270$, $P = 0.002$). Corneal sub-basal nerve plexus tortuosity negatively correlated with Schimer test I ($r = 20.179$; $P = 0.019$).

Patients in pSS and non-SS *Sicca* groups were compared with control subjects, and ROC curves were used to identify cut-off points with maximum sensitivity and specificity

concerning to corneal sub-basal nerve plexus density and length. Cut-off values that best discriminated patients from normal controls were 36.5 nerves/mm^2 for nerve density (80.2% sensitivity and 85.0% specificity) and 12.5 mm/mm^2 for nerve length (81.9% sensitivity and 85.0% specificity), respectively. The area under the curve values obtained were 0.877 (95% confidence interval, 0.811–0.942) for nerve density and 0.869 (95% confidence interval, 0.805–0.932) for nerve length.

Discussion

DED is a chronic ocular surface disorder, with a wide and expanding prevalence among the general population.[1,8] Although it is one of the most frequent ophthalmologic findings encountered in daily practice, the methods routinely used for its diagnosis and follow-up have low-to-moderate reproducibility and, sometimes, high variability, being influenced by ambient and patient conditions.[27–29] Therefore, the protocol for evaluation of DED should be reviewed.

TABLE 1. Patients' Clinical Features

	pSS	*Sicca*	**Control**	***P***
No. patients, n (%)	54 (39.7)	62 (45.6)	20 (14.7)	
Age (yr) *	57.8 (11.9)	60.7 (11.0)	50.9 (6.5)	0.003§
Gender, n (%)	54F (100.0)	62F (100.0)	20F (100.0)	
Symptoms Onset (yr) *	46.1 (11.2)	50.8 (10.5)	–	0.02‡
Diagnosis (yr) *	51.7 (11.7)	57.4 (10.9)	–	0.007‡
Xerostomia, n (%)	52 (96.3%)	60 (96.8)	–	NS
Xerophthalmia, n (%)	51 (94.4%)	59 (95.2)	–	NS
Minor salivary gland biopsy (FS≥ 1), n (%)	41/51 (78.8)	0 (0.0)	–	< 0.001‡
Unstimulated salivary flow (ml/min) [a]	38 (70.4)	38 (62.3)	–	NS
Abnormal Submaxillary gland scintigraphy, n (%)	22/27 (81.5)	38/48 (88.4)	–	NS
Abnormal Parotid scintigraphy, n (%)	20/27 (74.1)	30/48 (69.8)	–	NS
Low C3, n (%)	11/53 (20.8)	6/58 (10.3)	–	NS
Low C4, n (%)	4/53 (7.5)	1/58 (1.7)	–	NS
Leukopenia, n (%)	9 (16.7)	8 (12.9)	–	NS
Gammaglobulin ≥ 1.6 g/dL, n (%)	14 (25.9)	3/60 (5.0)	–	0.003†
Anti-Ro/SSA, n (%)	37 (68.5)	1 (1.6)	–	< 0.001†
Anti-La/SSB, n (%)	18/48 (37.5)	1/56 (1.8)	–	< 0.001†
Anti-nuclear antibodies, n (%)	50 (92.6%)	46 (74.2)	–	0.013†
Rheumatoid factor, n (%)	23/47 (48.9)	18/60 (30.0)	–	NS

Whenever there were missing values, percentages reflect the number of occurrences over the number of patients tested for the item.

* Mean and SD were reported.

†χ^2 test with Monte Carlo estimation.

‡*t* test.

§F-test (one-way ANOVA).

ANOVA, analysis of variance; FS, focus score; non-SS *Sicca*, non-Sjögren's syndrome *Sicca* patients; NS, nonsignificant; pSS, primary Sjögren's syndrome.

In this cross-sectional study, 116 subjects with *Sicca* manifestations, associated either with pSS or "idiopathic" *Sicca* syndrome, and 20 normal control subjects were evaluated for DED. Conventional and standardized dry eye tests,[8] namely, the Schirmer test I, TBUT, and CSS were performed. Additionally, tear meniscus and corneal sub-basal nerve plexus morphologies were assessed by AS-OCT and in vivo confocal microscopy, respectively, aiming to determine if the latter are sensitive tests to detect DED. To the best of our knowledge, this is the first study evaluating simultaneously conventional DED ocular tests, tear meniscus, and corneal sub-basal nerve plexus, both in pSS and non-SS *Sicca* patients.

TABLE 2. Patients' ophthalmic features

	pSS	**Non-SS *Sicca***	**Control**	***P***
Schirmer's test I (mm)*	5.6 (5.9)	7.1 (6.3)	22.3 (8.3)	< 0.001 ‡
TBUT (Low)	37 (68.5)	32 (51.6)	0 (0.0%)	< 0.001§
CSS †	83.9	62.8	44.5	< 0.001‖
Tear meniscus height (mm)*	0.29 (0.23)	0.34 (0.25)	0.43 (0.11)	n.s.
Tear meniscus area (mm^2)*	0.05 (0.06)	0.06 (0.05)	0.06 (0.03)	n.s.
Sub-basal nerve plexus density (n/mm^2)*	28.1 (12.2)	26.3 (9.2)	43.9 (12.9)	< 0.001†
Sub-basal nerve plexus length (mm/mm^2)*	10.3 (6.6)	9.4 (3.4)	15.4 (5.1)	< 0.001‡
Sub-basal nerve plexus tortuosity†	77.7	65.3	53.6	0.018‖

*Mean and SD were reported.
†Mean rank was reported for ordinal variables.
‡F-test (1-way ANOVA).
§χ^2 test with Monte Carlo estimation.
‖Kruskal–Wallis test.
ANOVA, analysis of variance; non-SS *Sicca*, non-Sjögren's syndrome *Sicca* patients; pSS, primary Sjögren's syndrome

TABLE 3. Pairwise comparisons

	pSS *vs* Control *P*	**Non-SS *Sicca* *vs* Control *P***	**pSS *vs* Non-SS *Sicca* *P***
Schirmer's test I (mm)	< 0.001	< 0.001	0.454
CSS	< 0.001	0.127	0.008
Sub-basal nerve plexus density (n/mm^2)	< 0.001	< 0.001	0.676
Sub-basal nerve plexus length (mm/mm^2)	0.001	< 0.001	0.642
Sub-basal nerve plexus tortuosity	0.031	0.512	0.103

pSS, primary Sjögren's syndrome; Non-SS *Sicca*, Non-Sjögren's syndrome *Sicca* patients.

We must emphasize that the patients included in our study were selected from Rheumatology units, where the recognition of pSS among DED patients is of crucial interest because of the associated organ-specific systemic manifestations and potential complications. It is known that some patients with DED not fulfilling pSS criteria may have mild or incomplete forms of the disease, without specific immunologic markers for pSS, which sometimes may also be absent in pSS. The identification of non-SS *Sicca* patients is important because, although they have similar objective findings in conventional tests, those patients probably do not suffer from autoimmune disease and may be managed exclusively in an ophthalmology clinic.[30–34] Therefore, it is of great importance to identify and validate novel objective tests in a clinical setting that could help in the discrimination between autoimmunerelated and nonautoimmune-related DED.

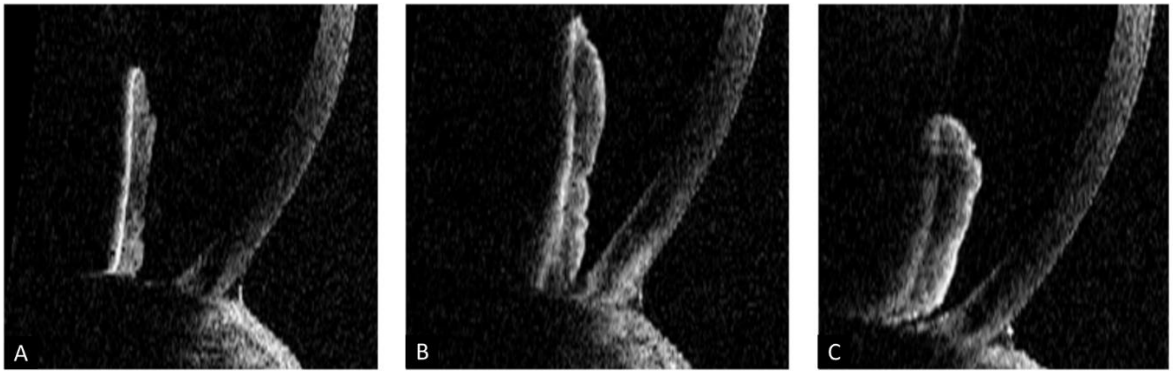

FIGURE 1. Anterior segment optical coherence tomographic images of the lower tear meniscus in a patient with pSS (A), in a non-SS *Sicca* patient (B), and in a normal control subject (C). A vertical scan across the corneal apex obtained immediately after a blink showed similar cross-sectional appearance of the tear meniscus. Visante OCT (Carl Zeiss Meditec, Dublin, CA).

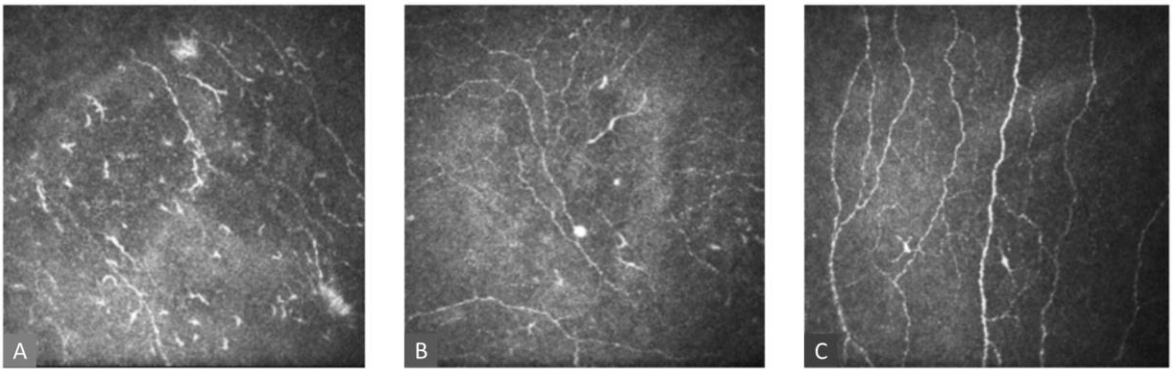

FIGURE 2. Corneal in vivo confocal microscopy images of the corneal sub-basal nerve plexus in a patient with pSS (A), in a non-SS *Sicca* patient (B), and in a normal control subject (C), showing reduced nerve density and length and increased nerve tortuosity. Heidelberg Retina Tomograph III, Rostock Cornea Module images 400×400 μm.

As expected, when comparing pSS and non-SS *Sicca* patients with normal control subjects, decreased Schirmer test I and TBUT scores and an increase in CSS were evident.

Concerning the corneal sub-basal nerve plexus, nerve density and nerve length were significantly lower, and nerve tortuosity was significantly higher in pSS and non-SS *Sicca* groups in contrast to the normal control group. Additionally, the changes in sub-basal nerve plexus morphology presented a good association with Schirmer test I and TBUT scores.

Cornea is the most highly innervated body tissue, being dependent on nerves' integrity for its structural maintenance and function.[35] Its microstructure can be evaluated by in vivo confocal microscopy, which enables a quantitative and objective assessment of high-resolution and repeatable images of corneal cells and nerves.[23,36] In fact, previous studies using this technique have demonstrated corneal sub-basal nerve plexus variations in DED[4,7] and that those variations may be related to disease severity, turning corneal sub-basal nerve plexus in vivo confocal microscopy potentially useful for the diagnosis and follow-up of DED.[16] However, it is important to remember that the decrease in nerves' density and length and the increase in tortuosity seem to represent indicators of nerve fiber damage, present not only in DED but also in other prevalent diseases, such as diabetes mellitus.[7,25,37–42] In addition, because it is an emerging tool, it is still of limited applicability in daily practice, taking into account the time and costs associated with this examination. Meanwhile, published data have shown promising results that seem to support the use of in vivo confocal microscopy, at least for now, in a more research setting, aiding in differential diagnosis, risk stratification, and follow-up, and in the knowledge of potential therapeutic approaches for disease control.[4,39,40] Also, the development of automated in vivo confocal microscopy data analysis with specific software, not yet commercially available to all, can eventually improve accuracy and reduce interpretation time, as well as extend the evaluation of DED to nonophthalmic providers.[43]

Cut-off values for sub-basal nerve plexus parameters are still required to be validated.[41] In our study, a cut-off of 36.5 nerves/mm2 in nerve density and 12.5 mm/mm2 in nerve length presented 80.2% to 81.9% sensitivity and 85% specificity for *Sicca* syndrome; the area under the curve obtained by ROC analysis showed that both density and length of corneal sub-basal nerve plexus may enable the detection of *Sicca* syndrome, when compared with normal control subjects.

On the other hand, no significant differences were found in tear meniscus height and area between the subjects in the 3 groups. This can be related with a possible variation in the time between the blink and the acquisition of the images on the optical coherence tomography.[44] Tear meniscus may be objectively assessed by optical coherence tomography,[13,45] but, as in Schirmer test I, tear meniscus might be also affected by external or patient variations, such as the presence of conjunctivochalasis and lid margin disorders.[19] Also, as the Visante OCT uses a time-domain technology, imaging resolution might still be unsatisfactory to detect changes within the tear film, especially in patients with DED.[10,46] Those reasons may explain the discrepancy of our results in relation to those

presented in studies already published.[19,28] AS-OCT based on Fourier-domain technology and the keratograph may have a greater sensitivity to analyze the tear meniscus.[47,48]

Finally, although significant differences between pSS and non-SS *Sicca* patients were found in CSS, the remaining ocular examinations used in our study did not discriminate between autoimmune-related and nonautoimmune-related DED. Therefore, further analysis is needed to explore associations between these ocular tests and specific clinical and immunologic features in pSS and non-SS *Sicca* patients that could support their utility in subsets of patients in both groups.

TABLE 4. Associations between patients' ophthalmic features

	1	2	3	4	5	6	7	8
Xerophtalmia								
Schirmer test†	0.064							
TBUT (Low)‡‖	–0.034	0.565***						
CSS§	–0.037	–0.502***	0.629***					
Tear meniscus height	0.048	0.298***	0.377***	–0.227**				
Tear meniscus are	0.023	0.147*	0.210*	–0.166*	0.421***			
Sub-basal nerve plexus density	0.038	0.364***	0.247**	–0.135	0.186*	–0.057		
Sub-basal nerve plexus length	0.065	0.270**	0.150	–0.175*	0.155	–0.038	0.659***	
Sub-basal nerve plexus tortuosity‖ [b]	0.028	–0.179*	0.139	0.104	–0.101	–0.033	–0.059	–0.054

*$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

N = 136.

†Pearson correlation coefficient was used to correlate metric variables.

‡Eta coefficient was used to associate nominal and metric variables.

§Spearman Rho coefficient was used to correlate ordinal and metric variables.

‖Cramer V coefficient was used to associate nominal and ordinal variables.

There are some limitations to the present methodology that must be mentioned. First, the recruitment of patients from Rheumatology units limits the etiology of DED in our sample to inflammatory conditions affecting tear production, which could limit the representativity of the findings to a specific clinical setting. Second, although all patients presented *Sicca* symptoms, they were not previously confirmed to have DED. Thus, the inclusion of patients who did not meet all the criteria for DED in the same group, as those with definitive DED, may have affected the results, especially for tear meniscus measurements. Additionally, because our population was representative of daily clinical practice and taking into account the restriction of eligibility criteria, the size of the normal control group is relatively small. Nevertheless, all the results were checked and guaranteed. Another potential limitation in the constitution of the control group is the fact that those individuals were not screened for pSS related autoantibodies. However, all control subjects

were previously subjected to a detailed clinical interrogatory screening, confirming that they did not present subjective or objective evidence of glandular dysfunction suspicious of pSS.[49] Although the presence of autoantibodies could precede disease onset by several years, the prevalence of anti-SSA and anti-SSB antibodies in asymptomatic individuals has been reported to be very low.[50–52] Therefore, we consider highly unlikely the presence of any latent pSS in the normal control group. Finally, to limit eventual confounding factors in the outcome analysis, patients with sSS were excluded. Though, some of the data published in the literature do not show significant differences between pSS and sSS regarding conventional dry eye tests and corneal in vivo confocal microscopy.[39]

In summary, new biomarkers for a better diagnosis and follow-up of patients with *Sicca* syndrome are needed. Corneal in vivo confocal microscopy may complement the more conventional modalities for DED evaluation in *Sicca* syndrome. Noninvasive high-resolution images of the living cornea, easily repeatable over time, enable the assessment of the corneal nerves to serve as potential biomarkers of corneal nerve fiber damage associated with DED. Further studies are still needed to prove the value of corneal in vivo confocal microscopy in the differential diagnosis of DED from other causes of corneal sub-basal nerve plexus changes and in identifying improvement of DED.

References

1. Farrand KF, Fridman M, Stillman IÖ, et al. Prevalence of diagnosed dry eye disease in the United States among adults aged 18 years and older. *Am J Ophthalmol.* 2017;182:90–98.
2. Whitcher JP, Shiboski CH, Shiboski SC, et al. A Simplified Quantitative Method for Assessing Keratoconjunctivitis *Sicca* from the Sjögren's Syndrome International Registry. *Am J Ophthalmol*. 2012;149:405–415.
3. Vitali C, Bombardieri S, Jonsson R, et al. Classification criteria for Sjögren's syndrome: a revised version of the Erupean criteria proposed by the American-European Consensus Group. *Ann Rheum Dis*. 2002;61:554–558.
4. Gabbriellini G, Baldini C, Varanini V, et al. In vivo confocal scanning laser microscopy in patients with primary Sjögren's syndrome: a monocentric experience. *Mod Rheumato*l. 2015;25:585–589.
5. Sullivan BD, Crews LA, Sönmez B, et al. Clinical utility of objective tests for dry eye disease. *Cornea*. 2012;31:1000–1008.
6. Gallo A, Baldini C, Teos L, et al. Emerging trends in Sjögren's syndrome: basic and translational research. *Clin Exp Rheumatol*. 2012; 30:779–784.
7. Benítez del Castillo JM, Wasfy MAS, Fernandez C, et al. An in vivo confocal masked study on corneal epithelium and subbasal nerves in patients with dry eye. *Invest Ophthalmol Vis Sci*. 2004;45:3030–3035.
8. The epidemiology of dry eye disease: report of the epidemiology subcommittee of the international Dry Eye WorkShop (2007). *Ocul Surf.* 2007;5:93–107.
9. Mainstone JC, Bruce AS, Golding TR. Tear meniscus measurement in the diagnosis of dry eye. *Curr Eye Res*. 1996;15:653–661.
10. Ang M, Baskaran M, Werkmeister RM, et al. Anterior segment optical coherence tomography. *Prog Retin Eye Res*. 2018;66:132–156.

11. Bai Y, Nichols JJ. Advances in thickness measurements and dynamic visualization of the tear film using non-invasive optical approaches. *Prog Retin Eye Res*. 2017;58:28–44.
12. Ibrahim OMA, Dogru M, Kojima T, et al. OCT assessment of tear meniscus after punctal occlusion in dry eye disease. *Optom Vis Sci*. 2012; 89:18–20.
13. Shen M, Wang J, Tao A, et al. Diurnal variation of upper and lower tear menisci. *Am J Ophthalmol*. 2008;145:801–806.
14. Penner V, Rocha G. Use of the Visante for anterior segment ocular coherence tomography. *Tech Ophthalmol*. 2007;5:67–77.
15. Jalbert I. In vivo confocal microscopy of the human cornea. *Br J Ophthalmol*. 2003;87:225–236.
16. Labbé A, Liang Q, Wang Z, et al. Corneal nerve structure and function in patients with non-sjögren dry eye: clinical correlations. *Invest Ophthalmol Vis Sci*. 2013;54:5144–5150.
17. Azizi S, Uçak T, Yas¸ar I, et al. Evaluation of the corneal layers in meibomian-gland-dysfunction-related dry eye by in vivo slit-scanning confocal microscopy. *Semin Ophthalmol*. 2017;32:377–383.
18. Patel DV, McGhee CNJ. In vivo laser scanning confocal microscopy confirms that the human corneal sub-basal nerve plexus is a highly dynamic structure*. Invest Ophthalmol Vis Sci.* 2008;49:3409–3412.
19. Ibrahim OMA, Dogru M, Takano Y, et al. Application of visante optical coherence tomography tear meniscus height measurement in the diagnosis of dry eye disease. *Ophthalmology*. 2010;117:1923–1929.
20. Lemp MA. Report of the national eye institute/industry workshop on clinical trials in dry eye. *CLAO J.* 1995;21:221–232.
21. Sook Chun Y, Park IK. Reliability of 4 clinical grading systems for corneal staining. *Am J Ophthalmol*. 2014;157:1097–1102.
22. Tavakoli M, Malik RA. Corneal confocal microscopy: a novel noninvasive technique to quantify small fibre pathology in peripheral neuropathies*. J Vis Exp*. 2011;47:1–7.
23. Kalteniece A, Ferdousi M, Adam S, et al. Corneal confocal microscopy is a rapid reproducible ophthalmic technique for quantifying corneal nerve abnormalities*. PLoS One.* 2017;12:1–10.
24. Petroll WM, Robertson DM. In vivo confocal microscopy of the cornea: new developments in image acquisition, reconstruction and analysis using the HRT-Rostock Corneal Module. *Ocul Surf*. 2015;13:187–203.
25. Malik RA, Kallinikos P, Abbott CA, et al. Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. *Diabetologia*. 2003;46:683–688.
26. Oliveira-Soto L, Efron N. Morphology of corneal nerves using confocal microscopy. *Cornea*. 2001;20:374–384.
27. Nichols KK, Nichols JJ, Mitchell GL. The lack of association between signs and symptoms in patients with dry eye disease. *Cornea*. 2004;23: 762–770.
28. Akiyama R, Usui T, Yamagami S. Diagnosis of dry eye by tear meniscus measurements using anterior segment swept source optical. *Cornea*. 2015;34:115–120.
29. Serruya LG, Nogueira DC, Hida RY. Schirmer test performed with open and closed eyes: variations in normal individuals. *Arq Bras Oftalmol*. 2009;72:65–67.
30. Guellec D, Cornec D, Jousse-Joulin S, et al. Diagnostic value of labial minor salivary gland biopsy for Sjögren's syndrome: a systematic review. *Autoimmun Rev.* 2013;12:416–420.
31. Trier NH, Nielsen IØ, Friis T, et al. Comparison of antibody assays for detection of autoantibodies to Ro 52, Ro 60 and La associated with primary Sjögren's syndrome*. J Immunol Methods*. 2016;433:44–50.
32. Akpek E, Klimava A, Thorne J. Evaluation of patients with dry eye for presence of underlying Sjögren's syndrome. *Cornea*. 2009;28:493–497.
33. Barcelos F, Martins C, Papoila A, et al. Association between memory Bcells and clinical and immunological features of primary Sjögren's syndrome and *Sicca* patients. *Rheumatol Int.* 2018;38:1063–1073.

34. Mathias JR, Dodd ME, Walters KB, et al. Primary Sjögren's syndrome is characterized by distinct phenotypic and transcriptional profiles of IgD+ unswitched memory cells. *Arthritis Rheumatol*. 2014;66:2558–2569.
35. Dua HS, Said DG, Messmer EM, et al. Neurotrophic keratopathy. Prog Retin Eye Res. 2018;66:107–131.
36. Chan TCY, Wan KH, Shih KC, et al. Advances in dry eye imaging: the present and beyond. *Br J Ophthalmol*. 2018;102:295–301.
37. El-Fayoumi D, Youssef MM, Khafagy MM, et al. Assessment of corneal and tear film parameters in rheumatoid arthritis patients using anterior segment spectral domain optical coherence tomography. *Ocul Immunol Inflamm.* 2016;3948:1–7.
38. Müller LJ, Marfurt CF, Kruse F, et al. Corneal nerves: structure, contents and function. *Exp Eye Res*. 2003;76:521–542.
39. Villani E, Galimberti D, Viola F, et al. The cornea in Sjögren's syndrome: an in vivo confocal study. *Invest Ophthalmol Vis Sci.* 2007; 48:2017–2022.
40. Villani E, Magnani F, Viola F, et al. In vivo confocal evaluation of the ocular surface. *Optom Vis Sci.* 2013;90:576–586.
41. Dabbah MA, Graham J, Petropoulos IN, et al. Automatic analysis of diabetic peripheral neuropathy using multi-scale quantitative morphology of nerve fibres in corneal confocal microscopy imaging. *Med Image Anal*. 2011;15:738–747.
42. Cruzat A, Pavan-Langston D, Hamrah P. In vivo confocal microscopy of corneal nerves: analysis and clinical correlation. *Semin Ophthalmol*. 2011;25:171–177.
43. Malik R. ACCMetrics—corneal nerve fibre analyser. Click2Go The University of Manchester's online licensing platform. Available at: https://www.click2go.umip.com/i/s_w/accmetrics_v2.html . Accessed August 25, 2018.
44. Palakuru JR, Wang J, Aquavella JV. Effect of blinking on tear dynamics. *Invest Ophthalmol Vis Sci.* 2007;48:3032–3037.
45. Wang J, Aquavella J, Palakuru J, et al. Repeated measurements of dynamic tear distribution on the ocular surface after instillation of artificial tears. *Invest Ophthalmol Vis Sci.* 2006;47:3325–3329.
46. Cui L, Shen M, Wang J, et al. Age-related changes in tear menisci imaged by optical coherence tomography. *Optom Vis Sci.* 2011;88:1214– 1219.
47. Baek J, Doh SH, Chung SK. Comparison of tear meniscus height measurements obtained with the keratograph and fourier domain optical coherence tomography in dry eye. *Cornea*. 2015;34:1209–1213.
48. Leitgeb R, Hitzenberger C, Fercher A. Performance of fourier domain vs time domain optical coherence tomography. *Opt Express*. 2003;11:889.
49. Brito-Zerón P, Theander E, Baldini C, et al. Early diagnosis of primary Sjögrens syndrome: EULAR-SS task force clinical recommendations. *Expert Rev Clin Immunol.* 2016;12:137–156.
50. Guo YP, Wang CG, Liu X, et al. The prevalence of antinuclear antibodies in the general population of China: a cross-sectional study. *Curr Ther Res Clin Exp.* 2014;76:116–119.
51. Fernandez SAV, Lobo AZC, Oliveira ZNP, et al. Prevalence of antinuclear autoantibodies in the serum of normal blood dornors. *Rev Hosp Clin Fac Med Sao Paulo.* 2003;58:315–319.
52. Theander E, Jonsson R, Sjöström B, et al. Prediction of Sjögren's syndrome years before diagnosis and identification of patients with early onset and severe disease course by autoantibody profiling. *Arthritis Rheumatol.* 2015;67:2427–2436.

CAPÍTULO VI (B). Corneal Sub-basal Nerve Plexus Assessment and its Association with Phenotypic Features and Lymphocyte subsets in Sjögren's Syndrome | *Avaliação do plexo nervoso corneano sub-basal e da sua associação com as características fenotípicas e subpopulações linfocitárias na Síndrome de Sjögren.*

Corneal Sub-basal Nerve Plexus Assessment and its Association with Phenotypic Features and Lymphocyte subsets in Sjögren's Syndrome

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Abstract

Purpose

To assess and compare corneal sub-basal nerve plexus morphology with circulating lymphocyte subsets, immunologic status and disease activity in Sjögren Syndrome (SjS) patients.

Methods

Fifty-five SjS patients, 63 *Sicca* patients (not fulfilling SjS criteria), 18 rheumatoid arthritis (RA) patients and 20 healthy controls (HC) were included. Systemic disease activity in SjS was assessed with the ESSDAI score. Lymphocyte subpopulations were studied with flow cytometry. Corneal confocal microscopy and ImageJ software were used to characterize corneal sub-basal nerve plexus in terms of nerve density (CNFD), length (CNFL), and tortuosity (CNFT). Conventional dry eye tests were also performed.

Results

CNFL and CNFD were lower in SjS, *Sicca* and RA groups, compared to HC ($p < 0.001$ for both SjS and *Sicca*); CNFL $p = 0.005$, CNFD $p = 0.018$ in RA). CNFT was higher in SjS, followed by *Sicca*, RA and HC. A negative correlation was found between ESSDAI score and CNFL ($r = -0.735$, $p = 0.012$). CNFL correlated negatively with $IL21^{+}CD8^{+}$T-cells ($r = -0.279$, $p = 0.039$) and a positively with total memory ($r = 0.299$, $p = 0.027$), unswitched memory ($r = 0.281$, $p = 0.038$) and $CD24^{Hi}CD27^{+}$ ($r = 0.278$, $p = 0.040$) B-cells. CNFD showed a tendency to significance in its negative correlation with ESSDAI ($r = -0.592$, $p = 0.071$) and in its positive correlation with switched memory B-Cells ($r = 0.644$, $p = 0.068$).

Conclusions

This is the first study aiming to correlate ocular findings with lymphocyte subsets in SjS. The associations founded between CNFL and CNFD and disease activity, $IL21^+$ follicular T-cells and some B-cell subsets suggest that corneal nerve damage may parallel systemic disease activity and inflammatory cells' dynamics.

Keywords Corneal confocal microscopy · Corneal sub-basal nerve plexus · Dry Eye · EULAR · Sjögren's Syndrome Disease Activity Index · Flow Cytometry · Lymphocyte subsets · Sjögren's Syndrome

1. Background

Sjögren's syndrome (SjS) is a chronic systemic autoimmune disease characterized by lymphocytic infiltration and damage of the exocrine glands, predominantly affecting the salivary and lacrimal glands. Dry eye and dry mouth are cardinal features of SjS, usually being the presenting symptoms (Kassan & Moutsopoulos 2004). In SjS, dry eye is characterized by decreased tear flow due to lacrimal gland inflammatory damage (Bron et al. 2017). Lacrimal gland lymphocyte infiltration in SjS correlates with the degree of glandular dysfunction (Tsubota et al. 1996), and the resulting aqueous deficiency leads to tear hyperosmolarity. In turn, hyperosmolarity stimulates ocular surface cells to produce inflammatory cytokines (Interleukin IL-1, IL-6 and Tumor Necrosis Factor TNF-α,) and proteases (De Paiva et al. 2006), which leads to additional recruitment of inflammatory cells. The apoptosis of surface epithelial cells (Yeh et al. 2003) and non-apoptotic death of corneal epithelial cells (Sahin et al. 2012) further contributes to this inflammatory vicious cycle.

Both innate (macrophages and NK-cells) and adaptative ($CD4^+$ T-cells – particularly Th1 and Th17) immune responses are involved in SjS pathogenesis (Baudouin 2001). The infiltration of exocrine glands by IL-21-producing $CXCR5^+$ follicular helper T-cells (Tfh) (Szabo et al. 2013) is crucial to B-cell survival and ectopic formation of germinal-centre (GC)-like structures, and contributes to perpetuating glandular dysfunction. CXCR5 is also expressed in CD8+ T-cells, pointing to the existence of a follicular cytotoxic T-cell subset (Tfc) (Quigley et al. 2007).

In fact, despite the common inflammatory pathway in all dry eye disease (DED) patients, recent studies reveal a greater upregulation of cytokines in tear fluid in SjS compared to non-SjS dry eye (Chen et al. 2019, Lee et al. 2013). They corroborated the interplay between innate and adaptive immunity in SjS dry eye pathogenesis. B-cells also

appear to be implicated in ocular manifestations of SjS, although their role is less clear (Stern et al. 2012).

Classical DED evaluations, such as the Schirmer's test, tear break-up time and corneal staining measurement have a low-to-moderate reproducibility and are often influenced by external factors (Nichols et al. 2004). Therefore, new tools for corneal assessment have been used lately, namely *in vivo* confocal microscopy (IVCM), which evaluates corneal sub-basal nerve plexus morphology. Unmyelinated corneal nerves are particularly prone to degeneration, also occurring due to immune or inflammatory conditions (Petropoulos et al. 2019). The damage and subsequent regrowth of these nerves have been described in DED (Belmonte et al. 2017). In SjS, several studies demonstrated significant differences in corneal sub-basal nerve plexus morphology (density, length, and tortuosity) compared to healthy individuals (Benítez-del-Castillo et al. 2007, Cardigos et al. 2019, Tuisku et al. 2008).

Our purpose was to evaluate corneal sub-basal nerve plexus and to explore possible correlations between corneal nerves degeneration, immune parameters, and disease activity in SjS patients.

2. Methods

2.1. Population

We included adult patients from the Rheumatology Department of *Instituto Português de Reumatologia* and *Hospital Cuf Descobertas*, with confirmed or suspected SjS. Patients fulfilling the AECG criteria formed the SjS group, whereas patients with *Sicca* symptoms not fulfilling AECG criteria formed the "*Sicca*" group. AECG criteria consist of 2 clinical items (ocular and oral symptoms), 2 objective items (ocular signs and oral diagnostic tests) and 2 immunological items (presence of focal lymphocytic infiltrates in the minor salivary gland biopsy, and positivity for anti-SSA or anti-SSB antibodies). To fulfil these criteria, at least 4 of the 6 items are necessary, being mandatory to have at least one of the immunological items. However, in a couple of patients, the salivary gland histopathology item was unfulfilled due to lack of minor glands in the biopsy, in which case the presence of anti-SSA/SSB antibodies allowed classification. To prevent overlap between the SjS and *Sicca* groups, all patients included as *Sicca* had a screening for anti-SSA/SSB antibodies and a valid minor salivary gland biopsy.

The AECG criteria also define several exclusion criteria: current anticholinergic therapy, hepatitis C and human immunodeficiency virus infection, pre-existing lymphoma, sarcoidosis, graft-versus-host disease, and history of head and neck radiation treatment.

Additional exclusion criteria considered were IgG_4-related disease, other systemic or organ-specific autoimmune diseases, diabetes mellitus or other possible causes of peripheral neuropathy, neurodegenerative diseases, ocular surface disease other than DED, contact lens use, previous ophthalmic surgery, and treatment with drugs of known corneal toxicity.

Patients with Rheumatoid Arthritis (RA) without *Sicca* symptoms were included as a control group with another autoimmune disease. A healthy control (HC) group was selected from the Ophthalmology outpatient clinic.

Disease activity in SjS was assessed with the EULAR SjS Disease Activity Index (ESSDAI) (Seror et al. 2010), which consists of 12 domains (constitutional, lymphadenopathy, glandular, articular, cutaneous, pulmonary, renal, peripheral nervous system, central nervous system, muscular, hematologic and biologic), each divided into 3–4 levels of activity. Clinically active disease was defined as activity in any ESSDAI domain, except the hematologic and biologic. Additionally, some patients with musculoskeletal inflammatory involvement were considered as active disease even though they didn't score in the ESSDAI articular domain.

Informed consent was obtained from all participants. The study was approved by the Ethics committees of both recruiting institutions and NOVA Medical School (no.17/2016/CEFCM).

2.2. Ocular examinations

All ocular examinations were performed by the same examiner, who was unaware of the subject's condition/group, under standardized conditions of room illumination (low illumination), temperature (20-25°C) and relative air humidity (40–60%) (Whitcher et al. 2010).

2.2.1. Conventional DED Tests

Conventional ocular tests for DED evaluation followed the previously defined sequence (Lemp 1995). After performing Schirmer's test I, a drop of 0.5% fluorescein was applied and two minutes later, tear break-up time (TBUT)TBUT was measured. A TBUT < 10 seconds was considered abnormal and consistent with dry eye. Finally, corneal staining score (CSS) was assessed by fluorescein staining and classified according to the Oxford grading scale (Bron, Evans & Smith 2003).

2.2.2. *IVCM* - Corneal Sub-basal Nerve Plexus Assessment

Corneal sub-basal nerve plexus assessment was performed through IVCM (Heidelberg® Retina Tomograph II, Rostock Cornea Module), according to the protocol established by Tavakoli *et al* (Tavakoli & Malik 2011).

Three to five best-focused, non-overlapping and most representative images were analysed, using ImageJ software, plugin NeuronJ. Central corneal images were selected based on the live imaging given by the camera attached to the microscope and the nerves' vertical orientation (Kalteniece et al. 2017, Petroll & Robertson 2015). As previously reported, sub-basal nerve plexus was characterized in terms of nerve density (CNFD),

length (CNFL), and tortuosity (CNFT). CNFD was defined as the total number of major nerves per square millimeter of corneal tissue; CNFL, was defined as the total length of all nerve fibers and branches, in mm per square millimetre of corneal tissue (Malik et al. 2003); and CNFT was classified as grade 0: almost straight; grade 1: slightly tortuous; grade 2: moderately tortuous; grade 3: quite tortuous; grade 4: very tortuous (Oliveira-Soto & Efron 2001).

2.3. Flow cytometry measurements

To perform immunophenotyping, peripheral blood samples were collected into EDTA-containing tubes, processed and analyzed within 24 hours after collection.

Pre-validated panels of monoclonal antibodies were used to characterize lymphocyte subpopulations, including CD3[2], CD4[2], CD19[1], CD24[1], CD25[1], CD27[1], CD38[1], CD127[1], CCR6[2], CCR7[1], CXCR3[2], CXCR5[2], Anti-IgD[1], and Anti-IgM[2] ([1]from Biolegend, San Diego, CA, USA; [2]from BD Biosciences, San Jose, CA, USA).

Samples were acquired in a 4-color BD FACS Calibur™ cytometer (BD Biosciences), and cells were analyzed with CellQuestPro™ software (BD Biosciences). The staining protocol was described elsewhere (Barcelos et al. 2018). The study addressed several subsets of T-cells, including Regulatory T-cells (Tregs) and Follicular T-cells (Tfh), as well as distinct naïve and memory B-cell subsets.

Each subset was evaluated in percentages and absolute counts, for which a single-platform strategy was used, with BD Trucount tubes™ (BD Biosciences).

Complete gating strategies are described in supplementary data.

2.4. Functional assays for the evaluation of IL21-producing T-cells

T-cells' functional capacities were addressed, particularly IL21 secretion, a cytokine typically associated with Tfh/Tfc subsets. IL17 production was assessed to identify IL21-producing Th17 cells.

Heparinised peripheral blood samples were collected, and cells were stimulated with PMA and ionomycin, for 5h at 37°C in a 5% CO_2 atmosphere in the presence of brefeldin A. After stimulation, cells were lysed, washed, and incubated with anti-CD3 and anti-CD8 for surface staining. For intracellular stain, cells were treated according to the protocol defined by the manufacturer for the BD Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (BD Biosciences) and marked with anti-IL21 and anti-IL17, after cell fixation and permeabilization. For each patient, stimulated and unstimulated tubes were run in parallel to assure proper stimulation and staining controls. Again, a 4-color BD FACS Calibur™ cytometer (BD Biosciences) and Cell Quest Pro™ software (BD Biosciences) were used for acquisition and analysis. Gating strategies are presented in supplementary data.

Table 1 Patient's Characteristics

	SjS n = 55	*Sicca* n = 63
Age (years, mean ± SD)	57.8 (11.8)	60.8 (10.9)
Age at diagnosis (years, mean ± SD)	51.8 (11.6)	57.5 (10.8)
Symptom duration (years, mean ± SD)	11.7 (7.7)	9.9 (5.0)
Ocular symptoms, n (%)	52 (94.5)	60 (95.2)
Oral symptoms, n (%)	53 (96.4)	61 (96.8)
Ocular signs, n (%)	34 (61.8)	33 (52.4)
Oral signs, n (%)	40 (72.7)	43 (68.3)
Extraglandular disease (ever), n (%)	23 (41.8)	23 (36.5)
Clinically active disease, n (%)	27 (49.1)	9 (14.3)
ESSDAI ≥5, n (%)	9 (16.4)	NA
Joint symptoms, n (%)	24 (43.6)	32 (50.8)
Skin, n (%)	16 (29.1)	17 (27.0)
Other Extraglandular involvement, n (%)	8 (14.5)	0 (0)
Raynaud's phenomenon, n (%)	8 (14.5)	16 (25.4)
Focus score ≥1, n (%)	42 (79.2)[a]	0 (0)
Anti-SSA, n (%)	37 (67.3)	0 (0)
Anti-SSB, n (%)	11 (37.5)[b]	0 (0)
ANA ≥ 1/320, n (%)	44 (80.0)	35 (55.6)
ANA ≥ 1/640, n (%)	31 (56.4)	16 (25.4)
RF,n (%)	23 (47.9)[b]	18 (29.5)[c]
Gammaglobulin ≥ 1.6 g/dl, n (%)	12 (21.8)	3 (4.8)[d]
Therapy (any), n (%)	30 (54.5)	29 (46.0)
Glucocorticoids, n (%)	18 (32.7)	17 (27.0)
Hydroxychloroquine, n (%)	19 (34.5)	19 (30.2)
Immunossupressants, n (%)	9 (16.4)	8 (12.7)

[a] n = 53; [b] n = 48; [c] n = 58; [d] n = 62.

SjS, Sjögren's syndrome; Anti-SSA/SSB, anti-Sjögren's syndrome A/B antibody; ANA, antinuclear antibody; RF, rheumatoid factor

2.5. Statistical analysis

All data were analyzed using GraphPadPrism™ software, version 8 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was concluded when *P*-value < 0.05. The normality of distributions was assessed using the D'Agostino and Pearson test. Categorical variables were expressed as numbers and percentages and analyzed using Fisher's exact test. Continuous variables were presented as mean (standard deviation) or median (25^{th}-75^{th} percentile), as applicable. The Mann-Whitney *U* test was used to compare every 2 independent groups. For the assessment of correlations, Spearman correlation coefficients were calculated.

3. Results

3.1. Patients' characteristics

We included fifty-five SjS patients, 63 patients in the *Sicca* group, 18 RA patients (mean age of 55.3 ± 13.7 years), and 20 subjects in the HC group (mean age of 51.0 ± 6.5 years). SjS and *Sicca* patients' demographic and clinical features are shown in Table 1.

3.2. Ocular assessment Schirmer's I test mean values were significantly lower in the SjS, *Sicca* and RA groups, compared to HC ($p < 0.001$) and the frequency of a lower TBUT was also higher in those groups ($p < 0.001$) (Table 2). No significant differences were found between SjS, *Sicca* and RA patients concerning Schirmer's-I and TBUT. Figure 1A presents data regarding CSS.

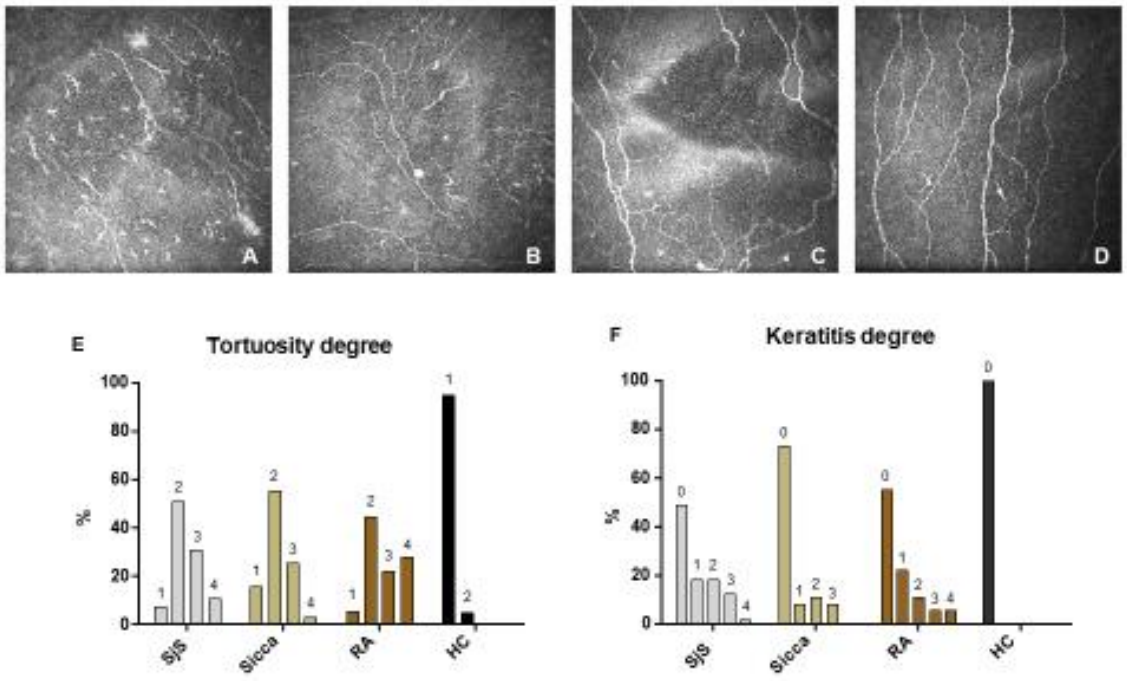

Figure 1 Ocular Examinations In vivo confocal microscopy images of the corneal sub-basal nerve plexus in a patient with SjS (A), in a Sicca group patient (B), in a patient with RA (C) and in a HC subject (D), showing reduced nerve density and length and increased nerve tortuosity in the former three compared to the healthy control. Heidelberg Retina Tomograph II, Rostock Cornea Module images 400 x 400µm.
Bar plot with the distribution of corneal nerve tortuosity (E) and keratitis (F) degrees within each group. SjS, Sjögren's syndrome. RA, Rheumatoid Arthritis. HC, Healthy Controls. Bar plot with the distribution of corneal nerve tortuosity (E) and keratitis (F) degrees within each group. SjS, Sjögren's syndrome. RA, Rheumatoid Arthritis. HC, Healthy Controls.

Table 2 Ophthalmic Assessment. Comparison between SjS, *Sicca*, RA and HC

	SjS n = 55	***Sicca*** n = 63	**Rheumatoid Arthritis** n = 18	**Healthy Controls** n = 20	**Group comparison (*p*-values)[b]**					
					SjS vs *Sicca*	**SjS vs** HC	**SjS vs** RA	***Sicca* vs** RA	***Sicca* vs** HC	**RA vs HC**
Oral and ocular objective features[a]										
Ocular Assessment										
Schirmer test I (mm)	6 (6,05)	7.22 (5,61)	7,00 (6,00)	21,55 (7,74)	0,178	**<0,001**	0,501	0,78	**<0,001**	**<0,001**
TBUT (low, %)	70,9	52,4	77,8	0	0,058 [c]		0,763 [c]	0,063 [c]		
Tear meniscus height (mm)	0,34 (0,21)	0,42 (0,23)	0,29 (0,22)	0,45 (0,07)	0,068	0,064	0,342	**0,033**	0,694	**0,009**
Tear meniscus area (mm^2)	0,06 (0,06)	0,07 (0,05)	0,03 (0,03)	0,07 (0,02)	0,100	0,060	0,128	**0,007**	0,773	**0,001**
Sub-basal nerve plexus density (mm/mm^2)	10,26 (6,49)	9,08 (3,01)	11,97 (4,79)	15,35 (5,14)	0,657	**<0,001**	**0,040**	**0,010**	**<0,001**	**0,018**
Sub-basal nerve plexus length (mm/mm^2)	28,13 (12,94)	26,23 (9,13)	32,97 (12,46)	43,92 (12,92)	0,822	**<0,001**	0,120	**0,044**	**<0,001**	**0,005**

a mean and SD, unless otherwise stated

b Mann-Whitney test, unless otherwise stated

c Fisher's exact test

CNFD and CNFL were significantly lower in SjS, *Sicca* and RA groups, compared to HC ($p < 0.001$ for SjS and *Sicca* in CNFD and CNFL; RA: $p = 0.018$ for CNFD and $p = 0.005$ for CNFL). No differences were found between SjS and *Sicca* patients regarding IVCM measurements, but CNFD was significantly lower in both groups compared to the RA group ($p \leq 0.040$). CNFL was also significantly lower in *Sicca* patients compared to RA ($p = 0.044$) (Table 2). SjS, *Sicca* and RA patients presented higher values of CNFT compared with HCs, without significant differences between them (Figure 1B).

In SjS patients with higher disease activity (ESSDAI≥5), a negative correlation was found between the ESSDAI and both the CNFL ($r = -0.735$, $p = 0.012$) and the CNFD ($r = -0.592$, $p = 0.071$) (Figure 2).

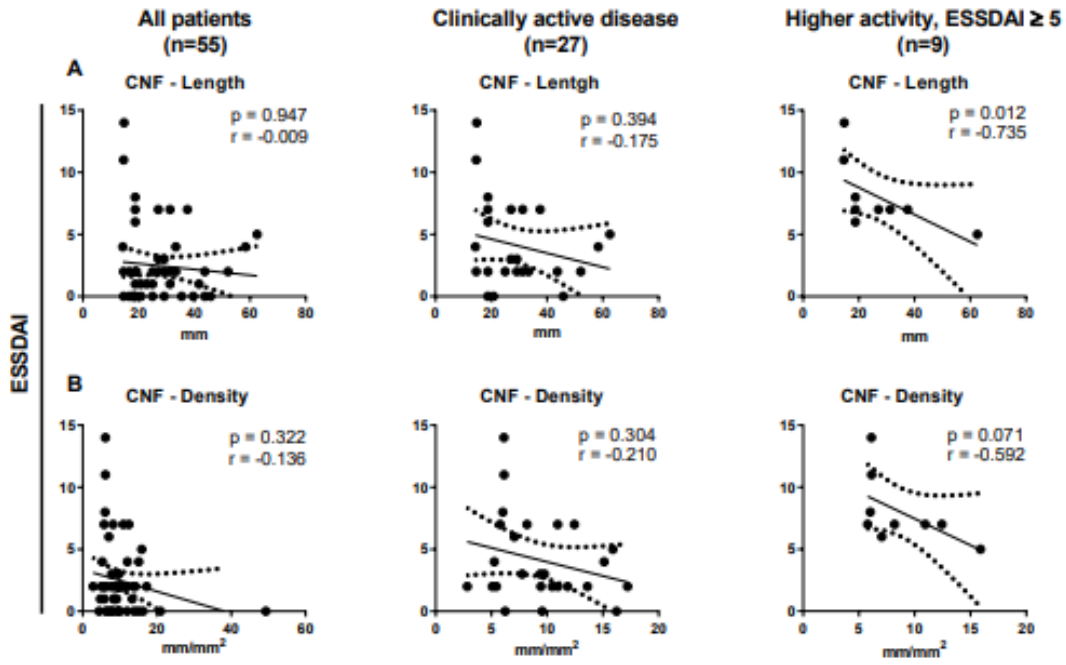

Figure 2 ESSDAI score correlations with CNF Length (A) and CNF Density (B) in all patients, patients with clinically active disease, and patients with ESSDAI score ≥ 5. Spearman correlation coefficients, 95% confidence interval, and p-values are indicated. CNF, Corneal Nerve Fibre.

3.3. Lymphocyte subsets

3.3.1. $CD4^+$ and $CD8^+$ T-cell subsets

Comparing T-cells subsets between SjS patients and HC, we observed that both $CD4^+$T-cell percentages and absolute counts were lower in SjS patients ($p = 0.003$ for percentages; $p < 0.001$ for absolute counts), whereas $CD8^+$ T-cell percentages were higher in SjS ($p = 0.006$) without differences in absolute counts (Table 3, Supplementary Table 1). Compared to *Sicca* patients, SjS patients also presented lower $CD4^+$ T-cells percentages ($p = 0.002$) and absolute counts ($p = 0.044$). No differences were found between SjS and RA patients.

Tregs absolute counts were lower in all patients' groups when compared to HC, although with similar percentages. In SjS patients, a negative correlation was found between the ESSDAI score and Tregs numbers ($r = -0.341$, $p = 0.011$), which was stronger when considering patients with moderate/high disease activity (ESSDAI≥5) ($r = -0.862$, $p = 0.001$) (Figure 3).

