UNESP - Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

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Frequência de centros germinativos e caracterização de células dendríticas mieloides e plasmocitoides na síndrome de Sjögren

Araraquara

2021

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Dissertação apresentada à Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara para obtenção do título de Mestre em Ciências Odontológicas, Área de Diagnóstico e Cirurgia.

Orientador: Prof. Dr. Jorge Esquiche León

Araraquara
2021

S586f

Silva, Evânio Vilela da
Frequência de centros germinativos e caracterização de células dendríticas mieloides e plasmocitoides na síndrome de Sjögren / Evânio Vilela da Silva. -- Araraquara, 2021
64 f.

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara
Orientador: Jorge Esquiche León

1. Síndrome de Sjögren. 2. Glândulas salivares menores. 3. Centro germinativo. 4. Células dendríticas. 5. Imuno-histoquímica. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Odontologia, Araraquara. Dados fornecidos pelo autor(a).

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Comissão julgadora

Dissertação para obtenção do grau de Mestre em Ciências Odontológicas

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Araraquara, 05 de março de 2021.

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Dedico este trabalho ao meu irmão, **Henrique**. Tu és amor, luz, fortaleza e leveza em minha vida. Amo-te!

AGRADECIMENTOS

Aqui estou à agradecer por mais uma conquista em minha vida. Eu paro, penso e reflito todas as batalhas travadas no caminho até chegar aqui, e encho-me de felicidades por inúmeras coisas boas que me foram agraciadas. Para tal, sempre estive cercado da presença divina de Deus e pessoas especiais, as quais foram fundamentais para que a caminhada se tornasse mais leve; e eu jamais poderia deixar de demonstrá-los a minha gratidão.

Agradeço a **Deus**, por tua presença em minha vida, sempre me abençoando com muita fé, coragem e esperança. Por me mostrar os caminhos da vida, permitir sonhar, me levantar nos momentos difíceis e ser meu refúgio e fortaleza. A ti, Senhor, toda minha devoção.

Aos meus pais, **Cláudia** e **Eraldo**, por todo amor, ensinamentos, confiança e abdicações para que pudesse me oportunizar crescer. Mãe, tu és meu maior exemplo de mãe, mulher, determinação e coragem. Pai, você é força, persistência e simplicidade. Eu, sou a junção de cada detalhe contido em vocês. Eu amo muito vocês!

Aos meus irmãos, **Camilla** e **Henrique,** pelo amor, união e compartilhamento de uma história juntos. Minha irmã, que alegria eu tenho de te ver feliz buscando seus objetivos; seja muito feliz. Meu irmão, Ah meu irmão! você é a maior riqueza da nossa família, um presente de Deus. Tua pureza me encanta e me motiva. Sempre irei te defender das circunstâncias da vida. Essa conquista é nossa!

Aos meus familiares, avós paternos, **Antônio** e **Irene** (in memoriam), avós maternos **Sebastião** (in memoriam) e **Zulmira,** por todo afeto, carinho, oração e ensinamentos que me foram atribuídos; quantas lembranças boas carrego em meu coração. Aos meus tios, em especial **Edsônia, Eliane, Everaldo, Gorete, Ivanilda, Marilene, Marlene** e **Marinês,** e primos, por participar da minha trajetória sempre me incentivando de alguma forma. Gratidão!

Aos meus professores da graduação na Universidade Federal de Sergipe, em especial a professora **Ignez Aurora**, minha maior inspiração na Odontologia, por todas as oportunidades de aprender e vivenciar a Odontologia, pela amizade e conselhos, sempre me mostrando um mundo de possibilidades; e a professora **Sílvia**

Sousa, sempre prestativa, com ensinamentos valiosos, me apresentando o mundo da histopatologia, até então desconhecido.