Regarding follicular T-cells subsets, no differences were found in the percentages of $CXCR5^+CD4^+$Tfh cells when comparing SjS patients with any of the other groups. However, Tfh1 cells percentages ($CXCR3^+CCR6^-CXCR5^+CD4^+$) were increased in SjS when compared to *Sicca* ($p = 0.009$), RA ($p = 0.064$) and HC ($p = 0.011$) (Table 3, Supplementary Table 1). Although SjS patients presented lower absolute numbers of Tfh cells compared to both RA

(p = 0.068) and HC (p < 0.001), the absolute counts of the Tfh1 subset were similar between the 3 groups of patients (SjS, *Sicca*, RA), but lower in SjS (p = 0.006) and *Sicca* (p = 0.009) when compared to HC.

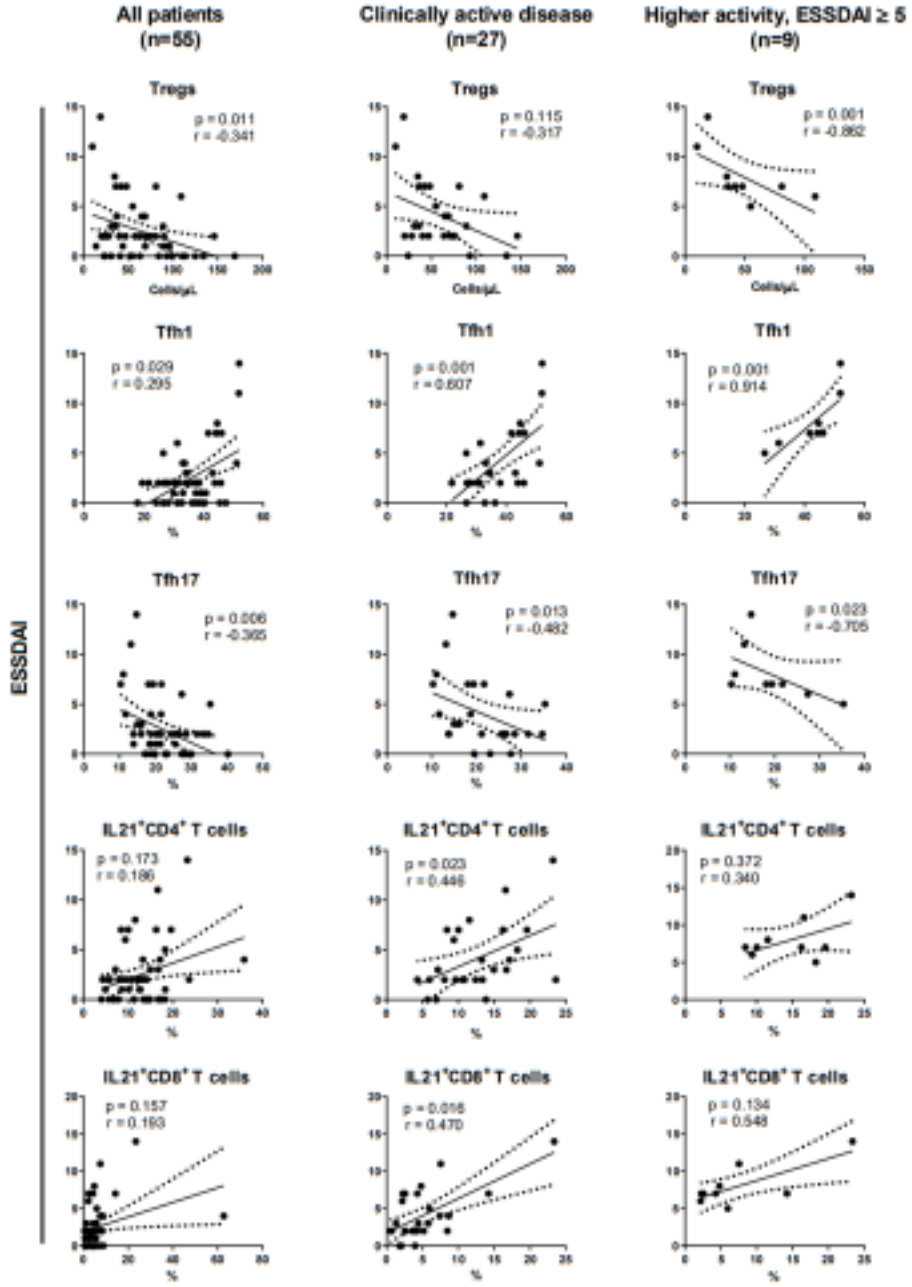

Figure 3 ESSDAI score correlations with T-cell subsets in all patients (n = 55), patients with clinically active disease, and patients with ESSDAI score ≥ 5 (n = 9). Spearman correlation coefficients, 95% confidence interval, and p-values are indicated.

Table 3 Comparison of Lymphocyte subsets and ratios (percentages) in SjS, *Sicca*, RA (Rheumatoid Arthritis) and HC (Healthy Controls)

					Group comparison (*p*-values)					
Lymphocyte %, median (25th-75th percentile)	**SjS n = 55**	***Sicca* n = 63**	**Rheumatoid Arthritis n = 18**	**Healthy Controls n = 20**	**SjS vs *Sicca***	**SjS vs RA**	**SjS vs HC**	***Sicca* vs HC**	***Sicca* vs RA**	**RA vs HC**
Lymphocytes	31,5 (24,5-37,2)	32,2 (25,5-36,4)	23,9 (18,8-30,6)	35,8 (29,3-39,8)	0,723	**0,022**	**0,039**	**0,043**	**0,004**	**<0,001**
B cells	9,7 (6,7-13,4)	11,2 (8,5-14,8)	6,1 (4,1-7,1)	10,8 (8,4-14,4)	0,106	**<0,001**	0,321	0,803	**<0,001**	**<0,001**
T cells	75,0 (69,1-78,4)	71,6 (67,2-77,1)	80,3 (72,6-83,4)	74,7 (70,1-79,3)	0,116	**0,044**	0,684	0,079	**0,004**	0,092
CD4+	61,2 (53,0-67,3)	65,9 (60,5-70,2)	65,3 (49,0-72,6)	68,4 (60,8-75,8)	**0,002**	0,278	**0,003**	0,378	0,553	0,274
Th1	39,0 (29,0-46,2)	39,8 (30,4-47,3)	33,8 (28,9-47,2)	36,8 (30,2-47,1)	0,701	0,541	0,885	0,626	0,421	0,647
Th17	20,6 (13,9-29,2)	22,4 (16,4-28,7)	23,4 (14,8-29,0)	24,4 (16,3-32,9)	0,427	0,794	0,286	0,601	0,878	0,567
Tregs	8,2 (6,5-10,5)	7,6 (7,0-8,9)	7,6 (6,2-9,3)	7,2 (6,7-8,7)	0,144	0,348	0,241	0,948	0,675	0,981
CD8+	38,4 (32,0-47,0)	34,1 (29,8-39,5)	34,8 (27,4-51,0)	31,6 (23,0-39,2)	**0,005**	0,355	**0,006**	0,345	0,554	0,262
CXCR5+CD4+ T-cells	18,6 (14,7-23,7)	18,2 (14,9-22,3)	20,0 (15,6-24,8)	20,6 (16,9-22,7)	0,804	0,572	0,55	0,291	0,362	0,935
Expression of CCR3 and CCR6 [a]										
Tfh1 (CCR3+CCR6-)	36,2 (30,3-40,8)	31,7 (28,9-36,4)	29,1 (25,7-39,0)	30,6 (25,2-35,2)	**0,009**	0,064	**0,011**	0,283	0,435	0,649
Tfh17 (CCR3-CCR6+)	21,4 (18,1-26,8)	23,0 (19,0-27,0)	24,4 (16,4-29,0)	20,9 (18,3-30,4)	0,287	0,475	0,646	0,794	0,798	0,934
Surface expression of CCR7 [b]										
Tfh Naïve (CCR7+)	18,1 (14,6-23,3)	18,0 (14,8-21,7)	19,7 (15,3-23,9)	20,2 (16,6-22,0)	0,980	0,515	0,515	0,298	0,425	0,981
Tfh Differentiated (CCR7-)	0,39 (0,26-0,89)	0,3 (0,18-0,57)	0,42 (0,20-0,71)	0,36 (0,22-0,67)	**0,025**	0,887	0,558	0,238	0,21	0,834
CXCR5+CD8+ T-cells										
Tfc Naïve (CCR7+)	1,9 (1,2-2,8)	2,0 (1,4-3,4)	1,2 (0,8-2,5)	2,0 (1,3-2,8)	0,369	0,087	0,715	0,694	**0,021**	0,075

(Cont.)

(Cont.)

Lymphocyte %, median (25^{th}-75^{th} percentile)	**SjS n = 55**	***Sicca* n = 63**	**Rheumatoid Arthritis n = 18**	**Healthy Controls n = 20**	**SjS vs *Sicca***	**SjS vs RA**	**SjS vs HC**	***Sicca* vs HC**	***Sicca* vs RA**	**RA vs HC**
Tfc Differentiated (CCR7-)	0,72 (0,44-1,30)	0,67 (0,45-1,10)	0,59 (0,25-1,25)	0,75 (0,51-0,99)	0,795	0,561	0,960	0,718	0,494	0,608
IL-21 producing CD4+ T-cells										
IL21+	12,5 (8,4-15,0)	9,6 (7,5-12,7)	9,5 (8,2-11,7)	9,5 (6,3-10,9)	**0,007**	0,064	**0,013**	0,454	0,837	0,628
IL21+IL17+	0,67 (0,54-0,96)	0,73 (0,55-1,00)	0,63 (0,33-0,85)	0,68 (0.24-1,07)	0,724	0,313	0,448	0,445	0,177	0,902
IL-21 producing CD8+ T-cells										
IL21+	4,1 (2,5-5,7)	3,1 (2,1-4,4)	2,4 (1,2-3,2)	2,6 (1,0-4,4)	0,071	**0,002**	**0,022**	0,200	**0,018**	0,447
IL21+IL17+	0,29 (0,13-0,46)	0,23 (0,16-0,60)	0,16 (0,09-0,54)	0,33 (0,15-0,75)	0,935	0,323	0,290	0,396	0,128	0,113
B-cells subsets										
IgD and CD27 surface expression										
Naïve	66,6 (52,0-77,5)	67,1 (47,8-75,2)	55,8 (27,6-66,3)	53,8 (42,6-74,2)	0,555	**0,044**	0,065	0,235	0,072	0,618
Memory	29,5 (20,1-44,2)	31,1 (22,7-45,4)	32,2 (24,2-59,7)	44,3 (23,6-54,7)	0,399	0,214	**0,029**	0,169	0,488	0,618
Unswitched memory	13,6 (8,7-22,0)	17,5 (10,9-25,5)	16,3 (10,3-28,4)	21,6 (13,7-32,2)	0,166	0,488	**0,028**	0,195	0,833	0,249
Switched memory	14,5 (9,89-21,3)	14,7 (10,7-21,7)	17,4 (13,7-30,7)	18,7 (13,7-27,5)	0,802	0,127	0,133	0,119	0,155	0,979
Transitional CD24HiCD38Hi	5,7 (3,1-9,5)	4,0 (2,2-5,8)	3,0 (1,0-6,1)	3,2 (2,5-6,5)	**0,026**	**0,037**	0,119	0,778	0,255	0,389
CD24HiCD27+	16,9 (11,9-25,6)	21,4 (13,9-35,8)	23,2 (12,1-38,6)	32,0 (20,0-42,5)	**0,045**	0,281	**0,003**	0,139	0,855	0,287
Plasmablasts $IgM^{-/+}$ $CD38^{++}$	2,0 (0,9-3,5)	1,2 (0,8-1,9)	1,4 (1,3-2,9)	1,4 (1,0-1,9)	**0,006**	0,459	0,063	0,507	0,052	0,373

Exploring the association between Tfh subsets and disease activity, we found a positive correlation between the ESSDAI and Tfh1 percentages, (r = 0.295, p = 0.029), which was more significant in patients with active disease (r = 0.607, r = 0.001), and among those, stronger when considering patients with ESSDAI≥5 (r = 0.914, r = 0.001) (Figure 3. Conversely, a negative correlation was found between the ESSDAI and Tfh17 percentages

(r = -0.365, p = 0.006), which was also stronger in patients with active disease (r = -0.482, p = 0.013) or with ESSDAI≥5 (r = -0.705, p = 0.023).

As for CXCR5+CD8+Tfc cells, no differences were found in percentages and absolute counts when comparing SjS with *Sicca*, RA or HC.

3.3.2. IL21 production by follicular T-cells

In the functional evaluation for IL21 production, SjS patients were found to have increased percentages of IL21-secreting $CD4^+$T-cells when compared to *Sicca* (p = 0.007), RA (p = 0.064) and HC (p = 0.013), although without significant differences in absolute counts. IL21-secreting $CD8^+$ T-cells percentages were also increased in SjS patients when compared to *Sicca* (p = 0.071), RA (p = 0.002) and HC (p = 0.022), with a corresponding increase in absolute counts which reached significance when comparing SjS with RA patients (p = 0.016). Data are shown in Table 3 and Supplementary Table 1.

Moreover, when considering SjS patients with clinically active disease, a positive correlation was found between the ESSDAI score and either $IL21^+CD4^+$ (r = 0.446, p = 0.023) and $IL21^+CD8^+$ T-cells (r = 0.470, p = 0.016) percentages (Figure 3).

3.3.3. B-cell subsets

The percentages of IgD^+CD27^-B-cells (naïve) were higher in SjS patients when compared to RA (p = 0.044) and HC (p = 0.065), whereas memory B-cells ($CD27^+$) percentages were lower in SjS comparing to HC (p = 0.029).

Transitional $IgM^{++}CD38^{++}$B-cells were increased in SjS patients with a numerical trend to significance when compared to *Sicca* (p = 0.064).

Among B-cells subsets known for being enriched in regulatory cells, $CD24^{Hi}CD38^{Hi}$B-cells were increased in SjS patients, with significant differences when compared to *Sicca* (p = 0.026) and RA (p = 0.037). Conversely, a significant decrease in $CD24^{Hi}CD27^+$B-cells was found in SjS patients, when compared to *Sicca* (p = 0.045), and HC (p = 0.003). Results are summarized in Table 3 and Supplementary Table 1.

Finally, we explored the association between B-cells subsets and disease activity in SjS patients and found significant negative correlations between the ESSDAI and absolute counts of memory B-cells (r = -0.316, p = 0.019), switched memory B-cells (r = -0.315, p = 0.019), and $CD24^{Hi}CD27^+$B-cells (r = -0.270, p = 0.047) (Figure 4). A stronger negative correlation was found between the ESSDAI and switched memory B-cells (r = -0.651, p = 0.041) and $CD24^{Hi}CD27^+$B-cells (r = -0.644, p = 0.045) when considering only patients with ESSDAI≥5.

3.4. Association between Ophthalmic parameters and Lymphocyte subsets.

We then explored the association between ocular and immune parameters.

Regarding B-cells, positive correlations were found between CNFL and absolute counts of total memory (r = 0.299, p = 0.027), unswitched memory (r = 0.281, p = 0.038) and $CD24^{Hi}CD27^{+}$ B-cells (r = 0.278, p = 0.040), and a tendency for a positive correlation between CNFD and switched memory B-cells counts (r = 0.644, p = 0.068) (Figure 5).

Considering T-cells, a negative correlation was found between CNFL and $IL21^{+}CD8^{+}$ T-cells counts (r = -0.279, p = 0.039). Moreover, when considering only patients with ESSDAI≥5, a tendency for a positive correlation between CNFL and Tregs counts was found (r = 0.644, p = 0.069), as well as a strong negative correlation between Tfh1 and both CNFL and CNFD (r = -0.760, p = 0.017, and r-0.700, p = 0.043, respectively (Figure 5).

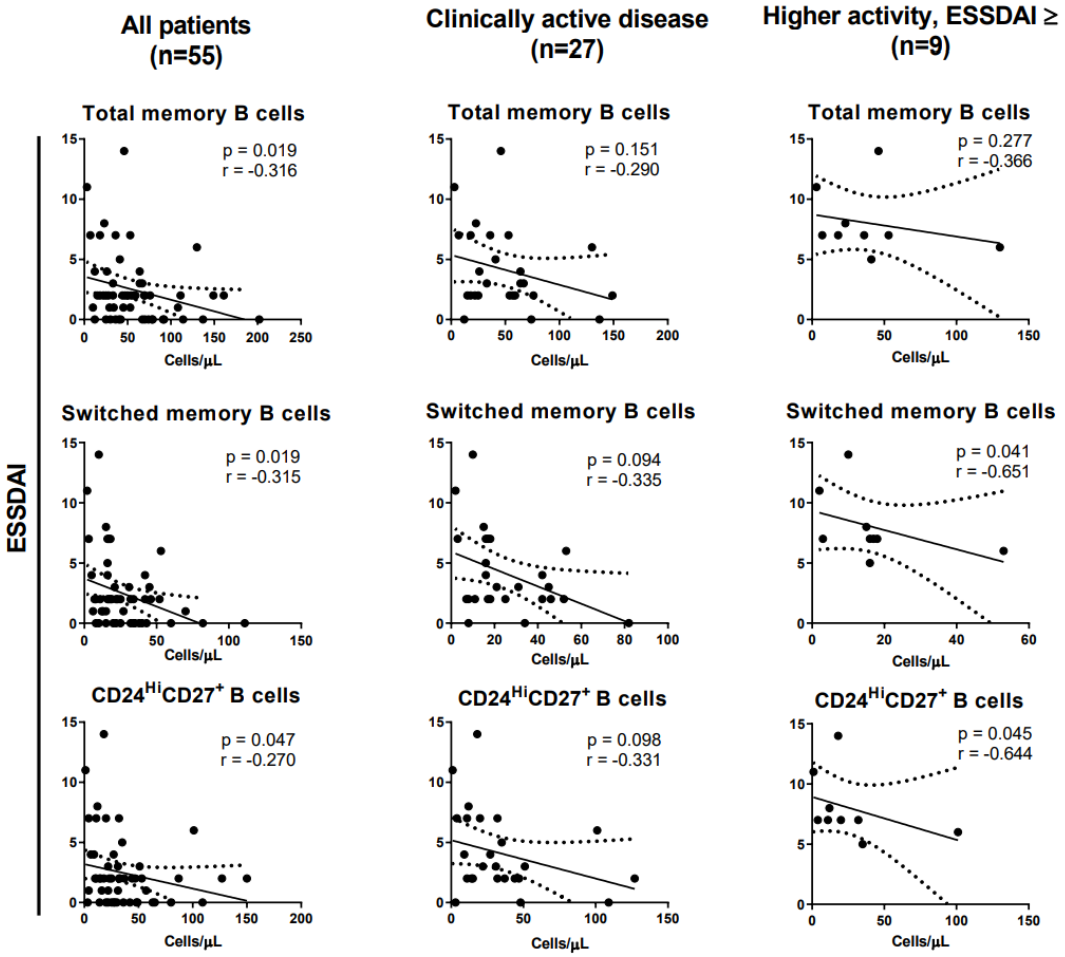

Figure 4 ESSDAI score correlations with B-cell subsets in all patients, patients with clinically active disease, and patients with ESSDAI score ≥ 5. Spearman correlation coefficients, 95% confidence interval, and p-values are indicated.

Discussion

Our study aimed to characterize ocular objective findings in SjS patients, particularly the study of corneal innervation through confocal microscopy, and to explore their association with clinical and immunological features of the disease. Thus, changes in circulating

lymphocyte subsets were studied to assess their relationship with ocular and clinical manifestations, as well as disease activity.

Although we found no differences in the percentages of circulating $CXCR5^+$ follicular T-cells in SjS patients, our study supports the evidence of an increased follicular compartment in SjS when considering $IL21^+CD4^+$ and $IL21^+CD8^+$ T-cells subsets. A modest positive correlation was found between ESSDAI and $IL21^+CD8^+$ T-cells in the whole SjS group. However, greater correlations were found between disease activity and both Tfh and Tfc cells when considering only patients with clinically active disease, which was even more robust considering patients with higher disease activity (ESSDAI≥5).

We also found, among $CXCR5^+CD4^+$ T-cells, a higher proportion of the Tfh1 subset in SjS, which also correlated with ESSDAI. This observation may be in concordance with the accepted Th1-derived responses (Saito et al. 2018) in the pathogenesis of SjS.

Our data on the association between disease activity and follicular T-cells not only are in accordance with the proposed role of Tfh cells in the pathogenesis of SjS (Pontarini, Lucchesi & Bombardieri 2018, Verstappen et al. 2017) but also suggest, for the first time, the involvement of Tfc.

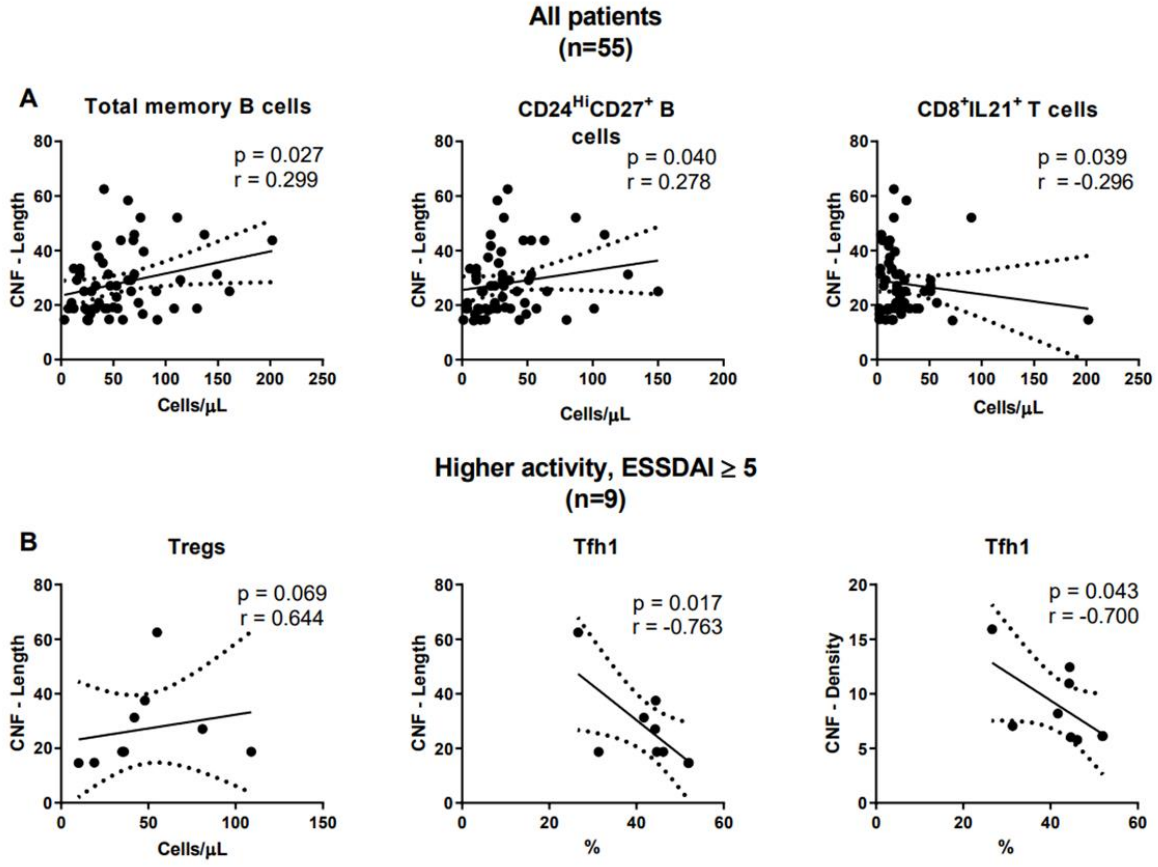

Figure 5 CNF Length and Density correlations with T and B-cell subsets in all patients (A), and in patients with ESSDAI score ≥ 5 (B). Spearman correlation coefficients, 95% confidence interval, and p-values are indicated. CNF, Corneal Nerve Fibre.

We confirmed a decrease in absolute numbers of circulating T-cells, which is a well-described feature of SjS (Talal et al. 1974). This could indicate retention of these cells at target organs, as supported by previous studies showing a T-cell predominance in lymphocytic infiltrates of exocrine glands (Singh & Cohen 2012). The noteworthy exception was the higher numbers of $IL21^{+}CD8^{+}$ T-cells, which showed a numerical trend to significance, compared to the other study groups, reinforcing the suggestion of a role of these cells is SjS pathology.

Our cohort also evidenced the distinctive B-cells profile classically considered for SjS-lower levels of memory subsets and increased naïve and transitional compartments, as previously reported (Barcelos et al. 2018, Roberts et al. 2014). These B-cell disturbances in SjS may represent the presence of a more immature component in transit to target-organs and a shift in maturation towards the plasma cell lineage. Also, the increased frequency of circulating follicular T-cells in SjS may represent the escape of some cells from target-organs where they are undergoing expansion and supporting GC-like structures.

A hallmark feature of SJS is decreased tear flow, but several other conditions may also impair lacrimal gland function (Conrady, Joos & Patel 2016). Ocular assessment by classical tests (Schirmer's-II, TBUT) revealed that SjS, *Sicca*, and RA patients had lower levels compared to HC.

It is known that tear composition is affected by the lacrimal gland inflammatory environment and that corneal integrity and innervation are compromised in DED (Tsubota et al. 2017). Therefore, the development of novel and more specific tests to assess ocular involvement in SjS is an active field of research.

In this study, SjS and *Sicca* patients presented a significantly lower CNFD and CNFL, as well as a higher CNFT, compared to HC, which has already been described (Belmonte et al. 2017, Cardigos et al. 2019, Tuisku et al. 2008). Interestingly, our non-*Sicca* RA group presented lower CNFL and CNFD than HC, and higher CNFT, suggesting an influence of the inflammatory environment in corneal nerve damage and regrowth. Despite that, the differences found in corneal nerve morphology between both pSS and *Sicca* patients and RA patients may represent the impact of DED on cornea innervation.

Although corneal nerves characterization could be a promising instrument to evaluate ocular signs of SjS, the relationship between their changes and disease phenotype, systemic disease activity, and lymphocytic profile had not been explored before. Exploring the association between disease activity and corneal nerve parameters, we found a strong negative correlation between the ESSDAI and CNFL and CNFD in patients with higher disease activity (ESSDAI≥5). In fact, patients with higher ESSDAI presented worst ocular outcomes, i.e., lower CNFD and CNFL. These data suggest that corneal nerve damage may parallel systemic disease activity, driven by the immune environment in these patients, since patients with higher disease activity presented higher levels of Tfc cells, but lower Tregs, and even $CD24^{Hi}CD27^{+}$B-cell subsets. Ocular dysfunction, along with disease activity,

could then relate to the lack of the protective immunossupressive subsets, such as Tregs and regulatory B-cells within the $CD24^{Hi}CD27^{+}$ compartment.

The absence of a more evident association between disease activity and corneal nerve parameters in the whole SjS population may be due to the presence of many stable patients with long-standing disease and undergoing systemic therapy, who had inactive disease or very low ESSDAI scores. The assessment of SjS patients with less evolved disease and not undergoing therapy could better clarify the association between corneal nerve parameters, disease activity, and lymphocyte subset distribution. Moreover, the possibility to assess local tissues could also help to clarify the immune cells movements between different target sites.

Regarding our study limitations, our study included patients with variable disease duration, which could influence some findings. The effect of therapies in lymphocyte populations is also elusive and unquantified. SjS patients (as well as *Sicca* and RA patients) were under different therapeutic strategies and this might affect lymphocytic populations and, consequently, the correlations observed between lymphocytic populations and ocular features. Also, we evaluated exclusively peripheral blood lymphocyte subsets, even though the salivary and lachrymal glands are major target-organs.

A future study would aim to verify the induction of lymphocyte subsets by ocular surface cells, to demonstrate if ocular cells can induce autoimmune epithelitis the same way exocrine glands do, demonstrating that ocular cells are also active participants in the destructive autoimmune cycle of SjS (Manoussakis & Kapsogeorgou 2010).

This is the first study conducted to correlate ocular findings with lymphocyte subsets in SjS. DED damage to the sub-basal corneal nerve plexus was evidenced, particularly in patients with SjS, which was greater with longer duration and disease activity. Moreover, the association between disease activity and some B-cell subsets with CNFD and CNFL favours the hypothesis that corneal nerve damage may parallel systemic disease activity. Evaluating ocular findings, particularly the cornea, not only for SjS diagnosis but also to assess treatment efficacy might be increasingly explored considering the easy access to this tissue. Further prospective longitudinal studies may clarify these results.

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References

Barcelos F, Martins C, Papoila A, et al. (2018): Association between memory B-cells and clinical and immunological features of primary Sjögren's syndrome and *Sicca* patients. *Rheumatol Int* **38**: 1063–1073.

Baudouin C (2001): The Pathology of Dry Eye. *Surv Ophthalmol* **45**: S211–S220.

Belmonte C, Nichols JJ, Cox SM, et al. (2017): TFOS DEWS II pain and sensation report. *Ocul Surf* **15**: 404–437.

Benítez-del-Castillo JM, Acosta MC, Wassfi MA, Díaz-Valle D, Gegúndez JA, Fernandez C & García-S´snchez J (2007): Relation between Corneal Innervation with Confocal Microscopy and Corneal Sensitivity with Noncontact Esthesiometry in Patients with Dry Eye. *Investig Opthalmology Vis Sci* **48**: 173.

Bron AJ, de Paiva CS, Chauhan SK, et al. (2017): TFOS DEWS II pathophysiology report. *Ocul Surf* **15**: 438–510.

Bron AJ, Evans VE & Smith JA (2003): Grading Of Corneal and Conjunctival Staining in the Context of Other Dry Eye Tests. *Cornea* **22**: 640–650.

Cardigos J, Barcelos F, Carvalho H, Hipólito D, Crisóstomo S, Vaz-Patto J & Alves N (2019): Tear Meniscus and Corneal Sub-basal Nerve Plexus Assessment in Primary Sjögren Syndrome and *Sicca* Syndrome Patients. *Cornea* **38**: 221–228.

Chen X, Aqrawi LA, Utheim TP, et al. (2019): Elevated cytokine levels in tears and saliva of patients with primary Sjögren's syndrome correlate with clinical ocular and oral manifestations. *Sci Rep* **9**: 7319.

Conrady CD, Joos ZP & Patel BCK (2016): Review: The Lacrimal Gland and Its Role in Dry Eye. *J Ophthalmol* **2016**: 1–11.

De Paiva CS, Corrales RM, Villarreal AL, Farley WJ, Li D-Q, Stern ME & Pflugfelder SC (2006): Corticosteroid and doxycycline suppress MMP-9 and inflammatory cytokine expression, MAPK activation in the corneal epithelium in experimental dry eye. *Exp Eye Res* **83**: 526–35.

Kalteniece A, Ferdousi M, Adam S, Schofield J, Azmi S, Petropoulos I, Soran H & Malik RA (2017): Corneal confocal microscopy is a rapid reproducible ophthalmic technique for quantifying corneal nerve abnormalities. (Bhattacharya S, Ed.) *PLoS One* **12**: e0183040.

Kassan SS & Moutsopoulos HM (2004): Clinical Manifestations and Early Diagnosis of Sjögren Syndrome. *Arch Intern* Med **164**: 1275.

Lee SY, Han SJ, Nam SM, Yoon SC, Ahn JM, Kim T-I, Kim EK & Seo KY (2013): Analysis of Tear Cytokines and Clinical Correlations in Sjögren Syndrome Dry Eye Patients and Non–Sjögren Syndrome Dry Eye Patients. *Am J Ophthalmol* **156**: 247-253.e1.

Lemp MA (1995): Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. *CLAO J* **21**: 221–32.

Malik RA, Kallinikos P, Abbott CA, van Schie CHM, Morgan P, Efron N & Boulton AJM (2003): Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. *Diabetologia* **46**: 683–688.

Manoussakis MN & Kapsogeorgou EK (2010): The role of intrinsic epithelial activation in the pathogenesis of Sjögren's syndrome. *J Autoimmun* **35**: 219–224.

Nichols KK, Nichols JJ, MPH M & Mitchell GL (2004): The Lack of Association Between Signs and Symptoms in Patients With Dry Eye Disease. *Cornea* **23**: 762–770.

Oliveira-Soto L & Efron N (2001): Morphology of Corneal Nerves Using Confocal Microscopy. *Cornea* **20**: 374–384.

Petroll WM & Robertson DM (2015): In Vivo Confocal Microscopy of the Cornea: New Developments in Image Acquisition, Reconstruction, and Analysis Using the HRT-Rostock Corneal Module. *Ocul Surf* **13**: 187–203.

Petropoulos IN, Ponirakis G, Khan A, Gad H, Almuhannadi H, Brines M, Cerami A & Malik RA (2019): Corneal confocal microscopy: ready for prime time. *Clin Exp Optom* 1–13.

Pontarini E, Lucchesi D & Bombardieri M (2018): Current views on the pathogenesis of Sjögren's syndrome.

Curr Opin Rheumatol **30**: 215–221.

Quigley MF, Gonzalez VD, Granath A, Andersson J & Sandberg JK (2007): CXCR5+ CCR7– CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. *Eur J Immunol* **37**: 3352–3362.

Roberts MEP, Kaminski D, Jenks SA, et al. (2014): Primary Sjögren's Syndrome Is Characterized by Distinct Phenotypic and Transcriptional Profiles of IgD+ Unswitched Memory B Cells. *Arthritis Rheumatol* **66**: 2558–2569.

Sahin A, Kam WR, Darabad RR, Topilow K & Sullivan DA (2012): Regulation of Leukotriene B 4 Secretion by Human Corneal, Conjunctival, and Meibomian Gland Epithelial Cells. *Arch Ophthalmol* **130**: 1013.

Saito M, Otsuka K, Ushio A, Yamada A, Arakaki R, Kudo Y & Ishimaru N (2018): Unique Phenotypes and Functions of Follicular Helper T Cells and Regulatory T Cells in Sjögren's Syndrome. *Curr Rheumatol* Rev **14**: 239–245.

Seror R, Ravaud P, Bowman SJ, et al. (2010): EULAR Sjögren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjögren's syndrome. Ann *Rheum Dis* **69**: 1103–1109.

Singh N & Cohen PL (2012): The T cell in Sjögren's syndrome: Force majeure, not spectateur. *J Autoimmun* **39**: 229–233.

Stern ME, Schaumburg CS, Siemasko KF, et al. (2012): Autoantibodies Contribute to the Immunopathogenesis of Experimental Dry Eye Disease. *Investig Opthalmology Vis Sci* **53**: 2062.

Szabo K, Papp G, Barath S, Gyimesi E, Szanto A & Zeher M (2013): Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. *Clin Immunol* **147**: 95–104.

Talal N, Sylvester RA, Daniels TE, Greenspan JS & Williams RC (1974): T and B Lymphocytes in Peripheral Blood and Tissue Lesions in Sjögren's Syndrome. *J Clin Invest* **53**: 180–189.

Tavakoli M & Malik RA (2011): Corneal Confocal Microscopy: A Novel Non-invasive Technique to Quantify Small Fibre Pathology in Peripheral Neuropathies. *J Vis Exp*.

Tsubota K, Xu KP, Fujihara T, Katagiri S & Takeuchi T (1996): Decreased reflex tearing is associated with lymphocytic infiltration in lacrimal glands. *J Rheumatol* **23**: 313–320.

Tsubota K, Yokoi N, Shimazaki J, et al. (2017): New Perspectives on Dry Eye Definition and Diagnosis: A Consensus Report by the Asia Dry Eye Society. *Ocul Surf* **15**: 65–76.

Tuisku IS, Konttinen YT, Konttinen LM & Tervo TM (2008): Alterations in corneal sensitivity and nerve morphology in patients with primary Sjögren's syndrome. *Exp Eye Res* **86**: 879–885.

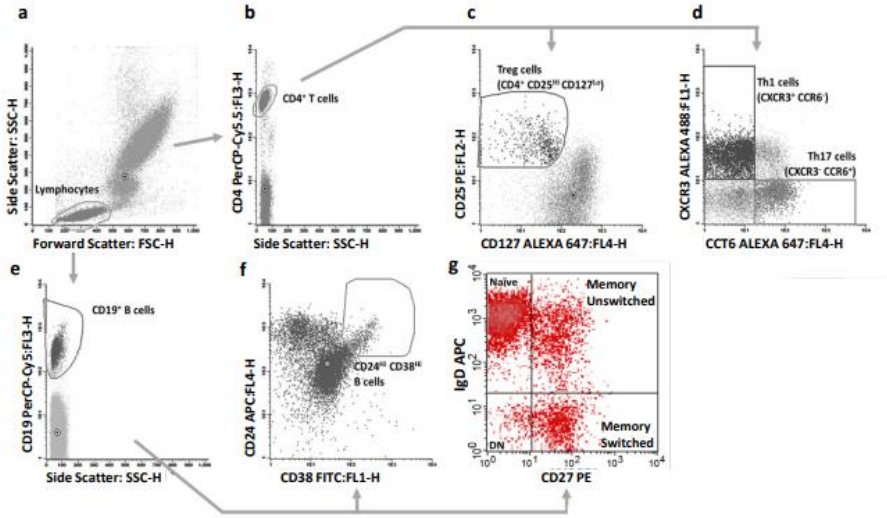
Verstappen GM, Kroese FGM, Meiners PM, et al. (2017): B Cell Depletion Therapy Normalizes Circulating Follicular Th Cells in Primary Sjögren Syndrome. *J Rheumatol* **44**: 49–58.

Whitcher JP, Shiboski CH, Shiboski SC, et al. (2010): A Simplified Quantitative Method for Assessing Keratoconjunctivitis *Sicca* From the Sjögren's Syndrome International Registry. *Am J Ophthalmol* **149**: 405–415.

Yeh S, Song XJ, Farley W, Li D-Q, Stern ME & Pflugfelder SC (2003): Apoptosis of Ocular Surface Cells in Experimentally Induced Dry Eye. *Investig Opthalmology Vis Sci* **44**: 124.

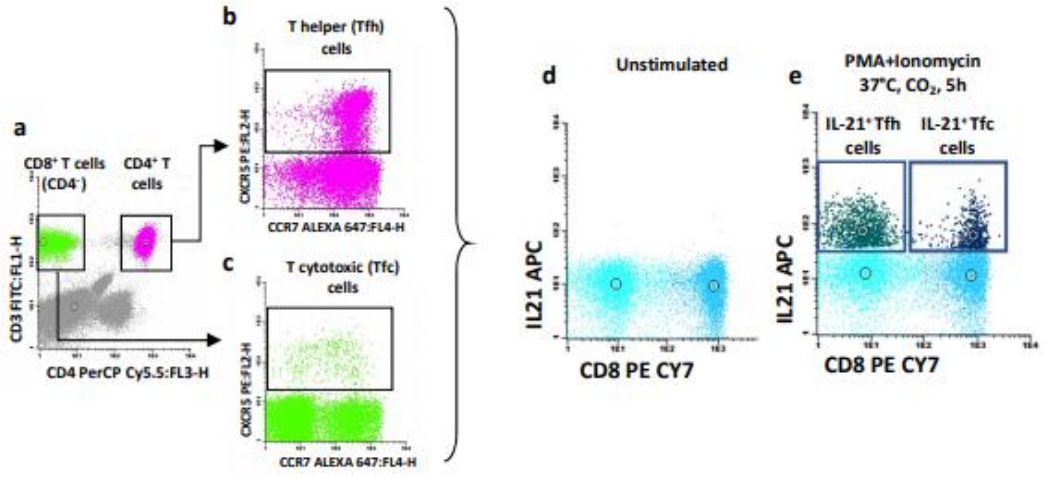
Supplementary Data

Supplementary Figure 1 Gating strategy for the identification of distinct T and B-cell subsets.



(a) Lymphocytes were identified according to forward and side-scatter characteristics. (b and e) $CD4^+$ T and B cells were identified as $CD4^+$ (b) and $CD19^+$ (e) cells within the lymphocyte gate, respectively. (c) Treg subset was identified according to the expression of CD25 and CD127 within the $CD4^+$ T cells gate. (d) T helper (Th) subsets were identified according to the expression of CXCR3 and CCR6 in Th1 ($CXCR3^+CCR6^-$) and Th17 ($CXCR3^-CCR6^+$) cells. (f) B cells with a regulatory phenotype were identified as $CD24^{Hi}CD38^{Hi}$ cells within the B lymphocyte gate. (g) B cell subsets were identified according to the expression of IgD and CD27 in naive, IgD^+CD27^-; unswitched memory/marginal zone like (MZ), IgD^+CD27^+; switched memory, IgD^-CD27^+; and double-negative (DN) memory B cells: IgD^-CD27^-.

Supplementary Figure 2 Identification of follicular T cell subsets.



a-c) Gating strategy for the identification of follicular-related ($CXCR5^+CCD7^{-/+}$) cytotoxic (b) and helper (c) T cells. (d and e) IL-21-producing $CXCR5^+$ $CD4^+$ and $CD8^+$ T cells were analysed for the expression of IL-21 after a 5-h incubation period without stimulation (d) and after stimulation (e) with PMA (Phorbol Myristate Acetate) and ionomycin, for 5h at 37°C in a 5% CO_2 atmosphere in the presence of brefeldin A. Unstimulated tubes were used to assess IL-21 positivity.

Supplementary Table 1 Comparison of lymphocyte subsets and ratios (absolute counts) in Sjögren (SjS), *Sicca*, Rheumatoid Arthritis (RA) and healthy controls (HC).

					Group comparisons (*p*-values)					
Lymphocyte %, median (25th-75th percentile)	SjS n=55	*Sicca* n=63	Rheumatoid Arthritis n=18	Healthy Controls n=20	SjS vs Sicca	SjS vs RA	SjS vs HC	Sicca vs HC	Sicca vs RA	RA vs HC
Lymphocytes	1517 (1132-2363)	1930 (1491-2288)	1935 (1532-2250)	2228 (1987-3179)	0,130	**0,001**	0,192	**0,009**	0,817	**0,030**
B cells	177 (85-261)	220 (140-295)	129 (77-148)	252 (168-434)	0,095	**0,016**	**0,011**	0,119	**<0,001**	**<0,001**
T cells	1143 (757-1784)	1348 (1035-1677)	1369 (1244-1726)	1705 (1416-2418)	0,226	**0,001**	0,093	**0,003**	0,332	0,076
CD4+	735 (424-1071)	880 (603-1176)	908 (686-1115)	1097 (998-1491)	**0,044**	**<0,001**	0,100	**0,002**	0,797	**0,022**
Th1	259 (168-410)	359 (214-468)	289 (240-388)	451 (329-596)	**0,035**	**<0,001**	0,264	**0,013**	0,566	**0,008**
Th17	125 (86-224)	198 (124-262)	187 (149-250)	275 (196-401)	**0,027**	**<0,001**	0,111	**0,009**	0,884	**0,011**
Tregs	65 (36-89)	69 (45-85)	69 (55-81)	92 (66-109)	0,490	**0,002**	0,440	**0,003**	0,725	**0,013**
CD8+	434 (318-636)	439 (299-573)	533 (340-683)	548 (391-753)	0,584	0,131	0,348	**0,034**	0,167	0,492
CXCR5+CD4+ T-cells	134 (82-203)	166 (118-202)	176 (134-224)	237 (186-288)	0,152	**<0,001**	0,068	**0,001**	0,383	**0,016**
Expression of CCR3 and CCR6 [a]										
Tfh1 (CCR3+CCR6-)	51 (25-71)	55 (34-71)	55 (42-75)	79 (47-84)	0,437	**0,006**	0,382	**0,009**	0,721	0,062
Tfh17 (CCR3-CCR6+)	28 (16-47)	36 (22-53)	36 (27-55)	52 (35-82)	**0,050**	**<0,001**	0,056	**0,004**	0,539	0,058
Surface expression of CCR7 [b]										
Tfh Naïve (CCR7+)	25 (14-37)	28 (18-41)	34 (21-47)	48 (30-68)	0,452	**0,001**	0,115	**0,003**	0,323	0,104
Tfh Differentiated (CCR7-)	0,6 (0,3-1,1)	0,4 (0,2-1,0)	0,7 (0,4-1,4)	1 (0,6-1,6)	0,392	0,034	0,240	**0,008**	0,128	0,387
CXCR5+CD8+ T-cells										
Tfc Naïve (CCR7+)	9 (5-14)	9 (5-12)	6 (4-13)	11 (6-18)	0,986	0,292	0,319	0,293	0,274	0,096
Tfc Differentiated (CCR7-)	3 (2-5)	3 (2-5)	3 (2-7)	5 (3-6)	0,844	0,274	0,926	0,166	0,909	0,400
IL-21 producing CD4+ T-cells										
IL21+	86 (51-122)	85 (57-111)	91 (64-107)	110 (60-152)	0,808	0,225	0,763	0,215	0,675	0,287
IL21+IL17+	5 (3-8)	6 (4-8)	6 (3-8)	7 (4-12)	0,074	0,182	0,931	0,537	0,309	0,319
IL-21 producing CD8+ T-cells										
IL21+	18 (10-26)	14 (8-21)	9 (6-16)	12 (6-25)	0,080	0,167	**0,016**	0,701	0,128	0,538
IL21+IL17+	1 (1-2)	1 (1-2)	1 (1-2)	2 (1-4)	0,930	0,126	0,969	0,084	0,964	0,225
B-cells subsets										
IgD and CD27 surface expression										
Naïve	106 (60-195)	136 (71-194)	58 (12-95)	142 (86-247)	0,204	0,107	**0,011**	0,435	**<0,001**	**<0,001**
Memory	47 (28-74)	65 (40-93)	39 (22-53)	101 (65-177)	**0,026**	**<0,001**	0,275	**0,006**	**0,001**	**<0,001**
Unswitched memory	22 (11-37)	34 (20-47)	17 (11-29)	54 (29-83)	**0,014**	**0,001**	0,250	**0,022**	**0,002**	**<0,001**
Switched memory	22 (15-35)	30 (19-46)	19 (12-33)	45 (37-81)	**0,042**	**<0,001**	0,475	**0,004**	**0,036**	**<0,001**
Transitional CD24HiCD38Hi	8 (3-18)	8 (4-13)	3 (1-5)	10 (5-21)	0,688	0,590	**0,002**	0,356	**0,001**	**0,001**
CD24HiCD27+	28 (15-48)	44 (27-73)	22 (13-40)	78 (48-135)	**0,002**	**<0,001**	0,448	**0,011**	**0,004**	**<0,001**
Plasmablasts $IgM^{-/+}$ $CD38^{++}$	3 (1-6)	2 (1-4)	2 (1-3)	4 (2-5)	0,413	0,306	0,082	**0,025**	0,140	**0,002**

[a] percentages among Tfh. CCR3+CCR6+ and CCR3-CCR6- cells not shown; [b] includes Tfh1, Tfh17 and other subsets

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CAPÍTULO VII. Lymphocyte subpopulations in the classification of Sjögren's syndrome | *Subpopulações linfocitárias na classificação da Síndrome de Sjögren.*

Lymphocyte subpopulations in the classification of Sjögren's syndrome

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Abstract

Background

Classification criteria are paramount in clinical and epidemiological studies of Sjögren's Syndrome (SjS). Since not all patients with clinical diagnosis fulfil classification criteria, the search for novel biomarkers is an active research area. We aimed to evaluate the added value of the inclusion of lymphocyte subpopulations regarding discrimination between SjS and non-Sjögren *Sicca* patients.

Methods

We have included 62 patients with clinical diagnosis of SjS determined by expert opinion (Gold Standard – GS) and 63 *Sicca* patients. Circulating B and T-cell subsets were characterized by flow cytometry. The 2002 American-European Consensus Grou*p* (AECG) and the 2016 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) SjS classification criteria were compared with clinical diagnosis. The added discriminative ability of joining lymphocytic populations to classification criteria was assessed by the area under the Receiver-Operating-Characteristic Curve (AUC) estimated by multivariable Firth's bias-reduced logistic regression. De Long's test was used to compare AUCs. A level of significance $\alpha = 0.05$ was considered.

Results

The agreement between AECG and ACR/EULAR criteria was 95.2%. Considering the GS, we obtained an AUC = 0.952 (95%CI:0.916-0.989) for the AECG criteria, and an UC = 0.921 (95%CI:0.875-0.966) for the ACR/EULAR criteria. Adding Tfh and Bm1 subsets to AECG criteria, performance of the multivariable model increased, attaining an AUC = 0.985 (95%CI:0.968-1.000) ($p = 0.021$). For ACR/EULAR criteria, Th1/Breg-like $CD24^{hi}CD27^{+}$ and switched memory B-cells (absolute counts) maximized AUC to 0.953 (95%CI: 0.916-0.990; $p = 0.043$).

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Conclusions
Lymphocyte subpopulations may assist in discriminating SjS from *Sicca* patients as the discriminative ability between these two groups of patients increases when specific lymphocyte subsets are added to the classification criteria.

Keywords: lymphocyte subpopulation, Sjögren's syndrome, non-Sjögren *Sicca* syndrome, classification criteria

Key messages:
Lymphocyte subsets show distinctive features in Sjögren's Syndrome patients compared to non-Sjögren Sicca patients.
Inclusion of immune cell subsets can improve the performance of classification criteria for Sjögren's Syndrome

Introduction

Sjögren's Syndrome (SjS) is a chronic systemic immune-mediated inflammatory disease that occurs due to lymphocytic infiltration and consequent lesion of exocrine glands[1]. The most common symptoms are xerostomia and xerophthalmia but extra-glandular symptoms occur in u*p* to 50% of patients[2].

In addition to symptoms of dryness, objective signs are sought to confirm salivary or lachrymal gland dysfunction[3,4]. For dry eye, these include evidence of keratoconjunctivitis *sicca* (quantified as a score, like the Ocular Staining Score[5]) and Schirmer's test[4]. Decreased salivary glands (SG) function may be assessed by sialometry or indirectly suspected through sonographic changes of the SG[6]. The main serological markers of SjS are anti-SSA and anti-SSB antibodies, present in 50-70% and 33-50% of patients, respectively[7]. The most specific finding for SjS diagnosis, and a major item in the latest classification criteria, is the demonstration of focal lymphocytic infiltration on histology of minor labial SG biopsy[8].

Although not designed for this purpose, classification criteria are useful in clinical practice as a guidance to support diagnosis. The 2002 American-European Consensus Grou*p* (AECG) SjS classification criteria[9] have been widely used since their presentation, with over 1500 references[10]. The new classification criteria proposed in 2016 by the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR)[11] were intended to replace the AECG criteria (Supplementary Table 1).

Nevertheless, despite the high sensitivity and specificity of both criteria sets, not all patients with a clinical diagnosis of SjS will fulfil classification criteria[12].

Efforts to increase the performance of SjS classification criteria have been made. Regarding the 2016 ACR/EULAR classification criteria, studies already assessed the effect of the inclusion of salivary gland sonography features in these criteria[13]. Regarding the

previous 2002 AECG classification criteria, Binard studied the effect of adding a naive-to-mature B-cells ratio to the criteria, reporting an increase in their performance for the diagnosis[14]. However, in a later study, these parameters performed poorly in a distinct clinical setting[15].

B-cells hyperactivity is the hallmark of SjS, playing a major role in pathogenesis and clinical evolution[16]. Changes in the distribution of peripheral blood B-cell subsets in SjS are typical, with increased naïve B-cells and decreased memory B-cells, particularly unswitched memory[17].

T-cells infiltrate target-organs, such as SG and lachrymal glands, produce pro-inflammatory cytokines and interact with B-cells in germinal centre (GC)-like structures, resulting in B-cell activation[18]. Th1 cells were the first $CD4^+$ effector subset to be recognized and associated with autoimmunity and their role in the pathogenesis of SjS is well described as the accumulation of IFNγ-producing Th1 cells in the salivary glands contribute to epithelial cell damage and, consequently, diminished saliva secretion[18].

Follicular helper T-cells (Tfh) have recently emerged as a key player in SjS pathogenesis since Tfh cells are a major source of IL-21, a cytokine that mediates B-cell differentiation and proliferation and promotes the ectopic formation of GC-like structures[19].

Th17 and Treg cells play opposite roles in autoimmune and inflammatory diseases. While Th17 cells promote autoimmunity, Tregs aim to control it and therefore play a crucial role in maintaining self-tolerance and controlling the expansion and activation of autoreactive $CD4^+$ effector T-cells[20].

The role of B-cell subpopulations as a biological marker for diagnosis and classification of SjS has been previously assessed[15]. T-cells also participate in SjS pathogenesis[18], but to our knowledge, no study has been made regarding their use as a biomarker in classification criteria.

We aimed to measure the performance of ACR/EULAR and AECG criteria when compared to the clinical diagnosis (considered the gold-standard, GS), and to evaluate the added value, regarding the discriminative ability to differentiate between SjS from non-Sjögren sicca patients, resulting from including lymphocytes subpopulations in both criteria. For that purpose, a comparison of B and T-cells subsets distribution between SjS and non-Sjögren *Sicca* patients was also performed.

Materials and Methods

Patient selection

Patients with confirmed or suspected SjS were consecutively selected from a SjS dedicated outpatient clinic. All took a multidisciplinary evaluation according to the AECG and ACR/EULAR criteria, including a labial gland biopsy, screening for anti-SSA antibodies, ocular evaluation, and unstimulated salivary flow assessment.

Clinical files were reviewed by the treating rheumatologists (FB, JVP) with patient identity occultation, and by an additional experienced rheumatologist (JCB). A clinical diagnosis was obtained according to at least 2 of the 3 expert physicians and considered the gold standard (GS) in this study. If a clinical diagnosis of SjS was established, the patient was included in the SjS group. Otherwise, the patient was included in the *Sicca* group.

The exclusion criteria applied to all participants were age under 18 years, hepatitis-C or human immunodeficiency virus infection, pre-existing lymphoma, sarcoidosis, graft-versus-host disease, IgG4-related disease, history of head and neck radiation treatment, current anticholinergic therapy, the use of B-cell-depleting therapies or other biologic disease-modifying anti-rheumatic drugs, and the presence of another systemic autoimmune disease.

Written informed consent was obtained from all participants. The study was approved by the Ethics committees of *Instituto Português de Reumatologia*, *Hospital Cuf Descobertas*, and NOVA Medical School (no. 17/2016/CEFCM).

Flow cytometry procedures

For the immunophenotyping protocols, peripheral blood samples collected in EDTA-coated tubes were processed and analyzed within 24h of collection. A pre-validated panel of monoclonal antibodies was used for the characterization of T and B-cell subsets, including CD3, CD4, CD8, CD19, CD24, CD27, CD38, CCR6, CCR7, CXCR3, CXCR5, Anti-IgD, and Anti-IgM. A lyse-wash protocol was performed for both T and B-cell characterization. A lyse-no wash single platform strategy with BD Trucount tubes (*BD Biosciences, San Diego CA, USA*) was used to obtain absolute counts of all cell subsets. All samples were acquired in a 4-colour BD FACS-Calibur cytometer (*BD Biosciences*).

CellQuest Pro™ (*BD Biosciences*) software was used for acquisition and analysis purposes, and Infinicyt™ 2.0 (*Cytognos S.L., Salamanca, Spain*) software was also used for more differentiated subset analysis.

Whenever appropriate, fluorescence-minus-one control tubes were prepared to assess the positivity of dimer expressions. The subsets analyzed, and the respective gating strategies, are displayed in Figure 1.

Within T-cells, we characterized $CD4^+$ and $CD8^+$ (CD4-) subsets, including Th1 (CXCR3+CCR6-), Th17 (CXCR3−CCR6+), Tregs ($CD4^+CD25^{hi}CD127^{lo}$), follicular-like $CXCR5^+$ $CD4^+$ (which we designated Tfh) and $CXCR5^+$ $CD8^+$ (designated Tfc). In the $CXCR5^+$ Tfh subset, we also identified Tfh1 (CXCR3+CCR6-) and Tfh17 (CXCR3−CCR6+).

B-cells' subsets were addressed according to the IgD/CD27 classification, where the expression of CD27 and IgD identifies naïve B-cells ($CD27^-IgD^+$), $CD27^+$ memory B-cells, unswitched ($CD27^+IgD^+$) and switched memory B-cells, ($CD27^+IgD^-$), and IgD^-CD27^- ("double negative") B-cells[21]. An alternative B-cell classification partially overlaps with the IgD/CD27, and identifies B-cell from Bm1 to Bm5[22]: Bm1 includes transitional and naïve B-cells (IgD^+CD38^-), which become Bm2 (IgD^+CD38^+) upon activation and progress to the germinal

centre founder Bm2' (IgD^+CD38^{++}), Bm3 centrocytes and Bm4 centrocytes (IgD^-CD38^+) that differentiate in the germinal centre into either plasma cells, or early and late memory B-cells (eBm5: IgD^-CD38^+ and Bm5: IgD^-CD38^-, respectively).

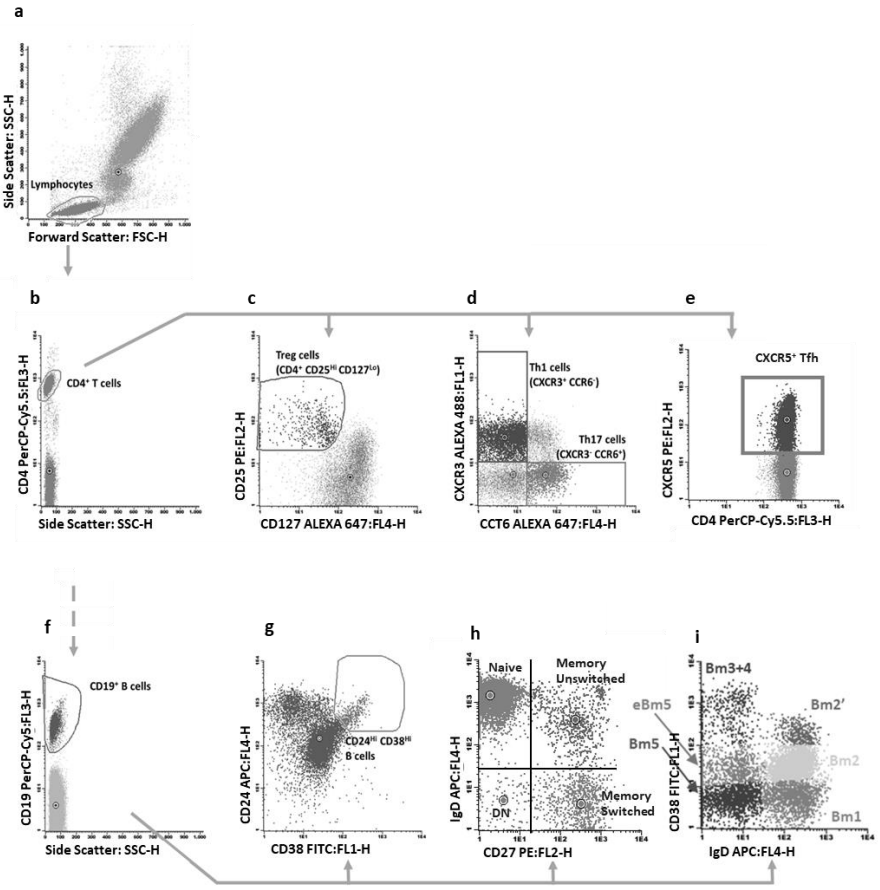

Figure 1 Gating strategy for the identification of distinct T and B-cell subsets
(a) Lymphocytes were identified according to forward and side-scatter characteristics. (b and f) $CD4^+$ T and B cells were identified as $CD4^+$ (b) and $CD19^+$ (f) cells within the lymphocyte gate, respectively. (c) Treg subset was identified according to the expression of CD25 and CD127 within the $CD4^+$ T cells gate. (d) T helper (Th) subsets were identified according to the expression of CXCR3 and CCR6 in Th1 ($CXCR3^+CCR6^-$) and Th17 ($CXCR3^-CCR6^+$) cells. (e) Follicular CD4 T cells were identified according to the expression of CXCR5. (g) In the B lymphocyte gate, we identified $CD24^{Hi}CD38^{Hi}$ B-cells, which include cells with a regulatory phenotype. (h) B cell subsets were identified according to the expression of IgD and CD27 in naive, IgD^+CD27^-; unswitched memory/marginal zone like (MZ), IgD^+CD27^+; switched memory, IgD^-CD27^+; and double-negative (DN) memory B cells: IgD^-CD27^-. (i) B cells were further characterized according to the Bm1-5 classification into Bm1 (IgD^+CD38^-), Bm2 (IgD^+CD38^+) Bm2' (IgD^+CD38^{++}), Bm3 and Bm4 (IgD^-CD38^+) and early and late memory B-cells (eBm5: IgD^-CD38^+ and Bm5: IgD^-CD38^-, respectively).

Statistical analysis

An exploratory analysis was carried out for all variables. Continuous variables were described with mean and standard deviation (SD) or median and inter-quantile range (IQR: 25^{th} percentile-75^{th} percentile), as appropriate. Categorical data were presented as

frequencies and percentages. Mann-Whitney non-parametric test was used to compare lymphocyte subsets or combinations, between SjS and non-Sjögren *Sicca* patients.

Univariable and multivariable analyses were performed using Firth's bias-reduced logistic regression models, used to deal with the problem of separation inherent to the data. Two univariable models (one for each classification criteria) considered clinical diagnosis (GS) as the dependent variable and each classification criteria as the independent variable. From these univariable models, two multivariable models resulted from adding the lymphocyte subsets or combinations. The discriminative ability, to distinguish between SjS and non-Sjögren sicca patients, of the univariable and multivariable models was compared through the Area Under the Receiver Operating Characteristic Curve (AUC), using DeLong test. A level of significance $\alpha = 0.05$ was used but, due to the exploratory nature of this study, results with p-values < 0.100 were still considered relevant. Data were analyzed using R software[23].

Table 1 SjS and *Sicca* Patients' characteristics.

	SjS n = 62	***Sicca*** n = 63
Age, mean (SD) (years)	58.1 (12.1)	60.9 (10.3)
Symptom duration, mean (SD) (years)	11.7 (7.6)	9.7 (4.9)
Ocular symptoms, n (%)	59 (95.2)	60 (95.2)
Oral symptoms, n (%)	60 (96.8)	60 (95.2)
Low Schirmer's, n (%)	34 (55.7) [a]	29 (46.8) [b]
Positive Corneal Staining, n (%)	20 (32.8) [a]	11 (17.7) [b]
Low Unstimulated Salivary Flow, n (%)	41 (66.1)	38 (30.3)
Focus score ≥ 1, n (%)	43 (71.7) [c]	0 (0)
Anti-SSA, n (%)	37 (67.3)	1 (1.6)
Anti-SSB, n (%)	18 (32.7) [d]	1 (1.8) [e]

a n = 61. b n = 62. c n = 60. d n = 55. e n = 56.
SjS, Sjögren's syndrome; SSA/SSB, Sjögren's syndrome antigen A and B.
Patient's characteristics are represented as the number of occurrences (n) and percentages (%). Whenever there were missing values, percentages reflect the number of occurrences over the number of patients tested for the item (a n = 61. b n = 62. c n = 60. d n = 55. e n = 56). SjS, primary Sjögren's syndrome; y, years; SSA/SSB, Sjögren's syndrome A/B antibody.

Results

Sixty-two patients were included in the SjS grou*p* and 63 in the *Sicca* group. Clinical and demographic data of SjS and *Sicca* are presented in Table 1.

Lymphocyte subsets

Complete results are presented in Table 2 and Supplementary Table 2.

Table 2 Comparison of T and B-cell subsets percentages in SjS and *Sicca* groups

Lymphocyte subsets or combinations Cells %, median (25th-75th percentile)	**SjS** n = 62	***Sicca*** n = 63	**SjS vs *Sicca* *p* values**
Lymphocytes	30.6 (11.0)	31.4 (8.7)	0.745
T-cells	75.0 (68.2, 78.0)	72.2 (67.6, 77.0)	0.185
$CD4^+$	61.2 (53.7, 68.1)	65.9 (60.3, 70.0)	**0.009**
$CD8^+$	38.9 (11.4)	34.0 (9.1)	**0.012**
B-cells	10.2 (7.5, 14.1)	11.2 (8.5, 14.6)	0.411
T-cells' subpopulations			
Th1	38.7 (29.7, 45.6)	40.4 (30.2, 47.4)	0.555
Th17	19.8 (14.2, 28.4)	22.3 (16.6, 28.6)	0.293
Treg	8.4 (6.6, 10.0)	7.6 (6.9, 8.7)	**0.059**
Tfh ($CXCR5^+CD4^+$)	19.4 (5.8)	18.4 (4.7)	0.397
Tfh1	35.4 (7.8)	32.3 (6.9)	**0.025**
Tfh17	21.9 (6.6)	23.2 (5.6)	0.165
Tfc ($CXCR5^+CD8^+$)	2.6 (1.9, 4.2)	2.63 (1.88, 4.07)	0.865
B-cell's subpopulations			
IgD/CD27			
Naive	68.5 (52.5, 77.7)	66.6 (48.6, 75.1)	0.369
Memory	29.3 (20.0, 44.1)	31.4 (23.3, 45.2)	0.239
Unswitched	13.6 (8.9, 21.6)	17.5 (11.2, 24.5)	**0.084**
Switched	14.4 (9.7, 21.9)	14.7 (10.9, 21.6)	0.724
Bm1-5			
Bm1	10.0 (5.6, 14.9)	13.9 (8.1, 19.5)	**0.003**
Bm2	61.4 (49.9, 67.2)	59.8 (47.5, 66.5)	0.673
Bm2'	8.5 (5.9)	5.9 (3.3)	**0.046**
Bm3+Bm4	1.34 (0.93, 3.28)	1.36 (0.87, 1.85)	0.348
eBm5	8.5 (6.1, 12.5)	8.6 (6.3, 12.6)	0.841
Bm5	6.9 (4.4, 12.4)	7.8 (5.1, 10.6)	0.927
Bm2+Bm2'	71.2 (56.2, 79.2)	67.1 (52.0, 73.4)	0.146
eBm5+Bm5	16.8 (10.8, 24.9)	16.7 (12.4, 25.2)	0.884
Bm2+Bm2'/Bm5+eBm5	3.9 (2.2, 7.2)	4.1 (2.0, 5.9)	0.641
$CD24^{hi}CD38^{hi}$	5.6 (2.2, 8.5)	4.1 (2.7, 5.3)	0.061
$CD24^{hi}CD27^+$	16.9 (12.2, 25.4)	21.3 (14.3, 35.4)	**0.024**
T-cell/B-cell Ratios			
$T_h1/CD24^{hi}CD27^+$	2.23 (1.22, 3.60)	1.60 (1.09, 2.70)	**0.033**
T_h17/Tregs	0.26 (0.18, 0.40)	0.35 (0.22, 0.44)	**0.021**

Bold numbers represent significance (p-values < 0.05) or relevance (p-values < 0.100).
SjS, Sjögren's syndrome. Mann-Whitney nonparametric U test was used for group's comparison. Results are presented as medians and 25th and 75th quartiles. Statistically significant results are indicated in bold.
SjS, Sjögren's syndrome.

$CD4^+$ and $CD8^+$ T-cell subsets

SjS patients presented lower CD4+ T-cells' percentages and absolute counts compared to *Sicca* ($p = 0.009$ and $p = 0.051$), and higher CD8+ T-cells percentages ($p = 0.012$).

Tregs counts were similar in both patients' groups (p = 0.436), although SjS patients presented evidence, although week, of higher percentages (p = 0.059), compared to *Sicca*.

Tfh and Tfc cells were similar in both groups of patients. Concerning Tfh subsets, Tfh1 percentages were higher in SjS than in *Sicca* (p = 0.025), and Tfh17 absolute counts were decreased in SjS (p = 0.05) (Table 2, Supplementary Table 2).

B-cell subsets according to IgD and CD27 classification

SjS patients had lower absolute counts of B-cells in comparison to *Sicca* patients (p = 0.152), and a significant decrease was found in the absolute counts of $CD19^{+}CD27^{+}$ memory B-cells' in SjS (50 vs 66 cells/µl, p = 0.021), more pronounced in the $CD19^{+}IgD^{+}CD27^{+}$ unswitched memory B-cells subset (23 vs 35 cells/µl, p = 0.009) (Table 2 and Sup$p$ 2). $CD19^{+}IgD^{+}CD27^{+}$ unswitched memory B-cells' percentages were also lower in SjS patients, with a trend to significance compared to *Sicca* (13.6% vs 17.5%, p = 0.084). Regarding naïve B-cells, there were no significant differences between SjS and *Sicca* patients (Table 2, Supplementary Table 2)

Bm1-Bm5 classification of mature B cells

The percentages and absolute counts of Bm1 cells were lower in SjS patients than in *Sicca* (p = 0.003 and p<0.001, respectively) (Table 2, Supplementary Table 2).

Regarding Bm2, no significant differences were found between groups. SjS patients presented higher Bm2´cells' percentages compared to *Sicca* (p = 0.046), without differences in counts.

SjS patients presented lower absolute counts of eBm5 and Bm5 cells compared to *Sicca*, with a trend to significance (p = 0.089 and p = 0.079, respectively), whereas percentages were similar.

No differences were found in the Bm2+Bm2'/Bm5+eBm5 ratio between SjS and *Sicca* patients.

Other B-cell subsets

When analysing subsets related to regulatory B-cells, $CD24^{hi}CD27^{+}$ B-cells were decreased in SjS compared to *Sicca* (p = 0.001 for absolute counts and p = 0.024 for percentages).

$CD24^{hi}CD38^{hi}$ B-cells absolute counts were similar in both groups, and weak evidence of higher percentages was identified in SjS patients compared to *Sicca* patients (5.6% vs 4.1%, p = 0.061).

Performance of T and B-cells' subsets in the discrimination between SjS and *Sicca* patients

Lymphocyte subsets were compared between SjS and *Sicca*, and AUC's were estimated considering the expert clinical diagnosis as the gold standard (Table 3).

Considering subsets' percentages, most AUC's obtained were lower than 0.60, with the highest being 0.66 for Bm1 cells, and 0.62 for Tfh1 and $CD24^{hi}CD27^{+}$. For absolute counts, higher AUC values were obtained for the Bm1 (0.675) and $CD24^{hi}CD27^{+}$(0.668) B-cells (Supplementary Table 3).

Table 3 *Area Under the Curve* (AUC) *receiver operating characteristic curve* (ROC) of the comparison of T and B-cell subsets percentages in SjS (clinical diagnosis) and *Sicca* groups (GS vs *Sicca*). Added-value of the addition of each cell subset to the AECG (AUC and p-value) and the ACR/EULAR criteria (AUC and p-value)

Lymphocyte subsets or combinations AUC, 95%CI	**GS vs *Sicca* AUC**	**Added to 2002 AECG**		**Added to 2016 ACR/EULAR**	
		AUC	***p* values**	**AUC**	***p* values**
Lymphocytes					
T-cells	0.57 (0.47 - 0.67)	0.958 (0.919 - 0.998)	0.625	0.932 (0.884 - 0.98)	0.440
B-cells	0.54 (0.44 - 0.65)	0.985 (0.968 - 1)	**0.0227**	0.944 (0.900 - 0.988)	0.163
T-cell subpopulations					
Th1	0.53 (0.43 - 0.63)	0.959 (0.921 - 0.996)	0.542	0.931 (0.882 - 0.979)	0.496
Th17	0.56 (0.45 - 0.66)	0.950 (0.904 - 0.996)	0.825	0.922 (0.867 - 0.978)	0.915
Treg	0.60 (0.50 - 0.70)	0.978 (0.951 - 1)	**0.081**	0.932 (0.877 - 0.987)	0.580
Tfh ($CXCR5^{+}$)	0.54 (0.44 - 0.65)	0.977 (0.953 - 1)	**0.053**	0.944 (0.898 - 0.989)	0.190
Tfh1	0.62 (0.52 - 0.72)	0.956 (0.919 - 0.993)	0.640	0.924 (0.872 - 0.976)	0.824
Tfh17	0.57 (0.47 - 0.67)	0.957 (0.916 - 0.999)	0.714	0.925 (0.870 - 0.980)	0.804
Tfc ($CXCR5^{+}CD8^{+}$)	0.51 (0.41 - 0.61)	0.954 (0.914 - 0.995)	0.860	0.922 (0.868 - 0.975)	0.945
B-cells' subpopulations					
IgD/CD27					
Naive	0.55 (0.45 - 0.65)	0.964 (0.928 - 1)	0.352	0.938 (0.892 - 0.984)	0.272
Memory	0.56 (0.46 - 0.66)	0.965 (0.930 - 1)	0.307	0.939 (0.894 - 0.984)	0.232
Unswitched	0.59 (0.49 - 0.69)	0.968 (0.936 - 1)	0.206	0.933 (0.885 - 0.981)	0.404

(Cont.)

(Cont.)

Lymphocyte subsets or combinations AUC, 95%CI	**GS vs *Sicca* AUC**	**Added to 2002 AECG**		**Added to 2016 ACR/EULAR**	
		AUC	***p* values**	**AUC**	***p* values**
Switched	0.52 (0.42 - 0.62)	0.960 (0.920 – 1)	0.640	0.939 (0.892 - 0.986)	0.269
Bm1-5					
Bm1	0.66 (0.56 - 0.75)	0.975 (0.95 - 1)	**0.062**	0.943 (0.903 - 0.982)	**0.087**
Bm2	0.52 (0.42 - 0.62)	0.967 (0.935 - 1)	0.207	0.948 (0.908 - 0.987)	**0.077**
Bm2’	0.60 (0.50 - 0.71)	0.963 (0.925 - 1)	0.422	0.935 (0.887 - 0.983)	0.362
Bm3+Bm4	0.45 (0.35 - 0.55)	0.966 (0.934 - 0.997)	0.199	0.945 (0.903 - 0.987)	0.125
eBm5	0.51 (0.41 - 0.61)	0.947 (0.900 - 0.994)	0.668	0.941 (0.896 - 0.986)	0.215
Bm5	0.51 (0.40 - 0.61)	0.957 (0.915 - 0.999)	0.726		0.477
Bm2+Bm2’	0.58 (0.47 - 0.68)	0.965 (0.930 - 1)	0.307	0.943 (0.900 - 0.985)	0.139
eBm5+Bm5	0.49 (0.39 - 0.60)	0.958 (0.916 - 0.999)	0.688	0.936 (0.888 - 0.983)	0.343
Bm2+Bm2’/Bm5+eBm5	0.48 (0.37 - 0.58)	0.960 (0.920 - 0.999)	0.570	0.939 (0.894 - 0.985)	0.238
$CD24^{hi}CD38^{hi}$	0.60 (0.49 - 0.70)	0.960 (0.918 - 1)	0.604	0.939 (0.891 - 0.987)	0.295
$CD24^{hi}CD27^{+}$	0.62 (0.52 - 0.72)	0.966 (0.932 - 1)	0.275	0.938 (0.894 - 0.982)	0.226
T-cells/B-cells Ratios					
$T_h1/CD24^{hi}CD27^{+}$	0.61 (0.51 - 0.71)	0.967 (0.933 - 1)	0.257	0.939 (0.896 - 0.982)	0.180
T_h17/Tregs	0.62 (0.52 - 0.72)	0.967 (0.934 - 1)	0.223	0.952 (0.912 - 0.992)	**0.075**

Bold numbers represent significance (p-values < 0.05) or relevance (p-values < 0.100).

GS, Gold-Standard; AUC, area under the Receiver-Operating-Characteristic Curve; IC: Interval of Confidence, AECG, American-European Consensus Grou*p* classification criteria; ACR/EULAR, American College of Rheumatology/European League Against Rheumatism classification criteria.

Additionally, the $Th1/CD24^{hi}CD27^{+}$ B-cells ratio was increased in SjS, compared to *Sicca* patients ($p = 0.033$), and for the Th17/Treg ratio, a significantly lower value was also found in SjS patients ($p = 0.021$).

Performance of AECG and ACR/EULAR classification criteria compared to expert clinical diagnosis

The AECG and ACR/EULAR criteria were compared with the GS to assess the discriminatory power between patients with SjS and *Sicca*, resulting in agreement rates of 95.2% and

90.3%, respectively. Both classification criteria agreed in 95.2% of cases. An analysis of the discriminatory ability of the classification criteria was also carried out taking into account the GS, with an AUC = 0.952 (95%CI: 0.916-0.989) for the AECG criteria and an AUC = 0.921 (95%CI: 0.853-0.954) for the 2016 ACR/EULAR criteria.

Added value on the discrimination between Sjögren and *Sicca* patients resulting from the inclusion of lymphocytes subpopulations in both classification criteria

Following a univariable analysis where the expert clinical diagnosis was considered as the dependent variable, some of the lymphocyte subpopulations, namely Tfh, memory B-cells (total and with switch), Bm1, $CD24^{hi}CD27^{+}$ B-cells, and the Th1/Breg $CD24^{hi}CD27^{+}$ ratio were selected for the multivariable analysis.

Regarding the multivariable model where those lymphocyte subpopulations were added to the 2002 AECG criteria, only the variables Tfh and Bm1 (both in percentages) remained in the final model, and an AUC of 0.985 (95%CI: 0.968-1.000; $p = 0.021$) was achieved. For the 2016 ACR/EULAR criteria, the variables Th1/Breg $CD24^{hi}CD27^{+}$ and absolute counts of switched memory B-cells (dichotomized with a cut-off = 25 cells/µl) maximized the AUC of the multivariable model to 0.953 (95%CI: 0.916-0.990; $p = 0.043$).

In both cases, the discrimination ability (SjS vs *Sicca*) of the two multivariable models led to results with statistical significance ($p = 0.021$ for the AECG, and $p = 0.043$ for the ACR/EULAR criteria).

Discussion

SjS diagnosis and classification remains a challenge, especially at the onset of the illness, when patients may have milder phenotypes of the disease or uncommon presentations[24]. In the absence of SjS diagnostic criteria, clinicians often use classification criteria for guidance in making the diagnosis, but these are not designed to be used for clinical diagnosis or applied to individual patients but intended to identify well-defined homogenous cohorts for clinical research[25]. SjS criteria have been revised with improved methodology, although there is still a margin for improvement[11,26].

Several authors report the need to improve the value of these criteria, mainly to allow an early diagnosis, because not all patients who have clinical, serological, imaging and/or functional characteristics suggestive of SjS effectively fulfil criteria, and can be erroneously managed[12,27].

One of our goals was to measure the performance of AECG and ACR/EULAR criteria compared to the gold standard for SjS diagnosis. Here we found an agreement rate of 95.2% of 2002 AECG in discriminating between SjS and *Sicca* which was in line with the

study of Zaho et al[28]. Concerning the 2016 ACR/EULAR criteria, we found a lower agreement rate, of 92.0%, in discriminating between SjS and *Sicca*.

Generally, the ACR/EULAR criteria had been reported to be slightly more sensitive than the AECG criteria. A study conducted in Japan that compared the performance of 2016 ACR/EULAR, the 2002 AECG criteria, the 2012 ACR criteria and the Japanese criteria considering physician diagnosis as reference standard concluded that ACR/EULAR criteria were the most sensitive but also the least specific of the three criteria[29]. A Korean study[30] which compared the ACR/EULAR criteria, the 2012 ACR criteria and the AECG criteria also showed that the ACR/EULAR had higher sensitivity and lower specificity compared with both previous criteria sets. Billings[10] compared the ACR/EULAR and the AECG criteria, which showed similar performance, without evidence of the superiority of the ACR/EULAR set. Le Goff[31] also found excellent agreement between both criteria sets, with the ACR/EULAR criteria being slightly more sensitive, allowing the classification of some patients with early disease and prominent systemic features.

Several groups have suggested that the performance of the ACR/EULAR criteria could be improved with the inclusion of SG ultrasonography[13,26,31]. Considering the distinctive lymphocyte profile in SjS[32], we sought to explore the added value of these variables to the ACR/EULAR and AECG SjS classification criteria.

Our SjS cohort had decreased B-cells' counts and evidenced the distinctive B-cells profile classically described in the disease – lower levels of memory subsets and increased naïve and transitional subsets, as previously reported using the IgD/CD27 classification[32,33]. Regarding the Bm1-Bm5 classification, Bm2 and Bm2' have been reported to be increased in SjS, whereas Bm5 and eBm5 were decreased[34], a tendency we have confirmed in our study, and we highlight as well a decrease in percentages and absolute counts of Bm1 cells in SjS.

The increase in naïve mature B-cells in SjS has been attributed to the impairment of early B-cell tolerance checkpoints[16]. The mobilization of self-reactive naïve B-cells from the bone marrow to the periphery is increased in SjS[16], and recently Glauzy[35] found that among the expanded naïve B-cells in SjS, most clones were polyreactive, pointing to their emergence from defective central and peripheral B-cell tolerance checkpoints in SjS patients. Also, there is increased migration of memory B-cells to the affected SG[36], accompanied by a shift in B-cell differentiation towards plasma cells[37].

In our study, $CD24^{hi}CD27^{+}$ B-cells, a population of memory B-cells known to be enriched in regulatory cells and the human equivalent of B10 cels[38], were significantly decreased in SjS patients, although our results are difficult to interpret as functional assays for IL-10-production were not performed. Interest in $CD24^{hi}CD27^{+}$ regulatory B cells has grown lately, and this subset has been implicated in other rheumatic diseases, such as Systemic Lupus Erythematosus (SLE)[39] and Rheumatoid Arthritis (RA)[40,41]. Nevertheless, some data suggest that changes in the "mother population", $CD24^{hi}CD27^{+}$ B-cells, may correlate with changes in the $IL10^{+}CD24^{hi}CD27^{+}$ B-cells, therefore providing a simpler

method to address Bregs in a clinical setting[39,41]. In the same line, we have previously reported a negative correlation between $CD24^{hi}CD27^{+}$ B-cells and disease activity in anti-SSA-positive SjS patients[42].

The increased Th1/$CD24^{hi}CD27^{+}$ B-cells ratio in our SjS patients may represent an imbalance between Breg-enriched populations and effector Th1 cells.

In SjS patients, we also found T-cell lymphopenia. Lymphopenia in SjS has been attributed to the migration of peripheral blood $CD4^{+}$ T-cells to the exocrine glands[43] and therefore may traduce a more active disease profile. Increased peripheral Tfh cells have been reported in SjS patients[44], although in our SjS population only the Tfh1 subset presented increased percentages compared to sicca, whereas Tfh17 presented lower percentages and absolute counts. Additionally, Tfh1 had one of the highest AUC in discriminating SjS from *Sicca*. This was not surprising considering the crucial role of Tfh cells in regulating immune responses, allowing B cell differentiation towards memory B cells and plasma cells within secondary lymphoid follicles[45].

Regarding regulatory T-cells' (Tregs), our SjS patients showed a tendency to increased percentages. Tregs frequency in SjS is variable in the literature, but SjS patients seem to present Tregs deficiencies[46], contributing to disease pathogenesis. Therefore, an increased number of Treg cells in SjS does not mean that these cells can suppress the immune response. Additionally, the decreased Th17/Treg ratio found in our SjS population was due to a relative increase in Tregs, as opposed to Th17, whose numbers were in line with the global T-cell lymphopenia.

We also aimed to evaluate the added value on discrimination between SjS and *Sicca* of the inclusion of lymphocytes subpopulations in both criteria.

It has been previously reported that a high (Bm2+Bm2')/(eBm5+Bm5) ratio is more frequent in SjS both compared to RA, SLE, *Sicca* patients and healthy subjects, and could constitute a diagnostic tool[14,15]. However, Cornec et al[15] reported that evaluating this ratio, although valuable for the individual patient, had a small diagnostic weight compared to other items of AECG CC, as adding an item consisting of a (Bm2+Bm2')/(eBm5+Bm5) ratio ≥ 5 did not significantly modify the performance of the criteria.

Mingueneau[43] recently published the first attempt of a global evaluation, using 34 distinct protein markers characterized by mass cytometry and using bioinformatics analysis to recognize multiple distinct cell subsets. A blood 4-cell disease signature including plasmacytoid dendritic cell, $CD4^{+}$ T cell, memory B cell, and human leukocyte antigen (HLA)-$DR^{+}CD4^{+}$ T cells was defined in this manner, and was able to diagnose SjS with good accuracy in two different cohorts of patients (AUC 0.86–0.89).

Using conventional cytometry, we have identified several lymphocyte subsets that showed a reasonable accuracy in distinguishing SjS from *Sicca* patients. When added to the classification criteria, some of those lymphocyte subsets were able to increase the performance of the criteria. In our study, the variables Th1/Breg $CD24^{hi}CD27^{+}$ and B-memory with switch (dichotomized with a cut-off = 25 cells/µl) maximized the AUC of the

2016 ACR/EULAR CC to 0.953. The value of the 2002 AECG CC was also maximized when considering the variables Bm1 and Tfh (both in percentages), thus increasing the AUC to 0.985.

We acknowledge some limitations in our study that may affect the reproducibility, namely patients characteristics such as age, sex, disease duration, current and previous therapies, although our patients were recruited from an outpatient clinic and are representative of the clinical setting. However, the small size of our sample may have limited the obtention of more robust results. Also, the use of clinical diagnosis as gold standard may originate an incorporation bias due to the familiarity of the experts with the two specific criteria sets under study.

Conclusion

To the best of our knowledge, this is the first study that addresses the effect of lymphocyte subset profiling in the performance of the 2016 ACR EULAR criteria. Our study confirmed the high degree of agreement between both classification criteria and clinical diagnosis, with 2002 AECG criteria having superior performance than 2016 ACR/EULAR criteria. The two models that included each set of criteria separately had an increase in their performance regarding discrimination between SjS and *Sicca* patients when specific lymphocyte subpopulations were considered. Although our exploratory study suggests, in a novel approach, the potential use of subsets such as unswitched memory B cells to improve the performance of classification criteria, further studies, preferentially multicentric, are needed to confirm if specific populations could be included as a new weighted item in the ACG/EULAR criteria.

Supplementary Tables

Supplementary Table 1 2002 AECG and 2016 ACR/EULAR criteria

#	2002 AECG	2016 ACR/EULAR	Score
1	Ocular symptoms: a positive response to at least one of the questions: Have you had daily, persistent, troublesome dry eyes for more than 3 months? Do you have a recurrent sensation of sand or gravel in the eyes? Do you use tear substitute more than 3 times a day?		
2	Oral symptoms: a positive response to at least one of the questions: Have you had a daily feeling of dry mouth for more than 3 months? Have you had recurrently and persistently swollen salivary glands as an adult? Do you frequently drink liquids to aid in swallowing dry food?		
3	Objective ocular signs – a positive result for at least one of the following two tests: Schirmer's I test, performed without anesthesia (≤5 mm in 5 min) Rose Bengal score or other ocular dye score (≥ 4 according to van Bijsterveld's scoring system)	Objective ocular signs: A positive result for Schirmer's I test, performed without anesthesia (≤5 mm in 5 min) Keratoconjunctivitis sicca with: Ocular staining score ≥ 3 or van Bijsterveld's scoring system ≥ 4	**1** **1**
4	Histopathology: in minor salivary glands (obtained through normal appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score ≥ 1, defined as number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue	Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥ 1 focus/ 4 mm^2	**3**
5	Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests: Unstimulated whole salivary flow (≤1.5 ml in 15 min) Parotid sialography showing the presence of diffuse sialectasias (punctuate, cavitary, or destructive pattern), without evidence of obstruction in major ducts Salivary scintigraphy showing delayed uptake, reduced concentration and /or delayed excretion of tracer	Salivary gland involvement: objective evidence of salivary gland involvement defined by Unstimulated whole salivary flow (≤1.5 ml in 15 min)	**1**

(Cont.)

(Cont.)

6	Autoantibodies: presence in the serum of the following antibodies: Antibodies to Ro (SSA) and/or La (SSB) antigens	Autoantibodies: presence in the serum of the following antibodies: Antibodies to Ro (SSA)	**3**
	Classification rules		
	The presence of any 4 of the 6 items is indicative of primary SS, as long as either item 4 (Histopathology) or 6 (Serology) is positive	The classification of SS, which applies to individuals with signs/symptoms that may be suggestive of SS, will be met in patients who have ≥ 4 points	

Supplementary Table 2 Comparison of T and B-cell subsets absolute counts in SjS and *Sicca* groups

Lymphocyte subsets or combinations of subsets Cells/µL, median (25th-75th percentile)	**SjS** n = 62	***Sicca*** n = 63	**Group's comparisons (*p* values)** **SjS vs *Sicca***
Lymphocytes	1679 (1135, 2350)	2006 (1533, 2277)	**0.089**
T-cells	1192 (768, 1754)	1429 (1076, 1672)	0.166
$CD4^+$	785 (437, 1074)	889 (708, 11595)	**0.051**
$CD8^+$	431 (319, 632)	457 (343, 577)	0.950
B-cells	187 (113, 264)	224 (140, 303)	0.152
T-cell subsets			
Th1	260 (161, 418)	359 (222, 467)	**0.028**
Th17	125 (87, 224)	200 (135, 264)	**0.010**
Treg	64 (38, 89)	69 (47, 84)	0.436
Tfh ($CXCR5^+CD4^+$)	135 (83, 205)	169 (121, 199)	0.177
Tfh1	51 (26, 73)	55 (34, 67)	0.600
Tfh17	27 (16, 49)	35 (24, 50)	**0.050**
Tfc ($CXCR5^+CD8^+$)	13 (7, 19)	12 (9, 17)	0.894
B-cell subsets			
IgD/CD27			
Naive	122 (63, 195)	135 (72, 203)	0.380
Memory	50 (29, 76)	66 (43, 97)	**0.021**
Unswitched	23 (12, 38)	35 (21, 54)	**0.009**
Switched	22 (15, 38)	30 (20, 47)	**0.045**
Bm1-5			
Bm1	18 (10, 28)	29 (18, 43)	**<0.001**
Bm2	109 (59, 172)	124 (72, 182)	0.331
Bm2'	11 (6, 29)	11 (6, 19)	0.562
Bm3+Bm4	3 (1, 5)	3 (2,4)	0.767
eBm5	15 (9, 23)	18 (11, 28)	**0.089**
Bm5	11 (8, 19)	15 (10, 22)	**0.079**
Bm2+Bm2'	128 (69, 195)	139 (81, 198)	0.479
eBm5+Bm5	26 (18, 44)	34 (23)	**0.071**
Bm2+Bm2'/Bm5+eBm5	4 (2, 7)	4 (2, 6)	0.476
$CD24^{hi}CD38^{hi}$	8 (4, 18)	9 (4, 13)	0.726
$CD24^{hi}CD27^+$	30 (17, 49)	47 (28, 77)	**0.001**
T-cell/B-cell ratios			
$T_h1/CD24^{hi}CD27^+$	9.75 (5.88, 16.12)	7.07 (4.39, 10.06)	**0.003**
T_h17/Tregs	0.26 (0.18, 0.39)	0.34 (0.22, 0.44)	**0.025**

Bold numbers represent significance (p-values < 0.05) or relevance (p-values < 0.100).
SjS, Sjögren's syndrome.

Supplementary Table 3 *Area Under the Curve* (AUC) *receiver operating characteristic curve* (ROC) of the comparison of T and B-cell subsets absolute counts in SjS (clinical diagnosis) and *Sicca* groups (GS vs *Sicca*). Added value of the addition of each cell subset to the AECG (AUC and p-value) and to the ACR/EULAR criteria (AUC and *p*-value).

Lymphocyte subsets or combinations of subsets AUC, 95% CI	**GS vs *Sicca***	**Added to 2002 AECG**		**Added to 2016 ACR/EULAR**	
	AUC	**AUC**	***p* values**	**AUC**	***p* values**
Lymphocytes					
T-cells	0.581 (0.477-0.685)	0.973 (0.938-1)	0.411	0.936(0.887 - 0.984)	0.361
B-cells	0.590 (0.489-0.690)	0.964 (0.930-0.999)	0.630	0.917(0.863 - 0.971)	0.719
T-cell subsets					
Th1	0.615 (0.515-0.715)	0.972 (0.938-1)	0.423	0.932 (0.881-0.983)	0.441
Th17	0.630 (0.530-0.729)	0.950 (0.900-1)	0.967	0.930 (0.877-0.982)	0.479
Treg	0.555 (0.453-0.658)	0.972 (0.936-1)	0.446	0.938 (0.981-0.894)	0.325
Tfh ($CXCR5^+$)	0.585 (0.482-0.687)	0.954 (0.904-1)	0.948	0.921 (0.861-0.982)	0.658
Tfh1	0.540 (0.437-0.643)	0.961 (0.919-1)	0.742	0.918 (0.859-0.978	0.707
Tfh17	0.614 (0.512-0.715)	0.953 (0.902-1)	0.955	0.900 (0.830-0.970)	0.941
Tfc ($CXCR5^+CD8^+$)	0.502 (0.399-0.605)	0.968 (0.931-1)	0.289	0.925 (0.873-0.977)	0.238
B-cell subsets					
IgD/CD27					
Naive	0.561 (0.459-0.662)	0.971 (0.943-0.998)	0.423	0.904 (0.844-0.965)	0.982
Memory	0.626 (0.526-0.725)	0.967 (0.930-1)	0.568	0.937 (0.891-0.983)	0.334
Unswitched	0.637 (0.539-0.735)	0.969 (0.936-1)	0.498	0.932 (0.884-0.980)	0.420
Switched	0.614 (0.514-0.714)	0.954 (0.909-1)	0.928	0.941 (0.897-0.986)	0.271
Bm1-5					
Bm1	0.675 (0.580-0.770)	0.964 (0.927-1)	0.637	0.923 (0.871-0.976)	0.593
Bm2	0.566 (0.526-0.725)	0.970 (0.941-0.999)	0.442	0.903 (0.842-0.965)	0.998
Bm2'	0.515 (0.411-0.618)	0.962 (0.924-1)	0.704	0.916 (0.860-0.972)	0.742
Bm3+Bm4	0.471 (0.368-0.573)	0.970 (0.937-1)	0.476	0.940(0.895-0.985)	0.289
eBm5	0.599 (0.499-0.700)	0.961 (0.922-1)	0.728	0.934 (0.886-0.981)	0.395
Bm5	0.594 (0.493-0.696)	0.956 (0.910-1)	0.890	0.397 (0.890-0.983)	0.344
Bm2+Bm2'	0.552 (0.450-0.654)	0.970 (0.941-0.999)	0.452	0.904 (0.844-0.965)	0.980
eBm5+Bm5	0.602 (0.501-0.703)	0.958 (0.916-1)	0.821	0.936 (0.890-0.982)	0.352
$CD24^{hi}CD38^{hi}$	0.497 (0.392-0.601)	0.961 (0.921-1)	0.744	0.925 (0.871-0.979)	0.567
$CD24^{hi}CD27^+$	0.668 (0.572-0.764)	0.967 (0.932-1)	0.560	0.932 (0.885-0.980)	0.410
T-cell/B-cell ratios					
$T_h1/CD24^{hi}CD27^+$	0.638 (0.539-0.736)	0.960 (0.923-0.997)	0.741	0.895 (0.832-0.958)	0.837
T_h17/Tregs	0.609 (0.509-0.709)	0.967 (0.932-1)	0.562	0.937 (0.889-0.984)	0.347

GS, Gold-Standard; AUC, area under the Receiver-Operating-Characteristic Curve; IC: Interval of Confidence; GS, Gold-Standard; AUC, area under the Receiver-Operating-Characteristic Curve; IC: Interval of Confidence, AECG, American-European Consensus Grou*p* classification criteria; ACR/EULAR, American College of Rheumatology/European League Against Rheumatism classification criteria.

References

1. Ramos-Casals M, Brito-Zeron P, Siso-Almirall A, Bosch X. Primary Sjogren syndrome. Bmj [Internet] 2012 [cited 2018 May 11]; 344: e3821–e3821. Available from: file:///C:/Users/fduar_000/Desktop/ramoscasals2012.pdf
2. Mavragani CP, Moutsopoulos HM, Fragoulis GE, Moutsopoulos HM. Sjögren's Syndrome. Annu Rev Pathol Mech Dis [Internet] 2014 [cited 2017 Oct 1]; 9: 273–285. Available from: file:///C:/Users/fduar_000/Desktop/mavragani2014.pdf
3. Aljanobi H, Sabharwal A, Krishnakumar B, Kramer JM. Is it Sjögren's syndrome or burning mouth syndrome? Distinct pathoses with similar oral symptoms. Oral Surg Oral Med Oral Pathol Oral Radiol [Internet] 2017; 123: 482–495. Available from: http://dx.doi.org/10.1016/j.oooo.2017.01.005
4. Tsubota K, Yokoi N, Shimazaki J, Watanabe H, Dogru M, Yamada M, et al. New Perspectives on Dry Eye Definition and Diagnosis: A Consensus Report by the Asia Dry Eye Society [Internet]. Vol. 15, Ocular Surface. 2017 [cited 2019 Feb 16]. p. 65–76. Available from: www.theocularsurface.com
5. Whitcher JP, Shiboski CH, Shiboski SC, Heidenreich AM, Kitagawa K, Zhang S, et al. A Simplified Quantitative Method for Assessing Keratoconjunctivitis Sicca From the Sjögren's Syndrome International Registry. Am J Ophthalmol [Internet] 2010 [cited 2019 Apr 25]; 149: 405–415. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3459675/pdf/nihms152739.pdf
6. Jousse-Joulin S, Milic V, Jonsson M V, Plagou A, Theander E, Luciano N, et al. Is salivary gland ultrasonography a useful tool in Sjögren's syndrome? A systematic review. Rheumatology (Oxford) [Internet] 2016 [cited 2016 Jun 12]; 55: 789–800. Available from: http://rheumatology.oxfordjournals.org/
7. Kyriakidis NC, Kapsogeorgou EK, Tzioufas AG. A comprehensive review of autoantibodies in primary Sjögren's syndrome: Clinical phenotypes and regulatory mechanisms [Internet]. Vol. 51, Journal of Autoimmunity. 2014 [cited 2017 Oct 1]. p. 67–74. Available from: file:///C:/Users/fduar_000/Desktop/kyriakidis2014.pdf
8. Guellec D, Cornec D, Jousse-Joulin S, Marhadour T, Marcorelles P, Pers JO, et al. Diagnostic value of labial minor salivary gland biopsy for Sjögren's syndrome: A systematic review [Internet]. Vol. 12, Autoimmunity Reviews. 2013 [cited 2017 Oct 14]. p. 416–420. Available from: file:///C:/Users/fduar_000/Desktop/guellec2013.pdf
9. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM A, Al. E et, Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis [Internet] 2002; 61: 554–558. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12006334%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1754137
10. Billings M, Amin Hadavand M, Alevizos I. Comparative analysis of the 2016 ACR-EULAR and the 2002 AECG classification criteria for Sjögren's syndrome: Findings from the NIH cohort. Oral Dis [Internet] 2018 [cited 2018 Apr 29]; 24: 184–190. Available from: http://doi.wiley.com/10.1111/odi.12772
11. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis Rheumatol 2017; 69: 35–45.
12. Brito-Zerón P, Retamozo S, Ramos-Casals M. Phenotyping Sjögren's syndrome: Towards a personalised management of the disease. Clin Exp Rheumatol 2018; 36: S198–S209.
13. van Nimwegen JF, Mossel E, Delli K, van Ginkel MS, Stel AJ, Kroese FGM, et al. Incorporation of Salivary Gland Ultrasonography Into the American College of Rheumatology/European League Against Rheumatism Criteria for Primary Sjögren's Syndrome. Arthritis Care Res 2020; 72: 583–590.
14. Binard A, Le Pottier L, Devauchelle-Pensec V, Saraux A, Youinou P, Pers J-O. Is the blood B-cell subset profile diagnostic for Sjögren's syndrome? Ann Rheum Dis [Internet] 2009 [cited 2016 Aug 6]; 68: 1447–1452. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18782791
15. Cornec D, Saraux A, Pers J-O, Jousse-Joulin S, Marhadour T, Roguedas-Contios A-M, et al. Diagnostic accuracy of blood B-cell subset profiling and autoimmunity markers in Sjögren's syndrome. Arthritis Res Ther [Internet] 2014 [cited 2016 May 27]; 16: R15. Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3978459&tool=pmcentrez&rendertype=abstract
16. Corsiero E, Sutcliffe N, Pitzalis C, Bombardieri M, Reddy J. Accumulation of self-reactive naïve and memory B cell reveals sequential defects in B cell tolerance checkpoints in Sjögren's Syndrome. PLoS One [Internet] 2014 [cited 2017 Mar 12]; 9. Available from: http://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0114575&type=printable
17. Bohnhorst JØ, Thoen JE, Natvig JB, Thompson KM. Significantly depressed percentage of CD27+ (memory) B cells among peripheral blood B cells in patients with primary Sjogren's syndrome. Scand J Immunol 2001; 54: 421–427.
18. Verstappen GM, Kroese FGM, Bootsma H. T cells in primary Sjögren's syndrome: targets for early intervention. Rheumatology 2019; : 1–11.
19. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. J Exp Med 2010; 207: 365–378.
20. Noack M, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. Autoimmun Rev [Internet] 2014 [cited 2017 May 7]; 13: 668–677. Available from: file:///C:/Users/fduar_000/Desktop/noack2014.pdf
21. Klein U, Rajewsky K, Küppers R. Human Immunoglobulin (Ig)M+ IgD+ Peripheral Blood B Cells Expressing the CD27 Cell Surface Antigen Carry Somatically Mutated Variable Region Genes: CD27 as a General Marker for Somatically Mutated (Memory) B Cells. J Exp Med [Internet] 1998; 188: 1679–1689. Available from: http://www.jem.org
22. Bohnhorst JØ, Bjørgan MB, Thoen JE, Natvig JB, Thompson KM. Bm1–Bm5 Classification of Peripheral Blood B Cells Reveals Circulating Germinal Center Founder Cells in Healthy Individuals and Disturbance in the B Cell Subpopulations in Patients with Primary Sjögren's Syndrome. J Immunol [Internet] 2001 [cited 2017 Jul 11]; 167: 3610–3618. Available from: http://www.jimmunol.org/content/167/7/3610
23. R Core Team. R Core Team 2020 R: A language and environment for statistical computing. R foundation for statistical computing. https://www.R-project.org/. 2020; : 2020. Available from: http://www.r-project.org/
24. Gálvez J, Sáiz E, López P, Pina MF, Carrillo A, Nieto A, et al. Diagnostic evaluation and classification criteria in Sjögren's Syndrome. Jt Bone Spine [Internet] 2009 [cited 2016 May 27]; 76: 44–49. Available from: http://www.sciencedirect.com/science/article/pii/S1297319X08001991
25. June RR, Aggarwal R. The use and abuse of diagnostic/classification criteria. Best Pract Res Clin Rheumatol [Internet] 2014 [cited 2017 May 21]; 28: 921–934. Available from: http://ac.els-cdn.com/S152169421500011X/1-s2.0-S152169421500011X-main.pdf?_tid=2dd5efe8-3e43-11e7-b93b-00000aab0f6b&acdnat=1495384571_a3bf2af6e0854e304c6e9d2365c34b0b
26. Jousse-Joulin S, Gatineau F, Baldini C, Baer A, Barone F, Bootsma H, et al. Weight of salivary gland ultrasonography compared to other items of the 2016 ACR/EULAR classification criteria for Primary Sjögren's syndrome. J Intern Med 2020; 287: 180–188.
27. Noaiseh G, Baer AN. Toward better outcomes in Sjögren's syndrome: The promise of a stratified medicine approach. Best Pract Res Clin Rheumatol 2020; 34.
28. Zhao Y, Zheng WJ, Zhou W, Zeng XF, Zhang FC, Tang FL, et al. The evaluation of the international classification criteria (2002) for primary Sjögren's syndrome in Chinese patients. APLAR J Rheumatol 2005; 8: 184–187.
29. Tsuboi H, Hagiwara S, Asashima H, Takahashi H, Hirota T, Noma H, et al. Comparison of performance of the 2016 ACR-EULAR classification criteria for primary Sjögren's syndrome with other sets of criteria in Japanese patients. Ann Rheum Dis [Internet] 2017 [cited 2018 Apr 29]; 76: 1980–1985. Available from: http://ard.bmj.com/content/annrheumdis/76/12/1980.full.pdf
30. Lee J, Koh JH, Kim J-W, Sung Y-K, Lee S-S, Choe JY, et al. Performance of the 2016 ACR-EULAR classification criteria for primary Sjogren's syndrome in a Korean cohort. Rheumatol Int [Internet] 2018; 38: 1651–1660. Available from: http://link.springer.com/10.1007/s00296-018-4109-y
31. Le Goff M, Cornec D, Jousse-Joulin S, Guellec D, Costa S, Marhadour T, et al. Comparison of 2002 AECG and 2016 ACR/EULAR classification criteria and added value of salivary gland ultrasonography in a patient cohort with suspected primary Sjögren's syndrome. Arthritis Res Ther [Internet] 2017 [cited 2018 Apr 29]; 19: 269. Available from: https://arthritis-research.biomedcentral.com/track/pdf/10.1186/s13075-017-1475-x

32. Barcelos F, Martins C, Papoila A, Geraldes C, Cardigos J, Nunes G, et al. Association between memory B-cells and clinical and immunological features of primary Sjögren's syndrome and Sicca patients. Rheumatol Int [Internet] 2018 [cited 2018 Jul 17]; 38: 1063–1073. Available from: https://doi.org/10.1007/s00296-018-4018-0
33. Roberts MEP, Kaminski D, Jenks SA, Maguire C, Ching K, Burbelo PD, et al. Primary Sjögren's syndrome is characterized by distinct phenotypic and transcriptional profiles of IgD+ unswitched memory B cells. Arthritis Rheumatol [Internet] 2014 [cited 2016 Aug 11]; 66: 2558–2569. Available from: http://doi.wiley.com/10.1002/art.38734
34. Carvajal Alegria G, Gazeau P, Hillion S, Daïen CI, Cornec DYK. Could Lymphocyte Profiling be Useful to Diagnose Systemic Autoimmune Diseases? Clin Rev Allergy Immunol [Internet] 2017; 53: 219–236. Available from: http://link.springer.com/10.1007/s12016-017-8608-5
35. Glauzy S, Sng J, Bannock JM, Gottenberg J-EE, Korganow A-SS, Cacoub P, et al. Defective Early B Cell Tolerance Checkpoints in Sjögren's Syndrome Patients. Arthritis Rheumatol [Internet] 2017 [cited 2018 Mar 13]; 69: 2203–2208. Available from: http://doi.wiley.com/10.1002/art.40215
36. Hansen A, Odendahl M, Reiter K, Jacobi AM, Feist E, Scholze J, et al. Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome. Arthritis Rheum [Internet] 2002 [cited 2017 Jun 10]; 46: 2160–2171. Available from: file:///C:/Users/fduar_000/Desktop/Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome.pdf
37. Szyszko EA, Brokstad KA, Øijordsbakken G, Jonsson M V., Jonsson R, Skarstein K. Salivary glands of primary Sjögren's syndrome patients express factors vital for plasma cell survival. Arthritis Res Ther [Internet] 2011 [cited 2020 Jul 25]; 13: 1–18. Available from: http://arthritis-research.com/content/13/1/R2
38. Mauri C, Menon M. Human regulatory B cells in health and disease: Therapeutic potential. Vol. 127, Journal of Clinical Investigation. 2017. p. 772–779.
39. Jin L, Weiqian C, Lihuan Y. Peripheral CD24hi CD27+ CD19+ B cells subset as a potential biomarker in naive systemic lupus erythematosus. Int J Rheum Dis [Internet] 2013 [cited 2018 Jul 17]; 16: 698–708. Available from: file:///C:/Users/fduar_000/Desktop/jin2013.pdf
40. Daien CI, Gailhac S, Mura T, Audo R, Combe B, Hahne M, et al. Regulatory B10 cells are decreased in patients with rheumatoid arthritis and are inversely correlated with disease activity. Arthritis Rheumatol 2014; 66: 2037–2046.
41. Salomon S, Guignant C, Morel P, Flahaut G, Brault C, Gourguechon C, et al. Th17 and CD24hiCD27+regulatory B lymphocytes are biomarkers of response to biologics in rheumatoid arthritis. Arthritis Res Ther 2017; 19: 1–10.
42. Barcelos F, Martins C, Madeira N, Ângelo-Dias M, Cardigos J, Alves N, et al. Lymphocyte subpopulations in Sjögren's syndrome are distinct in anti-SSA-positive patients and related to disease activity. Clin Rheumatol 2021; .
43. Mingueneau M, Boudaoud S, Haskett S, Reynolds TL, Nocturne G, Norton E, et al. Cytometry by time-of-flight immunophenotyping identifies a blood Sjögren's signature correlating with disease activity and glandular inflammation. J Allergy Clin Immunol [Internet] 2016 [cited 2016 Jun 13]; 137: 1809–1821. Available from: http://dx.doi.org/10.1016/j.jaci.2016.01.024
44. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. Clin Immunol [Internet] 2013 [cited 2016 Aug 11]; 147: 95–104. Available from: http://dx.doi.org/10.1016/j.clim.2013.02.024
45. Szabó K, Papp G, Szántó A, Tarr T, Zeher M. A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjögren's syndrome and systemic lupus erythematosus. Clin Exp Immunol 2016; 183: 76–89.
46. Alunno A, Carubbi F, Bistoni O, Caterbi S, Bartoloni E, Mirabelli G, et al. T regulatory and T helper 17 cells in primary Sjögren's syndrome: Facts and perspectives [Internet]. Vol. 2015, Mediators of Inflammation. 2015 [cited 2017 Mar 12]. p. 1–10. Available from: http://dx.doi.org/10.1155/2015/243723

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Authors' Contribution F Barcelos conceived the original research idea, while all of the authors designed the study and created the study protocol. F Barcelos, N Madeira and J Vaz-Patto recruited the patients and collected the data. J Cardigos and N Alves recruited healthy controls and collected the data. C Martins and R Monteiro analyzed the blood samples using flow cytometry. C Brás-Geraldes and A-L Papoila performed the statistical analysis. JC Branco and L-M Borrego supervised all the work and the research protocol. All of the authors contributed to data analysis and interpretation. F Barcelos drafted the manuscript, and all of the authors revised it and contributed to it intellectually. All of the authors have approved the final version of the manuscript.