Aos meus amigos **Brunelly**, **Letícia Pacheco, Lucas, Ludmilla**, **Mellany**, **Nailson**, **Norma Jean**, **Rafaela, Raquel, Rayle, Rayssa**, **Sthefanne** e **Yasmin**, que estão sempre se fazendo presentes, enviando boas energias. Vocês são muito importantes! E à minha querida amiga, **Fernanda Felix,** por compartilhar uma amizade nutrida de boas conversas, discussões de casos e vivências da pós-graduação. Obrigado por tanto!

Ao meu orientador, professor **Jorge León,** por todos os ensinamentos e oportunidades. Sua expertise no diagnóstico clinicopatológico é supreendente; aprendo bastante com você. Grato, mais uma vez, pelas constantes contribuições para o meu amadurecimento pessoal e profissional.

À equipe do Diagnóstico Bucal da FOAr, as professores, **Andreia Bufalino, Cláudia, Elaine, Luciana** e **Mirian,** por todas as contribuições na sedimentação do conhecimento científico e humanizado; e os amigos que compõe à equipe, **Audrey, Camila, Maria Leticia** e **Túlio,** os quais tive e tenho a honra de compartilhar grande alegrias, admiração, ciência e respeito. À vocês, todo sucesso na caminhada.

Aos demais amigos de pós-graduação da FOAr/UNESP, **Analú, Básia, Bianca, Déborah, Eduardo, Lucas Portela** e **Renato,** pela convivência, compartilhamento de alegrias e perrengues da vida de pós-graduando; muito sucesso para vocês. De forma particular, agradeço à minha amiga, **Jessica Katarine**, por toda amizade, carinho e respeito que foi se consolidando nessa trajetória; te admiro muito, és de uma força gigante, nunca esqueça disso. Grato por tanto! À querida, **Laís Grifoni**, pela amizade construída, receptvidade e vivência compartilhada. Te desejo muito sucesso! Ao meu amigo, **Luiz Henrique**, pela amizade, bondade, companheirismo e por compartilhar de tantas boas histórias, desde a aprovação no mestrado até aqui, de uma forma tão leve. Tenho muito respeito e admiração por você.

Na Faculdade de Odontologia de Ribeirão Preto, onde realizei parte do meu mestrado, tive a surpresa e alegria de contar com mais pessoas, as quais nutro uma relação de harmonia, e que foram fundamentais para chegada até aqui. Em especial, ao meu amigo, **Heitor¸** por toda dedicação, companheirismo, conversas, disposição em ajudar e por todos ensinamentos na prática da patologia; muita prosperidade, você

merece. À minha amiga, **Xiomara,** por toda generosidade, determinação e leveza que carrega consigo. Que alegria poder compartilhar essa trajetória contigo. E a minha amiga, **Beatriz,** pelas conversas, alegrias e determinação que tornam os dias mais leves na rotina da pós-graduação. Muito sucesso em sua vida.

Aos docentes e servidores administrativos da **Faculdade de Odontologia de Araraquara - Universidade Estadual Paulista**, aqui representados pelo Diretor Professor **Edson Alves de Campos.** Que orgulho tenho de fazer parte desta instituição.

Ao **Programa de Pós-Graduação em Ciências Odontológicas**, pela oportunidade de cursar o mestrado, aqui representado pela Coordenadora professora **Fernanda Lourenção Brighenti.**

À **Seção Técnica de Pós-graduação (STPG)**, em especial ao **Cristiano Afonso**, por toda eficiência e disposição para sanar quaisquer questões burocráticas pertinentes ao programa de pós-graduação.

À **Faculdade de Odontologia de de Ribeirão Preto – Universidade de São Paulo,** pela contribuição nesta trajetória.

E, por fim, à **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES)** pelo apoio na realização deste trabalho - Código de financiamento 001.

[...]
“A vida é uma corrida
que não se corre sozinho.
E vencer não é chegar,
é aproveitar o caminho
sentindo o cheiro das flores
e aprendendo com as dores
causadas por cada espinho.”