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CAPÍTULO VIII. Discussão Geral

A Síndrome de Sjögren primária (SSP) é uma doença inflamatória crónica, de origem autoimune, que se carateriza pela infiltração linfocitária e lesão das glândulas exócrinas [395]. O envolvimento das glândulas salivares e lacrimais origina as queixas secas que a caraterizam [396]. No seu decurso podem ocorrer manifestações extra-glandulares ou sistémicas, potencialmente graves, em cerca de metade dos doentes [397].

O diagnóstico diferencial entre uma síndrome seca de origem autoimune (SSP) ou não-autoimune (complexo Sicca) é o principal desafio no diagnóstico da SSP, tendo extrema importância no prognóstico e na terapêutica.

O diagnóstico da SSP, na prática clínica, baseia-se na confirmação objetiva da redução da função secretória lacrimal ou salivar num doente com queixas secas, e na comprovação obrigatória da existência de um distúrbio imunológico subjacente.

Atualmente, os principais elementos para confirmar o componente autoimune da SSP são a presença de anticorpos anti-SSA e anti-SSB [3], e de infiltrados linfocitários focais na biópsia de glândula salivar *minor* (labial) [4,5]. Nem sempre é fácil confirmar o diagnóstico, sobretudo nas fases precoces da doença, pelo que são desejáveis novos biomarcadores que traduzam a disfunção imune presente na SSP [773].

Na patogénese da SSP, a hiperatividade das células B suportada por células T é responsável por algumas das caraterísticas clínicas e imunológicas mais reconhecíveis da doença [437,810]. Já foi sugerido que perfis de linfócitos específicos poderiam auxiliar no diagnóstico da SSP, tendo como modelo os critérios de classificação AECG [773,811].

O objetivo principal deste projeto de investigação consistiu na exploração das subpopulações linfocitárias (caraterizadas por citometria de fluxo), como biomarcador auxiliar no diagnóstico e classificação da SSP.

Foram incluídos 57 doentes com SSP classificados de acordo com os critérios do AECG [13], 68 doentes com síndrome Sicca sem critérios de SSP, 20 doentes com AR, e 24 indivíduos saudáveis sem queixas secas.

No **Capítulo III** caraterizam-se e comparam-se as subpopulações de linfócitos B de doentes com SSP e Sicca, e de controlos saudáveis. Estabeleceram-se os pontos de cut-off nos valores das distribuições celulares que melhor distinguiram entre SSP e controlos, e

compararam-se as caraterísticas fenotípicas de doentes com SSP e Sicca acima e abaixo do cut-off das subpopulações mais relevantes.

O racional deste estudo prende-se com estudos anteriores nos quais foi documentada a existência de alterações de várias subpopulações linfocitárias B na SSP, em especial a redução das células B de memória circulantes e o aumento de células B naïve,[19,541,812], tendo mesmo sido postulado por Carvajal que a caraterização do perfil linfocitário na SSP pode ter utilidade no diagnóstico [8].

No nosso estudo, em que foi utilizada a classificação IgD/CD27 [812], verificou-se a existência de uma redução dos números absolutos e percentagens de células B de memória (sobretudo as sem switch) e aumento das células B naïve, em comparação com os controlos, já descrita por outros autores [18,436,541,542,812].

Estes valores refletem provavelmente falhas nos *checkpoints* de tolerância das células B, levando a um aumento da mobilização de células B auto-reativas da medula óssea para a periferia [436] e à migração de células B de memória para as glândulas salivares [18,435].

A avaliação das células B de acordo com a classificação Bm1-Bm5, proposta por Bohnhorst [19], confirmou o aumento das subpopulações Bm2 e Bm2´, e a diminuição das eBm5 e Bm5, mas menos acentuada que o descrito por nos trabalhos de Binard [811] e de Cornec [813]. Na razão entre as Bm2+Bm2' e as eBm5+Bm5 obteve-se um cut-off ideal na discriminação entre SSP e controlos de ≥3,8 considerando os números absolutos de células, e ≥3,2 considerando as percentagens, distante do cut-off ≥5 obtido por Binard [811], e com menor sensibilidade e especificidade.

Investigámos cut-offs nos valores das populações linfocitárias B de acordo com dois cenários, um em que se maximizava a especificidade, e outro em se determinava o ponto de corte referente à sensibilidade e especificidade ótimas. Optámos pelo que maximizava a especificidade, de modo a reduzir a probabilidade de falsos positivos na comparação entre SSP e controlos saudáveis. Foi obtida melhor discriminação com a utilização dos números absolutos. Doentes com SSP abaixo de um cut-off de ≤58 células de memória/µl ou de <23,5 células de memória com *switch*/µl) apresentavam maior frequência de caraterísticas fenotípicas da doença, como FS≥1, anticorpos anti-SSA e anti-SSB, ANA e FR, e aumento das imunoglobulinas. Esta associação de menores valores de células B de memória à disfunção autoimune na SSP está de acordo com a hipótese de que a diferenciação das células B periféricas que migram para as glândulas salivares favorecem a formação de plasmócitos e a consequente produção de autoanticorpos [543,812].

Verificámos também que os doentes Sicca, considerando a classificação IgD/CD27, apresentaram percentagens de células B naïve e de memória semelhantes às de doentes com SSP e distintas das dos controlos, enquanto que os valores absolutos destas populações foram superiores aos da SSP, mas inferiores aos controlos. Doentes com síndrome Sicca constituem um desafio diagnóstico, pois muitas das suas caraterísticas clínicas sobrepõem-se à SSP, mas carecem dos marcadores imunológicos de SSP, nomeadamente os anticorpos anti-SSA e o infiltrado focal nas glândulas salivares. O perfil linfocitário intermédio entre SSP e controlos, evidente nos doentes Sicca, sugere a presença de disfunção imunológica, podendo alguns deles corresponder a formas ligeiras ou precoces de SSP.

No grupo Sicca, cerca de 40% dos doentes apresentavam contagens de células B de memória com *switch* abaixo do cut-off estabelecido para a SSP, reforçando a hipótese de que alguns destes indivíduos possam efetivamente ter SSP. Estes dados estão em linha com o trabalho de Roberts e colaboradores [541], que descreveu a existência de percentagens inferiores de células B de memória em doentes Sicca comparados com controlos, tendo identificado um subgrupo de doentes Sicca com um perfil transcriptómico semelhante à SSP.

O nosso estudo foi o primeiro estudo realizado que procurou identificar os valores de cut-off nas subpopulações de linfócitos B definidas pela classificação IgD/CD27. Deste modo, comprovámos a existência de um cut-off nas contagens de células B de memória que claramente distingue SSP de controlos saudáveis, ou seja, patológico de normal. Aplicando um cut-off semelhante aos doentes Sicca, poderá vir a ser possível identificar indivíduos cujo perfil se assemelhe ao da SSP, e que de facto representem formas ligeiras ou precoces da doença.

Adicionalmente, demonstrámos que em doentes com SSP a presença de menores contagens de células B de memória estava associada a uma evolução mais prolongada e a doença mais ativa, o que abre perspetivas para que a avaliação desta população celular possa ter também um papel prognóstico.

As células T foliculares efectoras (Tfh) têm um importante papel na patogénese da SSP, uma vez que infiltram os órgão afetados e promovem a hiperatividade das células B [452]. As interações entre as células T e as células B ativadas ocorrem em estruturas semelhantes a centros germinativos ectópicos (CGE) formados nos órgãos-alvo, como as GS [814]. As células Tfh são grandes produtoras de IL-21, uma citocina fundamental para a sobrevivência das células B e a formação de CGE [815].

Na SSP, está descrito um aumento das células Tfh circulantes e uma expansão da sua diferenciação nas GS [21,22]. Para além das células T CD4+, as células T CD8+ também expressam CXCR5 (*chemokine receptor X5*), que induz a atração celular para as zonas B de estruturas linfoides, indicando a existência de células foliculares citotóxicas (Tfc) [515]. A marcada expansão de células Tfh e a simultânea presença de células Tfc nas glândulas salivares na SSP pode relacionar-se com mecanismos de resposta a um agente infecioso sialotrópico, como o EBV [456]. Assim, aquando da caraterização das células T foliculares no **Capítulo IV**, foi avaliado também o perfil serológico do EBV, um vírus fortemente suspeito na iniciação dos processos autoimunes da SSP, e comparadas as subpopulações linfocitárias de acordo com os distintos perfis.

Neste estudo confirmámos o perfil de células B típico da SSP, com expansão das células B naïve circulantes e redução das B de memória [816], e demonstrámos importantes diferenças nas subpopulações de linfócitos T foliculares entre doentes com SSA e indivíduos com AR ou controlos saudáveis. Apesar dos números absolutos de células T $CXCR5^+$ estarem diminuídos na SSP, encontrámos um aumento das células T produtoras de IL-21, quer $CD4^+$ (Tfh), quer $CD8^+$ (Tfc).

As células Tfc estavam aumentadas nos doentes com SSP com maior atividade da doença, tendo sido possível identificar uma correlação positiva entre os números absolutos de células T $IL21^+CD8^+$ e o ESSDAI, sugerindo um papel destas células na patogénese da doença. Em linha com os nossos resultados, Gong já havia associado o aumento da IL-21 à atividade sistémica da SSP [137], no entanto o nosso estudo parece realçar o papel das Tfc neste cenário. Curiosamente, o aumento das células T produtoras de IL-21 lembra o perfil de uma infeção viral crónica, como sugerido em modelos animais [817].

Em relação aos marcadores serológicos de EBV, os doentes com SSP apresentaram maior prevalência de anti-EBV EA-D IgG, em comparação com doentes com AR e controlos saudáveis, sendo estes resultados consistentes com a literatura. Os nossos doentes com SSP apresentaram prevalências de anti-EBV EA-D IgG sobreponíveis às encontradas por Pasoto e colaboradores [818]. Os grupos de Croia [456]. e Kivity [819] descrevem também uma maior prevalência de anti-EBV EA-D IgG na SSP. Adicionalmente, Croia e colaboradores sugeriram que a infeção por EBV pode contribuir para a expansão e diferenciação de células B em estruturas linfoides secundárias na SSP [456].

Reconhecendo que os doentes com SSP apresentavam aumento da prevalência de anti-EBV EA-D IgG e uma prevalência importante de anti-EBNA IgG, procedemos à avaliação clínica e das subpopulações linfocitárias de acordo com o perfil serológico do EBV. Para isso, subdividimos os doentes com SSP em 3 grupos: G1, infeção por EBV prévia (EA-IgG–, EBNA IgG+); G2, infeção recente/reativação (EA IgG+, EBNA IgG+/-); G3, ausência de marcadores de infeção por EBV ativa (EA IgG–, EBNA IgG–) de acordo com o proposto por Maślińska [455].

Em relação à atividade sistémica da doença, os doentes com serologia positiva para EBV (G1 e G2) apresentavam ESSDAI superior ao de doentes sem evidência de infeção (G3). Não se encontraram diferenças entre os grupos G1 e G2 em domínios específicos do ESSDAI nem na sua pontuação total, mas no grupo G2 a frequência de doentes com doença ativa era superior à dos doentes com infeção antiga, embora sem significado estatístico.

Doentes com evidência de infeção por EBV, em especial aqueles com infeção recente/reativação apresentavam um perfil imunológico distinto, com maior representação das subpopulações T pro-inflamatórias Th1 e Tfh1, o que pode indicar a influência viral na diferenciação preferencial em células Tfh1, conforme postulado por Fahey [817]. Adicionalmente, doentes com infeção por EBV recente/reativação apresentavam níveis superiores de células B de transição e de plasmablastos, o que pode indicar a influência do EBV na modulação das respostas imunes na SSP e na expressão clínica, conforme sugerido pela manifestação mais precoce da doença e maior frequência de doença ativa nestes indivíduos. Em linha com o aumento destas células, e suportando a influência da infeção recente/reativação, os doentes do grupo G2 apresentavam níveis de IgG mais elevados (mas sem atingir significado estatístico).

Apesar do EBV EA (*early antigen*) ter já sido correlacionado com a produção de anticorpos por outros autores [819], o nosso estudo explora pela primeira vez a associação entre os padrões serológicos do EBV e o perfil de subpopulações linfocitárias na SSP.

Em síntese, os nossos resultados suportam a presença de uma amplificação do ambiente folicular na SSP, que se traduz pela secreção aumentada de IL-21 por células T $CD4^+$ e $CD8^+$ (foliculares).

Este estudo também é pioneiro na abordagem das células Tfc ($CXCR5^+CD8^+$e IL-21^+CD8^+) no contexto de autoimunidade, uma vez que a associação entre estas células e a atividade da doença na SSP poderá ser um indicador do seu envolvimento na patogénese da epitelite autoimune.

Na SSP, a presença de anticorpos anti-SSA associa-se a caraterísticas fenotípicas distintas, como pior função glandular, maior infiltração linfocitária glandular, tumefação glandular, manifestações extra-glandulares (fenómeno de Raynaud, artrite, vasculite, envolvimento renal, neuropatia periférica), citopénias e marcadores imunológicos positivos (ANA, FR, crioglobulinas) [636]. Assim, tendo em conta esta maior atividade autoimune, no **Capítulo V** pretendemos investigar se existiam diferenças nas populações linfocitárias de doentes com SSP e anti-SSA positivo (SSA^+SSP), em comparação com doentes seronegativos (SSA^-SSP), doentes com síndrome Sicca e controlos saudáveis.

Pretendeu-se também caraterizar eventuais associações de subpopulações linfocitárias com a atividade sistémica da doença nos doentes SSA^+SSP e SSA^-SSP.

Assinalamos que o explorámos pela primeira vez a relação entre as células $CXCR5^+CD8^+$ (Tfc) e a positividade para anti-SSA na SSP. Nos doentes com SSP encontrámos não só linfopénia T, mas também redução dos números de células B, o que se verificava também nos doentes Sicca, embora em menor grau.

Os doentes SSA^+SSP apresentaram maiores proporções de células T $CD4^+$ e $CD8^+$ produtoras de IL-21, uma acentuada redução das células B de memória e um aumento das B naïve B, em comparação com controlos saudáveis. Além disso, os doentes SSA^+SSP apresentaram maior atividade de doença, e verificou-se a existência de associações entre o ESSDAI e várias subpopulações linfocitárias, incluindo as populações T $IL\text{-}21^+CD4^+$, $IL\text{-}21^+CD8^+$ e Tregs.

A linfopénia T na SSP pode dever-se à migração das células $CD4^+$ periféricas para as glândulas salivares [773] e assim refletir a disfunção autoimune e um perfil de doença mais ativo. Ambos os grupos, SSA^+SSP e SSA^-SSP, apresentaram menores contagens de células T $CD4^+$ em comparação com doentes Sicca e controlos, e foi interessante verificar que os doentes Sicca tinham menores contagens $CD4^+$ que os controlos. Os nossos resultados estão em linha com estudos prévios publicados por Mandl [615] e Sudzius [526].

A correlação positiva entre o ESSDAI e as percentagens de células $CXCR5^+Th1$ (Tfh1), observada apenas nos doentes SSA^+SSP, e ainda mais significativa nos doentes com atividade de doença moderada/elevada (ESSDAI ≥5), sugere que os mecanismos patogénicos subjacentes à dinâmica das células T e às manifestações da doença diferem de acordo com a presença de anticorpos anti-SSA.

As menores frequências de células $CXCR5^+Th17$ circulantes e a sua associação negativa com o ESSDAI verificada em doentes SSA^+SSP pode dever-se a uma maior acumulação destas células nas GS destes doentes [820] ou a uma polarização da diferenciação Tfh a favor do subtipo Tfh1.

Em relação às Tregs, encontrámos uma forte correlação negativa entre os seus números e o ESSDAI em doentes com SSA^+SSP apresentando atividade de doença moderada/elevada. Considerando estes dados e as observações prévias de Christodoulou [423], os menores números de Tregs circulantes podem refletir a sua acumulação nas GS, proporcional à maior atividade da doença.

Para melhor caraterizar as células T com fenótipo de célula folicular, avaliámos a produção de IL-21 pelas células T $CD4^+$ e $CD8^+$. Esta citocina, produzida pelas células T foliculares, regula os processos imunes nos centro germinativos, como a ativação das células B e a produção de autoanticorpos [432].

Os doentes SSA^+SSP apresentaram maiores percentagens de células T $CD4^+$ produtoras de IL-21 ($IL\text{-}21^+CD4^+$) em comparação com os restantes grupos, e maiores percentagens de células $IL\text{-}21^+CD8^+$ em comparação com os Sicca e controlos. Estes resultados sugerem que doentes SSA^+SSP com função folicular aumentada poderão desenvolver reações de centro germinativo mais intensas. Os nossos resultados estão em linha com um estudo recente de Pontarini e colaboradores [821], que demonstraram que as células Tfh circulantes $CXCR5^+ICOS^+PD\text{-}1^+$ secretoras de elevados níveis de IL-21 e IFN-γ estavam aumentadas em doentes com SSP positivos para anti-SSA e anti-SSB, em especial os que apresentavam estruturas linfoides ectópicas, e se correlacionavam o *focus score* das GS. Na mesma linha, Gao e colaboradores demonstraram o papel das células T $CD8^+$ no desenvolvimento e progressão das lesões das GS em doentes com SSP [822]. A forte correlação positiva entre o ESSDAI e os níveis de células $IL\text{-}21^+CD4^+$ e $IL\text{-}21^+CD8^+$ nos doentes SSA^+SSP com doença clinicamente ativa, que não se verificou nos doentes SSA^-SSP, suporta uma maior ativação B nestes doentes. Adicionalmente, os nossos resultados suportam o provável papel importante das células Tfc no desenvolvimento do ambiente inflamatório e perfil clinicamente ativo dos doentes SSA^+SSP.

Avaliando as subpopulações B, várias diferenças entre os doentes SSA^+SSP e SSA^-SSP apoiam a presença de maior desregulação imune nos primeiros, como a mais elevada proporção de células B naïve B e a menor proporção de células B de memória, sendo a maior mobilização de células naïve da medula e retenção das B de memória nas GS a explicação mais plausível [436]. Esta alteração da dinâmica das populações B, bem como a diferenciação terminal preferencial em plasmócitos nos doentes SSA^+SSP [549] é apoiada pela maior frequência de tumefação das GS e hipergamaglobulinémia nestes doentes, sendo a própria presença de anti-SSA sugestiva de uma maior resposta humoral [823]. No entanto, uma vez que as células $IgM^{-/+}CD38^{Hi}$, precursores dos plasmócitos, estavam aumentadas nos doentes SSA^+SSP em comparação com os Sicca e controlos, mas sem diferenças face aos SSA^-SSP, sugere a intervenção de outros fatores na resposta humoral.

Nos doentes SSA^+SSP encontrámos uma diminuição das células B $CD24^{Hi}CD27^+$ em comparação com os SSA^-SSP, uma subpopulação que, tal como as B de transição $CD24^{Hi}CD38^{Hi}$ (em menor número, mas aumentadas nos SSA^+SSP), é enriquecida em células Breg [235]. No entanto, não podemos avaliar em concreto o perfil das Breg dado não se ter efetuado o estudo da produção de IL-10, citocina que carateriza funcionalmente as células B reguladoras. Os trabalhos de Jin e colaboradores no LES [824] e de Salomon e colaboradores

na AR [825] sugerem que as alterações nas células B $CD24^{hi}CD27^{+}$ se podem correlacionar com as alterações nas células com fenótico regulador, $IL10^{+}CD24^{hi}CD27^{+}$, constituindo assim um método mais simples para o estudo das Bregs num contexto clínico. Nesta linha, encontrámos uma correlação negativa entre as células B $CD24^{hi}CD27^{+}$ e a atividade da doença nos doentes $SSA^{+}SSP$.

Estas alterações, sobretudo das células $CD24^{Hi}CD27^{+}$, podem refletir a influência de distúrbios nos números e função das Breg na maior expressão clínica e disfunção imune de doentes $SSA^{+}SSP$.

Em resumo, os nossos resultados indicam que os doentes $SSA^{+}SSP$ apresentam maior atividade de doença, incluindo marcadores de ativação B como hipergamaglobulinémia, FR e tumefação das GS. Globalmente, o perfil linfocitário observado nestes doentes sugere que os mecanismos autoimunes subjacentes diferem de acordo com a presença de anti-SSA. O maior desequilíbrio entre células B naïve e B de memória reforça a importância da expansão do compartimento T folicular e da produção de IL-21 na atividade das células B. Além disso, a associação entre a atividade da doença e as células T $IL\text{-}21^{+}CD4^{+}$ e $IL\text{-}21^{+}CD8^{+}$ (foliculares), para além de poder traduzir o envolvimento destas células num fenótipo mais ativo da doença, poderá indicar um futuro papel da caraterização destas células no prognóstico e monitorização da doença.

No **Capítulo VI (A)** são descritos os resultados da avaliação com duas novas técnicas diagnósticas não invasivas: a avaliação do menisco lacrimal por AS-OCT (*anterior segment optical coherence tomography*) [667] e a avaliação do plexo nervoso corneano sub-basal por microscopia confocal [826] em comparação com os testes convencionais (teste de Schirmer I, o teste de quebra do filme lacrimal (*tear breakup time* - TBUT) e marcação da córnea com corantes vitais), com o objetivo de avaliar características distintas entre doentes com SSP, Sicca e controlos saudáveis.

A microscopia confocal *in vivo* permite a avaliação quantitativa e objetiva de imagens de alta resolução das células e nervos da córnea [827,828] O dano e subsequente regeneração destas fibras está descrito na síndrome de olho seco [829]. As fibras nervosas não-mielinizadas da córnea são particularmente vulneráveis à degeneração, que ocorre também no contexto de inflamação ou autoimunidade [830]. Na SSP, foram demonstradas diferenças na morfologia do plexo nervoso corneano sub-basal (densidade, comprimento e tortuosidade) em comparação com indivíduos saudáveis [829,831], pelo que esta técnica pode vir a ser útil não só na deteção da doença, mas também na monitorização da resposta ao tratamento [676]. No **Capítulo VIb** explorámos e descrevemos possíveis associações entre a morfologia do plexo nervoso corneano sub-basal e as subpopulações linfocitárias e a atividade da doença na SSP.

Na avaliação objetiva efetuada no **Capítulo VI (A)**, os testes convencionais de olho seco estavam alterados em ambos os grupos de indivíduos com queixas secas (SSP e Sicca), em comparação com os controlos, como esperado.

As caraterísticas do menisco lacrimal (área e altura) não apresentaram diferenças entre grupos.

Em relação à morfologia do plexo nervoso corneano sub-basal, a densidade e comprimento das fibras foi significativamente menor nos doentes com olho seco, e a tortuosidade significativamente maior, em comparação com o grupo controlo. Doentes com SSP e Sicca apresentaram parâmetros morfológicos do plexo corneano semelhantes. Adicionalmente, as alterações do plexo nervoso corneano sub-basal apresentaram uma boa associação com o teste de Schirmer e o tempo de quebra do filme lacrimal.

A imagem da córnea por microscopia confocal demonstrou um bom desempenho na identificação de indivíduos com olho seco, face a indivíduos saudáveis sem queixas secas. Adicionalmente, o facto de ser não-invasiva e menos dependente de fatores ambientais e relacionados com o doente torna-a um instrumento promissor na avaliação objetiva da xeroftalmia.

No entanto, é preciso ter em conta que outras doenças com potencial neuropático, como a diabetes, podem também levar a alterações da inervação corneana. Assim, é necessária a identificação de eventuais perfis morfológicos que possam distinguir entre o dano induzido pela hipossecreção lacrimal, pelo efeito de mediadores inflamatórios locais, e por doenças como a diabetes [832–835].

O estudo apresentado no **Capítulo VI (B)** foi o primeiro na literatura a avaliar a relação entre as caraterísticas morfológicas do plexo corneano sub-basal e a distribuição das subpopulações linfocitárias na SSP.

Em relação às alterações morfológicas do plexo nervoso corneano sub-basal atribuíveis ao olho, foram mais evidentes nos doentes com SSP com maior duração e atividade da doença.

Explorando mais detalhadamente a associação entra a atividade da doença na SSP e as caraterísticas dos nervos corneanos, encontrou-se uma forte correlação negativa entre o ESSDAI e comprimento (CNFL) e a densidade das fibras (CNFD) em doentes com maior atividade da doença (ESSDAI ≥5), doentes estes que apresentavam os piores parâmetros oculares (menor CNFD e CNFL).

Adicionalmente, quando considerados apenas os doentes com ESSDAI≥5, encontrou-se uma correlação positiva entre o CNFL e o número de Tregs, bem como uma forte correlação negativa entre as Tfh1 e ambos os parâmetros morfológicos do plexo corneano (CNFL e CNFD.

Em relação às células B, verificaram-se correlações positivas entre o CNFL e os números absolutos de células de memória (totais, com switch e sem switch) e de células B $CD24^{Hi}CD27^{+}$.

Considerando as células T, encontrou-se uma correlação negativa entre o CNFL e os números absolutos de células $IL21^{+}CD8^{+}$, que pode apoiar a influência da disfunção imune na lesão da córnea.

Os nossos dados sugerem que a lesão dos nervos corneanos é mais acentuada em doentes com maior atividade sistémica da doença, o que pode sugerir uma evolução paralela à atividade inflamatória da SSP. Adicionalmente, piores parâmetros morfológicos do plexo corneano parecem relacionar-se com a maior desregulação imune nestes doentes, com níveis mais elevados de Tfc e mais baixos de Tregs e de células B $CD24^{Hi}CD27^{+}$.

Em conclusão, considerando as associações da CNFL e da CNFD com a atividade da doença, com as células T foliculares $IL21^{+}$ e com as subpopulações B de memória, parece existir evidência que indica a evolução paralela do dano às fibras nervosas da córnea e a atividade imunológica da SSP.

Deste modo, a avaliação morfológica do plexo nervoso corneano sub-basal em doentes com SSP pode fornecer indícios do grau de disfunção imunológica e da dinâmica das populações linfocitárias. Para esse fim, é necessário o desenvolvimento de um sistema de pontuação das alterações morfológicas, bem como a identificação de eventuais padrões morfológicos típicos da SSP.

O diagnóstico e classificação da SSP é ainda desafiante, sobretudo nas fase iniciais da doença, em que os doentes podem apresentar fenótipos mais ligeiros ou manifestações incomuns [836]. Na prática clínica, os critérios de classificação são habitualmente utilizados como orientação para o diagnóstico, apesar de não serem elaborados com vista à aplicação ao doente individual, mas sim para a seleção de grupos homogéneos para fins de investigação [728]. Os critérios de classificação da SSP têm sido revistos, recorrendo a novas metodologias, mas há ainda margem para aperfeiçoamento [15,737].

O papel das células B como biomarcador para o diagnóstico e classificação da SSP foi previamente avaliado em associação aos critérios AECG [811,813]. Apesar das células T serem

elementos fundamentais na patogénese da SSP [452], até à data não tinham sido avaliadas como potencial biomarcador em critérios de classificação. Adicionalmente, não tinha ainda sido abordado o efeito da adição de subpopulações linfocitárias aos critérios ACR/EULAR. No **Capítulo VII** pretendemos avaliar se a inclusão de subpopulações linfocitárias aumentava o poder discriminativo dos critérios AECG e ACR/EULAR na distinção entre SSP e Sicca. Para isso, recorremos à definição dos casos de SSP de acordo com o diagnóstico clínico (definido como o padrão-ouro), e comparámos a distribuição das subpopulações linfocitárias B e T de doentes com SSP e Sicca. Algumas das subpopulações que demonstraram uma aceitável precisão na distinção entre SSP e Sicca foram incluídas no modelo multivariado para avaliar se melhoravam o desempenho dos critérios de classificação.

Este estudo avaliou pela primeira vez o efeito da caraterização do perfil linfocitário no desempenho dos critérios de classificação ACR/EULAR.

Nos doentes com SSP confirmou-se o perfil típico de subpopulações de linfócitos B habitualmente descrito na doença através da utilização da classificação IgD/CD27 – diminuição dos linfócitos B de memória e aumento dos linfócitos B naïve (Roberts et al. 2014).

Em relação à classificação das células B de Bm1 a Bm5, na SSP é habitual o aumento das populações Bm2 e Bm2' e a diminuição das eBm5 e Bm5, conforme revisto por Carvajal [8]. No nosso estudo confirmámos este perfil, e adicionalmente destacamos a redução das percentagens e valores absolutos das células Bm1.

Destacamos também a significativa diminuição da subpopulação B caraterizada por $CD24^{hi}CD27^{+}$, que sabemos ser enriquecida em células B reguladoras [235]. Tendo em conta a correlação negativa entre estas células e a atividade da doença, que descrevemos no Capítulo V, o aumento da razão Th1/$CD24^{hi}CD27^{+}$ nos nossos doentes com SSP pode refletir o desequilíbrio entre o compartimento B regulador e as células efetoras Th1.

O grupo de Szabó descreveu um aumento das células Tfh na SSP [21], mas no nosso grupo SSP apenas as células Tfh1 apresentaram aumento das percentagens em relação aos Sicca, enquanto as Tfh17 estavam diminuídas. Efetivamente, as células Tfh1 demonstraram uma das maiores AUC na discriminação entre SSP e Sicca.

As células Tregs apresentaram uma tendência para valores aumentados em comparação com os Sicca. Conforme revisto por Alunno e colaboradores [20], as frequências de Tregs na SSP são variáveis na literatura, mas deficiências da sua função parecem contribuir para a patogénese da doença. Adicionalmente, constatou-se a presença de uma

razão Th17/Treg diminuída, explicada pelo relativo aumento das Tregs e pela diminuição das Th17, que seguia a tendência para linfopénia nestes doentes.

Ao avaliar o desempenho dos critérios ACR/EULAR em comparação com o diagnóstico clínico, obtivemos uma concordância de 92,0% na discriminação entre SSP e Sicca. Em relação ao desempenho dos critérios AECG, a concordância com o padrão-ouro foi de 95,2%, em linha com o estudo de Zhao e colaboradores [838].

Pretendemos de seguida avaliar o valor acrescentado ao poder discriminativo entre SSP e Sicca com a inclusão de populações linfocitárias em ambos os critérios.

Após termos identificado diferenças em várias subpopulações linfocitárias entre SSP e Sicca, aquelas que demonstraram uma maior AUC na distinção entre ambos os grupos foram incluídas no modelo multivariado. Assim, demonstrámos que algumas populações linfocitárias permitiam aumentar o poder discriminativo dos critérios de classificação. Em relação aos critérios ACR/EULAR, as variáveis Th1/Breg $CD24^{hi}CD27^{+}$ e as células B de memória com switch (dicotomizadas com um cut-off=25 células/µl) maximizaram a AUC para 0,953. Considerando os critérios AECG, a AUC foi maximizada para 0,985 quando consideradas as percentagens de Bm1 e Tfh.

Demonstrámos assim, neste estudo exploratório, que é possível aumentar o poder discriminativo de ambos os critérios de classificação com a inclusão no modelo de populações linfocitárias. A nossa abordagem foi diferente da de Binard e colaboradores (Binard et al. 2009), que utilizaram a razão (Bm2+Bm2')/(eBm5+Bm5) nos critérios AECG, com melhoria do desempenho, embora num estudo subsequente Cornec e colaboradores [813] obtiveram um reduzido valor diagnóstico comparado com os restantes itens dos critérios AECG. Por sua vez, o grupo de Mingueneau [773], recorrendo a uma avaliação de 34 biomarcadores por citometria em massa e bioinformática, identificou um perfil distinto de 4 tipos celulares (células dendríticas plasmacitóides, células T $CD4^{+}$, células B de memória, e células T HLA-$DR^{+}CD4^{+}$) que identificava doentes com SSP com boa precisão (AUC 0.86–0.89). No entanto, esta técnica não é, na atualidade, de utilização rotineira.

No nosso estudo verificámos que a inclusão de subpopulações linfocitárias no modelo levou a um aumento na capacidade discriminativa dos critérios EACG e ACR/EULAR. Tendo em conta que estes últimos se baseiam num conjunto de itens aos quais é atribuída uma pontuação ponderada, o nosso estudo abre perspetivas para uma posterior análise com maior número e diversidade de doentes, com vista a confirmar a seleção das populações linfocitárias a incluir nos critérios ACR/EULAR, e determinar a pontuação que lhes seria atribuído.

Limitações do projeto de investigação

Estamos cientes de que este Projeto de Investigação apresenta algumas limitações, umas de índole global, e outras de natureza específica.

Em relação às **limitações de índole global**, reconhecemos que a dimensão da amostra foi reduzida, quando se constata a heterogeneidade dos grupos de doentes no que respeita à idade, duração da doença, perfil de manifestações clínicas e serologia dos autoanticorpos.

Esta variabilidade reflete o contexto clínico em que o recrutamento se processou, sendo representativa da prática assistencial em Reumatologia.

Outro aspeto a considerar é o efeito das terapêuticas imunomoduladoras nos processos imunes e na distribuição das populações linfocitárias. Uma vez que os doentes com SSP e Sicca foram recrutados da consulta de Reumatologia, muitos encontravam-se sob terapêutica, maioritariamente corticoesteróides em baixa dose e hidroxicloroquina. No entanto, o estudo de doentes com SSP sem este tipo de medicação enquadrar-se-á em dois cenários: doentes em fase precoce da doença ou do acompanhamento reumatológico, ou doentes sem doença grave que não necessitassem de terapêutica imunomoduladora. Ambos os cenários teriam desvantagens: o primeiro obrigaria à realização de um estudo multicêntrico de modo a se obter uma amostra de dimensão aceitável, enquanto o segundo cenário representaria um viés de seleção a favor de formas mais leves da doença.

O seguimento prospetivo dos nossos doentes, com avaliações clínicas regulares e a repetição subsequente da caraterização das populações linfocitárias e das avaliações poderia esclarecer melhor a dinâmica destas células em doentes sob fármacos imunomoduladores.

Também assinalamos a dificuldade na obtenção de um grupo controlo saudável sem queixas secas cuja idade não diferisse da dos doentes com SSP ou Sicca. Apesar de grandes diferenças de idade se poderem traduzir em diferentes dinâmicas celulares, consideramos que no nosso estudo a diferença entre doentes e controlos, apesar de estatisticamente significativa, não terá sido relevante na interpretação dos nossos resultados.

Por fim, a caraterização das populações linfocitárias foi realizada exclusivamente no sangue periférico. Reconhecemos que as populações circulantes poderão não refletir fielmente os números e interações das células imunes nas glândulas exócrinas, nem identificar as vias migratórias entre órgãos-alvo distintos. Seria importante a caraterizar em simultâneo as populações linfocitárias presentes nos tecidos-alvo, como as glândulas

salivares, para melhor caraterizar a disfunção imune na SSP e validar o significado do perfil linfocitário circulatório.

Quanto às **limitações específicas** de cada trabalho, temos a referir:

Em relação à caraterização do perfil serológico para o EBV, seriam importantes estudos moleculares como a pesquisa de RNA viral para melhor determinar a presença de infeção ativa e respetiva carga viral.

Adicionalmente, a avaliação de tecido das glândulas salivares poderia revelar sinais da presença de EBV nestes órgãos.

Finalmente, sendo o EBV um vírus com elevada prevalência na população adulta, seria necessária uma amostra maior de doentes e controlos para se caraterizar as diferenças imunológicas entre os indivíduos sem história de infeção e os indivíduos portadores de infeção classificados de acordo com o grau de atividade.

Em relação à exploração da relação entre a presença do anticorpo anti- SSA e o perfil linfocitário, seria importante a determinação dos níveis de anticorpos, bem como o acesso às populações linfocitárias das glândulas salivares.

Uma limitação aplicável a todos os nossos estudos de populações linfocitárias, mas que tem mais relevo nas associações entre a presença de anti-SSA, a atividade da doença e as populações celulares, é identificação das populações reguladoras.

Apesar da caraterização das células Treg ser possível através de marcadores de superfície, a identificação do marcador de linhagem Foxp3 carateriza melhor o fenótipo regulador, mas a sua localização intracelular dificulta a aplicabilidade clínica.

Em relação às células Breg, a identificação das populações-mãe (como as CD24CD27) pode transmitir uma impressão vaga sobre a dinâmica das células B reguladoras incluídas nessa subpopulação. Seria necessária a avaliação da produção de IL-10 para identificar as células com fenótipo regulador, dado não possuírem marcadores de linhagem.

Adicionalmente, quer se utilizem marcadores de linhagem ou se avalie a capacidade produtora de uma citocina específica, a ausência de estudos para avaliar a função supressora das células Treg e Breg pode negligenciar deficiências funcionais destas células na SSP.

Relativamente à caraterização da morfologia do plexo nervoso corneano sub-basal e a sua relação com os processos imunes na SSP, seria útil a avaliação do ambiente inflamatório lacrimal, nomeadamente através do estudo de citocinas e células inflamatórias da lágrima, para melhor compreender as relações entre a inflamação sistémica, local e o dano ocular.

Relativamente à avaliação do valor acrescentado da inclusão de subpopulações linfocitárias no poder discriminativo dos critérios AECG e ACR/EULAR face ao diagnóstico clínico, para além das limitações relacionadas com a dimensão da amostra, assinalamos a variabilidade clínica e as terapêuticas em curso, que podem afetar as distribuições linfocitárias. Deste modo, estes resultados necessitam de validação numa coorte independente, multicêntrica e de maior dimensão.

Além disso, pelas suas caraterísticas, existe o potencial de variabilidade nos protocolos de citometria de fluxo, sendo necessária uma padronização de modo a facilitar a utilização das subpopulações linfocitárias como biomarcador na SSP.

Perspetivas Futuras

Neste estudo demonstrámos que é possível a determinação de um *cut-off* nas contagens de células B de memória que claramente distingue a SSP de controlos saudáveis, ou seja, patológico de normal.

Aplicando um *cut-off* semelhante aos doentes Sicca, poderá vir a ser possível identificar indivíduos cujo perfil se assemelhe ao da SSP, e que de facto representem formas ligeiras ou precoces da doença. Esta é uma possível aplicação como auxiliar de diagnóstico e terapêutica.

Adicionalmente, demonstrámos que em doentes com SSP a presença de menores contagens de células B de memória estava associada a evolução mais prolongada e doença mais ativa, o que abre perspetivas de que a avaliação desta população celular possa ter também um papel prognóstico.

A sugestão do envolvimento das Tfc na patogénese d SSP, com um perfil de amplificação do ambiente folicular similar a uma resposta viral e maior prevalência de marcadores serológicos de EBV em doentes com maior atividade de doença justifica estudos complementares acerca do papel destas células na autoimunidade e dos seus agentes ativadores. A confirmar-se a ação da estimulação crónica por EBV na manutenção

deste perfil Tfc ativo, futuras abordagens terapêuticas poderão contemplar a interrupção da ativação linfocitária induzida por estes agentes.

Independentemente da sua eventual associação a um estímulo viral, a promoção do ambiente folicular relacionado com a hiperatividade das células T foliculares pode ser avaliado através de estudos funcionais por citometria de fluxo, e constituir um indicador de atividade de doença, prognóstico e monitorização da resposta à terapêutica.

A avaliação da avaliação da morfologia do plexo nervoso corneano sub-basal por microscopia confocal é sensível na deteção do dano das fibras nervosas associado ao olho seco, e tem boa correlação com os testes convencionais de olho seco.

Quando a técnica vier a estar padronizada, e caso se confirme a existência de padrões morfológicos específicos da SSP, a avaliação do plexo nervoso corneano sub-basal poderá ser um instrumento não-invasivo, estável e de fácil reprodução e avaliação. Acresce que num futuro próximo análises de imagens deste tipo poderão ser realizadas facilmente por programas informáticos.

A homogeneização de protocolos de citometria de fluxo pode permitir a realização de avaliações multicêntricas da distribuição das subpopulações de linfócitos na SSP. Nessa perspetiva, a possibilidade de se vir a utilizar o perfil linfocitário como adjuvante dos critérios de classificação é uma possibilidade em aberto.

As perspetivas de **aplicabilidade imediata** que identificámos são as seguintes:

1) Variações nas populações linfocitárias podem ajudar a identificar indivíduos com maior atividade de doença ou com maior risco de manifestações graves

2) A estratificação clínica dos doentes, de acordo com o binómio atividade/impacto, pode ser facilitada pela presença de alterações na distribuição das populações linfocitárias

3) A decisão quanto ao início e à escolha de uma terapêutica imunomoduladora, bem como a aferição do seu efeito no controlo dos processos autoimunes pode ser facilitada pelo perfil de subpopulações linfocitárias.

REFERÊNCIAS

1. Brito-Zerón P, Theander E, Baldini C, Seror R, Retamozo S, Quartuccio L, et al. Early diagnosis of primary Sjögren's syndrome: EULAR-SS task force clinical recommendations. Expert Rev Clin Immunol [Internet] 2016 [cited 2016 Jun 13]; 12: 1–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26691952

2. Parisis D, Chivasso C, Perret J, Soyfoo MS, Delporte C. Current State of Knowledge on Primary Sjögren's Syndrome, an Autoimmune Exocrinopathy. J Clin Med 2020; 9: 2299.

3. Kyriakidis NC, Kapsogeorgou EK, Tzioufas AG. A comprehensive review of autoantibodies in primary Sjögren's syndrome: Clinical phenotypes and regulatory mechanisms [Internet]. Vol. 51, Journal of Autoimmunity. 2014 [cited 2017 Oct 1]. p. 67–74. Available from: file:///C:/Users/fduar_000/Desktop/kyriakidis2014.pdf

4. Guellec D, Cornec D, Jousse-Joulin S, Marhadour T, Marcorelles P, Pers JO, et al. Diagnostic value of labial minor salivary gland biopsy for Sjögren's syndrome: A systematic review [Internet]. Vol. 12, Autoimmunity Reviews. 2013 [cited 2017 Oct 14]. p. 416–420. Available from: file:///C:/Users/fduar_000/Desktop/guellec2013.pdf

5. Fisher BA, Brown RM, Bowman SJ, Barone F. A review of salivary gland histopathology in primary Sjögren's syndrome with a focus on its potential as a clinical trials biomarker. Ann Rheum Dis [Internet] 2015 [cited 2016 Jun 13]; 74: 1–6. Available from: http://ard.bmj.com/lookup/doi/10.1136/annrheumdis-2015-207499

6. Nichols KK, Nichols JJ, Mitchell GL. The lack of association between signs and symptoms in patients with dry eye disease. Cornea 2004; 23: 762–770.

7. Sack U, Boldt A, Mallouk N, Gruber R, Krenn V, Berger-Depincé AE, et al. Cellular analyses in the monitoring of autoimmune diseases [Internet]. Vol. 15, Autoimmunity Reviews. 2016 [cited 2017 Jul 11]. p. 883–889. Available from: file:///C:/Users/fduar_000/Desktop/sack2016.pdf

8. Carvajal Alegria G, Gazeau P, Hillion S, Daïen CI, Cornec DYK. Could Lymphocyte Profiling be Useful to Diagnose Systemic Autoimmune Diseases? Clin Rev Allergy Immunol [Internet] 2017; 53: 219–236. Available from: http://link.springer.com/10.1007/s12016-017-8608-5

9. Shen L, Suresh L, Lindemann M, Xuan J, Kowal P, Malyavantham K, et al. Novel autoantibodies in Sjogren's syndrome. Clin Immunol 2012; 145: 251–255.

10. Jousse-Joulin S, Milic V, Jonsson M V, Plagou A, Theander E, Luciano N, et al. Is salivary gland ultrasonography a useful tool in Sjögren's syndrome? A systematic review. Rheumatology (Oxford) [Internet] 2016 [cited 2016 Jun 12]; 55: 789–800. Available from: http://rheumatology.oxfordjournals.org/

11. Aggarwal R, Ringold S, Khanna D, Neogi T, Johnson SR, Miller A, et al. Distinctions between

diagnostic and classification criteria? Arthritis Care Res 2015; 67: 891–897.

12. Ramos-Casals M, Brito-Zerón P, Bombardieri S, Bootsma H, De Vita S, Dörner T, et al. EULAR recommendations for the management of Sjögren's syndrome with topical and systemic therapies. Ann Rheum Dis 2020; 79: 3–18.

13. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM A, Al. E et, Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis [Internet] 2002; 61: 554–558. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12006334%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1754137

14. Billings M, Amin Hadavand M, Alevizos I. Comparative analysis of the 2016 ACR-EULAR and the 2002 AECG classification criteria for Sjögren's syndrome: Findings from the NIH cohort. Oral Dis [Internet] 2018 [cited 2018 Apr 29]; 24: 184–190. Available from: http://doi.wiley.com/10.1111/odi.12772

15. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis Rheumatol 2017; 69: 35–45.

16. Brito-Zerón P, Retamozo S, Ramos-Casals M. Phenotyping Sjögren's syndrome: Towards a personalised management of the disease. Clin Exp Rheumatol 2018; 36: S198–S209.

17. López-Pintor RM, Fernández Castro M, Hernández G. Oral Involvement in Patients With Primary Sjögren's Syndrome. Multidisciplinary Care by Dentists and Rheumatologists. Reumatol Clínica (English Ed [Internet] 2015; 11: 387–394. Available from: http://www.reumatologiaclinica.org/en/oral-involvement-in-patients-with/articulo/S2173574315001069/

18. Hansen A, Odendahl M, Reiter K, Jacobi AM, Feist E, Scholze J, et al. Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome. Arthritis Rheum [Internet] 2002 [cited 2017 Jun 10]; 46: 2160–2171. Available from: file:///C:/Users/fduar_000/Desktop/Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome.pdf

19. Bohnhorst JØ, Bjørgan MB, Thoen JE, Natvig JB, Thompson KM. Bm1–Bm5 Classification of Peripheral Blood B Cells Reveals Circulating Germinal Center Founder Cells in Healthy Individuals and Disturbance in the B Cell Subpopulations in Patients with Primary Sjögren's Syndrome. J Immunol [Internet] 2001 [cited 2017 Jul 11]; 167: 3610–3618. Available from: http://www.jimmunol.org/content/167/7/3610

20. Alunno A, Carubbi F, Bistoni O, Caterbi S, Bartoloni E, Mirabelli G, et al. T regulatory and T helper 17 cells in primary Sjögren's syndrome: Facts and perspectives [Internet]. Vol. 2015, Mediators of Inflammation. 2015 [cited 2017 Mar 12]. p. 1–10. Available from: http://dx.doi.org/10.1155/2015/243723

21. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. Clin Immunol [Internet] 2013 [cited 2016 Aug 11]; 147: 95–104. Available from:

http://dx.doi.org/10.1016/j.clim.2013.02.024

22. Li X yan X yi, Wu Z biao, Ding J, Zheng Z hui, Li X yan X yi, Chen L na, et al. Role of the frequency of blood CD4+CXCR5+CCR6+T cells in autoimmunity in patients with Sjögren's syndrome. Biochem Biophys Res Commun [Internet] 2012 [cited 2017 Feb 28]; 422: 238–244. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0006291X12008157

23. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. Allergy, Asthma Clin Immunol [Internet] 2018; 14: 1–10. Available from: https://doi.org/10.1186/s13223-018-0278-1

24. Poon MML, Farber DL. The Whole Body as the System in Systems Immunology. iScience [Internet] 2020; 23: 101509. Available from: https://doi.org/10.1016/j.isci.2020.101509

25. Turvey SE, Broide DH. Innate immunity. J Allergy Clin Immunol [Internet] 2010; 125: S24–S32. Available from: http://dx.doi.org/10.1016/j.jaci.2009.07.016

26. Ginhoux F, Guilliams M. Tissue-Resident Macrophage Ontogeny and Homeostasis. Immunity [Internet] 2016; 44: 439–449. Available from: http://dx.doi.org/10.1016/j.immuni.2016.02.024

27. Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, de Bruijn M, Rodewald HR, et al. The Origin of Tissue-Resident Macrophages: When an Erythro-myeloid Progenitor Is an Erythro-myeloid Progenitor. Immunity [Internet] 2015; 43: 1023–1024. Available from: http://dx.doi.org/10.1016/j.immuni.2015.11.022

28. Ginhoux F, Jung S. Monocytes and macrophages: Developmental pathways and tissue homeostasis. Nat Rev Immunol [Internet] 2014; 14: 392–404. Available from: http://dx.doi.org/10.1038/nri3671

29. Lavin Y, Winter D, Blecher-Gonen R, David E, Keren-Shaul H, Merad M, et al. Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. Cell [Internet] 2014; 159: 1312–1326. Available from: http://dx.doi.org/10.1016/j.cell.2014.11.018

30. Nagelkerke SQ, Bruggeman CW, Den Haan JMM, Mul EPJ, Van Den Berg TK, Van Bruggen R, et al. Red pulp macrophages in the human spleen are a distinct cell population with a unique expression of Fc-g receptors. Blood Adv 2018; 2: 941–963.

31. Audiger C, Rahman MJ, Yun TJ, Tarbell K V, Lesage S. The Importance of Dendritic Cells in Maintaining Immune Tolerance. J Immunol 2017; 198: 2223–2231.

32. Austyn JM. Dendritic Cells in the Immune System-History, Lineages, Tissues, Tolerance, and Immunity. Myeloid Cells Heal Dis 2017; : 155–207.

33. Bigley V, McGovern N, Milne P, Dickinson R, Pagan S, Cookson S, et al. Langerin-expressing dendritic cells in human tissues are related to CD1c + dendritic cells and distinct from Langerhans cells and CD141 high XCR1 + dendritic cells. J Leukoc Biol 2015; 97: 627–634.

34. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: Ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 2013; 31: 563–604.

35. Dogra P, Rancan C, Ma W, Toth M, Senda T, Carpenter DJ, et al. Tissue Determinants of

Human NK Cell Development, Function, and Residence. Cell 2020; 180: 749-763.e13.

36. Yudanin NA, Schmitz F, Flamar AL, Thome JJC, Tait Wojno E, Moeller JB, et al. Spatial and Temporal Mapping of Human Innate Lymphoid Cells Reveals Elements of Tissue Specificity. Immunity 2019; 50: 505-519.e4.

37. Ishizuka IE, Chea S, Gudjonson H, Constantinides MG, Dinner AR, Bendelac A, et al. Single-cell analysis defines the divergence between the innate lymphoid cell lineage and lymphoid tissue-inducer cell lineage. Nat Immunol [Internet] 2016; 17: 269–276. Available from: http://dx.doi.org/10.1038/ni.3344

38. Bonilla FA, Oettgen HC. Adaptive immunity. J Allergy Clin Immunol [Internet] 2010; 125: S33–S40. Available from: http://dx.doi.org/10.1016/j.jaci.2009.09.017

39. Shen P, Fillatreau S. Antibody-independent functions of B cells: A focus on cytokines. Vol. 15, Nature Reviews Immunology. Nature Publishing Group; 2015. p. 441–451.

40. Lino AC, Dörner T, Bar-Or A, Fillatreau S. Cytokine-producing B cells: A translational view on their roles in human and mouse autoimmune diseases. Immunol Rev 2016; 269: 130–144.

41. Allman D, Pillai S. Peripheral B cell subsets. Vol. 20, Current Opinion in Immunology. Curr Opin Immunol; 2008. p. 149–157.

42. Carsetti R, Rosado MM, Wardemann H. Peripheral development of B cells in mouse and man. Vol. 197, Immunological Reviews. Immunol Rev; 2004. p. 179–191.

43. Montecino-Rodriguez E, Dorshkind K. B-1 B Cell Development in the Fetus and Adult. Vol. 36, Immunity. Cell Press; 2012. p. 13–21.

44. Baumgarth N. The double life of a B-1 cell: Self-reactivity selects for protective effector functions. Nat Rev Immunol 2011; 11: 34–46.

45. Samitas K, Lötvall J, Bossios A. B Cells: From early development to regulating allergic diseases. Vol. 58, Archivum Immunologiae et Therapiae Experimentalis. Arch Immunol Ther Exp (Warsz); 2010. p. 209–225.

46. Holodick NE, Rothstein TL. B cells in the aging immune system: Time to consider B-1 cells. Ann N Y Acad Sci 2015; 1362: 176–187.

47. Cerutti A, Cols M, Puga I. Marginal zone B cells: Virtues of innate-like antibody-producing lymphocytes. Vol. 13, Nature Reviews Immunology. Nat Rev Immunol; 2013. p. 118–132.

48. Weill J-C, Weller S, Reynaud C-A. Human Marginal Zone B Cells. Annu Rev Immunol 2009; 27: 267–285.

49. Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, et al. Human blood IgM “memory” B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. Blood 2004; 104: 3647–3654.

50. Berkowska MA, Driessen GJA, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood 2011; 118: 2150–2158.

51. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response

against T-independent blood-borne particulate antigens. Immunity 2001; 14: 617–629.

52. Vinuesa CG, Sze DMY, Cook MC, Toellner KM, Klaus GGB, Ball J, et al. Recirculating and germinal center B cells differentiate into cells responsive to polysaccharide antigens. Vol. 33, European Journal of Immunology. Eur J Immunol; 2003. p. 297–305.

53. Weller S, Bonnet M, Delagreverie H, Israel L, Chrabieh M, Maródi L, et al. IgM+IgD+CD27+ B cells are markedly reduced in IRAK-4-, MyD88-, and TIRAP- but not UNC-93B-deficient patients. Blood 2012; 120: 4992–5001.

54. Cinamon G, Zachariah MA, Lam OM, Foss FW, Cyster JG. Follicular shuttling of marginal zone B cells facilitates antigen transport. Nat Immunol 2008; 9: 54–62.

55. Ferguson AR, Youd ME, Corley RB. Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells. Int Immunol 2004; 16: 1411–1422.

56. Attanavanich K, Kearney JF. Marginal Zone, but Not Follicular B Cells, Are Potent Activators of Naive CD4 T Cells. J Immunol 2004; 172: 803–811.

57. Song HH, Cerny J. Functional Heterogeneity of Marginal Zone B Cells Revealed by Their Ability to Generate Both Early Antibody-forming Cells and Germinal Centers with Hypermutation and Memory in Response to a T-dependent Antigen. J Exp Med 2003; 198: 1923–1935.

58. Puga I, Cols M, Barra CM, He B, Cassis L, Gentile M, et al. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. Nat Immunol 2012; 13: 170–180.

59. MacLennan ICM, Toellner KM, Cunningham AF, Serre K, Sze DMY, Zúñiga E, et al. Extrafollicular antibody responses. Vol. 194, Immunological Reviews. Immunol Rev; 2003. p. 8–18.

60. He B, Xu W, Santini PA, Polydorides AD, Chiu A, Estrella J, et al. Intestinal Bacteria Trigger T Cell-Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL. Immunity 2007; 26: 812–826.

61. Gumperz JE, Christopher R, Makowska A, Lum D, Sugita M, Podrebarac T, et al. Murine CD1d-restricted T cell recognition of cellular lipids. Immunity 2000; 12: 211–221.

62. Leadbetter EA, Brigl M, Illarionov P, Cohen N, Luteran MC, Pillai S, et al. NK T cells provide lipid antigen-specific cognate help for B cells. Proc Natl Acad Sci U S A 2008; 105: 8339–8344.

63. Ghia P, Ten Boekel E, Rolink AG, Melchers F. B-cell development: A comparison between mouse and man. Vol. 19, Immunology Today. Immunol Today; 1998. p. 480–485.

64. LeBien TW. Fates of human B-cell precursors. Blood 2000; 96: 9–23.

65. Dorshkind K. Multilineage development from adult bone marrow cells. Vol. 3, Nature Immunology. Nature Publishing Group; 2002. p. 311–313.

66. Laiosa C V., Stadtfeld M, Graf T. Determinants of lymphoid-myeloid lineage diversification. Vol. 24, Annual Review of Immunology. Annual Reviews; 2006. p. 705–738.

67. Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. Vol. 6, Nature Reviews Immunology. Nat Rev Immunol; 2006. p. 107–116.

68. Tokoyoda K, Egawa T, Sugiyama T, Choi B Il, Nagasawa T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. Immunity 2004; 20: 707–718.

69. Scheeren FA, Van Lent AU, Nagasawa M, Weijer K, Spits H, Legrand N, et al. Thymic stromal lymphopoietin induces early human B-cell proliferation and differentiation. Eur J Immunol 2010; 40: 955–965.

70. Parrish YK, Baez I, Milford T-A, Benitez A, Galloway N, Rogerio JW, et al. IL-7 Dependence in Human B Lymphopoiesis Increases during Progression of Ontogeny from Cord Blood to Bone Marrow. J Immunol 2009; 182: 4255–4266.

71. Dias S, Silva H, Cumano A, Vieira P. Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. J Exp Med 2005; 201: 971–979.

72. Cobaleda C, Schebesta A, Delogu A, Busslinger M. Pax5: The guardian of B cell identity and function. Vol. 8, Nature Immunology. Nat Immunol; 2007. p. 463–470.

73. Nutt SL, Kee BL. The Transcriptional Regulation of B Cell Lineage Commitment. Vol. 26, Immunity. Cell Press; 2007. p. 715–725.

74. Busslinger M. Transcriptional Control of Early B Cell Development. Annu Rev Immunol 2004; 22: 55–79.

75. Welinder E, Åhsberg J, Sigvardsson M. B-lymphocyte commitment: Identifying the point of no return. Vol. 23, Seminars in Immunology. Semin Immunol; 2011. p. 335–340.

76. Allman D, Li J, Hardy RR. Commitment to the B lymphoid lineage occurs before D(H)-J(H) recombination. J Exp Med 1999; 189: 735–740.

77. Hoffman W, Lakkis FG, Chalasani G. B cells, antibodies, and more. Clin J Am Soc Nephrol 2016; 11: 137–154.

78. van Zelm MC, van der Burg M, de Ridder D, Barendregt BH, de Haas EFE, Reinders MJT, et al. Ig Gene Rearrangement Steps Are Initiated in Early Human Precursor B Cell Subsets and Correlate with Specific Transcription Factor Expression. J Immunol 2005; 175: 5912–5922.

79. Meffre E, Milili M, Blanco-Betancourt C, Antunes H, Nussenzweig MC, Schiff C. Immunoglobulin heavy chain expression shapes the B cell receptor repertoire in human B cell development. J Clin Invest 2001; 108: 879–886.

80. Desiderio S V., Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, et al. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984; 311: 752–755.

81. Benedict CL, Gilfillan S, Thai T-H, Kearney JF. Terminal deoxynucleotidyl transferase and repertoire development. Immunol Rev 2000; 175: 150–157.

82. Übelhart R, Werner M, Jumaa H. Assembly and Function of the Precursor B-Cell Receptor. Curr Top Microbiol Immunol [Internet] 2015; 393: 3–25. Available from: http://books.google.com/books?id=_DDwCqx6wpcC&printsec=frontcover&dq=unwritten+

rules+of+phd+research&hl=&cd=1&source=gbs_api%255Cnpapers2://publication/uuid/48967E01-55F9-4397-B941-310D9C5405FA%255Cnhttp://medcontent.metapress.com/index/A65RM03P4874243N.p

83. Herzog S, Reth M, Jumaa H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. Vol. 9, Nature Reviews Immunology. Nat Rev Immunol; 2009. p. 195–205.

84. Espeli M, Rossi B, Mancini SJC, Roche P, Gauthier L, Schiff C. Initiation of pre-B cell receptor signaling: Common and distinctive features in human and mouse. Vol. 18, Seminars in Immunology. Semin Immunol; 2006. p. 56–66.

85. Melchers F. The pre-B-cell receptor: Selector of fitting immunoglobulin heavy chains for the B-cell repertoire. Vol. 5, Nature Reviews Immunology. Nat Rev Immunol; 2005. p. 578–584.

86. Minegishi Y, Rohrer J, Coustan-Smith E, Lederman HM, Pappu R, Campana D, et al. An essential role for BLNK in human B cell development. Science (80-) 1999; 286: 1954–1957.

87. Conley ME, Parolini O, Rohrer J, Campana D. X-Linked Agammaglobulinemia: New Approaches to Old Questions based on the Identification of the Defective Gene. Immunol Rev 1994; 138: 5–21.

88. Chen XF, Wang WF, Zhang YD, Zhao W, Wu J, Chen TX. Clinical characteristics and genetic profiles of 174 patients with X-linked agammaglobulinemia: Report from Shanghai, China (2000-2015). Med (United States) 2016; 95.

89. Conley ME, Mathias D, Treadaway J, Minegishi Y, Rohrer J. Mutations in Btk in patients with presumed X-linked agammaglobulinemia. Am J Hum Genet 1998; 62: 1034–1043.

90. Geier JK, Schlissel MS. Pre-BCR signals and the control of Ig gene rearrangements. Vol. 18, Seminars in Immunology. Semin Immunol; 2006. p. 31–39.

91. Grawunder U, Leu TMJ, Schatz DG, Werner A, Rolink AG, Melchers F, et al. Down-regulation of RAG1 and RAG2 gene expression in PreB cells after functional immunoglobulin heavy chain rearrangement. Immunity 1995; 3: 601–608.

92. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science (80-) 1990; 248: 1517–1523.

93. Stadhouders R, de Bruijn MJW, Rother MB, Yuvaraj S, de Almeida CR, Kolovos P, et al. Pre-B Cell Receptor Signaling Induces Immunoglobulin κ Locus Accessibility by Functional Redistribution of Enhancer-Mediated Chromatin Interactions. Nemazee D, editor PLoS Biol 2014; 12: e1001791.

94. Takeda S, Sonoda E, Arakawa H. The kappa:lambda ratio of immature B cells. Vol. 17, Immunology today. Elsevier Current Trends; 1996. p. 200–201.

95. Hombach J, Tsubata T, Leclercq L, Stappert H, Reth M. Molecular components of the B-cell antigen receptor complex of the IgM class. Nature 1990; 343: 760–762.

96. Casellas R, Tien-An Yang Shih, Kleinewietfeld M, Rakonjac J, Nemazee D, Rajewsky K, et al. Contribution of receptor editing to the antibody repertoire. Science (80-) 2001; 291: 1541–1544.

97. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. Science (80-) 2003; 301: 1374–1377.

98. Nemazee D. Mechanisms of central tolerance for B cells. Vol. 17, Nature Reviews Immunology. Nature Publishing Group; 2017. p. 281–294.

99. Pelanda R, Torres RM. Central B-Cell tolerance: Where selection begins. Cold Spring Harb Perspect Biol 2012; 4.

100. Nemazee D. Receptor editing in lymphocyte development and central tolerance. Vol. 6, Nature Reviews Immunology. Nat Rev Immunol; 2006. p. 728–740.

101. Tiegs SL, Russell DM, Nemazee D. Receptor editing in self-reactive bone marrow B cells. J Exp Med 1993; 177: 1009–1020.

102. Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: From transgenic models to naturally occurring anergic B cells? Vol. 7, Nature Reviews Immunology. Nat Rev Immunol; 2007. p. 633–643.

103. Russell DM, Dembić Z, Morahan G, Miller JFAP, BÜrki K, Nemazee D. Peripheral deletion of self-reactive B cells. Nature 1991; 354: 308–311.

104. Levine MH, Haberman AM, Sant'Angelo DB, Hannum LG, Cancro MP, Janeway CA, et al. A B-cell receptor-specific selection step governs immature to mature B cell differentiation. Proc Natl Acad Sci U S A 2000; 97: 2743–2748.

105. Loder F, Mutschler B, Ray RJ, Paige CJ, Sideras P, Torres R, et al. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. J Exp Med 1999; 190: 75–89.

106. Ekland EH, Forster R, Lipp M, Cyster JG. Requirements for Follicular Exclusion and Competitive Elimination of Autoantigen-Binding B Cells. J Immunol 2004; 172: 4700–4708.

107. Rolink AG, Tschopp J, Schneider P, Melchers F. BAFF is a survival and maturation factor for mouse B cells. Eur J Immunol 2002; 32: 2004–2010.

108. Schiemann B, Gommerman JL, Vora K, Cachero TG, Shutga-Morskaya S, Dobles M, et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. Science (80-) 2001; 293: 2111–2114.

109. Cancro MP. Signalling crosstalk in B cells: Managing worth and need. Vol. 9, Nature Reviews Immunology. NIH Public Access; 2009. p. 657–661.

110. Meffre E, Wardemann H. B-cell tolerance checkpoints in health and autoimmunity. Vol. 20, Current Opinion in Immunology. Curr Opin Immunol; 2008. p. 632–638.

111. Groom J, Kalled SL, Cutler AH, Olson C, Woodcock SA, Schneider P, et al. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjögren's syndrome. J Clin Invest 2002; 109: 59–68.

112. Mariette X, Roux S, Zhang J, Bengoufa D, Lavie F, Zhou T, et al. The level of BLyS (BAFF) correlates with the titre of autoantibodies in human Sjögren's syndrome. Ann Rheum Dis 2003; 62: 168–171.

113. Lavie F, Miceli-Richard C, Quillard J, Roux S, Leclerc P, Mariette X. Expression of BAFF (BLyS) in T cells infiltrating labial salivary glands from patients with Sjögren's syndrome. J Pathol 2004; 202: 496–502.

114. Allman D, Lindsley RC, DeMuth W, Rudd K, Shinton SA, Hardy RR. Resolution of Three Nonproliferative Immature Splenic B Cell Subsets Reveals Multiple Selection Points During Peripheral B Cell Maturation. J Immunol 2001; 167: 6834–6840.

115. Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. Vol. 9, Nature Reviews Immunology. Nat Rev Immunol; 2009. p. 767–777.

116. Cuss AK, Avery DT, Cannons JL, Yu LJ, Nichols KE, Shaw PJ, et al. Expansion of Functionally Immature Transitional B Cells Is Associated with Human-Immunodeficient States Characterized by Impaired Humoral Immunity. J Immunol 2006; 176: 1506–1516.

117. Cariappa A, Tang M, Parng C, Nebelitskiy E, Carroll M, Georgopoulos K, et al. The follicular versus marginal zone B lymphocyte cell fate decision is regulated by Aiolos, Btk, and CD21. Immunity 2001; 14: 603–615.

118. Saito T, Chiba S, Ichikawa M, Kunisato A, Asai T, Shimizu K, et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. Immunity 2003; 18: 675–685.

119. Tanigaki K, Han H, Yamamoto N, Tashiro K, Ikegawa M, Kuroda K, et al. Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. Nat Immunol 2002; 3: 443–450.

120. Hozumi K, Negishi N, Suzuki D, Abe N, Sotomaru Y, Tamaoki N, et al. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. Nat Immunol 2004; 5: 638–644.

121. Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the Human Immunology Project. Vol. 12, Nature Reviews Immunology. Nat Rev Immunol; 2012. p. 191–200.

122. Cyster JG, Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. Vol. 30, Annual Review of Immunology. Annual Reviews; 2012. p. 69–94.

123. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Vol. 23, Annual Review of Immunology. Annual Reviews; 2005. p. 127–159.

124. Reif K, Ekland EH, Ohl L, Nakano H, Lipp M, Förster R, et al. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. Nature 2002; 416: 94–99.

125. Okada T, Cyster JG. B cell migration and interactions in the early phase of antibody responses. Vol. 18, Current Opinion in Immunology. Curr Opin Immunol; 2006. p. 278–285.

126. Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell 1996; 87: 1037–1047.

127. Cyster JG, Ansel KM, Reif K, Ekland EH, Hyman PL, Tang HL, et al. Follicular stromal cells

and lymphocyte homing to follicles. Vol. 176, Immunological Reviews. Blackwell Munksgaard; 2000. p. 181–193.

128. Katakai T, Suto H, Sugai M, Gonda H, Togawa A, Suematsu S, et al. Organizer-Like Reticular Stromal Cell Layer Common to Adult Secondary Lymphoid Organs. J Immunol 2008; 181: 6189–6200.

129. Allen CDC, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. Science (80-) 2007; 315: 528–531.

130. Cyster JG. B cell follicles and antigen encounters of the third kind. Vol. 11, Nature Immunology. Nature Publishing Group; 2010. p. 989–996.

131. Carroll MC. The role of complement and complement receptors in induction and regulation of immunity. Vol. 16, Annual Review of Immunology. Annual Reviews; 1998. p. 545–568.

132. Allen CDC, Cyster JG. Follicular dendritic cell networks of primary follicles and germinal centers: Phenotype and function. Vol. 20, Seminars in Immunology. Semin Immunol; 2008. p. 14–25.

133. Heinen E, Braun M, Coulie PG, Van Snick J, Moeremans M, Cormann N, et al. Transfer of immune complexes from lymphocytes to follicular dendritic cells. Eur J Immunol 1986; 16: 167–172.

134. Wykes M, Pombo A, Jenkins C, MacPherson GG. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. J Immunol [Internet] 1998; 161: 1313–1319. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9686593

135. Suzuki K, Grigorova I, Phan TG, Kelly LM, Cyster JG. Visualizing B cell capture of cognate antigen from follicular dendritic cells. J Exp Med 2009; 206: 1485–1493.

136. Koppel EA, Wieland CW, van den Berg VCM, Litjens M, Florquin S, van Kooyk Y, et al. Specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary Streptococcus pneumoniae infection. Eur J Immunol 2005; 35: 2962–2969.

137. Gong YZ, Nititham J, Taylor K, Miceli-Richard C, Sordet C, Wachsmann D, et al. Differentiation of follicular helper T cells by salivary gland epithelial cells in primary Sjögren's syndrome. J Autoimmun 2014; 51: 57–66.

138. Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, et al. The Role of ICOS in the CXCR5 + Follicular B Helper T Cell Maintenance In Vivo. J Immunol 2005; 175: 2340–2348.

139. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T Follicular Helper Cells Is Mediated by Interleukin-21 but Independent of T Helper 1, 2, or 17 Cell Lineages. Immunity 2008; 29: 138–149.

140. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med 2000; 192: 1545–1551.

141. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5

expression defines follicular homing T cells with B cell helper function. J Exp Med 2000; 192: 1553–1562.

142. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The Transcriptional Repressor Bcl-6 Directs T Follicular Helper Cell Lineage Commitment. Immunity 2009; 31: 457–468.

143. Haynes NM, Allen CDC, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in Follicular Th Cell Positioning and Appearance of a Programmed Cell Death Gene-1 High Germinal Center-Associated Subpopulation. J Immunol 2007; 179: 5099–5108.

144. Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, et al. Follicular Helper T Cell Differentiation Requires Continuous Antigen Presentation that Is Independent of Unique B Cell Signaling. Immunity 2010; 33: 241–253.

145. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf Cooperate To Instruct Human Follicular Helper CD4 T Cell Differentiation. J Immunol 2012; 188: 3734–3744.

146. Tsuboi H, Hagiwara S, Asashima H, Takahashi H, Hirota T, Noma H, et al. Comparison of performance of the 2016 ACR-EULAR classification criteria for primary Sjögren's syndrome with other sets of criteria in Japanese patients. Ann Rheum Dis [Internet] 2017 [cited 2018 Apr 29]; 76: 1980–1985. Available from: http://ard.bmj.com/content/annrheumdis/76/12/1980.full.pdf

147. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu Rev Immunol 2008; 26: 741–766.

148. Batista FD, Harwood NE. The who, how and where of antigen presentation to B cells. Vol. 9, Nature Reviews Immunology. Nat Rev Immunol; 2009. p. 15–27.

149. Harwood NE, Batista FD. Early Events in B Cell Activation. Annu Rev Immunol 2010; 28: 185–210.

150. Mongini PKA, Inman JK. Cytokine dependency on human B cell cycle progression elicited ligands which coengage BCR and the CD21/CD19/CD81 costimulatory complex. Cell Immunol 2001; 207: 127–140.

151. Crotty S. Follicular Helper CD4 T Cells (T FH). Annu Rev Immunol [Internet] 2011 [cited 2017 Feb 28]; 29: 621–663. Available from: http://www.annualreviews.org/doi/10.1146/annurev-immunol-031210-101400

152. Pereira JP, Kelly LM, Xu Y, Cyster JG. EBI2 mediates B cell segregation between the outer and centre follicle. Nature 2009; 460: 1122–1126.

153. Gatto D, Paus D, Basten A, Mackay CR, Brink R. Guidance of B Cells by the Orphan G Protein-Coupled Receptor EBI2 Shapes Humoral Immune Responses. Immunity 2009; 31: 259–269.

154. Kaminski DA, Wei C, Qian Y, Rosenberg AF, Sanz I. Advances in human B cell phenotypic profiling. Vol. 3, Frontiers in Immunology. Frontiers Media SA; 2012.

155. Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. Vol. 16, Nature Immunology. Nature Publishing Group; 2015. p. 142–152.

156. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. Vol. 209, Journal of Experimental Medicine. J Exp Med; 2012. p. 1241–1253.

157. Yu D, Vinuesa CG. The elusive identity of T follicular helper cells. Trends Immunol 2010; 31: 377–383.

158. Blink EJ, Light A, Kallies A, Nutt SL, Hodgkin PD, Tarlinton DM. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. J Exp Med 2005; 201: 545–554.

159. Allen CDC, Okada T, Cyster JG. Germinal-Center Organization and Cellular Dynamics. Vol. 27, Immunity. Immunity; 2007. p. 190–202.

160. Chan TD, Gatto D, Wood K, Camidge T, Basten A, Brink R. Antigen Affinity Controls Rapid T-Dependent Antibody Production by Driving the Expansion Rather than the Differentiation or Extrafollicular Migration of Early Plasmablasts. J Immunol 2009; 183: 3139–3149.

161. Finak G, Langweiler M, Jaimes M, Malek M, Taghiyar J, Korin Y, et al. Standardizing Flow Cytometry Immunophenotyping Analysis from the Human ImmunoPhenotyping Consortium. Sci Rep [Internet] 2016 [cited 2018 Aug 3]; 6. Available from: www.nature.com/scientificreports

162. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. Nat Rev Immunol 2015; 15: 160–171.

163. Shapiro-Shelef M, Calame KC. Regulation of plasma-cell development. Vol. 5, Nature Reviews Immunology. Nat Rev Immunol; 2005. p. 230–242.

164. Klein U, Casola S, Cattoretti G, Shen Q, Lia M, Mo T, et al. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. Nat Immunol 2006; 7: 773–782.

165. Nera KP, Kyläniemi MK, Lassila O. Regulation of B Cell to Plasma Cell Transition within the Follicular B Cell Response. Vol. 82, Scandinavian Journal of Immunology. Blackwell Publishing Ltd; 2015. p. 225–234.

166. Luther SA, Maillard I, Luthi F, Scarpellino L, Diggelmann H, Acha-Orbea H. Early neutralizing antibody response against mouse mammary tumor virus: Critical role of viral infection and superantigen-reactive T cells. J Immunol 1997; 159: 2807–2814.

167. Taylor JJ, Pape KA, Jenkins MK. A germinal center-independent pathway generates unswitched memory B cells early in the primary response. J Exp Med 2012; 209: 597–606.

168. MacLennan ICM. Germinal centers. Vol. 12, Annual Review of Immunology. Annual Reviews; 1994. p. 117–139.

169. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. Science (80-) 1997; 276: 589–592.

170. Ye BH, Cattoretti G, Shen Q, Zhang J, Hawe N, De Waard R, et al. The BCL-6 proto-oncogene controls germinal-centre formation and Th2- type inflammation. Nat Genet 1997; 16: 161–170.

171. Victora GD, Nussenzweig MC. Germinal centers. Vol. 30, Annual Review of Immunology.

Annual Reviews; 2012. p. 429–457.

172. Bannard O, Horton RM, Allen CDC, An J, Nagasawa T, Cyster JG. Germinal center centroblasts transition to a centrocyte phenotype according to a timed program and depend on the dark zone for effective selection. Immunity 2013; 39: 912–924.

173. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular Helper T Cells: Lineage and Location [Internet]. Vol. 30, Immunity. 2009 [cited 2017 Feb 28]. p. 324–335. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1074761309001149

174. Caron G, Le Gallou S, Lamy T, Tarte K, Fest T. CXCR4 Expression Functionally Discriminates Centroblasts versus Centrocytes within Human Germinal Center B Cells. J Immunol 2009; 182: 7595–7602.

175. Wilson PC, De Bouteiller O, Liu YJ, Potter K, Banchereau J, Capra JD, et al. Somatic hypermutation introduces insertions and deletions into immunoglobulin V genes. J Exp Med 1998; 187: 59–70.

176. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 2000; 102: 553–563.

177. Di Noia JM, Neuberger MS. Molecular mechanisms of antibody somatic hypermutation. Vol. 76, Annual Review of Biochemistry. Annual Reviews; 2007. p. 1–22.

178. Allen CDC, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol 2004; 5: 943–952.

179. Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell 2010; 143: 592–605.

180. Anderson SM, Khalil A, Uduman M, Hershberg U, Louzoun Y, Haberman AM, et al. Taking Advantage: High-Affinity B Cells in the Germinal Center Have Lower Death Rates, but Similar Rates of Division, Compared to Low-Affinity Cells. J Immunol 2009; 183: 7314–7325.

181. Gitlin AD, Shulman Z, Nussenzweig MC. Clonal selection in the germinal centre by regulated proliferation and hypermutation. Nature 2014; 509: 637–640.

182. De Silva NS, Klein U. Dynamics of B cells in germinal centres. Nat Rev Immunol 2015; 15: 137–148.

183. Chaudhuri J, Basu U, Zarrin A, Yan C, Franco S, Perlot T, et al. Evolution of the Immunoglobulin Heavy Chain Class Switch Recombination Mechanism. Vol. 94, Advances in Immunology. Adv Immunol; 2007. p. 157–214.

184. Nutt SL, Tarlinton DM. Give and take in the germinal center. Vol. 11, Nature Immunology. Nat Immunol; 2010. p. 464–466.

185. Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, et al. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. Nature 2015; 517: 214–218.

186. Shulman Z, Gitlin AD, Weinstein JS, Lainez B, Esplugues E, Flavell RA, et al. Germinal

centers: Dynamic signaling by T follicular helper cells during germinal center B cell selection. Science (80-) 2014; 345: 1058–1062.

187. Al-Saud BK, Al-Sum Z, Alassiri H, Al-Ghonaium A, Al-Muhsen S, Al-Dhekri H, et al. Clinical, immunological, and molecular characterization of Hyper-IgM syndrome due to CD40 deficiency in eleven patients. J Clin Immunol 2013; 33: 1325–1335.

188. Aghamohammadi A, Parvaneh N, Rezaei N, Moazzami K, Kashef S, Abolhassani H, et al. Clinical and laboratory findings in Hyper-IgM syndrome with novel CD40L and AICDA mutations. J Clin Immunol 2009; 29: 769–776.

189. Crotty S. A brief history of T cell help to B cells. Nat Rev Immunol 2015; 15: 185–189.

190. Avery DT, Bryant VL, Ma CS, de Waal Malefyt R, Tangye SG. IL-21-Induced Isotype Switching to IgG and IgA by Human Naive B Cells Is Differentially Regulated by IL-4. J Immunol 2008; 181: 1767–1779.

191. Stavnezer J. Immunoglobulin class switching. Curr Opin Immunol 1996; 8: 199–205.

192. Seo GY, Park SR, Kim PH. Analyses of TGF-β1-inducible Ig germ-line γ2b promoter activity: Involvement of Smads and NF-κB. Eur J Immunol 2009; 39: 1157–1166.

193. Woof JM, Ken MA. The function of immunoglobulin A in immunity. Vol. 208, Journal of Pathology. J Pathol; 2006. p. 270–282.

194. Wu LC, Zarrin AA. The production and regulation of IgE by the immune system. Vol. 14, Nature Reviews Immunology. Nature Publishing Group; 2014. p. 247–259.

195. Nimmerjahn F, Ravetch J V. Fcγ receptors as regulators of immune responses. Vol. 8, Nature Reviews Immunology. Nat Rev Immunol; 2008. p. 34–47.

196. Fagarasan S, Kawamoto S, Kanagawa O, Suzuki K. Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. Annu Rev Immunol 2010; 28: 243–273.

197. Stavnezer J, Guikema JEJ, Schrader CE. Mechanism and regulation of class switch recombination. Vol. 26, Annual Review of Immunology. Annual Reviews; 2008. p. 261–292.

198. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. Vol. 11, Nature Immunology. Nat Immunol; 2010. p. 114–120.

199. Kometani K, Kurosaki T. Differentiation and maintenance of long-lived plasma cells. Vol. 33, Current Opinion in Immunology. Elsevier Ltd; 2015. p. 64–69.

200. Hu CCA, Dougan SK, McGehee AM, Love JC, Ploegh HL. XBP-1 regulates signal transduction, transcription factors and bone marrow colonization in B cells. EMBO J 2009; 28: 1624–1636.

201. Chu VT, Fröhlich A, Steinhauser G, Scheel T, Roch T, Fillatreau S, et al. Eosinophils are required for the maintenance of plasma cells in the bone marrow. Nat Immunol 2011; 12: 151–159.

202. Winter O, Moser K, Mohr E, Zotos D, Kaminski H, Szyska M, et al. Megakaryocytes constitute a functional component of a plasma cell niche in the bone marrow. Blood 2010;

116: 1867–1875.

203. Belnoue E, Tougne C, Rochat A-F, Lambert P-H, Pinschewer DD, Siegrist C-A. Homing and Adhesion Patterns Determine the Cellular Composition of the Bone Marrow Plasma Cell Niche. J Immunol 2012; 188: 1283–1291.

204. Kurosaki T, Kometani K, Ise W. Memory B cells. Nat Rev Immunol 2015; 15: 149–159.

205. Schmidlin H, Diehl SA, Nagasawa M, Scheeren FA, Schotte R, Uittenbogaart CH, et al. Spi-B inhibits human plasma cell differentiation by repressing BLIMP1 and XBP-1 expression. Blood 2008; 112: 1804–1812.

206. Levels MJ, Fehres CM, van Baarsen LGM, van Uden NOP, Germar K, O'Toole TG, et al. BOB.1 controls memory B-cell fate in the germinal center reaction. J Autoimmun 2019; 101: 131–144.

207. Basso K, Klein U, Niu H, Stolovitzky GA, Tu Y, Califano A, et al. Tracking CD40 signaling during germinal center development. Blood 2004; 104: 4088–4096.

208. Maarof G, Bouchet-Delbos L, Gary-Gouy H, Durand-Gasselin I, Krzysiek R, Dalloul A. Interleukin-24 inhibits the plasma cell differentiation program in human germinal center B cells. Blood 2010; 115: 1718–1726.

209. Tangye SG, Avery DT, Deenick EK, Hodgkin PD. Intrinsic Differences in the Proliferation of Naive and Memory Human B Cells as a Mechanism for Enhanced Secondary Immune Responses. J Immunol 2003; 170: 686–694.

210. Ahmed R, Gray D. Immunological memory and protective immunity: Understanding their relation. Science (80-) 1996; 272: 54–60.

211. Seifert M, Küppers R. Human memory B cells. Vol. 30, Leukemia. Nature Publishing Group; 2016. p. 2283–2292.

212. Dogan I, Bertocci B, Vilmont V, Delbos F, Mégret J, Storck S, et al. Multiple layers of B cell memory with different effector functions. Nat Immunol 2009; 10: 1292–1299.

213. Pape KA, Taylor JJ, Maul RW, Gearhart PJ, Jenkins MK. Different B cell populations mediate early and late memory during an endogenous immune response. Science (80-) 2011; 331: 1203–1207.

214. Mcheyzer-Williams LJ, Milpied PJ, Okitsu SL, Mcheyzer-Williams MG. Class-switched memory B cells remodel BCRs within secondary germinal centers. Nat Immunol 2015; 16: 296–305.

215. Xiong H, Dolpady J, Wabl M, de Lafaille MAC, Lafaille JJ. Sequential class switching is required for the generation of high affinity IgE antibodies. J Exp Med 2012; 209: 353–364.

216. He JS, Meyer-Hermann M, Xiangying D, Zuan LY, Jones LA, Ramakrishna L, et al. The distinctive germinal center phase of IgE+ B lymphocytes limits their contribution to the classical memory response. J Exp Med 2013; 210: 2755–2771.

217. Defrance T, Taillardet M, Genestier L. T cell-independent B cell memory. Vol. 23, Current Opinion in Immunology. Curr Opin Immunol; 2011. p. 330–336.

218. Alugupalli KR, Leong JM, Woodland RT, Muramatsu M, Honjo T, Gerstein RM. B1b

lymphocytes confer T cell-independent long-lasting immunity. Immunity 2004; 21: 379–390.

219. Obukhanych T V., Nussenzweig MC. T-independent type II immune responses generate memory B cells. J Exp Med 2006; 203: 305–310.

220. Rosser EC, Mauri C. Regulatory B Cells: Origin, Phenotype, and Function. Immunity [Internet] 2015 [cited 2018 May 6]; 42: 607–612. Available from: http://dx.doi.org/10.1016/j.immuni.2015.04.005

221. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. Immunity [Internet] 2002 [cited 2018 May 8]; 16: 219–230. Available from: file:///C:/Users/fduar_000/Desktop/PIIS1074761302002741.pdf

222. Carter NA, Vasconcellos R, Rosser EC, Tulone C, Munoz-Suano A, Kamanaka M, et al. Mice Lacking Endogenous IL-10-Producing Regulatory B Cells Develop Exacerbated Disease and Present with an Increased Frequency of Th1/Th17 but a Decrease in Regulatory T Cells. J Immunol [Internet] 2011 [cited 2018 May 8]; 186: 5569–5579. Available from: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100284

223. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. Sci Transl Med [Internet] 2013 [cited 2016 Aug 11]; 5: 173ra23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23427243

224. Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19+CD24hiCD38hi B Cells Exhibit Regulatory Capacity in Healthy Individuals but Are Functionally Impaired in Systemic Lupus Erythematosus Patients. Immunity [Internet] 2010 [cited 2018 Mar 18]; 32: 129–140. Available from: file:///C:/Users/fduar_000/Desktop/1-s2.0-S1074761309005470-main.pdf

225. Matsumoto M, Baba A, Yokota T, Nishikawa H, Ohkawa Y, Kayama H, et al. Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. Immunity [Internet] 2014 [cited 2018 Feb 13]; 41: 1040–1051. Available from: http://dx.doi.org/10.1016/j.immuni.2014.10.016

226. Sun CM, Deriaud E, Leclerc C, Lo-Man R. Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. Immunity [Internet] 2005 [cited 2018 May 8]; 22: 467–477. Available from: file:///C:/Users/fduar_000/Desktop/PIIS1074761305000932.pdf

227. Tian J, Zekzer D, Hanssen L, Lu Y, Olcott A, Kaufman DL. Lipopolysaccharide-Activated B Cells Down-Regulate Th1 Immunity and Prevent Autoimmune Diabetes in Nonobese Diabetic Mice. J Immunol [Internet] 2001 [cited 2018 May 8]; 167: 1081–1089. Available from: http://www.jimmunol.org/content/167/2/1081

228. Parekh V V, Prasad DVR, Banerjee PP, Joshi BN, Kumar A, Mishra GC. B cells activated by lipopolysaccharide, but not by anti-Ig and anti-CD40 antibody, induce anergy in CD8+ T cells: role of TGF-beta 1. J Immunol [Internet] 2003 [cited 2018 May 8]; 170: 5897–5911. Available from: http://www.jimmunol.org/content/170/12/5897

229. Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E, et al. IL-35-

producing B cells are critical regulators of immunity during autoimmune and infectious diseases. Nature [Internet] 2014 [cited 2018 Mar 18]; 507: 366–370. Available from: file:///C:/Users/fduar_000/Desktop/nature12979.pdf

230. Wang R-X, Yu C-R, Dambuza IM, Mahdi RM, Dolinska MB, Sergeev Y V, et al. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. Nat Med [Internet] 2014 [cited 2018 May 8]; 20: 633–641. Available from: http://www.nature.com/authors/editorial_policies/license.html#terms

231. Bosma A, Abdel-Gadir A, Isenberg DA, Jury EC, Mauri C. Lipid-Antigen Presentation by CD1d+B Cells Is Essential for the Maintenance of Invariant Natural Killer T Cells. Immunity [Internet] 2012 [cited 2018 May 8]; 36: 477–490. Available from: file:///C:/Users/fduar_000/Desktop/PIIS1074761312000817.pdf

232. Maravillas-Montero JL, Acevedo-Ochoa E. Human b regulatory cells: The new players in autoimmune disease. Rev Investig Clin 2017; 69: 243–246.

233. Rudensky AY. Regulatory T cells and Foxp3. Immunol Rev [Internet] 2011 [cited 2018 May 8]; 241: 260–268. Available from: file:///C:/Users/fduar_000/Desktop/nihms-278191.pdf

234. Candando KM, Lykken JM, Tedder TF. B10 cell regulation of health and disease. Immunol Rev [Internet] 2014 [cited 2018 May 9]; 259: 259–272. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4049540/pdf/nihms570699.pdf

235. Mauri C, Menon M. Human regulatory B cells in health and disease: Therapeutic potential. Vol. 127, Journal of Clinical Investigation. 2017. p. 772–779.

236. Baba Y, Matsumoto M, Kurosaki T. Signals controlling the development and activity of regulatory B-lineage cells. Int Immunol [Internet] 2015 [cited 2018 May 9]; 27: 487–493. Available from: http://intimm.oxfordjournals.org/

237. Rosser EC, Oleinika K, Tonon S, Doyle R, Bosma A, Carter NA, et al. Regulatory B cells are induced by gut microbiota-driven interleukin-1β and interleukin-6 production. Nat Med [Internet] 2014 [cited 2018 May 9]; 20: 1334–1339. Available from: file:///C:/Users/fduar_000/Desktop/rosser2014.pdf

238. Yoshizaki A, Miyagaki T, Dilillo DJ, Matsushita T, Horikawa M, Kountikov EI, et al. Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. Nature [Internet] 2012 [cited 2018 May 9]; 491: 264–268. Available from: file:///C:/Users/fduar_000/Desktop/nihms-400295.pdf

239. Rafei M, Hsieh J, Zehntner S, Li M, Forner K, Birman E, et al. A granulocyte-macrophage colony-stimulating factor and interleukin-15 fusokine induces a regulatory B cell population with immune suppressive properties. Nat Med 2009; 15: 1038–1045.

240. Evans JG, Chavez-Rueda KA, Eddaoudi A, Meyer-Bahlburg A, Rawlings DJ, Ehrenstein MR, et al. Novel Suppressive Function of Transitional 2 B Cells in Experimental Arthritis. J Immunol [Internet] 2007 [cited 2018 May 9]; 178: 7868–7878. Available from: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.178.12.7868

241. Mota I, Martins C, Borrego LM. Regulatory B cells and allergy: Uncovering the link [Internet]. Vol. 27, Journal of Investigational Allergology and Clinical Immunology. 2017 [cited 2018 Feb 13]. p. 204–212. Available from: http://www.jiaci.org/revistas/vol27issue4_1.pdf

242. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nat Immunol 2002; 3: 944–950.

243. Mauri C, Menon M. The expanding family of regulatory B cells [Internet]. Vol. 27, International Immunology. 2015 [cited 2018 Feb 13]. p. 479–486. Available from: file:///C:/Users/fduar_000/Desktop/dxv038.pdf

244. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, et al. Characterization of a rare IL-10–competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood [Internet] 2011 [cited 2018 Feb 13]; 117: 530–541. Available from: file:///C:/Users/fduar_000/Desktop/530.full.pdf

245. Lindner S, Dahlke K, Sontheimer K, Hagn M, Kaltenmeier C, Barth TFE, et al. Interleukin 21-induced granzyme b-expressing b cells infiltrate tumors and regulate t cells. Cancer Res [Internet] 2013 [cited 2018 May 8]; 73: 2468–2479. Available from: file:///C:/Users/fduar_000/Desktop/2468.full.pdf

246. Liu J, Zhan W, Kim CJ, Clayton K, Zhao H, Lee E, et al. IL-10-producing B cells are induced early in HIV-1 infection and suppress HIV-1-specific T cell responses. PLoS One [Internet] 2014 [cited 2018 May 8]; 9. Available from: file:///C:/Users/fduar_000/Desktop/pone.0089236.pdf

247. Khoder A, Sarvaria A, Alsuliman A, Chew C, Sekine T, Cooper N, et al. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. Blood [Internet] 2014 [cited 2018 May 11]; 124: 2034–2045. Available from: file:///C:/Users/fduar_000/Desktop/2034.full.pdf

248. Simon Q, Pers JO, Cornec D, Le Pottier L, Mageed RA, Hillion S. In-depth characterization of CD24highCD38high transitional human B cells reveals different regulatory profiles. J Allergy Clin Immunol [Internet] 2016 [cited 2017 Jul 10]; 137: 1577-1584.e10. Available from: http://www.jacionline.org/article/S0091-6749(15)01352-4/pdf

249. De Masson A, Bouaziz JD, Le Buanec H, Robin M, O'Meara A, Parquet N, et al. CD24hiCD27+ and plasmablast-like regulatory B cells in human chronic graft-versus-host disease. Blood [Internet] 2015 [cited 2018 Feb 14]; 125: 1830–1839. Available from: file:///C:/Users/fduar_000/Desktop/1830.full.pdf

250. Knippenberg S, Peelen E, Smolders J, Thewissen M, Menheere P, Cohen Tervaert JW, et al. Reduction in IL-10 producing B cells (Breg) in multiple sclerosis is accompanied by a reduced naïve/memory Breg ratio during a relapse but not in remission. J Neuroimmunol [Internet] 2011 [cited 2018 Apr 10]; 239: 80–86. Available from: file:///C:/Users/fduar_000/Desktop/knippenberg2011.pdf

251. Menon M, Blair PA, Isenberg DA, Mauri C. A Regulatory Feedback between Plasmacytoid Dendritic Cells and Regulatory B Cells Is Aberrant in Systemic Lupus Erythematosus. Immunity [Internet] 2016 [cited 2018 Mar 18]; 44: 683–697. Available from: http://dx.doi.org/10.1016/j.immuni.2016.02.012

252. Heinemann K, Wilde B, Hoerning A, Tebbe B, Kribben A, Witzke O, et al. Decreased IL-10+ regulatory B cells (Bregs) in lupus nephritis patients. Scand J Rheumatol [Internet] 2016 [cited 2018 May 9]; 45: 312–316. Available from: http://www.tandfonline.com/action/journalInformation?journalCode=irhe20

253. Furuzawa-Carballeda J, Hernández-Molina G, Lima G, Rivera-Vicencio Y, Férez-Blando K, Llorente L. Peripheral regulatory cells immunophenotyping in Primary Sjögren's Syndrome: a cross-sectional study. Arthritis Res Ther [Internet] 2013 [cited 2017 Mar 4]; 15: R68. Available from: http://download.springer.com/static/pdf/799/art%253A10.1186%252Far4245.pdf?originUrl=http%3A%2F%2Farthritis-research.biomedcentral.com%2Farticle%2F10.1186%2Far4245&token2=exp=1488627813~acl=%2Fstatic%2Fpdf%2F799%2Fart%25253A10.1186%25252Far4245.pdf*~hmac=

254. Lin W, Jin L, Chen H, Wu Q, Fei Y, Zheng W, et al. B cell subsets and dysfunction of regulatory B cells in IgG4-related diseases and primary Sjögren's syndrome: the similarities and differences. Arthritis Res Ther [Internet] 2014 [cited 2017 Jun 10]; 16: R118. Available from: http://download.springer.com/static/pdf/771/art%253A10.1186%252Far4571.pdf?originUrl=http%3A%2F%2Farthritis-research.biomedcentral.com%2Farticle%2F10.1186%2Far4571&token2=exp=1497115302~acl=%2Fstatic%2Fpdf%2F771%2Fart%25253A10.1186%25252Far4571.pdf*~hmac=

255. Mavropoulos A, Liaskos C, Simopoulou T, Bogdanos DP, Sakkas LI. IL-10-producing regulatory B cells (B10 cells), IL-17 + T cells and autoantibodies in systemic sclerosis. Clin Immunol [Internet] 2017 [cited 2018 May 9]; 184: 26–32. Available from: file:///C:/Users/fduar_000/Desktop/mavropoulos2017.pdf

256. Mavropoulos A, Varna A, Zafiriou E, Liaskos C, Alexiou I, Roussaki-Schulze A, et al. IL-10 producing Bregs are impaired in psoriatic arthritis and psoriasis and inversely correlate with IL-17- and IFNγ-producing T cells. Clin Immunol [Internet] 2017 [cited 2018 May 9]; 184: 33–41. Available from: file:///C:/Users/fduar_000/Desktop/mavropoulos2017 (1).pdf

257. Rothenberg E V., Taghon T. Molecular genetics of T cell development. Annu Rev Immunol [Internet] 2005 [cited 2018 May 14]; 23: 601–649. Available from: file:///C:/Users/fduar_000/Desktop/rothenberg2005.pdf

258. Spits H. Development of αβ T cells in the human thymus. Nat Rev Immunol 2002; 2: 760–772.

259. Hale JS, Fink PJ. T-cell receptor revision: Friend or foe? Immunology 2010; 129: 467–473.

260. Pearse G. Normal Structure, Function and Histology of the Thymus. Toxicol Pathol 2006; 34: 504–514.

261. Anderson G, Lane PJL, Jenkinson EJ. Generating intrathymic microenvironments to establish T-cell tolerance. Nat Rev Immunol 2007; 7: 954–963.