* Bráulio Bessa

* Bessa B. Poesia que transforma. 2018.

Silva EV. Frequência de centros germinativos e caracterização de células dendríticas mieloides e plasmocitoides na síndrome de Sjögren [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2021.

RESUMO

A síndrome de Sjögren (SS) é uma doença autoimune crônica sistêmica que afeta principalmente as glândulas salivares e lacrimais, ocasionando xerostomia e ceratoconjuntivite *sicca*, respectivamente. A histopatologia de biópsias de glândulas salivares menores (GSm) exibindo sialadenite linfocítica focal (SLF) com um escore focal ≥1, é um critério diagnóstico importante para definir o dignóstico de SS. Em alguns casos, a SLF mostra a presença de centros germinativos (CGs). Alguns estudos sugerem que CGs na SLF pode ser preditivo para o desenvolvimento de linfoma em pacientes com SS. As células dendríticas (CDs) são eficientes células apresentadoras de antígenos e foram sugeridas agir modulando a ativação de células T e B na SS; no entanto, a sua contribuição precisa ser melhor esclarecida. Este estudo analisou a frequência de CGs e a presença de CDs mieloides e plasmocitoides em biópsias de GSm de pacientes com SS primária (SSp). Cinquenta casos de SSp (grupo SSp) e 31 casos de lesões orais reativas (não SS, não sicca, grupo controle) contendo também características típicas de SLF, foram avaliados por análise morfológica e imunoistoquímica (CD10, CD23 e Bcl-6), visando à detecção de CGs. Além desses, para caracterizar a presença de CDs, foram utilizados os seguintes anticorpos CD1a, CD207, CD123 e CD303. A análise morfológica revelou CGs em 20% (10/50) e 19% (6/31) do grupo SSp e controle, respectivamente. O número de focos foram significativamente maiores no grupo SSp do que no grupo controle com FS <1. A imunoistoquímica confirmou todos os achados morfológicos (CG mostrando positividade para CD23 e Bcl-6, com expressão variável de CD10) e adicionalmente em 3 e 1 casos do grupo SSp e controle, respectivamente. A análise imunoistoquímica para CDs, no grupo SSp, revelou uma população heterogênea de células de Langerhans (CLs) CD1a+ e CD207+ localizadas preferencialmente em padrão periductal, e de CDs plasmocitoides (CDp) CD123+ e CD303+ distribuídas na periferia dos infiltrados inflamatórios; enquanto que, no grupo controle, essas células foram escassas (CLs, $p<0,05$). Em conclusão, este trabalho mostra que a SLF também pode ser observada quando examinando lesões orais reativas, as quais apresentaram frequência de CGs semelhante às encontradas em pacientes com SSp. Ainda, a imunoexpressão de marcadores de CDs em pacientes com SSp sugere a participação dessas células na perpetuação do processo imunoinflamatório na SSp, contribuindo na elucidação de alvos imunoterapêuticos.

Palavras chave: Síndrome de Sjogren. Glândulas salivares menores. Centro germinativo. Células dendríticas. Imuno-histoquímica.

Silva EV. Frequency of germinal centers and characterization of myeloid and plasmacytoid dendritic cells in Sjögren's syndrome [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2021.