262. Takahama Y. Journey through the thymus: Stromal guides for T-cell development and selection. Nat Rev Immunol 2006; 6: 127–135.

263. Payne KJ, Crooks GM. Human hematopoietic lineage commitment. Immunol Rev 2002; 187: 48–64.

264. Terstappen LWMM, Huang S, Safford M, Lansdorp PM, Loken MR. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. Blood 1991; 77: 1218–1227.

265. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B. Isolation of a candidate

human hematopoietic stem-cell population. Proc Natl Acad Sci U S A 1992; 89: 2804–2808.

266. Spits H, Blom B, Jaleco AC, Weijer K, Verschuren MCM, Van Dongen JJM, et al. Early stages in the development of human T, natural killer and thymic dendritic cells. Immunol Rev 1998; 165: 75–86.

267. Blom B, Spits H. Development of human lymphoid cells. Annu Rev Immunol 2006; 24: 287–320.

268. Staal FJT, Weerkamp F, Langerak AW, Hendriks RW, Clevers HC. Transcriptional Control of T Lymphocyte Differentiation. Stem Cells 2001; 19: 165–179.

269. Blom B, Res P, Noteboom E, Weijer K, Spits H. Prethymic CD34+ progenitors capable of developing into T cells are not committed to the T cell lineage. J Immunol 1997; 158: 3571–3577.

270. Spits H, Couwenberg F, Bakker AQ, Weijer K, Uittenbogaart CH. Id2 and Id3 inhibit development of CD34+ stem cells into predendritic cell (pre-DC)2 but not into pre-DC1: Evidence for a lymphoid origin of pre-DC2. J Exp Med 2000; 192: 1775–1783.

271. Blom B, Verschuren MCM, Heemskerk MHM, Bakker AQ, van Gastel-Mol EJ, Wolvers-Tettero ILM, et al. TCR Gene Rearrangements and Expression of the Pre-T Cell Receptor Complex During Human T-Cell Differentiation. Blood 1999; 93: 3033–3043.

272. El Andaloussi A, Graves S, Meng F, Mandal M, Mashayekhi M, Aifantis I. Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus. Nat Immunol 2006; 7: 418–426.

273. Petrie HT, Zúñiga-Pflücker JC. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. Annu Rev Immunol 2007; 25: 649–679.

274. Artavanis-tsakonas S, Rand MD, Lake RJ. Notch Signaling: Cell Fate Control and Signal Integration in Development. Science 1999; 284: 770–776.

275. Allman D, Punt JA, Izon DJ, Aster JC, Pear WS. An invitation to T and more: Notch signaling in lymphopoiesis. Cell 2002; 109: S1.

276. Hozumi K, Mailhos C, Negishi N, Hirano KI, Yahata T, Ando K, et al. Delta-like 4 is indispensable in thymic environment specific for T cell development. J Exp Med 2008; 205: 2507–2513.

277. La Motte-Mohs RN, Herer E, Zúñiga-Pflücker JC. Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. Blood 2005; 105: 1431–1439.

278. De Smedt M, Hoebeke I, Plum J. Human bone marrow CD34 + progenitor cells mature to T cells on OP9-DL1 stromal cell line without thymus microenvironment. Blood Cells, Mol Dis 2004; 33: 227–232.

279. Lind EF, Prockop SE, Porritt HE, Petrie HT. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. J Exp Med 2001; 194: 127–134.

280. Jaleco AC, Neves H, Hooijberg E, Gameiro P, Clode N, Haury M, et al. Differential effects of

Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. J Exp Med 2001; 194: 991–1001.

281. Plum J, De Smedt M, Leclercq G, Verhasselt B, Vandekerckhove B. Interleukin-7 is a critical growth factor in early human T-cell development. Blood 1996; 88: 4239–4245.

282. Puel A, Ziegler SF, Buckley RH, Leonard WJ. Defective IL7R expression in T-B+NK+ severe combined immunodeficiency. Nat Genet 1998; 20: 394–397.

283. Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, et al. Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. Cell 1993; 73: 147–157.

284. Macchi P, Villa A, Giliani S, Sacco MG, Frattini A, Porta F, et al. Mutations of jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). Vol. 377, Nature. 1995. p. 65–68.

285. Kraft BDL, Weissman IL, Waller EK. Differentiation of CD3-4-8- Human Fetal Thymocytes In Vivo: Characterization of a CD3-4+8- Intermediate. J Exp Med 1993; 178: 265–277.

286. Dik WA, Pike-Overzet K, Weerkamp F, De Ridder D, De Haas EFE, Baert MRM, et al. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. J Exp Med 2005; 201: 1715–1723.

287. Kreslavsky T, Garbe AI, Krueger A, Von Boehmer H. T cell receptor-instructed -αβ versus γδ lineage commitment revealed by single-cell analysis. J Exp Med 2008; 205: 1173–1186.

288. Burtrum DB, Kim S, Dudley EC, Hayday AC, Petrie HT. TCR gene recombination and alpha beta-gamma delta lineage divergence: productive TCR-beta rearrangement is neither exclusive nor preclusive of gamma delta cell development. J Immunol [Internet] 1996; 157: 4293–4296. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8906802

289. Hayes SM, Shores EW, Love PE. An architectural perspective on signaling by the pre-, alphabeta and gammadelta T cell receptors. Immunol Rev [Internet] 2003; 191: 28–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12614349%5Cnhttp://onlinelibrary.wiley.com/store/10.1034/j.1600-065X.2003.00011.x/asset/j.1600-065X.2003.00011.x.pdf?v=1&t=hw39ow7k&s=927b6b049122974bef0cda04388b06c56e37c88b

290. Von Boehmer H, Fehling HJ. Structure and function of the pre-T cell receptor. Annu Rev Immunol 1997; 15: 433–452.

291. Hori T, Cupp J, Wrighton N, Lee F, Spits H. Identification of a novel human thymocyte subset with a phenotype of CD3- CD4+ CD8 alpha + beta-1. Possible progeny of the CD3- CD4- CD8- subset. J Immunol [Internet] 1991; 146: 4078–4084. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1828260

292. Verschuren MC, Wolvers-Tettero IL, Breit TM, Noordzij J, van Wering ER, van Dongen JJ. Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. J Immunol [Internet] 1997; 158: 1208–1216. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9013961

293. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. Annu Rev

Immunol 2003; 21: 139–176.

294. Vrisekoop N, Monteiro JP, Mandl JN, Germain RN. Revisiting thymic positive selection and the mature T cell repertoire for antigen. Immunity 2014; 41: 181–190.

295. Mathis D, Benoist C. Aire. Annu Rev Immunol [Internet] 2009; 27: 287–312. Available from: https://doi.org/10.1146/annurev.immunol.25.022106.141532

296. Singer A, Adoro S, Park JH. Lineage fate and intense debate: Myths, models and mechanisms of CD4- versus CD8-lineage choice. Nat Rev Immunol 2008; 8: 788–801.

297. von Boehmer H, Kisielow P, Kishi H, Scott B, Borgulya P, Teh HS. The Expression of CD4 and CD8 Accessory Molecules on Mature T Cells is not Random but Correlates with the Specificity of the αβ Receptor for Antigen. Immunol Rev 1989; 109: 143–152.

298. Reith W, Mach B. The Bare Lymphocyte Syndrome and the Regulation of MHC Expression. Annu Rev Immunol [Internet] 2001; 19: 331–373. Available from: https://doi.org/10.1146/annurev.immunol.19.1.331

299. Singer A. New perspectives on a developmental dilemma: The kinetic signaling model and the importance of signal duration for the CD4/CD8 lineage decision. Curr Opin Immunol 2002; 14: 207–215.

300. Brugnera E, Bhandoola A, Cibotti R, Yu Q, Guinter TI, Yamashita Y, et al. Coreceptor reversal in the thymus: Signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. Immunity 2000; 13: 59–71.

301. Yu Q, Erman B, Bhandoola A, Sharrow SO, Singer A. In vitro evidence that cytokine receptor signals are required for differentiation of double positive thymocytes into functionally mature CD8+ T cells. J Exp Med 2003; 197: 475–487.

302. Kappes DJ, He X, He X. Role of the transcription factor Th-POK in CD4:CD8 lineage commitment. Immunol Rev 2006; 209: 237–252.

303. Sato T, Ohno SI, Hayashi T, Sato C, Kohu K, Satake M, et al. Dual functions of runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. Immunity 2005; 22: 317–328.

304. Egawa T, Taniuchi I. Antagonistic interplay between ThPOK and Runx in lineage choice of thymocytes. Blood Cells, Mol Dis [Internet] 2009; 43: 27–29. Available from: http://dx.doi.org/10.1016/j.bcmd.2009.03.004

305. Aliahmad P, Kaye J. Development of all CD4 T lineages requires nuclear factor TOX. J Exp Med 2008; 205: 245–256.

306. Hernández-Hoyos G, Anderson MK, Wang C, Rothenberg E V., Alberola-Ila J. GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. Immunity 2003; 19: 83–94.

307. Harding F, McArthur J, Gross J, Raulet D, Allison J. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature 1992; 359: 710–713.

308. Pennock ND, White JT, Cross EW, Cheney EE, Tamburini BA, Kedl RM. T cell responses:

Naïve to memory and everything in between. Am J Physiol - Adv Physiol Educ 2013; 37: 273–283.

309. Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol 2003; 3: 984–993.

310. Gao GF, Rao Z, Bell JI. Molecular coordination of αβ T-cell receptors and coreceptors CD8 and CD4 in their recognition of peptide-MHC ligands. Trends Immunol 2002; 23: 408–413.

311. Acuto O, Michel F. CD28-mediated co-stimulation: A quantitative support for TCR signalling. Nat Rev Immunol 2003; 3: 939–951.

312. Kaliński P, Hilkens CMU, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: The concept of a third signal. Immunol Today 1999; 20: 561–567.

313. Zhu J, Paul WE. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. Immunol Rev [Internet] 2010; 238: 247–262. Available from: http://dx.doi.org/10.1016/j.earlhumdev.2015.09.003%5Cnhttp://dx.doi.org/10.1016/j.earlhumdev.2014.01.002%5Cnhttp://dx.doi.org/10.1016/S0378-3782(12)70006-3%5Cnhttp://www.sciencedirect.com/science/article/pii/S2341287914000763%5Cnhttp://dx.doi.org/10.1016/

314. Zhu J, Yamane H, Paul WE. Differentiation of Effector CD4 T Cell Populations. Annu Rev Immunol [Internet] 2010 [cited 2018 May 14]; 28: 445–489. Available from: file:///C:/Users/fduar_000/Desktop/nihms416711.pdf

315. Zhu J, Paul WE. CD4 T cells : fates , functions , and faults ASH 50th anniversary review CD4 T cells : fates , functions , and faults. Immunobiology 2009; 112: 1557–1569.

316. Lighvani AA, Frucht DM, Jankovic D, Yamane H, Aliberti J, Hissong BD, et al. T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells. Proc Natl Acad Sci U S A 2001; 98: 15137–15142.

317. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science (80-) 1993; 260: 547–549.

318. Geginat J, Paroni M, Facciotti F, Gruarin P, Kastirr I, Caprioli F, et al. The CD4-centered universe of human T cell subsets. Semin Immunol [Internet] 2013; 25: 252–262. Available from: http://dx.doi.org/10.1016/j.smim.2013.10.012

319. Paul WE, Zhu J. How are T H 2-type immune responses initiated and amplified? $watermark-text $watermark-text $watermark-text. Nat Rev Immunol [Internet] 2010; 10: 225–235. Available from: http://www.uniprot.org

320. Manel N, Unutmaz D, Littman DR. The differentiation of human TH-17 cells requires transforming growth factor-β and induction of the nuclear receptor RORγt. Nat Immunol 2008; 9: 641–649.

321. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006; 24: 179–189.

322. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather

than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 2003; 421: 744–748.

323. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. Annu Rev Immunol 2009; 27: 485–517.

324. Stockinger B, Veldhoen M, Martin B. Th17 T cells: Linking innate and adaptive immunity. Semin Immunol 2007; 19: 353–361.

325. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T Cells and Immune Tolerance [Internet]. Vol. 133, Cell. 2008 [cited 2018 Jul 29]. p. 775–787. Available from: file:///C:/Users/fduar_000/Desktop/1-s2.0-S0092867408006247-main.pdf

326. Josefowicz SZ, Lu L-F, Rudensky AY. Regulatory T Cells: Mechanisms of Differentiation and Function. Annu Rev Immunol [Internet] 2012 [cited 2018 Aug 3]; 30: 531–564. Available from: http://www.annualreviews.org/doi/10.1146/annurev.immunol.25.022106.141623

327. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic Self-Tolerance Maintained by Activated T Cells Expressing 11-2 Receptor a-Chains (CD25) Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune Diseases'. J Immunol [Internet] 1995 [cited 2018 Jul 30]; 155: 1151–1164. Available from: file:///C:/Users/fduar_000/Desktop/ji_1995_cd25_treg_0.pdf

328. Shevach EM. Certified professionals: CD4+CD25+ suppressor T cells. J Exp Med [Internet] 2001 [cited 2018 Jul 30]; 193: F41–F46. Available from: http://www.jem.org/cgi/content/full/193/11/F41

329. Hori SSN, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science [Internet] 2003 [cited 2018 Aug 1]; 299: 1057–1061. Available from: file:///C:/Users/fduar_000/Desktop/hori2003.pdf

330. Sakaguchi S. Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005; 6: 345–352.

331. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation [Internet]. Vol. 184, Journal of Experimental Medicine. 1996 [cited 2018 Aug 9]. Available from: http://www.jem.org/cgi/doi/10.1084/jem.184.2.387

332. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol 2001; 2: 301–306.

333. Kronenberg M, Rudensky A. Regulation of immunity by self-reactive T cells. Nature 2005; 435: 598–604.

334. Pennington DJ, Silva-Santos B, Silberzahn T, Escórcio-Correia M, Woodward MJ, Roberts SJ, et al. Early events in the thymus affect the balance of effector and regulatory T cells. Nature [Internet] 2006; 444: E4–E4. Available from: http://www.nature.com/articles/nature05368

335. Kitagawa Y, Sakaguchi S. Molecular control of regulatory T cell development and function. Curr Opin Immunol [Internet] 2017; 49: 64–70. Available from: http://dx.doi.org/10.1016/j.coi.2017.10.002

336. Panduro M, Benoist C, Mathis D. Tissue Tregs. Annu Rev Immunol [Internet] 2016 [cited 2018 Aug 4]; 34: 609–633. Available from: www.annualreviews.org

337. Lu L, Barbi J, Pan F, Hospital FA, Streets C, Division H, et al. The regulation of immune tolerance by FOXP3. Nat Rev Immunol 2018; 17: 703–717.

338. Takatori H, Kawashima H, Matsuki A, Meguro K, Tanaka S, Iwamoto T, et al. Helios enhances treg cell function in cooperation with FoxP3. Arthritis Rheumatol 2015; 67: 1491–1502.

339. Getnet D, Grosso JF, Goldberg M V, Harris TJ, Yen HR, Bruno TC, et al. A role for the transcription factor Helios in human CD4+CD25+regulatory T cells. Mol Immunol [Internet] 2010 [cited 2018 Aug 11]; 47: 1595–1600. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3060613/pdf/nihms-180581.pdf

340. Zabransky DJ, Nirschl CJ, Durham NM, Park B V., Ceccato CM, Bruno TC, et al. Phenotypic and functional properties of Helios + regulatory T cells. PLoS One 2012; 7: 1–10.

341. Szurek E, Cebula A, Wojciech L, Pietrzak M, Rempala G, Kisielow P, et al. Differences in expression level of Helios and neuropilin-1 do not distinguish thymus-derived from extrathymically-induced CD4+Foxp3+regulatory T cells. PLoS One 2015; 10: 1–16.

342. Li X, Zheng Y. Regulatory T cell identity: Formation and maintenance [Internet]. Vol. 36, Trends in Immunology. NIH Public Access; 2015 [cited 2018 Aug 3]. p. 344–353. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25981968

343. Mills KHG. Regulatory T cells: Friend or foe in immunity to infection? Nat Rev Immunol 2004; 4: 841–855.

344. Curotto de Lafaille MA, Lafaille JJ. Natural and Adaptive Foxp3+ Regulatory T Cells: More of the Same or a Division of Labor? [Internet]. Vol. 30, Immunity. 2009 [cited 2018 Aug 9]. p. 626–635. Available from: https://www.cell.com/action/showPdf?pii=S1074-7613%2809%2900199-X

345. Kanamori M, Nakatsukasa H, Okada M, Lu Q, Yoshimura A. Induced Regulatory T Cells: Their Development, Stability, and Applications. Trends Immunol [Internet] 2016; 37: 803–811. Available from: http://dx.doi.org/10.1016/j.it.2016.08.012

346. Zhang X, Olsen N, Zheng SG. The progress and prospect of regulatory T cells in autoimmune diseases. J Autoimmun [Internet] 2020; 111: 102461. Available from: https://doi.org/10.1016/j.jaut.2020.102461

347. Mohr A, Malhotra R, Mayer G, Gorochov G, Miyara M. Human FOXP3+ T regulatory cell heterogeneity. Clin Transl Immunol [Internet] 2018; 7: e1005. Available from: http://doi.wiley.com/10.1002/cti2.1005

348. Mizui M, Tsokos GC. Targeting regulatory T cells to treat patients with systemic lupus erythematosus. Front Immunol 2018; 9: 1–9.

349. Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of Treg-mediated T-cell suppression. Front Immunol [Internet] 2012 [cited 2018 Aug 3]; 3: 1–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22566933

350. Shevach EM. Mechanisms of Foxp3+ T Regulatory Cell-Mediated Suppression [Internet].

Vol. 30, Immunity. Elsevier; 2009 [cited 2018 Aug 3]. p. 636–645. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19464986

351. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory T cells: How do they suppress immune responses? [Internet]. Vol. 21, International Immunology. Oxford University Press; 2009 [cited 2018 Aug 3]. p. 1105–1111. Available from: https://academic.oup.com/intimm/article-lookup/doi/10.1093/intimm/dxp095

352. Bianchini R, Bistoni O, Alunno A, Petrillo MG, Ronchetti S, Sportoletti P, et al. CD4+CD25lowGITR+ cells: A novel human CD4+ T-cell population with regulatory activity. Eur J Immunol [Internet] 2011; 41: 2269–2278. Available from: http://doi.wiley.com/10.1002/eji.201040943

353. Fukaura H, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL, Hafler DA. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. [Internet]. Vol. 98, Journal of Clinical Investigation. 1996 [cited 2018 Aug 8]. Available from: http://www.jci.org/articles/view/118779

354. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, De Vries JE, et al. A CD4+T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997; 389: 737–742.

355. Cosmi L, Liotta F, Lazzeri E, Francalanci M, Angeli R, Mazzinghi B, et al. Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. Blood 2003; 102: 4107–4114.

356. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF. Tumor-Infiltrating γδ T Cells Suppress T and Dendritic Cell Function via Mechanisms Controlled by a Unique Toll-like Receptor Signaling Pathway. Immunity 2007; 27: 334–348.

357. Gol-Ara M, Jadidi-Niaragh F, Sadria R, Azizi G, Mirshafiey A. The Role of Different Subsets of Regulatory T Cells in Immunopathogenesis of Rheumatoid Arthritis. Arthritis [Internet] 2012; 2012: 1–16. Available from: http://www.hindawi.com/journals/arthritis/2012/805875/

358. Wollenberg I, Agua-Doce A, Hernández A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the Germinal Center Reaction by Foxp3 + Follicular Regulatory T Cells. J Immunol 2011; 187: 4553–4560.

359. Caridade M, Graca L, Ribeiro RM. Mechanisms underlying CD4+ Treg immune regulation in the adult: From experiments to models. Front Immunol 2013; 4: 1–9.

360. Wei Y, Feng J, Hou Z, Wang XM, Yu D. Flow cytometric analysis of circulating follicular helper T (Tfh) and follicular regulatory T (Tfr) populations in human blood. Methods Mol Biol [Internet] 2015 [cited 2017 Feb 28]; 1291: 199–207. Available from: http://link.springer.com/10.1007/978-1-4939-2498-1_17

361. Fazilleau N, Aloulou M. Several follicular regulatory T cell subsets with distinct phenotype and function emerge during germinal center reactions. Front Immunol 2018; 9: 1–9.

362. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003; 4: 330–336.

363. Fabien P, Michèle B, Guillaume M, Cécile B, Pierre P, Isabelle P, et al. A standardized flow cytometry procedure for the monitoring of regulatory T cells in clinical trials. Cytometry B Clin Cytom [Internet] 2018 [cited 2018 Jul 29]; : 348–355. Available from: http://doi.wiley.com/10.1002/cyto.b.21622%0Ahttp://www.ncbi.nlm.nih.gov/pubmed/29316248

364. Aerts NE, Dombrecht EJ, Ebo DG, Bridts CH, Stevens WJ, De Clerck LS. Activated T cells complicate the identification of regulatory T cells in rheumatoid arthritis. Cell Immunol 2008; 251: 109–115.

365. Yu N, Li XX, Song W, Li D, Yu D, Zeng X, et al. CD4+CD25+CD127low/− T Cells: A More Specific Treg Population in Human Peripheral Blood. Inflammation [Internet] 2012 [cited 2018 May 28]; 35: 1773–1780. Available from: http://link.springer.com/10.1007/s10753-012-9496-8

366. Rodríguez-Perea AL, Arcia ED, Rueda CM, Velilla PA. Phenotypical characterization of regulatory T cells in humans and rodents [Internet]. Vol. 185, Clinical and Experimental Immunology. 2016 [cited 2018 Jul 29]. p. 281–291. Available from: file:///C:/Users/fduar_000/Downloads/Rodr-guez-Perea_et_al-2016-Clinical_%2526_Experimental_Immunology.pdf

367. Crotty S. T Follicular Helper Cell Differentiation, Function, and Roles in Disease. Immunity [Internet] 2014 [cited 2016 Aug 11]; 41: 529–542. Available from: http://dx.doi.org/10.1016/j.immuni.2014.10.004

368. Goenka R, Barnett LG, Silver JS, O'Neill PJ, Hunter CA, Cancro MP, et al. Cutting Edge: Dendritic Cell-Restricted Antigen Presentation Initiates the Follicular Helper T Cell Program but Cannot Complete Ultimate Effector Differentiation. J Immunol 2011; 187: 1091–1095.

369. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. Immunity [Internet] 2011; 34: 932–946. Available from: http://dx.doi.org/10.1016/j.immuni.2011.03.023

370. Choi YS, Yang JA, Yusuf I, Johnston RJ, Greenbaum J, Peters B, et al. Bcl6 Expressing Follicular Helper CD4 T Cells Are Fate Committed Early and Have the Capacity To Form Memory. J Immunol 2013; 190: 4014–4026.

371. Tubo NJ, Pagán AJ, Taylor JJ, Nelson RW, Linehan JL, Ertelt JM, et al. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. Cell 2013; 153: 785–796.

372. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. Science (80-) [Internet] 2009; 325: 1001–1005. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf

373. Pepper M, Pagán AJ, Igyártó BZ, Taylor JJ, Jenkins MK. Opposing Signals from the Bcl6 Transcription Factor and the Interleukin-2 Receptor Generate T Helper 1 Central and Effector Memory Cells. Immunity 2011; 35: 583–595.

374. Choi YS, Eto D, Yang JA, Lao C, Crotty S. Cutting Edge: STAT1 Is Required for IL-6–Mediated Bcl6 Induction for Early Follicular Helper Cell Differentiation. J Immunol 2013; 190: 3049–

3053.

375. Ballesteros-Tato A, León B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 Inhibits Germinal Center Formation by Limiting T Follicular Helper Cell Differentiation. Immunity 2012; 36: 847–856.

376. Xu H, Li X, Liu D, Li J, Zhang X, Chen X, et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. Nature [Internet] 2013; 496: 523–527. Available from: http://dx.doi.org/10.1038/nature12058

377. Liang HE, Reinhardt RL, Bando JK, Sullivan BM, Ho IC, Locksley RM. Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. Nat Immunol 2012; 13: 58–66.

378. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. Nat Med 2011; 17: 975–982.

379. Hu J, Havenar-Daughton C, Crotty S. Modulation of SAP dependent T:B cell interactions as a strategy to improve vaccination. Curr Opin Virol 2013; 3: 363–370.

380. Shulman Z, Gitlin AD, Targ S, Jankovic M, Pasqual G, Nussenzweig MC, et al. T follicular helper cell dynamics in germinal centers. Science (80-) 2013; 341: 673–677.

381. Kitano M, Moriyama S, Ando Y, Hikida M, Mori Y, Kurosaki T, et al. Bcl6 Protein Expression Shapes Pre-Germinal Center B Cell Dynamics and Follicular Helper T Cell Heterogeneity. Immunity [Internet] 2011; 34: 961–972. Available from: http://dx.doi.org/10.1016/j.immuni.2011.03.025

382. Chevalier N, Jarrossay D, Ho E, Avery DT, Ma CS, Yu D, et al. CXCR5 Expressing Human Central Memory CD4 T Cells and Their Relevance for Humoral Immune Responses. J Immunol 2011; 186: 5556–5568.

383. Hale JS, Youngblood B, Latner DR, Mohammed AUR, Ye L, Akondy RS, et al. Distinct memory cd4+ t cells with commitment to t follicular helper- and t helper 1-cell lineages are generated after acute viral infection. Immunity [Internet] 2013; 38: 805–817. Available from: http://dx.doi.org/10.1016/j.immuni.2013.02.020

384. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science (80-) [Internet] 2009; 325: 1006–1010. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf

385. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human Blood CXCR5+CD4+ T Cells Are Counterparts of T Follicular Cells and Contain Specific Subsets that Differentially Support Antibody Secretion. Immunity [Internet] 2011 [cited 2017 Mar 7]; 34: 108–121. Available from: http://ac.els-cdn.com/S1074761310004917/1-s2.0-S1074761310004917-main.pdf?_tid=d38665a2-0320-11e7-9e9e-00000aacb35f&acdnat=1488882698_a6a5a754c9236d7e58245f20ce9ec330

386. Chung Y, Tanaka S, Chu F, Nurieva R. Follicular regulatory T (Tfr) cells with dual Foxp3 and Bcl6 expression suppress germinal center reactions. Nat Med [Internet] 2011; 17: 983–988. Available from: https://www.scienceopen.com/document_file/8d82c957-45e7-4853-a9c1-5a0dd862ebdc/PubMedCentral/8d82c957-45e7-4853-a9c1-5a0dd862ebdc.pdf

387. Huang Y, Chen Z, Wang H, Ba X, Shen P, Lin W, et al. Follicular regulatory T cells: a novel target for immunotherapy? Clin Transl Immunol 2020; 9: 1–17.

388. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. Arthritis Rheum [Internet] 2010 [cited 2017 Feb 28]; 62: 234–244. Available from: http://doi.wiley.com/10.1002/art.25032

389. Mittrücker HW, Visekruna A, Huber M. Heterogeneity in the Differentiation and Function of CD8+ T Cells. Arch Immunol Ther Exp (Warsz) 2014; 62: 449–458.

390. St. Paul M, Ohashi PS. The Roles of CD8+ T Cell Subsets in Antitumor Immunity. Trends Cell Biol [Internet] 2020; 30: 695–704. Available from: https://doi.org/10.1016/j.tcb.2020.06.003

391. Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. Nat Rev Immunol 2012; 12: 749–761.

392. Rouvier E, Luciani MF, Golstein P. Fas involvement in Ca2+-independent T cell-mediated cytotoxicity. J Exp Med 1993; 177: 195–200.

393. Yu Y, Ma X, Gong R, Zhu J, Wei L, Yao J. Recent advances in CD8+ regulatory t cell research (Review). Oncol Lett 2018; 15: 8187–8194.

394. Yu D, Ye L. A Portrait of CXCR5+ Follicular Cytotoxic CD8+ T cells. Trends Immunol 2018; 39: 965–979.

395. Moutsopoulos HM. Sjogren's Syndrome: Autoimmune Epithelitis. Clin Immunol Immunopathol 1994; 72: 162–165.

396. Brito-Zerón P, Baldini C, Bootsma H, Bowman SJSJ, Jonsson R, Mariette X, et al. Sjögren syndrome. Nat Rev Dis Prim [Internet] 2016 [cited 2017 Dec 2]; 2: 1–20. Available from: file:///C:/Users/fduar_000/Desktop/britozern2016.pdf

397. Skopouli FN, Dafni U, Ioannidis JP, Moutsopoulos HM. Clinical evolution, and morbidity and mortality of primary Sjögren's syndrome. Semin Arthritis Rheum [Internet] 2000 [cited 2018 May 13]; 29: 296–304. Available from: file:///C:/Users/fduar_000/Desktop/skopouli2000.pdf

398. He J, Ding Y, Feng M, Guo J, Sun X, Zhao J, et al. Characteristics of Sjögren's syndrome in rheumatoid arthritis. Rheumatol (United Kingdom) [Internet] 2013 [cited 2018 May 12]; 52: 1084–1089. Available from: file:///C:/Users/fduar_000/Desktop/kes374.pdf

399. Brown LE, Frits ML, Iannaccone CK, Weinblatt ME, Shadick NA, Liao KP. Clinical characteristics of RA patients with secondary SS and association with joint damage. Rheumatol (United Kingdom) [Internet] 2014 [cited 2018 May 12]; 54: 816–820. Available from: file:///C:/Users/fduar_000/Desktop/keu400.pdf

400. Manoussakis MN, Georgopoulou C, Zintzaras E, Spyropoulou M, Stavropoulou A, Skopouli FN, et al. Sjögren's Syndrome Associated with Systemic Lupus Erythematosus: Clinical and Laboratory Profiles and Comparison with Primary Sjögren's Syndrome. Arthritis Rheum [Internet] 2004 [cited 2018 May 12]; 50: 882–891. Available from: file:///C:/Users/fduar_000/Desktop/manoussakis2004.pdf

401. Baer AN, Maynard JW, Shaikh F, Magder LS, Petri M. Secondary Sjogren's Syndrome in Systemic Lupus Erythematosus Defines a Distinct Disease Subset. J Rheumatol [Internet] 2010 [cited 2018 May 12]; 37: 1143–1149. Available from: http://www.jrheum.org/cgi/doi/10.3899/jrheum.090804

402. Avouac J, Sordet C, Depinay C, Ardizonne M, Vacher-Lavenu MC, Sibilia J, et al. Systemic sclerosis-associated Sjögren's syndrome and relationship to the limited cutaneous subtype: Results of a prospective study of sicca syndrome in 133 consecutive patients. Arthritis Rheum [Internet] 2006 [cited 2018 May 12]; 54: 2243–2249. Available from: file:///C:/Users/fduar_000/Desktop/avouac2006.pdf

403. Salliot C, Mouthon L, Ardizzone M, Sibilia J, Guillevin L, Mariette X. Sjögren's syndrome is associated with and not secondary to systemic sclerosis. Rheumatology [Internet] 2007 [cited 2018 May 12]; 46: 321–326. Available from: file:///C:/Users/fduar_000/Desktop/salliot2006.pdf

404. Bloch KJ, Buchanan WW, Wohl MJ, Bunim JJ. Sjögren's syndrome: A clinical, pathological, and serological study of sixty-two cases. Med (United States) [Internet] 1965 [cited 2018 May 14]; 44: 187–231. Available from: http://ci.nii.ac.jp/naid/10005644173/en/

405. Theander E, Jacobsson LTH. Relationship of Sjögren's Syndrome to Other Connective Tissue and Autoimmune Disorders. Rheum Dis Clin North Am [Internet] 2008; 34: 935–947. Available from: http://dx.doi.org/10.1016/j.rdc.2008.08.009

406. Ramos-Casals M, Brito-Zerón P, Font J. The Overlap of Sjögren's Syndrome with Other Systemic Autoimmune Diseases. Semin Arthritis Rheum 2007; 36: 246–255.

407. Anaya JM, Rojas-Villarraga A, Mantilla RD, Arcos-Burgos M, Sarmiento-Monroy JC. Polyautoimmunity in Sjögren Syndrome [Internet]. Vol. 42, Rheumatic Disease Clinics of North America. 2016 [cited 2018 May 12]. p. 457–472. Available from: file:///C:/Users/fduar_000/Desktop/anaya2016.pdf

408. Sjogren H. Zur Kenntnis der Keratoconjunctivitis sicca [Internet]. Acta ophthalm. 1933 [cited 2018 May 14]. Available from: http://ci.nii.ac.jp/naid/10013193495/en/

409. Gougerot H. Insuffisance progressive et atrophie des glandes salivaires et muqueuses de la bouche, des conjonctives (et parfois des muqueuses, nasale, laryngee, vulvaire). Bull Soc Fr Derm Syph [Internet] 1925 [cited 2018 May 14]; 32: 376–379. Available from: http://ci.nii.ac.jp/naid/10011014912/en/

410. Mikulicz J. Über eine eigenartige symmetrische Erkrankung der Tränen und Mundspeicheldrüsen. Beitr Chir Fortsch Gewidmet Theodor Billroth Stuttgart [Internet] 1892 [cited 2018 May 14]; : 610–630. Available from: http://ci.nii.ac.jp/naid/10009399558/en/

411. Kamisawa T, Okamoto A. IgG4-related sclerosing disease. World J Gastroenterol 2008; 14: 3948–3955.

412. Masaki Y, Sugai S, Umehara H. IgG4-related diseases including Mikulicz's disease and sclerosing pancreatitis: Diagnostic insights. J Rheumatol 2010; 37: 1380–1385.

413. Clark G, Reichlin M, Tomasi TB. Characterization of a soluble cytoplasmic antigen reactive with sera from patients with systemic lupus erythmatosus. J Immunol [Internet] 1969 [cited 2018 May 14]; 102: 117–122. Available from:

http://ci.nii.ac.jp/naid/10027167792/en/

414. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjogren ' s disease. J Clin Pathol [Internet] 1968 [cited 2018 May 14]; 1933: 656–660. Available from: http://jcp.bmj.com/

415. Talal N, Bunim JJ. The development of malignant lymphoma in the course of Sjögren's syndrome. Am J Med [Internet] 1964 [cited 2018 May 14]; 36: 529–540. Available from: http://linkinghub.elsevier.com/retrieve/pii/0002934364901019

416. Qin B, Wang J, Yang Z, Yang M, Ma N, Huang F, et al. Epidemiology of primary Sjögren's syndrome: A systematic review and meta-analysis [Internet]. Vol. 74, Annals of the Rheumatic Diseases. 2015 [cited 2018 May 12]. p. 1983–1989. Available from: http://dx.doi.org/10.1136/

417. Kassan SS, Moutsopoulos HM. Clinical manifestations and early diagnosis of Sjögren syndrome. Arch Intern Med [Internet] 2004 [cited 2017 Jul 11]; 164: 1275–1284. Available from: http://archinte.jamanetwork.com/article.aspx?doi=10.1001/archinte.164.12.1275

418. Cornec D, Chiche L. Is primary Sjogren's syndrome an orphan disease? A critical appraisal of prevalence studies in Europe. Ann Rheum Dis [Internet] 2015 [cited 2016 May 27]; 74: e25–e25. Available from: http://ard.bmj.com/cgi/doi/10.1136/annrheumdis-2014-206860

419. Narváez J, Sánchez-Fernández SÁ, Seoane-Mato D, Díaz-González F, Bustabad S. Prevalence of Sjögren's syndrome in the general adult population in Spain: estimating the proportion of undiagnosed cases. Sci Rep [Internet] 2020; 10: 1–10. Available from: https://doi.org/10.1038/s41598-020-67462-z

420. Mavragani CP, Moutsopoulos HM. The geoepidemiology of Sjögren's syndrome. Autoimmun Rev [Internet] 2010 [cited 2018 May 12]; 9: A305–A310. Available from: file:///C:/Users/fduar_000/Desktop/mavragani2010.pdf

421. Nocturne G, Mariette X. Advances in understanding the pathogenesis of primary Sjögren's syndrome [Internet]. Vol. 9, Nature Reviews Rheumatology. 2013 [cited 2017 Dec 3]. p. 544–556. Available from: file:///C:/Users/fduar_000/Desktop/nocturne2013.pdf

422. Brkic Z, Maria NI, Van Helden-Meeuwsen CG, Van De Merwe JP, Van Daele PL, Dalm VA, et al. Prevalence of interferon type I signature in CD14 monocytes of patients with Sjögren's syndrome and association with disease activity and BAFF gene expression. Ann Rheum Dis 2013; 72: 728–735.

423. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos NM, Moutsopoulos HM. Foxp3+ T-Regulatory Cells in Sjögren's Syndrome. Am J Pathol [Internet] 2008 [cited 2018 Jan 16]; 173: 1389–1396. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0002944010615258

424. Katsifis GE, Rekka S, Moutsopoulos NM, Pillemer S, Wahl SM. Systemic and local interleukin-17 and linked cytokines associated with Sjögren's syndrome immunopathogenesis. Am J Pathol 2009; 175: 1167–1177.

425. Theander E, Vasaitis L, Baecklund E, Nordmark G, Warfvinge G, Liedholm R, et al. Lymphoid organisation in labial salivary gland biopsies is a possible predictor for the development of malignant lymphoma in primary Sjögren's syndrome. Ann Rheum Dis 2011; 70: 1363–1368.

426. Low HZ, Witte T. Aspects of innate immunity in Sjögren's syndrome. Arthritis Res Ther

2011; 13: 1–7.

427. Ainola M, Porola P, Takakubo Y, Przybyla B, Kouri VP, Tolvanen TA, et al. Activation of plasmacytoid dendritic cells by apoptotic particles – mechanism for the loss of immunological tolerance in Sjögren's syndrome. Clin Exp Immunol 2018; 191: 301–310.

428. Hillen MR, Pandit A, Blokland SLM, Hartgring SAY, Bekker CPJ, van der Heijden EHM, et al. Plasmacytoid DCs From Patients With Sjögren's Syndrome Are Transcriptionally Primed for Enhanced Pro-inflammatory Cytokine Production. Front Immunol 2019; 10: 1–15.

429. Kapsogeorgou EK, Abu-Helu RF, Moutsopoulos HM, Manoussakis MN. Salivary gland epithelial cell exosomes: A source of autoantigenic ribonucleoproteins. Arthritis Rheum 2005; 52: 1517–1521.

430. Singh N, Cohen PL. The T cell in Sjogren's syndrome: Force majeure, not spectateur. J Autoimmun [Internet] 2012 [cited 2012 Dec 8]; 39: 229–233. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22709856

431. Bombardieri M, Pitzalis C. Ectopic Lymphoid Neogenesis and Lymphoid Chemokines in Sjogren's Syndrome: At the Interplay between Chronic Inflammation, Autoimmunity and Lymphomagenesis. Curr Pharm Biotechnol 2012; 13: 1989–1996.

432. Long D, Chen Y, Wu H, Zhao M, Lu Q. Clinical significance and immunobiology of IL-21 in autoimmunity. Vol. 99, Journal of Autoimmunity. Academic Press; 2019. p. 1–14.

433. Papiris SA, Saetta M, Turato G, La Corte R, Trevisani L, Mapp CE, et al. CD4-positive T-lymphocytes infiltrate the bronchial mucosa of patients with Sjogren's syndrome. Am J Respir Crit Care Med 1997; 156: 637–641.

434. Hansen A, Lipsky PE, Dörner T. B cells in Sjögren's syndrome: indications for disturbed selection and differentiation in ectopic lymphoid tissue. Arthritis Res Ther [Internet] 2007; 9: 218. Available from: http://arthritis-research.biomedcentral.com/articles/10.1186/ar2210

435. Hansen A, Daridon C, Dörner T. What do we know about memory B cells in primary Sjögren's syndrome? [Internet]. Vol. 9, Autoimmunity Reviews. 2010 [cited 2017 Oct 14]. p. 600–603. Available from: file:///C:/Users/fduar_000/Desktop/hansen2010.pdf

436. Corsiero E, Sutcliffe N, Pitzalis C, Bombardieri M, Reddy J. Accumulation of self-reactive naïve and memory B cell reveals sequential defects in B cell tolerance checkpoints in Sjögren's Syndrome. PLoS One [Internet] 2014 [cited 2017 Mar 12]; 9. Available from: http://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0114575&type=printable

437. Kroese FGM, Abdulahad WH, Haacke E, Bos NA, Vissink A, Bootsma H. B-cell hyperactivity in primary Sjögren's syndrome. Expert Rev Clin Immunol [Internet] 2014 [cited 2017 Dec 3]; 10: 483–499. Available from: file:///C:/Users/fduar_000/Desktop/kroese2014.pdf

438. Tzioufas AG, Voulgarelis M. Update on Sjögren's syndrome autoimmune epithelitis: from classification to increased neoplasias. Best Pract Res Clin Rheumatol [Internet] 2007 [cited 2012 Dec 8]; 21: 989–1010. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18068857

439. Fragkioudaki S, Mavragani CP, Moutsopoulos HM. Predicting the risk for lymphoma

development in Sjogren syndrome: An easy tool for clinical use. Medicine (Baltimore) [Internet] 2016 [cited 2017 Oct 1]; 95: e3766. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27336863

440. Björk A, Mofors J, Wahren-Herlenius M. Environmental factors in the pathogenesis of primary Sjögren's syndrome. J Intern Med 2020; 287: 475–492.

441. Imgenberg-Kreuz J, Rasmussen A, Sivils K, Nordmark G. Genetics and epigenetics in primary Sjögren's syndrome. Rheumatology 2019; .

442. Cruz-Tapias P, Rojas-Villarraga A, Maier-Moore S, Anaya JM. HLA and Sjögren's syndrome susceptibility. A meta-analysis of worldwide studies. Autoimmun Rev [Internet] 2012; 11: 281–287. Available from: http://dx.doi.org/10.1016/j.autrev.2011.10.002

443. Lessard CJ, Li H, Adrianto I, Ice JA, Rasmussen A, Grundahl KM, et al. Variants at multiple loci implicated in both innate and adaptive immune responses are associated with Sjögren ' s syndrome. Nat Genet 2013; 45: 1284–1294.

444. Harris VM, Hal Scofield R, Sivils KL. Genetics in Sjögren's syndrome: Where we are and where we go. Clin Exp Rheumatol 2019; 37: S234–S239.

445. Gottenberg JE, Cagnard N, Lucchesi C, Letourneur F, Mistou S, Lazure T, et al. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren's syndrome. Proc Natl Acad Sci U S A 2006; 103: 2770–2775.

446. Yao Q, Song Z, Wang B, Qin Q, Zhang JA. Identifying Key Genes and Functionally Enriched Pathways in Sjögren's Syndrome by Weighted Gene Co-Expression Network Analysis. Front Genet 2019; 10: 1–10.

447. Liu Z, Li F, Pan A, Xue H, Jiang S, Zhu C, et al. Elevated CCL19/CCR7 Expression During the Disease Process of Primary Sjögren's Syndrome. Front Immunol 2019; 10: 795.

448. Vitali C, Dolcino M, Del Papa N, Minniti A, Pignataro F, Maglione W, et al. Gene Expression Profiles in Primary Sjögren's Syndrome With and Without Systemic Manifestations. ACR Open Rheumatol 2019; 1: 603–613.

449. Lucchesi D, Pitzalis C, Bombardieri M. EBV and other viruses as triggers of tertiary lymphoid structures in primary Sjögren's syndrome. Expert Rev Clin Immunol 2014; 10: 445–455.

450. Lugonja B, Yeo L, Milward MR, Smith D, Dietrich T, Chapple ILC, et al. Periodontitis prevalence and serum antibody reactivity to periodontal bacteria in primary Sjögren's syndrome: A pilot study. J Clin Periodontol 2016; 43: 26–33.

451. Mofors J, Arkema E V., Björk A, Westermark L, Kvarnström M, Forsblad-d'Elia H, et al. Infections increase the risk of developing Sjögren's syndrome. J Intern Med 2019; 285: 670–680.

452. Verstappen GM, Kroese FGM, Bootsma H. T cells in primary Sjögren's syndrome: targets for early intervention. Rheumatology 2019; : 1–11.

453. Igoe A, Scofield RH. Autoimmunity and infection in Sjögren's syndrome. Curr Opin Rheumatol 2013; 25: 480–487.

454. Khanolkar A, Fuller MJ, Zajac AJ. T cell responses to viral infections: Lessons from lymphocytic choriomeningitis virus. Immunol Res 2002; 26: 309–321.

455. Maślińska M. The role of Epstein-Barr virus infection in primary Sjögren's syndrome. Curr Opin Rheumatol 2019; 31: 475–483.

456. Croia C, Astorri E, Murray-Brown W, Willis A, Brokstad KA, Sutcliffe N, et al. Implication of Epstein-Barr virus infection in disease-specific autoreactive B cell activation in ectopic lymphoid structures of Sjögren's syndrome. Arthritis Rheumatol 2014; 66: 2545–2557.

457. Kemeny ME, Schedlowski M. Understanding the interaction between psychosocial stress and immune-related diseases: A stepwise progression. Brain Behav Immun 2007; 21: 1009–1018.

458. Porcelli B, Pozza A, Bizzaro N, Fagiolini A, Costantini MC, Terzuoli L, et al. Association between stressful life events and autoimmune diseases: A systematic review and meta-analysis of retrospective case-control studies. Autoimmun Rev [Internet] 2016; 15: 325–334. Available from: http://dx.doi.org/10.1016/j.autrev.2015.12.005

459. Karaiskos D, Mavragani CP, Makaroni S, Zinzaras E, Voulgarelis M, Rabavilas A, et al. Stress, coping strategies and social support in patients with primary Sjogren's syndrome prior to disease onset: A retrospective case-control study. Ann Rheum Dis 2009; 68: 40–46.

460. Tzioufas AG, Tsonis J, Moutsopoulos HM. Neuroendocrine dysfunction in Sjögren's syndrome. Neuroimmunomodulation 2008; 15: 37–45.

461. Fox RL, Stern M. Sjögren's syndrome: Mechanisms ofpathogenesis involve interaction of immune and neurosecretory systems. Scand J Rheumatol 2002; 31: 3–13.

462. Brandt JE, Priori R, Valesini G, Fairweather D. Sex differences in Sjögren's syndrome: A comprehensive review of immune mechanisms. Biol Sex Differ [Internet] 2015 [cited 2021 Jan 19]; 6: 1–13. Available from: http://dx.doi.org/10.1186/s13293-015-0037-7

463. McCoy SS, Sampene E, Baer AN. Association of Sjögren's Syndrome With Reduced Lifetime Sex Hormone Exposure: A Case–Control Study. Arthritis Care Res 2020; 72: 1315–1322.

464. Olsson T, Barcellos LF, Alfredsson L. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. Nat Rev Neurol [Internet] 2016; 13: 26–36. Available from: http://dx.doi.org/10.1038/nrneurol.2016.187

465. Olsson P, Bodewes ILA, Nilsson AM, Turesson C, Jacobsson LTH, Theander E, et al. Associations of cigarette smoking with disease phenotype and type I interferon expression in primary Sjögren's syndrome. Rheumatol Int [Internet] 2019; 39: 1575–1584. Available from: https://doi.org/10.1007/s00296-019-04335-3

466. Loftus E V. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology 2004; 126: 1504–1517.

467. Manthorpe R, Benoni C, Jacobsson L, Kirtava Z, Larsson Å, Liedholm R, et al. Lower frequency of focal lip sialadenitis (focus score) in smoking patients. Can tobacco diminish the salivary gland involvement as judged by histological examination and anti-SSA/Ro and anti-SSB/La antibodies in Sjogren's syndrome? Ann Rheum Dis 2000; 59: 54–60.

468. Karabulut G, Kitapcioglu G, Inal V, Kalfa M, Yargucu F, Keser G, et al. Cigarette smoking in

primary Sjögren's syndrome: Positive association only with ANA positivity. Mod Rheumatol 2011; 21: 602–607.

469. Stone DU, Fife D, Brown M, Earley KE, Radfar L, Kaufman CE, et al. Effect of tobacco smoking on the clinical, histopathological, and serological manifestations of sjögren's syndrome. PLoS One 2017; 12: 1–13.

470. Szabo G, Saha B. Alcohol's Effect on Host Defense. Alcohol Res 2015; 37: 159–170.

471. Mandrekar P, Catalano D, White B, Szabo G. Moderate alcohol intake in humans attenuates monocyte inflammatory responses: Inhibition of nuclear regulatory factor kappa B and induction of interleukin 10. Alcohol Clin Exp Res 2006; 30: 135–139.

472. Jin Z, Xiang C, Cai Q, Wei X, He J. Alcohol consumption as a preventive factor for developing rheumatoid arthritis: A dose-response meta-analysis of prospective studies. Ann Rheum Dis 2014; 73: 1962–1967.

473. Hedström AK, Hillert J, Olsson T, Alfredsson L. Alcohol as a modifiable lifestyle factor affecting multiple sclerosis risk. JAMA Neurol 2014; 71: 300–305.

474. Ben-Eli H, Aframian DJ, Ben-Chetrit E, Mevorach D, Kleinstern G, Paltiel O, et al. Shared medical and environmental risk factors in dry eye syndrome, Sjogren's syndrome, and B-cell non-Hodgkin lymphoma: A case-control study. J Immunol Res 2019; 2019.

475. Chaigne B, Lasfargues G, Marie I, Hüttenberger B, Lavigne C, Marchand-Adam S, et al. Primary Sjögren's syndrome and occupational risk factors: A case-control study. J Autoimmun 2015; 60: 80–85.

476. Miller FW, Alfredsson L, Costenbader KH, Kamen DL, Nelson LM, Norris JM, et al. Epidemiology of environmental exposures and human autoimmune diseases: Findings from a National Institute of Environmental Health Sciences Expert Panel Workshop. J Autoimmun 2012; 39: 259–271.

477. Cohen Tervaert JW, Colaris MJ, Van Der Hulst RR. Silicone breast implants and autoimmune rheumatic diseases: Myth or reality. Curr Opin Rheumatol 2017; 29: 348–354.

478. Mavragani CP, Crow MK. Activation of the type I interferon pathway in primary Sjogren's syndrome. J Autoimmun [Internet] 2010; 35: 225–231. Available from: http://dx.doi.org/10.1016/j.jaut.2010.06.012

479. Shimizu T, Nakamura H, Takatani A, Umeda M, Horai Y, Kurushima S, et al. Activation of Toll-like receptor 7 signaling in labial salivary glands of primary Sjögren's syndrome patients. Clin Exp Immunol 2019; 196: 39–51.

480. Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. Curr Opin Virol [Internet] 2011; 1: 519–525. Available from: http://dx.doi.org/10.1016/j.coviro.2011.10.008

481. Mavragani CP, Niewold TB, Moutsopoulos NM, Pillemer SR, Wahl SM, Crow MK. Augmented interferon-α pathway activation in patients with Sjögren's syndrome treated with etanercept. Arthritis Rheum 2007; 56: 3995–4004.

482. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating

dendritic cells in vivo. Immunity 2001; 14: 461–470.

483. Hervas-Stubbs S, Perez-Gracia JL, Rouzaut A, Sanmamed MF, Le Bon A, Melero I. Direct effects of type I interferons on cells of the immune system. Clin Cancer Res 2011; 17: 2619–2627.

484. Maria NI, Vogelsang P, Versnel MA. The clinical relevance of animal models in Sjögren's syndrome: The interferon signature from mouse to man. Arthritis Res Ther [Internet] 2015; 17: 68–74. Available from: https://doi.org/10.1016/j.coi.2020.10.009

485. Marketos N, Cinoku I, Rapti A, Mavragani CP. Type I interferon signature in Sjögren's syndrome: Pathophysiological and clinical implications. Clin Exp Rheumatol 2019; 37: S185–S191.

486. Rivière E, Pascaud J, Tchitchek N, Boudaoud S, Paoletti A, Ly B, et al. Salivary gland epithelial cells from patients with Sjögren's syndrome induce B-lymphocyte survival and activation. Ann Rheum Dis 2020; 79: 1468–1477.

487. Adamson TC, Fox RI, Frisman DM, Howell F V. Immunohistologic analysis of lymphoid infiltrates in primary Sjogren's syndrome using monoclonal antibodies. J Immunol 1983; 130: 203–208.

488. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos HM. Characteristics of the minor salivary gland infiltrates in Sjögren's syndrome. J Autoimmun 2010; 34: 400–407.

489. Yao Y, Ma JF, Chang C, Xu T, Gao CY, Gershwin ME, et al. Immunobiology of T Cells in Sjögren's Syndrome. Clin Rev Allergy Immunol 2020; .

490. Katsifis GE, Moutsopoulos NM, Wahl SM. T lymphocytes in Sjögren's syndrome: Contributors to and regulators of pathophysiology. Clin Rev Allergy Immunol 2007; 32: 252–264.

491. Karabiyik A, Peck AB, Nguyen CQ. The Important Role of T Cells and Receptor Expression in Sjögren's Syndrome. Scand J Immunol 2013; 78: 157–166.

492. Ohlsson M, Jonsson R, Brokstad KA. Subcellular redistribution and surface exposure of the Ro52, Ro60 and La48 autoantigens during apoptosis in human ductal epithelial cells: A possible mechanism in the pathogenesis of Sjögren's syndrome. Scand J Immunol 2002; 56: 456–469.

493. Davies ML, Taylor EJ, Gordon C, Young SP, Welsh K, Bunce M, et al. Candidate T cell epitopes of the human La/SSB autoantigen. Arthritis Rheum 2002; 46: 209–214.

494. Ogawa N, Ping L, Zhenjun L, Takada Y, Sugai S. Involvement of the interferon-γ-induced T cell-attracting chemokines, interferon-γ-inducible 10-kd protein (CXCL10) and monokine induced by interferon-γ (CXCL9), in the salivary gland lesions of patients with Sjögren's syndrome. Arthritis Rheum 2002; 46: 2730–2741.

495. Kong L, Ogawa N, Nakabayashi T, Liu GT, D'Souza E, Mcguff HS, et al. Fas and Fas ligand expression in the salivary glands of patients with primary Sjogren's syndrome. Arthritis Rheum 1997; 40: 87–97.

496. Reed JH, Verstappen GM, Rischmueller M, Bryant VL. When B cells break bad: development of pathogenic B cells in Sjögren's syndrome. Clin Exp Rheumatol 2020; 38:

S271–S282.

497. Mitsias DI, Tzioufas AG, Veiopoulou C, Zintzaras E, Tassios IK, Kogopoulou O, et al. The Th1/Th2 cytokine balance changes with the progress of the immunopathological lesion of Sjogren's syndrome. Clin Exp Immunol 2002; 128: 562–568.

498. Mosmann TR, Coffman RL. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989; 7: 145–173.

499. Ozaki Y, Amakawa R, Ito T, Iwai H, Tajima K, Uehira K, et al. Alteration of peripheral blood dendritic cells in patients with primary Sjögren's syndrome. Arthritis Rheum 2001; 44: 419–431.

500. Delaleu N, Nguyen CQ, Peck AB, Jonsson R. Sjögren's syndrome: Studying the disease in mice. Arthritis Res Ther 2011; 13: 1–16.

501. Donate A, Voigt A, Nguyen CQ. The value of animal models to study immunopathology of primary human Sjögren's syndrome symptoms. Expert Rev Clin Immunol 2014; 10: 469–481.

502. Moriyama M, Tanaka A, Maehara T, Furukawa S, Nakashima H, Nakamura S. T helper subsets in Sjögren's syndrome and IgG4-related dacryoadenitis and sialoadenitis: A critical review. J Autoimmun [Internet] 2014; 51: 81–88. Available from: http://dx.doi.org/10.1016/j.jaut.2013.07.007

503. Ohyama Y, Nakamura S, Matsuzaki G, Shinohara M, Hiroki A, Fujimura T, et al. Cytokine messenger RNA expression in the labial salivary glands of patients with Sjogren's syndrome. Arthritis Rheum 1996; 39: 1376–1384.

504. Verstappen GM, Corneth OBJ, Bootsma H, Kroese FGM. Th17 cells in primary Sjögren's syndrome: Pathogenicity and plasticity. J Autoimmun 2018; 87: 16–25.

505. Sakai A, Sugawara Y, Kuroishi T, Sasano T, Sugawara S. Identification of IL-18 and Th17 Cells in Salivary Glands of Patients with Sjögren's Syndrome, and Amplification of IL-17-Mediated Secretion of Inflammatory Cytokines from Salivary Gland Cells by IL-18. J Immunol 2008; 181: 2898–2906.

506. Fei Y, Zhang W, Lin D, Wu C, Li M, Zhao Y, et al. Clinical parameter and Th17 related to lymphocytes infiltrating degree of labial salivary gland in primary Sjögren's syndrome. Clin Rheumatol 2014; 33: 523–529.

507. Nguyen CQ, Hu MH, Li Y, Stewart C, Peck AB. Salivary gland tissue expression of interleukin-23 and interleukin-17 in Sjögren's syndrome: Findings in humans and mice. Arthritis Rheum 2008; 58: 734–743.

508. Barone F, Nayara S, Camposa J, Cloakea T, Withers DR, Toellner KM, et al. IL-22 regulates lymphoid chemokine production and assembly of tertiary lymphoid organs. Proc Natl Acad Sci U S A 2015; 112: 11024–11029.

509. Chen W, Yang F, Xu G, Ma J, Lin J. Follicular helper T cells and follicular regulatory T cells in the immunopathology of primary Sjögren's syndrome. J Leukoc Biol 2020; : 1–11.

510. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly-T FH cells in human health and disease. Nat Rev Immunol [Internet] 2013; 13: 412–426. Available from:

http://dx.doi.org/10.1038/nri3447

511. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. PLoS One 2011; 6.

512. Corsiero E, Nerviani A, Bombardieri M, Pitzalis C. Ectopic lymphoid structures: Powerhouse of autoimmunity. Front Immunol 2016; 7: 1–6.

513. Kang KY, Kim HOHY, Kwok SK, Ju JH, Park KS, Sun D Il, et al. Impact of interleukin-21 in the pathogenesis of primary Sjögren's syndrome: increased serum levels of interleukin-21 and its expression in the labial salivary glands. Arthritis Res Ther 2011; 13.

514. Szabo K, Papp G, Dezso B, Zeher M. The histopathology of labial salivary glands in primary sjögren's syndrome: Focusing on follicular helper t cells in the inflammatory infiltrates. Mediators Inflamm 2014; 2014.

515. Quigley MF, Gonzalez VD, Granath A, Andersson J, Sandberg JK. CXCR5+ CCR7- CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. Eur J Immunol 2007; 37: 3352–3362.

516. Chen Y, Yu M, Zheng Y, Fu G, Xin G, Zhu W, et al. CXCR5+PD-1+ follicular helper CD8 T cells control B cell tolerance. Nat Commun [Internet] 2019; 10: 1–15. Available from: http://dx.doi.org/10.1038/s41467-019-12446-5

517. Noack M, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. Autoimmun Rev [Internet] 2014 [cited 2017 May 7]; 13: 668–677. Available from: file:///C:/Users/fduar_000/Desktop/noack2014.pdf

518. Alunno A, Manetti M, Caterbi S, Ibba-Manneschi L, Bistoni O, Bartoloni E, et al. Altered immunoregulation in rheumatoid arthritis: The role of regulatory T cells and proinflammatory Th17 cells and therapeutic implications. Mediators Inflamm [Internet] 2015; 2015: 751793. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25918479

519. Yamada A, Ushio A, Arakaki R, Tsunematsu T, Kudo Y, Hayashi Y, et al. Impaired expansion of regulatory T cells in a neonatal thymectomy-induced autoimmune mouse model. Am J Pathol [Internet] 2015; 185: 2886–2897. Available from: http://dx.doi.org/10.1016/j.ajpath.2015.07.007

520. Schneider MA, Meingassner JG, Lipp M, Moore HD, Rot A. CCR7 is required for the in vivo function of CD4+ CD25 + regulatory T cells. J Exp Med 2007; 204: 735–745.

521. Ishimaru N, Nitta T, Arakaki R, Yamada A, Lipp M, Takahama Y, et al. In Situ patrolling of regulatory T cells is essential for protecting autoimmune exocrinopathy. PLoS One 2010; 5: 1–9.

522. Liu MF, Lin LH, Weng CT, Weng MY. Decreased CD4+CD25+bright T cells in peripheral blood of patients with primary Sjogren's syndrome. Lupus [Internet] 2008 [cited 2018 Jan 16]; 17: 34–39. Available from: http://lup.sagepub.com

523. Szodoray P, Papp G, Horvath IF, Barath S, Sipka S, Nakken B, et al. Cells with regulatory function of the innate and adaptive immune system in primary Sjögren's syndrome. Clin Exp Immunol [Internet] 2009 [cited 2017 Mar 18]; 157: 343–349. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2745027&tool=pmcentrez&r

endertype=abstract

524. Alunno A, Petrillo MG, Nocentini G, Bistoni O, Bartoloni E, Caterbi S, et al. Characterization of a new regulatory CD4+ T cell subset in primary Sjogren's syndrome. Rheumatology [Internet] 2013 [cited 2017 Mar 12]; 52: 1387–1396. Available from: https://academic.oup.com/rheumatology/article-lookup/doi/10.1093/rheumatology/ket179

525. Banica L, Besliu A, Pistol G, Stavaru C, Ionescu R, Forsea AM, et al. Quantification and molecular characterization of regulatory T cells in connective tissue diseases. Autoimmunity 2009; 42: 41–49.

526. Sudzius G, Mieliauskaite D, Siaurys A, Viliene R, Butrimiene I, Characiejus D, et al. Distribution of peripheral lymphocyte populations in primary Sjögren's syndrome patients. J Immunol Res [Internet] 2015 [cited 2017 Mar 12]; 2015: Article ID 854706. Available from: file:///C:/Users/fduar_000/Downloads/854706.pdf

527. Miao M, Hao Z, Guo Y, Zhang X, Zhang S, Luo J, et al. Short-term and low-dose IL-2 therapy restores the Th17/Treg balance in the peripheral blood of patients with primary Sjögren's syndrome. Ann Rheum Dis 2018; 0: 4–6.

528. Gottenberg J-E, Lavie F, Abbed K, Gasnault J, Le Nevot E, Delfraissy J-F, et al. CD4 CD25high regulatory T cells are not impaired in patients with primary Sjögren's syndrome. J Autoimmun [Internet] 2005 [cited 2018 Jan 16]; 24: 235–242. Available from: file:///C:/Users/fduar_000/Desktop/gottenberg2005.pdf

529. Sarigul M, Yazisiz V, Başsorgun CI, Ulker M, Avci AB, Erbasan F, et al. The numbers of Foxp3+ Treg cells are positively correlated with higher grade of infiltration at the salivary glands in primary Sjögren's syndrome. Lupus [Internet] 2010 [cited 2018 Jan 16]; 19: 138–145. Available from: http://lup.sagepub.com

530. Liu C, Guan Z, Zhao L, Song Y. Elevated level of circulating CD4+Helios+FoxP3+ cells in primary Sjogren's syndrome patients. Mod Rheumatol [Internet] 2017 [cited 2017 Mar 12]; 27: 630–637. Available from: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emexb&NEWS=N&AN=612277063

531. Sage PT, Sharpe AH. The multifaceted functions of follicular regulatory T cells. Vol. 67, Current Opinion in Immunology. 2020. p. 68–74.

532. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. Immunity [Internet] 2014; 41: 1026–1039. Available from: http://dx.doi.org/10.1016/j.immuni.2014.12.005

533. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. Immunity [Internet] 2014; 41: 1013–1025. Available from: http://dx.doi.org/10.1016/j.immuni.2014.12.006

534. Clement RL, Daccache J, Mohammed MT, Diallo A, Blazar BR, Kuchroo VK, et al. Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses. Nat Immunol [Internet] 2019; 20: 1360–1371. Available from:

http://dx.doi.org/10.1038/s41590-019-0472-4

535. Hansen A, Reiter K, Ziprian T, Jacobi A, Hoffmann A, Gosemann M, et al. Dysregulation of chemokine receptor expression and function by B cells of patients with primary Sjögren's syndrome. Arthritis Rheum 2005; 52: 2109–2119.

536. Ramos-Casals M, Font J. Primary Sjögren's syndrome: Current and emergent aetiopathogenic concepts. Rheumatology 2005; 44: 1354–1367.

537. Bikker A, Van Woerkom JM, Kruize AA, Wenting-van Wijk M, De Jager W, Bijlsma JWJ, et al. Increased expression of interleukin-7 in labial salivary glands of patients with primary sjögren's syndrome correlates with increased inflammation. Arthritis Rheum 2010; 62: 969–977.

538. Guerrier T, Youinou P, Pers JO, Jamin C. TLR9 drives the development of transitional B cells towards the marginal zone pathway and promotes autoimmunity. J Autoimmun 2012; 39: 173–179.

539. Tiller T, Tsuiji M, Yurasov S, Velinzon K, Nussenzweig MC, Wardemann H. Autoreactivity in Human IgG+ Memory B Cells. Immunity 2007; 26: 205–213.

540. Hansen A, Gosemann M, Pruss A, Reiter K, Ruzickova S, Lipsky PE, et al. Abnormalities in peripheral B cell memory of patients with primary Sjögren's syndrome. Arthritis Rheum [Internet] 2004 [cited 2017 Sep 10]; 50: 1897–1908. Available from: http://doi.wiley.com/10.1002/art.20276

541. Roberts MEP, Kaminski D, Jenks SA, Maguire C, Ching K, Burbelo PD, et al. Primary Sjögren's syndrome is characterized by distinct phenotypic and transcriptional profiles of IgD+ unswitched memory B cells. Arthritis Rheumatol [Internet] 2014 [cited 2016 Aug 11]; 66: 2558–2569. Available from: http://doi.wiley.com/10.1002/art.38734

542. Szabó K, Papp G, Szántó A, Tarr T, Zeher M. A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjögren's syndrome and systemic lupus erythematosus. Clin Exp Immunol 2016; 183: 76–89.

543. Hamza N, Bos N a, Kallenberg CGM. B-cell populations and sub-populations in Sjögren's syndrome. Presse Med [Internet] 2012 [cited 2012 Dec 8]; 41: e475-83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22841377

544. Bombardieri M, Lewis M, Pitzalis C. Ectopic lymphoid neogenesis in rheumatic autoimmune diseases. Vol. 13, Nature Reviews Rheumatology. Nature Publishing Group; 2017. p. 141–154.

545. Moisini I, Davidson A. BAFF: A local and systemic target in autoimmune diseases. Clin Exp Immunol 2009; 158: 155–163.

546. Daridon C, Devauchelle V, Hutin P, Le Berre R, Martins-Carvalho C, Bendaoud B, et al. Aberrant expression of BAFF by B lymphocytes infiltrating the salivary glands of patients with primary Sjögren's syndrome. Arthritis Rheum 2007; 56: 1134–1144.

547. Ittah M, Miceli-Richard C, Gottenberg JE, Lavie F, Lazure T, Ba N, et al. B cell-activating factor of the tumor necrosis factor family (BAFF) is expressed under stimulation by interferon in salivary gland epithelial cells in primary Sjögren's syndrome. Arthritis Res Ther 2006; 8: 1–9.

548. Tengnér P, Halse AK, Haga HJ, Jonsson R, Wahren-Herlenius M. Detection of anti-Ro/SSA and anti-La/SSB autoantibody-producing cells in salivary glands from patients with Sjogren's syndrome. Arthritis Rheum 1998; 41: 2238–2248.

549. Szyszko EA, Brokstad KA, Øijordsbakken G, Jonsson M V., Jonsson R, Skarstein K. Salivary glands of primary Sjögren's syndrome patients express factors vital for plasma cell survival. Arthritis Res Ther [Internet] 2011 [cited 2020 Jul 25]; 13: 1–18. Available from: http://arthritis-research.com/content/13/1/R2

550. Amft N, Curnow SJ, Scheel-Toellner D, Devadas A, Oates J, Crocker J, et al. Ectopic expression of the B cell-attracting chemokine BCA-1 (CXCL13) on endothelial cells and within lymphoid follicles contributes to the establishment of germinal center-like structures in Sjögren's syndrome. Arthritis Rheum 2001; 44: 2633–2641.

551. Barone F, Bombardieri M, Manzo A, Blades MC, Morgan PR, Challacombe SJ, et al. Association of CXCL13 and CCL21 expression with the progressive organization of lymphoid-like structures in Sjögren's syndrome. Arthritis Rheum 2005; 52: 1773–1784.

552. De Guinoa JS, Barrio L, Mellado M, Carrasco YR. CXCL13/CXCR5 signaling enhances BCR-triggered B-cell activation by shaping cell dynamics. Blood [Internet] 2011; 118: 1560–1569. Available from: http://dx.doi.org/10.1182/blood-2011-01-332106

553. Cupedo T, Mebius RE. Role of chemokines in the development of secondary and tertiary lymphoid tissues. Semin Immunol 2003; 15: 243–248.

554. Salomonsson S, Larsson P, Tengnér P, Mellquist E, Hjelmström P, Wahren-Herlenius M. Expression of the B cell-attracting chemokine CXCL13 in the target organ and autoantibody production in ectopic lymphoid tissue in the chronic inflammatory disease Sjögren's syndrome. Scand J Immunol 2002; 55: 336–342.

555. Aqrawi LA, Brokstad KA, Jakobsen K, Jonsson R, Skarstein K. Low number of memory B cells in the salivary glands of patients with primary Sjögren's syndrome. Autoimmunity 2012; 45: 547–555.

556. Salomonsson S, Wahren-Herlenius M. Local production of Ro/SSA and La/SSB autoantibodies in the target organ coincides with high levels of circulating antibodies in sera of patients with Sjögren's syndrome. Scand J Rheumatol 2003; 32: 79–82.

557. Ramos-Casals M, Brito-Zeron P, Siso-Almirall A, Bosch X. Primary Sjogren syndrome. Bmj [Internet] 2012 [cited 2018 May 11]; 344: e3821–e3821. Available from: file:///C:/Users/fduar_000/Desktop/ramoscasals2012.pdf

558. Fox PC, Busch KA, Baum BJ. Subjective reports of xerostomia and objective measures of salivary gland performance. J Am Dent Assoc 1987; 115: 581–584.

559. Farrand KF, Fridman M, Stillman IÖ, Schaumberg DA. Prevalence of Diagnosed Dry Eye Disease in the United States Among Adults Aged 18 Years and Older. Am J Ophthalmol 2017; 182: 90–98.

560. Bernacchi E, Amato L, Parodi A, Cottoni F, Rubegni P, De Pità O, et al. Sjögren's syndrome: A retrospective review of the cutaneous features of 93 patients by the Italian Group of Immunodermatology. Clin Exp Rheumatol 2004; 22: 55–62.

561. Midilli R, Gode S, Oder G, Kabasakal Y, Karci B. Nasal and paranasal involvement in primary

Sjögren's syndrome. Rhinology 2013; 51: 265–267.

562. Papiris SA, Maniati M, Constantopoulos SH, Roussos C, Moutsopoulos HM, Skopouli FN. Lung involvement in primary Sjogren's syndrome is mainly related to the small airway disease. Ann Rheum Dis 1999; 58: 61–64.

563. Priori R, Minniti A, Derme M, Antonazzo B, Brancatisano F, Ghirini S, et al. Quality of sexual life in women with primary Sjögren syndrome. J Rheumatol 2015; 42: 1427–1431.

564. ter Borg EJ, Kelder JC. Lower prevalence of extra-glandular manifestations and anti-SSB antibodies in patients with primary Sjögren's syndrome and widespread pain: Evidence for a relatively benign subset. Clin Exp Rheumatol 2014; 32: 349–353.

565. Karageorgas T, Fragioudaki S, Nezos A, Karaiskos D, Moutsopoulos HM, Mavragani CP. Fatigue in Primary Sjögren's Syndrome: Clinical, Laboratory, Psychometric, and Biologic Associations. Arthritis Care Res 2016; 68: 123–131.

566. Wang HC, Chang K, Lin CY, Chen YH, Lu PL. Periodic fever as the manifestation of primary Sjogren's syndrome: A case report and literature review. Clin Rheumatol 2012; 31: 1517–1519.

567. Voulgarelis M, Moutsopoulos HM. Mucosa-Associated Lymphoid Tissue Lymphoma in Sjögren's Syndrome: Risks, Management, and Prognosis. Rheum Dis Clin North Am 2008; 34: 921–933.

568. Ng WF, Bowman SJ. Primary Sjogren's syndrome: too dry and too tired. Rheumatology (Oxford) 2010; 49: 844–853.

569. Vitali C, Del Papa N, Moutsopoulos HM, Vitali C, Bombardieri S, Jonsson R, et al. Pain in primary Sjögren's syndrome. Best Pract Res Clin Rheumatol [Internet] 2015 [cited 2017 Aug 29]; 29: 63–70. Available from: file:///C:/Users/fduar_000/Desktop/Pain in primary Sjögren's syndrome.pdf

570. Segal BM, Pogatchnik B, Henn L, Rudser K, Sivils KM. Pain severity and neuropathic pain symptoms in primary Sjögren's syndrome: A comparison study of seropositive and seronegative Sjögren's syndrome patients. Arthritis Care Res 2013; 65: 1291–1298.

571. Brito-Zerón P, Kostov B, Solans R, Fraile G, Suárez-Cuervo C, Casanovas A, et al. Systemic activity and mortality in primary Sjögren syndrome: Predicting survival using the EULAR-SS Disease Activity Index (ESSDAI) in 1045 patients. Ann Rheum Dis 2016; 75: 348–355.

572. Baldini C, Pepe P, Quartuccio L, Priori R, Bartoloni E, Alunno A, et al. Primary sjögren's syndrome as a multi-organ disease: Impact of the serological profile on the clinical presentation of the disease in a large cohort of Italian patients. Rheumatol (United Kingdom) [Internet] 2014 [cited 2018 May 13]; 53: 839–844. Available from: file:///C:/Users/fduar_000/Desktop/baldini2013.pdf

573. Gottenberg JE, Seror R, Miceli-Richard C, Benessiano J, Devauchelle-Pensec V, Dieude P, et al. Serum Levels of Beta2-Microglobulin and Free Light Chains of Immunoglobulins Are Associated with Systemic Disease Activity in Primary Sjögren's Syndrome. Data at Enrollment in the Prospective ASSESS Cohort. PLoS One 2013; 8.

574. Ramos-Casals M, Brito-Zerón P, Solans R, Camps MT, Casanovas A, Sopeña B, et al. Systemic involvement in primary Sjögren's syndrome evaluated by the EULAR-SS disease

activity index: Analysis of 921 spanish patients (GEAS-SS registry). Rheumatol (United Kingdom) [Internet] 2014 [cited 2018 May 12]; 53: 321–331. Available from: http://rheumatology.oxfordjournals.org/

575. Ramos-Casals M, Brito-Zerón P, Seror R, Bootsma H, Bowman SJ, Dörner T, et al. Characterization of systemic disease in primary Sjögren's syndrome: EULAR-SS Task Force recommendations for articular, cutaneous, pulmonary and renal involvements. Rheumatol (United Kingdom) 2015; 54: 2230–2238.

576. Mirouse A, Seror R, Vicaut E, Mariette X, Dougados M, Fauchais AL, et al. Arthritis in primary Sjögren's syndrome: Characteristics, outcome and treatment from French multicenter retrospective study. Autoimmun Rev 2019; 18: 9–14.

577. Riente L, Scirè CA, Delle Sedie A, Baldini C, Filippucci E, Meenagh G, et al. Ultrasound imaging for the rheumatologist XXIII. Sonographic evaluation of hand joint involvement in primary Sjögren's syndrome. Clin Exp Rheumatol 2009; 27: 747–750.

578. Colafrancesco S, Priori R, Gattamelata A, Picarelli G, Minniti A, Brancatisano F, et al. Myositis in primary Sjögren's syndrome: Data from a multicentre cohort. Clin Exp Rheumatol 2015; 33: 457–464.

579. Generali E, Costanzo A, Mainetti C, Selmi C. Cutaneous and Mucosal Manifestations of Sjögren's Syndrome. Clin Rev Allergy Immunol 2017; 53: 357–370.

580. Quartuccio L, Isola M, Corazza L, Ramos-Casals M, Retamozo S, Ragab GM, et al. Validation of the classification criteria for cryoglobulinaemic vasculitis. Rheumatol (United Kingdom) 2014; 53: 2209–2213.

581. Retamozo S, Acar-Denizli N, Rasmussen A, Horváth IF, Baldini C, Priori R, et al. Systemic manifestations of primary Sjögren's syndrome out of the ESSDAI classification: Prevalence and clinical relevance in a large international, multi-ethnic cohort of patients. Clin Exp Rheumatol 2019; 37: S97–S106.

582. García-Carrasco M, Sisó A, Ramos-Casals M, Rosas J, De la Red G, Gil V, et al. Raynaud's phenomenon in primary Sjögren's syndrome. Prevalence and clinical characteristics in a series of 320 patients [Internet]. Vol. 29, Journal of Rheumatology. 2002 [cited 2021 Feb 12]. Available from: http://www.jrheum.org/content/29/4/726http://www.jrheum.org/cgi/alerts/etochttp://jrheum.com/subscribe.htmlhttp://jrheum.com/reprints.htmlwww.jrheum.orgwww.jrheum.orgDownloadedfrom

583. Liang M, Bao L, Xiong N, Jin B, Ni H, Zhang J, et al. Cardiac arrhythmias as the initial manifestation of adult primary Sjögren's syndrome: A case report and literature review. Int J Rheum Dis 2015; 18: 800–806.

584. Gupta S, Ferrada MA, Hasni SA. Pulmonary manifestations of primary sjögren's syndrome: Underlying immunological mechanisms, clinical presentation, and management. Front Immunol 2019; 10: 1–9.

585. Kelly C, Gardiner P, Pal B, Griffiths I. Lung function in primary Sjogren's syndrome: A cross sectional and longitudinal study. Thorax 1991; 46: 180–183.

586. Kampolis CF, Fragkioudaki S, Mavragani CP, Zormpala A, Samakovli A, Moutsopoulos HM. Prevalence and spectrum of symptomatic pulmonary involvement in primary Sjögren's

syndrome. Clin Exp Rheumatol 2018; 36: S94–S101.

587. Roca F, Dominique S, Schmidt J, Smail A, Duhaut P, Lévesque H, et al. Interstitial lung disease in primary Sjögren's syndrome. Autoimmun Rev 2017; 16: 48–54.

588. Gao H, Zhang XW, He J, Zhang J, An Y, Sun Y, et al. Prevalence, risk factors, and prognosis of interstitial lung disease in a large cohort of Chinese primary Sjögren syndrome patients: A case-control study. Med (United States) 2018; 97.

589. Popov Y, Salomon-Escoto K. Gastrointestinal and Hepatic Disease in Sjogren Syndrome. Rheum Dis Clin North Am [Internet] 2018; 44: 143–151. Available from: https://doi.org/10.1016/j.rdc.2017.09.010

590. Geyl S, Jacques J, Parreau S, Cypierre A, Tabouret T, Gondran G, et al. Gastroparesis may be the cause of unexplained dyspepsia in patients with primary Sjögren syndrome. Rev Med Interne 2018; 39: 427–430.

591. de Carvalho JF, Lerner A, Gonçalves CM, Shoenfeld Y. Sjögren syndrome associated with protein-losing enteropathy: case-based review. Clin Rheumatol 2020; .

592. Szodoray P, Barta Z, Lakos G, Szakáll S, Zeher M. Coeliac disease in Sjögren's syndrome - A study of 111 Hungarian patients. Rheumatol Int 2004; 24: 278–282.

593. Zeron PB, Retamozo S, Bové A, Kostov BA, Sisó A, Ramos-Casals M. Diagnosis of liver involvement in primary sjögren syndrome. J Clin Transl Hepatol 2013; 1: 94–102.

594. Selmi C, Gershwin ME. Chronic Autoimmune Epithelitis in Sjögren's Syndrome and Primary Biliary Cholangitis: A Comprehensive Review. Rheumatol Ther 2017; 4: 263–279.

595. De Santis M, Crotti C, Selmi C. Liver abnormalities in connective tissue diseases. Best Pract Res Clin Gastroenterol [Internet] 2013; 27: 543–551. Available from: http://dx.doi.org/10.1016/j.bpg.2013.06.016

596. Tsianos E V., Hoofnagle JH, Fox PC, Alspaugh M, Jones EA, Schafer DF, et al. Sjögren's syndrome in patients with primary biliary cirrhosis. Hepatology 1990; 11: 730–734.

597. Afzelius P, Fallentin EM, Larsen S, Møller S, Schiødt M. Pancreatic function and morphology in Sjögren's syndrome. Scand J Gastroenterol 2010; 45: 752–758.

598. Goules A V., Tatouli IP, Moutsopoulos HM, Tzioufas AG. Clinically significant renal involvement in primary sjögren's syndrome: Clinical presentation and outcome. Arthritis Rheum 2013; 65: 2945–2953.

599. Evans R, Zdebik A, Ciurtin C, Walsh SB. Renal involvement in primary Sjögren's syndrome. Rheumatol (United Kingdom) 2015; 54: 1541–1548.

600. Geng Y, Zhao Y, Zhang Z. Tubulointerstitial nephritis-induced hypophosphatemic osteomalacia in sjögren's syndrome: A case report and review of the literature. Clin Rheumatol 2018; 37: 257–263.

601. Darrieutort-Laffite C, André V, Hayem G, Saraux A, Le Guern V, Le Jeunne C, et al. Sjögren's syndrome complicated by interstitial cystitis: A case series and literature review. Jt Bone Spine [Internet] 2015; 82: 245–250. Available from: http://dx.doi.org/10.1016/j.jbspin.2014.12.007

602. Alunno A, Carubbi F, Bartoloni E, Cipriani P, Giacomelli R, Gerli R. The kaleidoscope of neurological manifestations in primary Sjögren's syndrome. Clin Exp Rheumatol 2019; 37: S192–S198.

603. Fauchais AL, Magy L, Vidal E. Central and peripheral neurological complications of primary Sjögren's syndrome. Press Medicale [Internet] 2012; 41: e485–e493. Available from: http://dx.doi.org/10.1016/j.lpm.2012.06.002

604. Teixeira FDD, Moreira I, Silva AM, Vasconcelos C, Farinha F, Santos E. Neurological involvement in primary sjögren's syndrome. Acta Reumatol Port 2013; 38: 29–36.

605. Alegria GC, Guellec D, Mariette X, Gottenberg JE, Dernis E, Dubost JJ, et al. Epidemiology of neurological manifestations in Sjögren's syndrome: Data from the French ASSESS Cohort. RMD Open 2016; 2: 1–8.

606. Pavlakis PP, Alexopoulos H, Kosmidis ML, Mamali I, Moutsopoulos HM, Tzioufas AG, et al. Peripheral neuropathies in Sjögren's syndrome: A critical update on clinical features and pathogenetic mechanisms. J Autoimmun [Internet] 2012; 39: 27–33. Available from: http://dx.doi.org/10.1016/j.jaut.2012.01.003

607. Lefaucheur JP, Wahab A, Planté-Bordeneuve V, Sène D, Ménard-Lefaucheur I, Rouie D, et al. Diagnosis of small fiber neuropathy: A comparative study of five neurophysiological tests. Neurophysiol Clin [Internet] 2015; 45: 445–455. Available from: http://dx.doi.org/10.1016/j.neucli.2015.09.012

608. Moreira I, Teixeira F, Martins Silva A, Vasconcelos C, Farinha F, Santos E. Frequent involvement of central nervous system in primary Sjögren syndrome. Rheumatol Int 2015; 35: 289–294.

609. Qiao L, Wang Q, Fei Y, Zhang W, Xu Y, Zhang Y, et al. The clinical characteristics of primary sjogren's syndrome with neuromyelitis optica spectrum disorder in China: A STROBE-compliant article. Med (United States) 2015; 94: 1–6.

610. Birnbaum J, Atri NM, Baer AN, Cimbro R, Montagne J, Casciola-Rosen L. Relationship Between Neuromyelitis Optica Spectrum Disorder and Sjögren's Syndrome: Central Nervous System Extraglandular Disease or Unrelated, Co-Occurring Autoimmunity? Arthritis Care Res 2017; 69: 1069–1075.

611. Tobn GJ, Pers JO, Devauchelle-Pensec V, Youinou P. Neurological disorders in primary Sjögren's syndrome. Autoimmune Dis 2012; 1.

612. Segal BM, Pogatchnik B, Holker E, Liu H, Sloan J, Rhodus N, et al. Primary Sjogren's syndrome: Cognitive symptoms, mood, and cognitive performance. Acta Neurol Scand [Internet] 2012; 125: 272–278. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf

613. Ramos-Casals M, Font J, García-Carrasco M, Brito MP, Rosas J, Calvo-Alen J, et al. Primary Sjögren syndrome: Hematologic patterns of disease expression. Medicine (Baltimore) 2002; 81: 281–292.

614. Quartuccio L, Isola M, Baldini C, Priori R, Bartoloni Bocci E, Carubbi F, et al. Biomarkers of lymphoma in Sjögren's syndrome and evaluation of the lymphoma risk in prelymphomatous conditions: Results of a multicenter study. J Autoimmun [Internet] 2014; 51: 75–80. Available from: http://dx.doi.org/10.1016/j.jaut.2013.10.002

615. Mandl T, Bredberg A, Jacobsson LTH, Manthorpe R, Henriksson G. CD4+ T-lymphocytopenia--a frequent finding in anti-SSA antibody seropositive patients with primary Sjögren's syndrome. J Rheumatol 2004; 31: 726–728.

616. Kapsogeorgou EK, Voulgarelis M, Tzioufas AG. Predictive markers of lymphomagenesis in Sjögren's syndrome: From clinical data to molecular stratification. J Autoimmun [Internet] 2019; 104: 102316. Available from: https://doi.org/10.1016/j.jaut.2019.102316

617. Brito-Zerón P, Soria N, Muñoz S, Bové A, Akasbi M, Belenguer R, et al. Prevalence and Clinical Relevance of Autoimmune Neutropenia in Patients with Primary Sjögren's Syndrome. Semin Arthritis Rheum [Internet] 2009; 38: 389–395. Available from: http://dx.doi.org/10.1016/j.semarthrit.2008.01.014

618. Sun R, Gu W, Ma Y, Wang J, Wu M. Relapsed/refractory acquired thrombotic thrombocytopenic purpura in a patient with Sjögren syndrome: Case report and review of the literature. Med (United States) 2018; 97.

619. Ramos-Casals M, Brito-Zerón P, Yagüe J, Akasbi M, Bautista R, Ruano M, et al. Hypocomplementaemia as an immunological marker of morbidity and mortality in patients with primary Sjögren's syndrome. Rheumatology 2005; 44: 89–94.

620. Theander E, Henriksson G, Ljungberg O, Mandl T, Manthorpe R, Jacobsson LTH. Lymphoma and other malignancies in primary Sjögren's syndrome: A cohort study on cancer incidence and lymphoma predictors. Ann Rheum Dis [Internet] 2006; 65: 796–803. Available from: www.annrheumdis.com

621. Retamozo S, Brito-Zerón P, Ramos-Casals M. Prognostic markers of lymphoma development in primary Sjögren syndrome. Lupus 2019; 28: 923–936.

622. Nocturne G, Mariette X. Sjögren Syndrome-associated lymphomas: An update on pathogenesis and management. Br J Haematol 2015; 168: 317–327.

623. Goules A V., Tzioufas AG. Lymphomagenesis in Sjögren's syndrome: Predictive biomarkers towards precision medicine. Autoimmun Rev [Internet] 2019; 18: 137–143. Available from: https://doi.org/10.1016/j.autrev.2018.08.007

624. Baer AN, Ambinder RF. Lymphoproliferative disease in Sjögren's syndrome. Sjogren's Syndr A Clin Handb 2019; : 129–152.

625. Skarlis C, Argyriou E, Mavragani CP. Lymphoma in Sjögren's Syndrome: Predictors and Therapeutic Options. Curr Treat Options Rheumatol 2020; 6: 1–17.

626. Bournia V-K, Vlachoyiannopoulos PG. Subgroups of Sjögren syndrome patients according to serological profiles. J Autoimmun [Internet] 2012 [cited 2016 May 27]; 39: 15–26. Available from: http://www.sciencedirect.com/science/article/pii/S0896841112000364

627. Goules A V., Argyropoulou OD, Pezoulas VC, Chatzis L, Critselis E, Gandolfo S, et al. Primary Sjögren's Syndrome of Early and Late Onset: Distinct Clinical Phenotypes and Lymphoma Development. Front Immunol 2020; 11: 3–12.

628. Anquetil C, Hachulla E, MacHuron F, Mariette X, Le Guern V, Vittecoq O, et al. Is early-onset primary Sjögren's syndrome a worse prognosis form of the disease? Rheumatol (United Kingdom) [Internet] 2019 [cited 2021 Jan 23]; 58: 1163–1167. Available from: https://academic.oup.com/rheumatology/article/58/7/1163/5250863

629. Theander E, Jonsson R, Sjöström B, Brokstad K, Olsson P, Henriksson G. Prediction of Sjögren's syndrome years before diagnosis and identification of patients with early onset and severe disease course by autoantibody profiling. Arthritis Rheumatol 2015; 67: 2427–2436.

630. Yayla ME, Karaman Z, Torgutalp M, Keleşoğlu Dinçer AB, Aydemir Gülöksüz EG, Sezer S, et al. Early onset primary Sjögren syndrome, clinical and laboratory characteristics. Clin Rheumatol 2020; 39: 2689–2696.

631. Goules A V, Argyropoulou OD, Pezoulas VC, Chatzis L, Critselis E, Gandolfo S, et al. Primary Sjögren's Syndrome of Early and Late Onset: Distinct Clinical Phenotypes and Lymphoma Development. Front Immunol [Internet] 2020; 11: 1. Available from: www.frontiersin.org

632. Baer AN, Walitt B. Update on Sjögren Syndrome and Other Causes of Sicca in Older Adults. Rheum Dis Clin North Am [Internet] 2018; 44: 419–436. Available from: https://doi.org/10.1016/j.rdc.2018.03.002

633. Ozdemir M, Temizdemir H. Age- and gender-related tear function changes in normal population. Eye 2010; 24: 79–83.

634. Wolff A, Joshi RK, Ekström J, Aframian D, Pedersen AML, Proctor G, et al. A Guide to Medications Inducing Salivary Gland Dysfunction, Xerostomia, and Subjective Sialorrhea: A Systematic Review Sponsored by the World Workshop on Oral Medicine VI. Drugs R D 2017; 17.

635. Manzo C, Maslinska M. Primary Sjögren ' s Syndrome in the Elderly : Does Age of Onset Make a Difference ? EMJ Rheumatol 2018; .

636. Ramos-Casals M, Solans R, Rosas J, Camps MT, Gil A, Del Pino-Montes J, et al. Primary Sjögren syndrome in Spain: Clinical and immunologic expression in 1010 patients. Medicine (Baltimore) 2008; 87: 210–219.

637. Chebbi W, Ben Salem W, Klii R, Kessomtini W, Jerbi S, Sfar MH. Primitive Sjögren syndrome in the elderly: clinical and immunological characteristics. Pan Afr Med J 2015; 20: 8.

638. Kvarnström M, Ottosson V, Nordmark B, Wahren-Herlenius M. Incident cases of primary Sjögren's syndrome during a 5-year period in Stockholm County: A descriptive study of the patients and their characteristics. Scand J Rheumatol 2015; 44: 135–142.

639. Park Y, Lee J, Park SH, Kwok SK. Male patients with primary Sjögren's syndrome: A distinct clinical subgroup? Int J Rheum Dis 2020; 23: 1388–1395.

640. Ramírez Sepúlveda JI, Kvarnström M, Brauner S, Baldini C, Wahren-Herlenius M. Difference in clinical presentation between women and men in incident primary Sjögren's syndrome. Biol Sex Differ 2017; 8: 1–8.

641. Ramírez Sepúlveda JI, Kvarnström M, Eriksson P, Mandl T, Norheim KB, Johnsen SJ, et al. Long-term follow-up in primary Sjögren's syndrome reveals differences in clinical presentation between female and male patients. Biol Sex Differ 2017; 8: 1–9.

642. Chatzis L, Pezoulas VC, Ferro F, Gandolfo S, Donati V, Binutti M, et al. Sjögren's Syndrome: The Clinical Spectrum of Male Patients. J Clin Med 2020; 9: 2620.

643. Baer AN, Walitt B. Sjögren Syndrome and Other Causes of Sicca in Older Adults. Clin

Geriatr Med 2017; 33: 87–103.

644. Aljanobi H, Sabharwal A, Krishnakumar B, Kramer JM. Is it Sjögren's syndrome or burning mouth syndrome? Distinct pathoses with similar oral symptoms. Oral Surg Oral Med Oral Pathol Oral Radiol [Internet] 2017; 123: 482–495. Available from: http://dx.doi.org/10.1016/j.oooo.2017.01.005

645. Tsubota K, Yokoi N, Shimazaki J, Watanabe H, Dogru M, Yamada M, et al. New Perspectives on Dry Eye Definition and Diagnosis: A Consensus Report by the Asia Dry Eye Society [Internet]. Vol. 15, Ocular Surface. 2017 [cited 2019 Feb 16]. p. 65–76. Available from: www.theocularsurface.com

646. Trier NH, Nielsen IØ, Friis T, Houen G, Theander E. Comparison of antibody assays for detection of autoantibodies to Ro 52, Ro 60 and La associated with primary Sjögren's syndrome. J Immunol Methods [Internet] 2016 [cited 2016 Jun 13]; 433: 44–50. Available from: http://dx.doi.org/10.1016/j.jim.2016.03.001

647. Daniels TE, Cox D, Shiboski CH, Schiødt M, Wu A, Lanfranchi H, et al. Associations between salivary gland histopathologic diagnoses and phenotypic features of Sjögren's syndrome among 1,726 registry participants. Arthritis Rheum [Internet] 2011 [cited 2017 Oct 14]; 63: 2021–2030. Available from: file:///C:/Users/fduar_000/Desktop/daniels2011.pdf

648. Dawes C. How much saliva is enough for avoidance of xerostomia? Caries Res 2004; 38: 236–240.

649. Guggenheimer J, Moore PA. Xerostomia: Etiology, recognition and treatment. J Am Dent Assoc 2003; 134: 61–69.

650. Percival RS, Challacombe S, Marsh PD. Flow Rates of Resting Whole and Stimulated Parotid Saliva in Relation to Age and Gender. J Dent Res 1994; 73: 1416–1420.

651. Ship JA, Pillemer SR, Baum BJ. Progress in geriatrics. Xerostomia and the geriatric patient. J Am Geriatr Soc 2002; 50: 535–543.

652. Navazesh M, Kumar SKS. Measuring salivary flow. J Am Dent Assoc [Internet] 2008; 139: 35S-40S. Available from: http://dx.doi.org/10.14219/jada.archive.2008.0353

653. Baldini C, Ferro F, Elefante E, Bombardieri S. Biomarkers for Sjögren's syndrome. Biomark Med [Internet] 2018 [cited 2018 Nov 26]; 12: 275–286. Available from: file:///C:/Users/fduar_000/Desktop/baldini2018.pdf

654. Baldini C, Luciano N, Tarantini G, Pascale R, Sernissi F, Mosca M, et al. Salivary gland ultrasonography: a highly specific tool for the early diagnosis of primary Sjögren's syndrome. Arthritis Res Ther [Internet] 2015 [cited 2016 Jun 13]; 17: 146. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4461980&tool=pmcentrez&rendertype=abstract

655. Mossel E, Delli K, Van Nimwegen JF, Stel AJ, Kroese FGM, Spijkervet FKL, et al. Ultrasonography of major salivary glands compared with parotid and labial gland biopsy and classification criteria in patients with clinically suspected primary Sjögren's syndrome. Ann Rheum Dis [Internet] 2017 [cited 2017 Dec 2]; 76: 1883–1889. Available from: file:///C:/Users/fduar_000/Desktop/Ultrasonography of major salivary glands compared with parotid and labial gland biopsy and classification criteria in patients with clinically suspected primary Sjögren's syndrome.pdf

656. Jazzar A, Manoharan A, Brown JE, Shirlaw PJ, Carpenter GH, Challacombe SJ, et al. Predictive value of ultrasound scoring in relation to clinical and histological parameters in xerostomia patients. Oral Dis 2019; 25: 150–157.

657. Moss SE, Klein R, Klein BEK. Prevalance of and risk factors for dry eye syndrome. Arch Ophthalmol 2000; 118: 1264–1268.

658. Gabbriellini G, Baldini C, Varanini V, Notarstefano C, Pepe P, Fanucci F, et al. In vivo confocal scanning laser microscopy in patients with primary sjögren's syndrome: A monocentric experience. Mod Rheumatol 2015; 25: 585–589.

659. Sullivan BD, Crews LA, Sönmez B, de la Paz MF, Comert E, Charoenrook V, et al. Clinical Utility of Objective Tests for Dry Eye Disease. Cornea 2012; 31: 1000–1008.

660. Whitcher JP, Shiboski CH, Shiboski SC, Heidenreich AM, Kitagawa K, Zhang S, et al. A Simplified Quantitative Method for Assessing Keratoconjunctivitis Sicca From the Sjögren's Syndrome International Registry. Am J Ophthalmol [Internet] 2010 [cited 2019 Apr 25]; 149: 405–415. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3459675/pdf/nihms152739.pdf

661. Akiyama R, Usui T, Yamagami S. Diagnosis of dry eye by tear meniscus measurements using anterior segment swept source optical coherence tomography. In: Cornea 2015. p. S115–S120.

662. Serruya LG, Nogueira DC, Hida RY. Comparação entre os testes de Schirmer em olhos abertos e fechados em indivíduos normais. Arq Bras Oftalmol 2009; 72: 65–67.

663. Cho P, Yap M. Schirmer test. I. A review. Vol. 70, Optometry and Vision Science. 1993. p. 152–156.

664. Bijsterveld V. Diagnostic tests in the sicca syndrome. Arch Ophthalmol [Internet] 1969 [cited 2019 Apr 25]; 82: 10–14. Available from: file:///C:/Users/fduar_000/Desktop/van Bijsterveld1969 Diagnostic Tests in the Sicca Syndrome.pdf

665. Bron AJ, Evans VE, Smith JA. Grading of corneal and conjunctival staining in the context of other dry eye tests. Cornea [Internet] 2003; 22: 640–650. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14508260

666. Shiboski SC, Shiboski CH, Criswell LA, Baer AN, Challacombe S, Lanfranchi H, et al. American College of Rheumatology Classification criteria for Sjögren Syndrome: A data-driven, expert consensus approach in the SICCA cohort. Arthritis Care Res [Internet] 2012 [cited 2017 Oct 1]; 64: 475–487. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3349440/pdf/nihms347776.pdf

667. Mainstone JC, Bruce AS, Golding TR. Tear meniscus measurement in the diagnosis of dry eye. Curr Eye Res 1996; 15: 653–661.

668. Ang M, Baskaran M, Werkmeister RM, Chua J, Schmidl D, Aranha dos Santos V, et al. Anterior segment optical coherence tomography. Prog Retin Eye Res 2018; 66: 132–156.

669. Bai Y, Nichols JJ. Advances in thickness measurements and dynamic visualization of the tear film using non-invasive optical approaches. Prog Retin Eye Res 2017; 58: 28–44.

670. Ibrahim OMA, Dogru M, Kojima T, Matsumoto Y, Wakamatsu TH, Tsubota K, et al. OCT

assessment of tear meniscus after punctal occlusion in dry eye disease. Optom Vis Sci 2012; 89: 18–20.

671. Shen M, Wang J, Tao A, Chen Q, Lin S, Qu J, et al. Diurnal Variation of Upper and Lower Tear Menisci. Am J Ophthalmol 2008; 145.

672. Penner V, Rocha G. Use of the Visante for anterior segment ocular coherence tomography. Vol. 5, Techniques in Ophthalmology. 2007. p. 67–77.

673. Jalbert I. In vivo confocal microscopy of the human cornea. Br J Ophthalmol 2003; 87: 225–236.

674. Labbé A, Liang Q, Wang Z, Zhang Y, Xu L, Baudouin C, et al. Corneal nerve structure and function in patients with non-sjögren dry eye: Clinical correlations. Investig Ophthalmol Vis Sci 2013; 54: 5144–5150.

675. Azizi S, Uçak T, Yaşar I, Karakurt Y, Erdogan E, Salman I. Evaluation of the Corneal Layers in Meibomian-Gland-Dysfunction-Related Dry Eye by In Vivo Slit-Scanning Confocal Microscopy. Semin Ophthalmol 2017; 32: 377–383.

676. Patel D V., McGhee CNJ. In vivo laser scanning confocal microscopy confirms that the human corneal sub-basal nerve plexus is a highly dynamic structure. Investig Ophthalmol Vis Sci 2008; 49: 3409–3412.

677. Shen L, Suresh L. Autoantibodies, detection methods and panels for diagnosis of Sjögren's syndrome. Clin Immunol [Internet] 2017 [cited 2017 Dec 2]; 182: 24–29. Available from: file:///C:/Users/fduar_000/Desktop/Autoantibodies, detection methods and panels for diagnosis of Sjögren's syndrome.pdf

678. Itoh K, Itoh Y, Frank MB. Protein heterogeneity in the human Ro/SSA ribonucleoproteins the 52- and 60-kD Ro/SSA autoantigens are encoded by separate genes. J Clin Invest 1991; 87: 177–186.

679. Lee AYS. A review of the role and clinical utility of anti-Ro52/TRIM21 in systemic autoimmunity. Rheumatol Int 2017; 37: 1323–1333.

680. Espinosa A, Dardalhon V, Brauner S, Ambrosi A, Higgs R, Quintana FJ, et al. Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway. J Exp Med 2009; 206: 1661–1671.