ABSTRACT

Sjögren's syndrome (SS) is a chronic autoimmune disease that mainly affects the salivary and lacrimal glands, causing xerostomia and ceratoconjunctivitis sicca, respectively. Histopathology of minor salivary glands (mGSs) biopsies showing the presence of focal lymphocytic sialadenitis (FLS) with focal score ≥1 is an important diagnostic criterion to define SS. In some cases, the FLS shows the presence of germinal centers (GCs).Some studies suggest that GCs in FLS may be predictive for lymphoma development in SS patients. Dendritic cells (CDs) are efficient antigen presenting cells and have been suggested to act by modulating the activation of T- and B-cells in SS, however, their contribution needs to be further clarified. This study analyzed the frequency of GC and the presence of myeloid and plasmacytoid DCs in mGSs biopsies of patients with primary SS (pSS).Fifty cases of primary SS (pSS group) and 31 cases of oral reactive lesions (non-SS non-sicca, control group) containing also typical FLS features, were assessed by morphological and immunohistochemical (CD10, CD23 and Bcl-6) analysis, aiming at the detection of GCs. In addition to these, to characterize the presence of DCs, the following antibodies CD1a, CD207, CD123 and CD303 were used. The morphological analysis revealed CGs in 20% (10/50) and 19% (6/31) of the pSS and control group, respectively. The FS and number of foci were significantly higher in pSS than control group with FS<1. Immunohistochemistry confirmed all morphological findings (GCs showing CD23 and Bcl-6 positivity, with variable CD10 expression) and additionally in 3 and 1 cases of the pSS and control group, respectively. Moreover, another 6 and 2 cases of the pSS and control group with FS≥1, respectively, showed positivity only for CD23. Immunohistochemical analysis for DCs, in the pSS group, revealed a heterogeneous population of CD1a+ and CD207+ Langerhans cells, preferably located in a periductal pattern, and of CD123+ and CD303+ plasmacytoid DCs (pDCs) distributed on the periphery of inflammatory infiltrates; whereas, in the control group, these cells are scarce (LCs, p<0,05). In conclusion, this study demonstrated that FLS can also be observed when assessing of reactive oral lesions, which showed similar frequency of GCs with those found in SSp patients. In addition, the immunoexpression of DCs in pSS patients suggest the participation of these cells in the perpetuation of the inflammatory process in pSS, contributing to the elucidation of immunotherapeutic targets.

Keywords: Sjogren’s syndrome. Salivary Glands, minor. Germinal center. Dendritic cells. Immunohistochemistry.

SUMÁRIO

1 INTRODUÇÃO

1.1 Síndrome de Sjögren

A síndrome de Sjögren (SS) é uma doença autoimune crônica sistêmica que afeta principalmente as glândulas salivares e lacrimais, ocasionando disfunção glandular, incluindo ceratoconjuntivite *sicca,* hipossalivação e sintomas de secura oral e ocular[1]. Estima-se que a prevalência na população em geral varia entre 0,5% a 3%, com incidência de 4 casos por 100.000 indivíduos. Além disso, a proporção de acometimento mulher/homem relatada é de 9:1, sendo predominantemente observada em adultos de meia-idade[2,3].

A doença é classificada em SS primária (SSp) e secundária (SSs); a SSp ocorre de forma isolada e a SSs se manifesta em associação com outras doenças autoimunes do tecido conjuntivo, como a artrite reumatoide e lúpus eritematoso sistêmico e esclerose sistêmica[3,4]. Manifestações extraglandulares, como mialgia, artralgia e fadiga não são achados incomuns (cerca de 5% a 10% dos pacientes com SSp), doenças cardiovasculares, insuficiência renal, doença pulmonar intersticial, e distúrbios imunológicos, como hipergamaglobulinemia, fator reumatoide, hipocomplementemia, crioglobulinemia e autoanticorpos direcionados às partículas da ribonucleoproteína SSA/Ro e SSB/La, são frequentemente detectados[5]. Além dessas manifestações, estudos mostram que pacientes com SS apresenta uma maior predisposição a desenvolver linfomas não-Hodgkin de células B, especificamente o linfoma de tecido linfoide associado à mucosa (MALT), quando comparado à população em geral[2,6,7].

Reconhece-se que a etiopatogênese da SS ainda não está esclarecida; no entanto, a associação entre predisposição genética, modificações epigenéticas, fatores ambientais e hormonais têm sido fortemente associados à resposta imune contra autoantígenos visualizada nessa síndrome[8,9]. A ativação imune contra as partículas de ribonucleoproteína SSA/Ro e SSB/La expressas pelo epitélio das glândulas exócrinas pode ser desencadeada por células dendríticas (CDs) presentes também nesse epitélio, que conduzem o acúmulo e a ativação das células do sistema imune, em especial das células T e B[10]. Além disso, as infecções virais, especialmente pelo vírus Esptein-Barr (VEB) e citomegalovírus (CMV), visualizadas em pacientes com SS montam uma assinatura de interferon tipo I (IFN-I) e estimulam as CDs,

ativando o sistema de resposta imune inata e contribuindo para a cronicidade do processo autoimune[8,9].

O diagnóstico da SS pode ser feito por meio de testes de secura ocular, sialometria, ultrassonaografia, cintilografia, biópsia de glândulas salivares menores (GSm) e detecção de autoanticorpos antirribonucleoproteína SSA/Ro e SSB/La no plasma sanguíneo[10]. Histologicamente, as biópsias de GSm de pacientes com SS exibem aglomerados linfocitários (focos) permeando o parênquima glandular, definido como sialadenite linfocítica focal (SLF). O escore focal (EF) define o número de focos, cada um deles contendo 50 ou mais linfócitos, em uma área glandular de 4mm² [11]. Notavelmente, GSm mostrando SLF com EF ≥1, é um critério diagnóstico importante para definir a SS[12,13]. Em certas situações, o infiltrado linfocitário que constitui os focos pode apresentar centros germinativos, com alguns estudos indicando valor prognóstico para este achado, com tendência para desenvolver linfoma tipo MALT[14].

1.2 Centros Germinativos

Os centros germinativos (CGs) são microambientes especializados dentro dos tecidos linfoides, onde as células B sofrem extensa proliferação, hipermutação somática e seleção de afinidade de antígeno. Estes CGs podem ser visualizados ao avaliar biópsias de GSm em pacientes com SS[5–7,15–19]. Apesar da importância da organização do infiltrado linfocitário em CGs nesses pacientes ainda não está completamente esclarecida, com alguns estudos favorecendo[7,14-16,20] ou não[21-23] o desenvolvimento de linfoma, suscita-se que eles reorganizem a arquitetura dos infiltrados inflamatórios, facilitem a comunicação entre os linfócitos e podem desta maneira estar relacionado ao desenvolvimento de linfoma[14].

Digno de nota, a relação entre a presença desses CGs e o risco acentuado de desenvolvimento de linfoma foi demonstrado em uma série de estudos[7,14–16,20]. Em um desses, foi observado que 4% (7/175) de uma coorte de SSp desenvolveu linfoma em um período de 10,6 anos, quando os casos foram comparativamente analisados a partir do status CG-positivo e CG-negativo no momento do diagnóstico, as porcentagens de desenvolvimento de linfoma foram de 14% e 0,8% respectivamente[7]. Acredita-se que tal associação seja resultado de uma estimulação crônica de células B em CGs presentes nesses pacientes[14]. No entanto, outros estudos indicam que GCs não são preditivos para o desenvolvimento de linfoma em pacientes com SS[21–23].

Somado a esse achado, a presença de CGs também se correlacionou com um maior escore focal (EF), redução da produção de saliva, níveis mais elevados de fator reumatoide e anticorpos anti-SSA e anti-SSB[1,14,17,19,24–26]. Ainda, os níveis de quimiocinas, mediadores pró-inflamatórios capazes de atrair células linfoides e mieloides, encontram-se aumentadas em pacientes com SS apresentando SLF com presença de CGs[14].

Estudos anteriores, através da coloração com hematoxilina e eosina (H&E) e alguns deles também por imunoistoquímica (IQ), revelaram que os CGs podem ser detectados em aproximadamente 25% das biópsias de GSm de pacientes com SSp[7,14,19], variando de 16,5% a 59%[15,27]. Além disso, outros estudos também avaliaram a presença de CGs em biópsias da glândula parótida[16,28–30]. Destes, enquanto alguns apresentaram resultados semelhantes com GSm[28,30], outros mostraram uma alta proporção (76% e 100%) de CGs nas glândulas parótidas do que GSm em pacientes com SSp[16,29].