681. Brauner S, Ivanchenko M, Thorlacius GE, Ambrosi A, Wahren-Herlenius M. The Sjögren's syndrome-associated autoantigen Ro52/TRIM21 modulates follicular B cell homeostasis and immunoglobulin production. Clin Exp Immunol 2018; 194: 315–326.

682. Kunishita Y, Yoshimi R, Kamiyama R, Kishimoto D, Yoshida K, Hashimoto E, et al. TRIM21 Dysfunction Enhances Aberrant B-Cell Differentiation in Autoimmune Pathogenesis. Front Immunol 2020; 11: 1–19.

683. Szczerba BM, Kaplonek P, Wolska N, Podsiadlowska A, Rybakowska PD, Dey P, et al. Interaction between innate immunity and Ro52-induced antibody causes Sjögren's syndrome-like disorder in mice. Ann Rheum Dis 2016; 75: 617–622.

684. Trzeciak M, Bagavant H, Papinska J, Deshmukh US. Immune response targeting Sjögren's syndrome antigen Ro52 suppresses tear production in female mice. Int J Mol Sci 2018; 19:

2935.

685. Boccitto M, Wolin SL. Ro60 and Y RNAs: structure, functions, and roles in autoimmunity. Crit Rev Biochem Mol Biol [Internet] 2019; 54: 133–152. Available from: https://doi.org/10.1080/10409238.2019.1608902

686. Xue D, Shi H, Smith JD, Chen X, Noe DA, Cedervall T, et al. A lupus-like syndrome develops in mice lacking the Ro 60-kDa protein, a major lupus autoantigen. Proc Natl Acad Sci U S A 2003; 100: 7503–7508.

687. Pruijn G, Slobbe R, van Venrooij W. Structure and Function of Ro and LA RNPs. Mol Biol Rep 1990; 14: 43–48.

688. Hsieh SC, Yu HS, Lin WW, Sun KH, Tsai CY, Huang DF, et al. Anti-SSB/La is one of the antineutrophil autoantibodies responsible for neutropenia and functional impairment of polymorphonuclear neutrophils in patients with systemic lupus erythematosus. Clin Exp Immunol 2003; 131: 506–516.

689. Boire G, Craft J. Human Ro ribonucleoprotein particles: Characterization of native structure and stable association with the La polypeptide. J Clin Invest 1990; 85: 1182–1190.

690. Scofield RH, Fayyaz A, Kurien BT, Koelsch KA. Prognostic value of Sjögren's syndrome autoantibodies. J Lab Precis Med 2018; 3: 92–92.

691. Tzioufas AG, Wassmuth R, Dafni UG, Guialis A, Haga HJ, Isenberg DA, et al. Clinical, immunological, and immunogenetic aspects of autoantibody production against Ro/SSA, La/SSB and their linear epitopes in primary Sjögren's syndrome (pSS): A European multicentre study. Ann Rheum Dis 2002; 61: 398–404.

692. Baer AN, DeMarco MMA, Shiboski SC, Lam MY, Challacombe S, Daniels TE, et al. The SSB-positive/SSA-negative antibody profile is not associated with key phenotypic features of Sjögren's syndrome. Ann Rheum Dis 2015; 74: 1557–1561.

693. Haneji N, Nakamura T, Takio K, Yanagi K, Higashiyama H, Saito I, et al. Identification of α-fodrin as a candidate autoantigen in primary Sjogren's syndrome. Science (80-) 1997; 276: 604–607.

694. Qin Q, Wang H, Wang HZ, Huang YL, Li H, Zhang WW, et al. Diagnostic accuracy of anti-alpha-fodrin antibodies for primary Sjögren's syndrome. Mod Rheumatol 2014; 24: 793–797.

695. Deng C, Hu C, Chen S, Li J, Wen X, Wu Z, et al. Meta-analysis of anti-muscarinic receptor type 3 antibodies for the diagnosis of Sjögren syndrome. PLoS One 2015; 10: 1–9.

696. Sumida T, Tsuboi H, Iizuka M, Asashima H, Matsumoto I. Anti-M3 muscarinic acetylcholine receptor antibodies in patients with Sjögren's syndrome. Mod Rheumatol 2013; 23: 841–845.

697. Namkoong E, Lee S woo, Kim N, Choi Y, Park K. Effect of anti-muscarinic autoantibodies on leukocyte function in Sjögren's syndrome. Mol Immunol [Internet] 2017; 90: 136–142. Available from: http://dx.doi.org/10.1016/j.molimm.2017.07.007

698. Suresh L, Malyavantham K, Shen L, Ambrus JL. Investigation of novel autoantibodies in Sjogren's syndrome utilizing Sera from the Sjogren's international collaborative clinical

alliance cohort. BMC Ophthalmol [Internet] 2015 [cited 2016 Jun 13]; 15: 38. Available from: http://www.biomedcentral.com/1471-2415/15/38

699. De Langhe E, Bossuyt X, Shen L, Malyavantham K, Ambrus JL, Suresh L. Evaluation of Autoantibodies in Patients with Primary and Secondary Sjogren's Syndrome. Open Rheumatol J 2017; 11: 10–15.

700. Xuan J, Wang Y, Xiong Y, Qian H, He Y, Shi G. Investigation of autoantibodies to SP-1 in Chinese patients with primary Sjögren's syndrome. Clin Immunol [Internet] 2018; 188: 58–63. Available from: http://dx.doi.org/10.1016/j.clim.2017.12.008

701. Nardi N, Brito-Zerón P, Ramos-Casals M, Aguiló S, Cervera R, Ingelmo M, et al. Circulating auto-antibodies against nuclear and non-nuclear antigens in primary Sjögren's syndrome. Clin Rheumatol 2006; 25: 341–346.

702. Martel C, Gondran G, Launay D, Lalloué F, Palat S, Lambert M, et al. Active immunological profile is associated with systemic Sjögren's syndrome. J Clin Immunol 2011; 31: 840–847.

703. Fauchais AL, Martel C, Gondran G, Lambert M, Launay D, Jauberteau MO, et al. Immunological profile in primary Sjögren syndrome. Clinical significance, prognosis and long-term evolution to other auto-immune disease. Autoimmun Rev 2010; 9: 595–599.

704. Soto ME, Hernández-Becerril N, Perez-Chiney AC, Hernández-Rizo A, Telich-Tarriba JE, Juárez-Orozco LE, et al. Predictive value of antinuclear antibodies in autoimmune diseases classified by clinical criteria: Analytical study in a specialized health institute, one year follow-up. Results Immunol [Internet] 2015; 5: 13–22. Available from: http://dx.doi.org/10.1016/j.rinim.2013.10.003

705. Ingegnoli F, Castelli R, Gualtierotti R. Rheumatoid factors: Clinical applications. Dis Markers 2013; 35: 727–734.

706. Pertovaara M, Pukkala E, Laippala P, Miettinen A, Pasternack A. A longitudinal cohort study of Finnish patients with primary Sjögren's syndrome: Clinical, immunological, and epidemiological aspects. Ann Rheum Dis 2001; 60: 467–472.

707. ter Borg E-J, Risselada AP, Kelder JC. Relation of systemic autoantibodies to the number of extraglandular manifestations in primary Sjögren's Syndrome: a retrospective analysis of 65 patients in the Netherlands. Semin Arthritis Rheum [Internet] 2011 [cited 2016 May 26]; 40: 547–551. Available from: http://www.sciencedirect.com/science/article/pii/S0049017210001459

708. Chung JK, Kim MK, Wee WR. Prognostic factors for the clinical severity of keratoconjunctivitis sicca in patients with Sjögren's syndrome. Br J Ophthalmol 2012; 96: 240–245.

709. Peen E, Mellbye OJ, Haga HJ. IgA rheumatoid factor in primary Sjögren's syndrome. Scand J Rheumatol 2009; 38: 46–49.

710. Maślińska M, Mańczak M, Kwiatkowska B. Usefulness of rheumatoid factor as an immunological and prognostic marker in PSS patients. Clin Rheumatol 2019; 38: 1301–1307.

711. Vitali C, Moutsopoulos HM, Bombardieri S. The European Community Study Group on Diagnostic Criteria for Sjögren's Syndrome. Sensitivity and specificity of tests for ocular and

oral involvement in Sjogren's syndrome. Ann Rheum Dis 1994; 53: 637–647.

712. Waterhouse JP, Doniach I. Post-Mortem Prevalence of Focal Lymphocytic Adenitis of the Submandibular Salivary Gland. J Pathol Bacteriol [Internet] 1966; 91: 53–64. Available from: doi: 10.1002/path.1700910107

713. Vitali C, Bombardieri S, Moutsopoulos HM, Balestrieri G, Bencivelli W, Bernstein RM, et al. Preliminary criteria for the classification of Sjögren's syndrome. Results of a prospective concerted action supported by the European community. Arthritis Rheum [Internet] 1993 [cited 2018 Apr 29]; 36: 340–347. Available from: file:///C:/Users/fduar_000/Desktop/art.1780360309.pdf

714. Stewart CM, Bhattacharyya I, Berg K, Cohen DM, Orlando C, Drew P, et al. Labial salivary gland biopsies in Sjögren's syndrome: still the gold standard? Oral Surgery, Oral Med Oral Pathol Oral Radiol Endodontology 2008; 106: 392–402.

715. Costa S, Quintin-Roué I, Lesourd A, Jousse-Joulin S, Berthelot JM, Hachulla E, et al. Reliability of histopathological salivary gland biopsy assessment in sjögren's syndrome: A multicentre cohort study. Rheumatol (United Kingdom) 2015; 54: 1056–1064.

716. Sharma R, Chaudhari KS, Kurien BT, Grundahl K, Radfar L, Lewis DM, et al. Sjögren syndrome without focal lymphocytic infiltration of the salivary glands. J Rheumatol 2020; 47: 394–399.

717. Vivas ÁJ, Bautista-Vargas M, Portacio S, Garcés-Palacio A, Urbano MA, Agualimpia A, et al. Reproducibility of minor salivary gland biopsy reports in Sjögren's syndrome and its correlation with disease biomarkers. Clin Rheumatol 2021; .

718. Wicheta S, Van der Groen T, Faquin WC, August M. Discrepancies in Interpretation of the Minor Salivary Gland Biopsy in the Diagnosis of Sjögren Syndrome. J Oral Maxillofac Surg [Internet] 2019; 77: 1628–1635. Available from: https://doi.org/10.1016/j.joms.2019.01.052

719. Morbini P, Manzo A, Caporali R, Epis O, Villa C, Tinelli C, et al. Multilevel examination of minor salivary gland biopsy for Sjogren's syndrome significantly improves diagnostic performance of AECG classification criteria. Arthritis Res Ther 2005; 7: 343–348.

720. Fisher BA, Jonsson R, Daniels T, Bombardieri M, Brown RM, Morgan P, et al. Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome. Ann Rheum Dis [Internet] 2017 [cited 2017 Oct 14]; 76: 1161–1168. Available from: http://ard.bmj.com/content/annrheumdis/76/7/1161.full.pdf

721. Carubbi F, Alunno A, Cipriani P, Di Benedetto P, Ruscitti P, Berardicurti O, et al. Is minor salivary gland biopsy more than a diagnostic tool in primary Sjögren's syndrome? Association between clinical, histopathological, and molecular features: A retrospective study. Semin Arthritis Rheum 2014; 44: 314–324.

722. Greenspan JS, Daniels TE, Talal N, Sylvester RA. The histopathology of Sjögren's syndrome in labial salivary gland biopsies. Oral Surgery, Oral Med Oral Pathol 1974; 37: 217–229.

723. Xanthou G, Tapinos NI, Polihronis M, Nezis IP, Margaritis LH, Moutsopoulos HM. CD4 cytotoxic and dendritic cells in the immunopathologic lesion of Sjogren's syndrome. Clin Exp Immunol 1999; 118: 154–163.

724. Pontarini E, Lucchesi D, Bombardieri M. Current views on the pathogenesis of Sjögren's syndrome. Curr Opin Rheumatol 2018; 30: 215–221.

725. Johnsen SJ, Brun JG, Gøransson LG, Johannesen TB, Haldorsen K, Harboe E, et al. Risk of non-Hodgkin's lymphoma in primary Sjögren's syndrome: A population-based study. Arthritis Care Res 2013; 65: 816–821.

726. Johnson SR, Goek ON, Singh-Grewal D, Vlad SC, Feldman BM, Felson DT, et al. Classification criteria in rheumatic diseases: A review of methodologie properties. Arthritis Care Res [Internet] 2007 [cited 2018 Sep 11]; 57: 1119–1133. Available from: file:///C:/Users/fduar_000/Desktop/johnson2007.pdf

727. Fries JF, Hochberg MC, Medsger TA, Hunder GG, Bombardier C. Criteria for rheumatic disease. different types and different functions. Arthritis Rheum 1994; 37: 454–462.

728. June RR, Aggarwal R. The use and abuse of diagnostic/classification criteria. Best Pract Res Clin Rheumatol [Internet] 2014 [cited 2017 May 21]; 28: 921–934. Available from: http://ac.els-cdn.com/S152169421500011X/1-s2.0-S152169421500011X-main.pdf?_tid=2dd5efe8-3e43-11e7-b93b-00000aab0f6b&acdnat=1495384571_a3bf2af6e0854e304c6e9d2365c34b0b

729. Miettinen OS. The modern scientific physician: 8. Educational preparation. Cmaj 2001; 165: 1501–1503.

730. Skopouli FN, Drosos AA, Papaioannou T, Moutsopoulos HM. Preliminary diagnostic criteria for Sjogren's syndrome. Scand J Rheumatol 1986; 15: 22–25.

731. Fox RI, Robinson CA, Curd JG, Kozin F, Howelly F V. Sjögren's syndrome. Proposed criteria for classification. Arthritis Rheum 1986; 29: 577–585.

732. Homma M, Tojo T, Akizuki M, Yamagata H. Criteria for Sjogren's syndrome in Japan. Scand J Rheumatol 1986; 61: 26–27.

733. Lee J, Koh JH, Kim J-W, Sung Y-K, Lee S-S, Choe JY, et al. Performance of the 2016 ACR-EULAR classification criteria for primary Sjogren's syndrome in a Korean cohort. Rheumatol Int [Internet] 2018; 38: 1651–1660. Available from: http://link.springer.com/10.1007/s00296-018-4109-y

734. Le Goff M, Cornec D, Jousse-Joulin S, Guellec D, Costa S, Marhadour T, et al. Comparison of 2002 AECG and 2016 ACR/EULAR classification criteria and added value of salivary gland ultrasonography in a patient cohort with suspected primary Sjögren's syndrome. Arthritis Res Ther [Internet] 2017 [cited 2018 Apr 29]; 19: 269. Available from: https://arthritis-research.biomedcentral.com/track/pdf/10.1186/s13075-017-1475-x

735. Takagi Y, Nakamura H, Sumi M, Shimizu T, Hirai Y, Horai Y, et al. Combined classification system based on ACR/EULAR and ultrasonographic scores for improving the diagnosis of Sjögren's syndrome. PLoS One 2018; 13: 1–15.

736. van Nimwegen JF, Mossel E, Delli K, van Ginkel MS, Stel AJ, Kroese FGM, et al. Incorporation of Salivary Gland Ultrasonography Into the American College of Rheumatology/European League Against Rheumatism Criteria for Primary Sjögren's Syndrome. Arthritis Care Res 2020; 72: 583–590.

737. Jousse-Joulin S, Gatineau F, Baldini C, Baer A, Barone F, Bootsma H, et al. Weight of

salivary gland ultrasonography compared to other items of the 2016 ACR/EULAR classification criteria for Primary Sjögren's syndrome. J Intern Med 2020; 287: 180–188.

738. Shiboski CHCH, Shiboski SCSC, Seror R, Criswell LALA, Labetoulle M, Lietman TMTM, et al. 2016 American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjögren's syndrome. Ann Rheum Dis [Internet] 2017; 76: 9–16. Available from: http://ard.bmj.com/lookup/doi/10.1136/annrheumdis-2016-210571

739. Noaiseh G, Baer AN. Toward better outcomes in Sjögren's syndrome: The promise of a stratified medicine approach. Best Pract Res Clin Rheumatol 2020; 34.

740. Krylova L, Isenberg D. Assessment of patients with primary Sjögren's syndrome-outcome over 10 years using the Sjögren's Syndrome Damage Index. Rheumatology 2010; 49: 1559–1562.

741. Vitali C, Palombi G, Baldini C, Benucci M, Bombardieri S, Covelli M, et al. Sjögren's syndrome disease damage index and disease activity index: Scoring systems for the assessment of disease damage and disease activity in Sjögren's syndrome, derived from an analysis of a cohort of Italian patients. Arthritis Rheum 2007; 56: 2223–2231.

742. Barry RJ, Sutcliffe N, Isenberg DA, Price E, Goldblatt F, Adler M, et al. The sjögren's syndrome damage Index - A damage index for use in clinical trials and observational studies in primary sjögren's syndrome. Rheumatology 2008; 47: 1193–1198.

743. Lackner A, Ficjan A, Stradner MH, Hermann J, Unger J, Stamm T, et al. It's more than dryness and fatigue: The patient perspective on health-related quality of life in Primary SjoÈgren's Syndrome - A qualitative study. PLoS One 2017; 12: 1–12.

744. Seror R, Gottenberg JE, Devauchelle-Pensec V, Dubost JJ, Le Guern V, Hayem G, et al. ESSDAI and ESSPRI: EULAR indexes for a complete picture of primary Sjögren's syndrome patients. Arthritis Care Res (Hoboken) [Internet] 2013; . Available from: http://www.ncbi.nlm.nih.gov/pubmed/23436737

745. Schmalz G, Patschan S, Patschan D, Ziebolz D. Oral-Health-Related Quality of Life in Adult Patients with Rheumatic Diseases—A Systematic Review. J Clin Med 2020; 9: 1172.

746. Slade GD. Derivation and validation of a short-form oral health impact profile. Community Dent Oral Epidemiol 1997; 25: 284–290.

747. Amaral JA, Sanches C, Marques D, Vaz Patto J, Barcelos F, Mata A. Validation of oral health impact profile-14 and its association with hypossialia in a Sjögren's Syndrome Portuguese population. Acta Reumatol Port 2018; 2018: 137–145.

748. Mangione CM, Lee PP, Gutierrez PR, Spritzer K, Coleman AL. Development of the 25-item National Eye Institute visual function questionnaire. Evidence-Based Eye Care 2002; 3: 58–59.

749. Schiffman RM, Christianson MD, Jacobsen G, Hirsch JD, Reis BL. Reliability and validity of the ocular surface disease index. Arch Ophthalmol 2000; 118: 615–621.

750. Özcura F, Aydin S, Helvaci MR. Ocular surface disease index for the diagnosis of dry eye syndrome. Ocul Immunol Inflamm 2007; 15: 389–393.

751. Amparo F, Schaumberg DA, Dana R. Comparison of Two Questionnaires for Dry Eye

Symptom Assessment: The Ocular Surface Disease Index and the Symptom Assessment in Dry Eye. Ophthalmology [Internet] 2015; 122: 1498–1503. Available from: http://dx.doi.org/10.1016/j.ophtha.2015.02.037

752. Bezzina OM, Gallagher P, Mitchell S, Bowman SJ, Griffiths B, Hindmarsh V, et al. Subjective and Objective Measures of Dryness Symptoms in Primary Sjögren's Syndrome – Capturing the discrepancy. Arthritis Care Res (Hoboken) 2016; .

753. Alunno A, Bartoloni E, Valentini V, La Paglia GMC, Valentini E, Leone MC, et al. Discrepancy between subjective symptoms, objective measures and disease activity indexes: The lesson of primary sjögren's syndrome. Clin Exp Rheumatol 2018; 36: S210–S214.

754. Lendrem D, Mitchell S, McMeekin P, Gompels L, Hackett K, Bowman S, et al. Do the EULAR Sjogren's syndrome outcome measures correlate with health status in primary Sjogren's syndrome? Rheumatology [Internet] 2015 [cited 2018 May 1]; 54: 655–659. Available from: https://academic.oup.com/rheumatology/article-lookup/doi/10.1093/rheumatology/keu361

755. Bowman SJ, Sutcliffe N, Isenberg DA, Goldblatt F, Adler M, Price E, et al. Sjögren's Systemic Clinical Activity Index (SCAI) - A systemic disease activity measure for use in clinical trials in primary Sjögren's syndrome. Rheumatology 2007; 46: 1845–1851.

756. Bowman SJ, Booth DA, Platts RG, Situnayake D, Gordon C, Hanu-Cernat L, et al. Measurement of fatigue and discomfort in primary Sjögren's syndrome using a new questionnaire tool. Rheumatology 2004; 43: 758–764.

757. Bowman SJ, Booth DA, Platts RG, Field A, Rostron J. Validation of the Sicca Symptoms Inventory for clinical studies of Sjögren's syndrome. J Rheumatol 2003; 30: 1259–1266.

758. Kersten P, White PJ, Tennant A. Is the pain visual analogue scale linear and responsive to change? An exploration using rasch analysis. PLoS One 2014; 9.

759. Seror R, Ravaud P, Mariette X, Bootsma H, Theander E, Hansen A, et al. EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI): Development of a consensus patient index for primary Sjögren's syndrome. Ann Rheum Dis 2011; 70: 968–972.

760. Seror R, Theander E, Brun JG, Ramos-Casals M, Valim V, Dörner T, et al. Validation of EULAR primary Sjögren's syndrome disease activity (ESSDAI) and Patient indexes (ESSPRI). Ann Rheum Dis 2015; 74: 859–866.

761. Seror R, Ravaud P, Bowman SJ, Baron G, Tzioufas A, Theander E, et al. EULAR Sjogren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjogren's syndrome. Ann Rheum Dis [Internet] 2010; 69: 1103–1109. Available from: http://ard.bmj.com/cgi/doi/10.1136/ard.2009.110619

762. Seror R, Bowman SJ, Brito-Zeron P, Theander E, Bootsma H, Tzioufas A, et al. EULAR Sjögren's syndrome disease activity index (ESSDAI): a user guide. RMD open [Internet] 2015 [cited 2017 Nov 12]; 1: e000022. Available from: http://dx.doi.org/10.1136/

763. Seror R, Rauz S, Gosset M, Bowman SJ. Disease activity and patient reported outcome measures in Sjögren's – what are the best tools to evaluate? Rheumatology 2019; .

764. Quartuccio L, Salvin S, Fabris M, Maset M, Pontarini E, Isola M, et al. Blys upregulation in Sjö gren's syndrome associated with lymphoproliferative disorders, higher ESSDAI score

and B-cell clonal expansion in the salivary glands. Rheumatol (United Kingdom) 2013; 52: 276–281.

765. Seror R, Bootsma H, Saraux A, Bowman SJ, Theander E, Brun JG, et al. Defining disease activity states and clinically meaningful improvement in primary Sjögren's syndrome with EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient-reported indexes (ESSPRI). Ann Rheum Dis [Internet] 2016 [cited 2018 Feb 27]; 75: 382–389. Available from: http://dx.doi.org/10.1136/

766. de Wolff L, Arends S, van Nimwegen JF, Bootsma H. Ten years of the ESSDAI: is it fit for purpose? Clin Exp Rheumatol 2020; 38: 283–290.

767. Lendrem D, Mitchell S, McMeekin P, Bowman S, Price E, T Pease C, et al. Health-related utility values of patients with primary Sjögren's syndrome and its predictors. Ann Rheum Dis 2014; 73: 1362–1368.

768. Cornec D, Devauchelle-Pensec V, Mariette X, Jousse-Joulin S, Berthelot JM, Perdriger A, et al. Development of the Sjögren's Syndrome Responder Index, a data-driven composite endpoint for assessing treatment efficacy. Rheumatol (United Kingdom) 2015; 54: 1699–1708.

769. Arends S, Van Nimwegen J, Verstappen G, Vissink A, Ray N, Bootsma H. Composite of Relevant Endpoints for Sjogren'S Syndrome (Cress). Ann Rheum Dis 2020; 79: 1022–1023.

770. Fisher BA, Everett CC, Rout J, O'Dwyer JL, Emery P, Pitzalis C, et al. Effect of rituximab on a salivary gland ultrasound score in primary Sjögren's syndrome: results of the TRACTISS randomised double-blind multicentre substudy. Ann Rheum Dis 2018; 77: 412–416.

771. Cornec D, Jousse-Joulin S, Costa S, Marhadour T, Marcorelles P, Berthelot JM, et al. High-grade salivary-gland involvement, assessed by histology or ultrasonography, is associated with a poor response to a single rituximab course in primary Sjögren's syndrome: Data from the TEARS randomized trial. PLoS One 2016; 11: 1–11.

772. Tarn JR, Howard-Tripp N, Lendrem DW, Mariette X, Saraux A, Devauchelle-Pensec V, et al. Symptom-based stratification of patients with primary Sjögren's syndrome: multi-dimensional characterisation of international observational cohorts and reanalyses of randomised clinical trials. Lancet Rheumatol 2019; 1: e85–e94.

773. Mingueneau M, Boudaoud S, Haskett S, Reynolds TL, Nocturne G, Norton E, et al. Cytometry by time-of-flight immunophenotyping identifies a blood Sjögren's signature correlating with disease activity and glandular inflammation. J Allergy Clin Immunol [Internet] 2016 [cited 2016 Jun 13]; 137: 1809–1821. Available from: http://dx.doi.org/10.1016/j.jaci.2016.01.024

774. van der Heijden EHM, Blokland SLM, Hillen MR, Lopes APP, van Vliet-Moret FM, Rosenberg AJWP, et al. Leflunomide–hydroxychloroquine combination therapy in patients with primary Sjögren's syndrome (RepurpSS-I): a placebo-controlled, double-blinded, randomised clinical trial. Lancet Rheumatol 2020; 2: e260–e269.

775. James JA, Guthridge JM, Chen H, Lu R, Bourn RL, Bean K, et al. Unique Sjögren's syndrome patient subsets defined by molecular features. Rheumatol (United Kingdom) 2020; 59: 860–868.

776. Price EJ, Rauz S, Tappuni AR, Sutcliffe N, Hackett KL, Barone F, et al. The British Society for

Rheumatology guideline for the management of adults with primary Sjögren's Syndrome. Rheumatology 2017; 56: e24–e48.

777. Vivino FB, Carsons SE, Foulks G, Daniels TE, Parke A, Brennan MT, et al. New Treatment Guidelines for Sjögren's Disease. Rheum Dis Clin North Am [Internet] 2016; 42: 531–551. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0889857X16300217%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/27431353

778. Zero DT, Brennan MT, Daniels TE, Papas A, Stewart C, Pinto A, et al. Clinical practice guidelines for oral management of Sjögren disease: Dental caries prevention. J Am Dent Assoc [Internet] 2016; 147: 295–305. Available from: http://dx.doi.org/10.1016/j.adaj.2015.11.008

779. Foulks GN, Forstot SL, Donshik PC, Forstot JZ, Goldstein MH, Lemp MA, et al. Clinical guidelines for management of dry eye associated with Sjögren disease. Ocul Surf 2015; 13: 118–132.

780. Furness S, Bryan G, Mcmillan R, Birchenough S, Worthington H V. Interventions for the management of dry mouth: Non-pharmacological interventions. Cochrane Database Syst Rev 2013; 2013.

781. Al Hamad A, Lodi G, Porter S, Fedele S, Mercadante V. Interventions for dry mouth and hyposalivation in Sjögren's syndrome: A systematic review and meta-analysis. Oral Dis 2019; 25: 1027–1047.

782. Furness S, Worthington H V, Bryan G, Birchenough S, McMillan R. Interventions for the management of dry mouth: non-systemic therapies. Cochrane Database Syst Rev 2011; : 2–4.

783. Carsons SE, Vivino FB, Parke A, Carteron N, Sankar V, Brasington R, et al. Treatment Guidelines for Rheumatologic Manifestations of Sjögren's - Use of Biologics, Management of Fatigue and Inflammatory Musculoskeletal Pain. Arthritis Care Res (Hoboken) [Internet] 2016 [cited 2016 Oct 5]; . Available from: http://doi.wiley.com/10.1002/acr.22968

784. Hernández-Molina G, Valim V, Secco A, Atisha-Fregoso Y, Guerra E, Adrover M, et al. Do antimalarials protect against damage accrual in primary Sjögren's syndrome? Results from a Latin-American retrospective cohort. Clin Exp Rheumatol 2018; 36: S182–S185.

785. Gottenberg JE, Ravaud P, Puéchal X, Le Guern V, Sibilia J, Goeb V, et al. Effects of hydroxychloroquine on symptomatic improvement in primary sjögren syndrome: The JOQUER randomized clinical trial. JAMA - J Am Med Assoc 2014; 312: 249–258.

786. Leverenz DL, St. Clair EW. Recent advances in the search for a targeted immunomodulatory therapy for primary sjögren's syndrome [version 1; peer review: 2 approved]. F1000Research 2019; 8: 1–12.

787. Pijpe J, Van Imhoff GW, Spijkervet FKL, Roodenburg JLN, Wolbink GJ, Mansour K, et al. Rituximab treatment in patients with primary Sjögren's syndrome: An open-label phase II study. Arthritis Rheum 2005; 52: 2740–2750.

788. Devauchelle-Pensec V, Pennec Y, Morvan J, Pers JO, Daridon C, Jousse-Joulin S, et al. Improvement of Sjögren's syndrome after two infusions of rituximab (anti-CD20). Arthritis Care Res 2007; 57: 310–317.

789. Dass S, Bowman SJ, Vital EM, Ikeda K, Pease CT, Hamburger J, et al. Reduction of fatigue in Sjögren syndrome with rituximab: Results of a randomised, double-blind, placebo-controlled pilot study. Ann Rheum Dis 2008; 67: 1541–1544.

790. Meijer JM, Meiners PM, Vissink A, Spijkervet FKL, Abdulahad W, Kamminga N, et al. Effectiveness of rituximab treatment in primary sjögren's syndrome: A randomized, double-blind, placebo-controlled trial. Vol. 62, Arthritis and Rheumatism. 2010. 960–968 p.

791. Everett S, Vishwanath S, Cavero V, Shen L, Suresh L, Malyavantham K, et al. Analysis of novel Sjogren's syndrome autoantibodies in patients with dry eyes. BMC Ophthalmol [Internet] 2017 [cited 2018 Jan 15]; 17. Available from: file:///C:/Users/fduar_000/Desktop/12886_2017_Article_412.pdf

792. Devauchelle-Pensec V, Mariette X, Jousse-Joulin S, Berthelot J-M, Perdriger A, Puéchal X, et al. Treatment of Primary Sjögren Syndrome With Rituximab. Ann Intern Med [Internet] 2014; 160: 233–242. Available from: http://www.uab.edu/medicine/rheumatology/images/AG_-_Devauchelle-Pensec_et_al_2014.pdf

793. Gandolfo S, De Vita S. Emerging drugs for primary Sjögren's syndrome. Expert Opin Emerg Drugs [Internet] 2019; 24: 121–132. Available from: https://doi.org/10.1080/14728214.2019.1634052

794. Felten R, Scher F, Sibilia J, Gottenberg JE, Arnaud L. The pipeline of targeted therapies under clinical development for primary Sjögren's syndrome: A systematic review of trials. Autoimmun Rev [Internet] 2019; 18: 576–582. Available from: https://doi.org/10.1016/j.autrev.2018.12.008

795. Karnell JL, Rieder SA, Ettinger R, Kolbeck R. Targeting the CD40-CD40L pathway in autoimmune diseases: Humoral immunity and beyond. Adv Drug Deliv Rev [Internet] 2019; 141: 92–103. Available from: https://doi.org/10.1016/j.addr.2018.12.005

796. Dörner T, Posch MG, Li Y, Petricoul O, Cabanski M, Milojevic JM, et al. Treatment of primary sjögren's syndrome with ianalumab (vay736) targeting b cells by baff receptor blockade coupled with enhanced, antibody-dependent cellular cytotoxicity. Ann Rheum Dis 2019; 78: 641–647.

797. Dörner T, Bowman SJ, Fox R, Mariette X, Papas A, Grader-Beck T, et al. Op0302 Ianalumab (Vay736), a Dual Mode of Action Biologic Combining Baff Receptor Inhibition With B Cell Depletion, Reaches Primary Endpoint for Treatment of Primary Sjogren'S Syndrome. Ann Rheum Dis 2020; 79: 187.1-188.

798. Posada J, Valadkhan S, Burge D, Davies K, Tarn J, Casement J, et al. Improvement of Severe Fatigue Following Nuclease Therapy in Patients With Primary Sjögren's Syndrome: A Randomized Clinical Trial. Arthritis Rheumatol 2021; 73: 143–150.

799. van der Heijden EHM, Hartgring SAY, Kruize AA, Radstake TRDJ, van Roon JAG. Additive immunosuppressive effect of leflunomide and hydroxychloroquine supports rationale for combination therapy for Sjögren's syndrome. Expert Rev Clin Immunol [Internet] 2019; 15: 801–808. Available from: https://doi.org/10.1080/1744666X.2019.1624527

800. Del Papa N, Vitali C. Management of primary Sjögren's syndrome: recent developments and new classification criteria. Ther Adv Musculoskel Dis 2018, 2018; 10: 39–54.

801. Pringle S, Wang X, Verstappen GMPJ, Terpstra JH, Zhang CK, He A, et al. Salivary Gland Stem Cells Age Prematurely in Primary Sjögren's Syndrome. Arthritis Rheumatol 2019; 71: 133–142.

802. Verstappen GM, Kroese FGM, Bootsma H. Stumbles in Sjögren's syndrome drug development: where to look for the next big leap? Expert Rev Clin Immunol [Internet] 2020; 16: 1043–1045. Available from: https://doi.org/10.1080/1744666X.2021.1831915

803. McCormick N, Marra CA, Aviña-Zubieta JA. Productivity Losses and Costs in the Less-Common Systemic Autoimmune Rheumatic Diseases. Curr Rheumatol Rep 2017; 19.

804. Meijer JM, Meiners PM, Slater JJRH, Spijkervet FKL, Kallenberg CGM, Vissink A, et al. Health-related quality of life, employment and disability in patients with sjögren's syndrome. Rheumatology 2009; 48: 1077–1082.

805. Mandl T, Jorgensen TS, Skougaard M, Olsson P, Kristensen LE. Work disability in newly diagnosed patients with primary sjögren syndrome. J Rheumatol 2017; 44: 209–215.

806. Singh AG, Singh S, Matteson EL. Rate, risk factors and causes of mortality in patients with sjögren's syndrome: A systematic review and meta-analysis of cohort studies. Rheumatol (United Kingdom) 2016; 55: 450–460.

807. Retamozo S, Gheitasi H, Quartuccio L, Kostov B, Corazza L, Bové A, et al. Cryoglobulinaemic vasculitis at diagnosis predicts mortality in primary Sjögren syndrome: Analysis of 515 patients. Rheumatol (United Kingdom) 2016; 55: 1443–1451.

808. Maciel G, Crowson CS, Matteson EL, Cornec D. Incidence and Mortality of Physician-Diagnosed Primary Sjögren Syndrome: Time Trends Over a 40-Year Period in a Population-Based US Cohort. Mayo Clin Proc [Internet] 2017; 92: 734–743. Available from: file:///C:/Users/Carla Carolina/Desktop/Artigos para acrescentar na qualificação/The impact of birth weight on cardiovascular disease risk in the.pdf

809. Horvath IF, Szanto A, Papp G, Zeher M. Clinical course, prognosis, and cause of death in primary Sjögren's syndrome. J Immunol Res 2014; 2014.

810. LeBien T, Tedder T. B lymphocytes: how they develop and function. Blood [Internet] 2008 [cited 2017 Dec 3]; 112: 1570–1580. Available from: file:///C:/Users/fduar_000/Desktop/1570.full.pdf

811. Binard A, Le Pottier L, Devauchelle-Pensec V, Saraux A, Youinou P, Pers J-O. Is the blood B-cell subset profile diagnostic for Sjögren's syndrome? Ann Rheum Dis [Internet] 2009 [cited 2016 Aug 6]; 68: 1447–1452. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18782791

812. Bohnhorst JØ, Thoen JE, Natvig JB, Thompson KM. Significantly depressed percentage of CD27+ (memory) B cells among peripheral blood B cells in patients with primary Sjogren's syndrome. Scand J Immunol 2001; 54: 421–427.

813. Cornec D, Saraux A, Pers J-O, Jousse-Joulin S, Marhadour T, Roguedas-Contios A-M, et al. Diagnostic accuracy of blood B-cell subset profiling and autoimmunity markers in Sjögren's syndrome. Arthritis Res Ther [Internet] 2014 [cited 2016 May 27]; 16: R15. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3978459&tool=pmcentrez&rendertype=abstract

814. Salomonsson S, Jonsson M V., Skarstein K, Brokstad KA, Hjelmström P, Wahren-Herlenius M, et al. Cellular Basis of Ectopic Germinal Center Formation and Autoantibody Production in the Target Organ of Patients With Sjögren's Syndrome. Arthritis Rheum 2003; 48: 3187–3201.

815. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. J Exp Med 2010; 207: 365–378.

816. Psianou K, Panagoulias I, Papanastasiou AD, de Lastic AL, Rodi M, Spantidea PI, et al. Clinical and immunological parameters of Sjögren's syndrome. Autoimmun Rev [Internet] 2018; 17: 1053–1064. Available from: https://doi.org/10.1016/j.autrev.2018.05.005

817. Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. J Exp Med 2011; 208: 987–999.

818. Pasoto SG, Natalino RR, Chakkour HP, Viana VDST, Bueno C, Leon EP, et al. EBV reactivation serological profile in primary Sjögren's syndrome: An underlying trigger of active articular involvement? Rheumatol Int 2013; 33: 1149–1157.

819. Kivity S, Arango MT, Ehrenfeld M, Tehori O, Shoenfeld Y, Anaya JM, et al. Infection and autoimmunity in Sjogren's syndrome: A clinical study and comprehensive review. J Autoimmun 2014; 51: 17–22.

820. Aqrawi LA, Ivanchenko M, Björk A, Ramírez Sepúlveda JI, Imgenberg-Kreuz J, Kvarnström M, et al. Diminished CXCR5 expression in peripheral blood of patients with Sjögren's syndrome may relate to both genotype and salivary gland homing. Clin Exp Immunol 2018; 192: 259–270.

821. Pontarini E, Murray-Brown WJ, Croia C, Lucchesi D, Conway J, Rivellese F, et al. Unique expansion of IL-21+ Tfh and Tph cells under control of ICOS identifies Sjögren's syndrome with ectopic germinal centres and MALT lymphoma. Ann Rheum Dis 2020; : 1588–1599.

822. Gao CY, Yao Y, Li L, Yang SH, Chu H, Tsuneyama K, et al. Tissue-Resident Memory CD8+ T Cells Acting as Mediators of Salivary Gland Damage in a Murine Model of Sjögren's Syndrome. Arthritis Rheumatol [Internet] 2019 [cited 2020 Jul 23]; 71: 121–132. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/art.40676

823. Maier-Moore JS, Koelsch KA, Smith K, Lessard CJ, Radfar L, Lewis D, et al. Antibody-secreting cell specificity in labial salivary glands reflects the clinical presentation and serology in patients with sjögren's syndrome. Arthritis Rheumatol 2014; 66: 3445–3456.

824. Jin L, Weiqian C, Lihuan Y. Peripheral CD24hi CD27+ CD19+ B cells subset as a potential biomarker in naive systemic lupus erythematosus. Int J Rheum Dis [Internet] 2013 [cited 2018 Jul 17]; 16: 698–708. Available from: file:///C:/Users/fduar_000/Desktop/jin2013.pdf

825. Salomon S, Guignant C, Morel P, Flahaut G, Brault C, Gourguechon C, et al. Th17 and CD24hiCD27+regulatory B lymphocytes are biomarkers of response to biologics in rheumatoid arthritis. Arthritis Res Ther 2017; 19: 1–10.

826. Benítez Del Castillo JM, Wasfy MAS, Fernandez C, Garcia-Sanchez J. An in vivo confocal masked study on corneal epithelium and subbasal nerves in patients with dry eye. Investig Ophthalmol Vis Sci 2004; .

827. Kalteniece A, Ferdousi M, Adam S, Schofield J, Azmi S, Petropoulos I, et al. Corneal confocal microscopy is a rapid reproducible ophthalmic technique for quantifying corneal nerve abnormalities. PLoS One 2017; 12: 1–10.

828. Chan TCY, Wan KH, Shih KC, Jhanji V. Advances in dry eye imaging: The present and beyond. Br J Ophthalmol 2018; 102: 295–301.

829. Benítez-Del-Castillo JM, Acosta MC, Wassfi MA, Díaz-Valle D, Gegúndez JA, Fernandez C, et al. Relation between corneal innervation with confocal microscopy and corneal sensitivity with noncontact esthesiometry in patients with dry eye. Investig Ophthalmol Vis Sci [Internet] 2007; 48: 173–181. Available from: http://iovs.arvojournals.org/article.aspx?doi=10.1167/iovs.06-0127

830. Petropoulos IN, Ponirakis G, Khan A, Gad H, Almuhannadi H, Brines M, et al. Corneal confocal microscopy: ready for prime time. Vol. 103, Clinical and Experimental Optometry. 2020. p. 265–277.

831. Tuisku IS, Konttinen YT, Konttinen LM, Tervo TM. Alterations in corneal sensitivity and nerve morphology in patients with primary Sjögren's syndrome. Exp Eye Res [Internet] 2008; 86: 879–885. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0014483508000882

832. El-Fayoumi D, Youssef MM, Khafagy MM, Badr El Dine N, Gaber W. Assessment of Corneal and Tear Film Parameters in Rheumatoid Arthritis Patients Using Anterior Segment Spectral Domain Optical Coherence Tomography. Ocul Immunol Inflamm 2018; 26: 632–638.

833. Villani E, Galimberti D, Viola F, Mapelli C, Ratiglia R. The cornea in Sjögren's syndrome: An in vivo confocal study. Investig Ophthalmol Vis Sci 2007; 48: 2017–2022.

834. Dabbah MA, Graham J, Petropoulos IN, Tavakoli M, Malik RA. Automatic analysis of diabetic peripheral neuropathy using multi-scale quantitative morphology of nerve fibres in corneal confocal microscopy imaging. Med Image Anal 2011; 15: 738–747.

835. Cruzat A, Pavan-Langston D, Hamrah P. In vivo confocal microscopy of corneal nerves: Analysis and clinical correlation. Semin Ophthalmol 2010; 25: 171–177.

836. Gálvez J, Sáiz E, López P, Pina MF, Carrillo A, Nieto A, et al. Diagnostic evaluation and classification criteria in Sjögren's Syndrome. Jt Bone Spine [Internet] 2009 [cited 2016 May 27]; 76: 44–49. Available from: http://www.sciencedirect.com/science/article/pii/S1297319X08001991

837. Barcelos F, Martins C, Papoila A, Geraldes C, Cardigos J, Nunes G, et al. Association between memory B-cells and clinical and immunological features of primary Sjögren's syndrome and Sicca patients. Rheumatol Int [Internet] 2018 [cited 2018 Jul 17]; 38: 1063–1073. Available from: https://doi.org/10.1007/s00296-018-4018-0

838. Zhao Y, Zheng WJ, Zhou W, Zeng XF, Zhang FC, Tang FL, et al. The evaluation of the international classification criteria (2002) for primary Sjögren's syndrome in Chinese patients. APLAR J Rheumatol 2005; 8: 184–187.

ANEXO 1

Table 3. The EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI): Domain and item definitions and weights.

Domain [Weight]	Activity level	Description
Constitutional [3] *Exclusion of fever of infectious origin and voluntary weight loss*	No = 0	Absence of the following symptoms
	Low = 1	Mild or intermittent fever (37.5°-38.5°C) / night sweats and/or involuntary weight loss of 5 to 10% of body weight
	Moderate = 2	Severe fever (>38.5°C) / night sweats and/or involuntary weight loss of >10% of body weight
Lymphadenopathy [4] *Exclusion of infection*	No = 0	Absence of the following features
	Low = 1	Lymphadenopathy ≥ 1 cm in any nodal region or ≥ 2 cm in inguinal region
	Moderate = 2	Lymphadenopathy ≥ 2 cm in any nodal region or ≥ 3 cm in inguinal region, and/or splenomegaly (clinically palpable or assessed by imaging)
	High = 3	Current malignant B-cell proliferative disorder
Glandular [2] *Exclusion of stone or infection*	No = 0	Absence of glandular swelling
	Low =1	Small glandular swelling with enlarged parotid (≤ 3 cm), or limited submandibular or lachrymal swelling
	Moderate = 2	Major glandular swelling with enlarged parotid (> 3 cm), or important submandibular or lachrymal swelling
Articular [2] *Exclusion of osteoarthritis*	No = 0	Absence of currently active articular involvement
	Low = 1	Arthralgias in hands, wrists, ankles and feet accompanied by morning stiffness (>30 min)
	Moderate = 2	1 to 5 (of 28 total count) synovitis
	High = 3	≥ 6 (of 28 total count) synovitis
Cutaneous [3] *Rate as "No activity" stable long-lasting features related to damage*	No = 0	Absence of currently active cutaneous involvement
	Low =1	Erythema multiforma
	Moderate = 2	Limited cutaneous vasculitis, including urticarial vasculitis, or purpura limited to feet and ankle, or subacute cutaneous lupus
	High = 3	Diffuse cutaneous vasculitis, including urticarial vasculitis, or diffuse purpura, or ulcers related to vasculitis
Pulmonary [5] *Rate as "No activity" stable long-lasting features related to damage, or respiratory involvement not related to the disease (tobacco use etc.)*	No =0	Absence of currently active pulmonary involvement
	Low = 1	Persistent cough or bronchial involvement with no radiographic abnormalities on radiography Or radiological or HRCT evidence of interstitial lung disease with: No breathlessness and normal lung function test.
	Moderate = 2	Moderately active pulmonary involvement, such as interstitial lung disease shown by HRCT with shortness of breath on exercise (NHYA II) or abnormal lung function tests restricted to: 70% >DL_{CO}≥ 40% or 80%>FVC≥60%
	High = 3	Highly active pulmonary involvement, such as interstitial lung disease shown by HRCT with shortness of breath at rest (NHYA III, IV) or with abnormal lung function tests: DL_{CO}< 40% or FVC< 60%
Renal [5] *Rate as "No activity" stable long-lasting features related to damage, and renal involvement not related to the disease.* *If biopsy has been performed, please rate activity based on* ***histological*** *features first*	No = 0	Absence of currently active renal involvement with proteinuria< 0.5 g/d, no hematuria, no leucocyturia, no acidosis, or long-lasting stable proteinuria due to damage
	Low = 1	Evidence of mild active renal involvement, limited to tubular acidosis without renal failure or glomerular involvement with proteinuria (between 0.5 and 1 g/d) and without hematuria or renal failure (GFR ≥60 ml/min)
	Moderate = 2	Moderately active renal involvement, such as tubular acidosis with renal failure (GFR <60 ml/min) or glomerular involvement with proteinuria between 1 and 1.5 g/d and without hematuria or renal failure (GFR ≥60 ml/min) or histological evidence of extra-membranous glomerulonephritis or important interstitial lymphoid infiltrate
	High = 3	Highly active renal involvement, such as glomerular involvement with proteinuria >1.5 g/d or hematuria or renal failure (GFR <60 ml/min), or histological evidence of proliferative glomerulonephritis or cryoglobulinemia related renal involvement

Muscular [6] *Exclusion of weakness due to corticosteroids*	No = 0	Absence of currently active muscular involvement
	Low = 1	Mild active myositis shown by abnormal EMG or biopsy with no weakness and creatine kinase (N <CK ≤ 2N)
	Moderate = 2	Moderately active myositis proven by abnormal EMG or biopsy with weakness (maximal deficit of 4/5), or elevated creatine kinase (2N <CK ≤4N),
	High = 3	Highly active myositis shown by abnormal EMG or biopsy with weakness (deficit ≤ 3/5) or elevated creatine kinase (>4N)
PNS [5] *Rate as "No activity" stable long-lasting features related to damage or PNS involvement not related to the disease*	No = 0	Absence of currently active PNS involvement
	Low = 1	Mild active peripheral nervous system involvement, such as pure sensory axonal polyneuropathy shown by NCS or trigeminal (V) neuralgia
	Moderate = 2	Moderately active peripheral nervous system involvement shown by NCS, such as axonal sensory-motor neuropathy with maximal motor deficit of 4/5, pure sensory neuropathy with presence of cryoglobulinemic vasculitis, ganglionopathy with symptoms restricted to mild/moderate ataxia, inflammatory demyelinating polyneuropathy (CIDP) with mild functional impairment (maximal motor deficit of 4/5or mild ataxia), Or cranial nerve involvement of peripheral origin (except trigeminal (V) neralgia)
	High = 3	Highly active PNS involvement shown by NCS, such as axonal sensory-motor neuropathy with motor deficit ≤3/5, peripheral nerve involvement due to vasculitis (mononeuritis multiplex etc.), severe ataxia due to ganglionopathy, inflammatory demyelinating polyneuropathy (CIDP) with severe functional impairment: motor deficit ≤3/5 or severe ataxia
CNS [5] *Rate as "No activity" stable long-lasting features related to damage or CNS involvement not related to the disease*	No = 0	Absence of currently active CNS involvement
	Low = 1	Moderately active CNS features, such as cranial nerve involvement of central origin, optic neuritis or multiple sclerosis-like syndrome with symptoms restricted to pure sensory impairment or proven cognitive impairment
	High = 3	Highly active CNS features, such as cerebral vasculitis with cerebrovascular accident or transient ischemic attack, seizures, transverse myelitis, lymphocytic meningitis, multiple sclerosis-like syndrome with motor deficit.
Hematological [2] *For anemia, neutropenia, and thrombopenia, only auto-immune cytopenia must be considered* *Exclusion of vitamin or iron deficiency, drug-induced cytopenia*	No = 0	Absence of auto-immune cytopenia
	Low = 1	Cytopenia of auto-immune origin with neutropenia (1000 < neutrophils < 1500/mm3), and/or anemia (10 < hemoglobin < 12 g/dl), and /or thrombocytopenia (100,000 < platelets < 150,000/mm3) Or lymphopenia (500 < lymphocytes < 1000/mm3)
	Moderate = 2	Cytopenia of auto-immune origin with neutropenia (500 ≤ neutrophils ≤ 1000/mm3), and/or anemia (8 ≤ hemoglobin ≤ 10 g/dl), and/or thrombocytopenia (50,000 ≤ platelets ≤ 100,000/mm3) Or lymphopenia (≤500/mm3)
	High = 3	Cytopenia of auto-immune origin with neutropenia (neutrophils < 500/mm3), and/or or anemia (hemoglobin < 8 g/dl) and/or thrombocytopenia (platelets <50,000/mm3)
Biological [1]	No = 0	Absence of any of the following biological feature
	Low = 1	Clonal component and/or hypocomplementemia (low C4 or C3 or CH50) and/or hypergammaglobulinemia or high IgG level between 16 and 20 g/L
	Moderate = 2	Presence of cryoglobulinemia and/or hypergammaglobulinemia or high IgG level > 20 g/L, and/or recent onset hypogammaglobulinemia or recent decrease of IgG level (<5 g/L)

CIDP= chronic inflammatory demyelinating polyneuropathy; CK= creatine kinase; CNS= central nervous system; DLCO= diffusing CO capacity; EMG= electromyogram; FVC= forced vital capacity; GFR= glomerular filtration rate; Hb= hemoglobin; HRCT= high-resolution computed tomography; IgG= immunoglobulin G; NCS= nerve conduction studies; NHYA= New York heart association classification; Plt= platelet; PNS=peripheral nervous system;