A análise de CGs, por IQ, foi determinada em alguns estudos levando em consideração a rede de células dendríticas foliculares (CDF), através da imunoexpressão de CD21[5,16,27,31], CD23 e CD35[1,24,32]. Outros, utilizaram o anticorpo Bcl-6, um fator de transcrição altamente expresso em células B dentro de CGs[21,30], bem como o uso de CD3/CD20[16,33], para melhor caracterização das populações de células B e T. Todavia, falta consenso nos critérios utilizados para identificação dos CGs, através da técnica de IQ[30], com alguns estudos indicando que focos isolados de CDF, sem a expressão de Bcl-6, não pode caracterizar estritamente um CG[21].

Como o papel dos CGs na SSp ainda não está totalmente esclarecido em relação ao seu impacto prognóstico[17], estudos relacionados à caracterização morfológica, identificação de marcadores, bem como elucidação das vias imunológicas e imunopatológicas, são necessários para um melhor entendimento da presença destas estruturas na patogênese da SSp[14].

1.3 Células Dendríticas (CDs)

As CDs são derivadas de células-tronco hematopoiéticas na medula óssea, integrantes do sistema de imunidade inata e eficientes células apresentadoras de antígenos (APCs). As CDs são capazes de estimular uma resposta imunitária celular contra células tumorais, desempenhar um papel importante na ativação e função de

linfócitos T e, ainda, auxiliar a manutenção da tolerância periférica. As CDs, quando sofrem processo de diferenciação são divididas em células dendríticas mieloides (CDmi) e plasmocitoides (CDp)[34,35]. Recentemente, as CDs foram sugeridas como candidatas à células mestras na ativação de células T e B na SS, no entanto, sua contribuição tem sido subavaliada[36].

1.3.1 Células dendríticas mieloides (CDmi)

As CDmi, ou clássicas, são células altamente especializadas encontradas em tecidos linfoides e, também, não linfoides. Essa células, em seu estado imaturo, capturam antígenos e migram através do sistema linfático para a zona de células T de órgãos linfoides secundários, onde ativam células T auxiliares / citotóxicas *naïve* e uma resposta imune adaptativa é iniciada[36]. Dependendo da forma como interagem, diferentes padrões de resposta são definidos, podendo variar desde a tolerância até a eliminação do antígeno ou o estabelecimento de estados imunopatológicos[37]. Quando presentes na pele e nas mucosas, as CDmi são conhecidas como células de Langerhans (CL) e células dendríticas submucosas (CDsub)[38]. As CL tem localização preferencial no epitélio, já as CDsub tem localização preferencial no tecido conjuntivo adjacente.

As CL apresentam um papel indispensável na imunovigilância, principalmente nas zonas de barreira de entrada de patógenos, tais como a pele, mucosas e tecido periodontal[39]. São caracterizadas pela expressão de S100, CD1a, CD45, moléculas do complexo principal de histocompatibilidade de classe II (MHC-II), E-caderina e a lectina do tipo C Langerin (CD207)[36]. Estudos mostraram que há um envolvimento das CL na indução da SS; bem como sua correlação com a intensidade e perpetuação do processo inflamatório[40]. Essas CDs foram observadas principalmente nas áreas periacinares e periductais próximas aos grandes infiltrados de linfócitos $CD4^{+}$[33,40,41], e apresentavam expressão imunofenotípica para CD1a[40-43], antígeno leucocitário anti-humano (HLA-DR+)[41] e S100+ [33].

Curiosamente, ao avaliar a presença dessas CL em biópsias de GSm e no sangue periférico de pacientes com SSp, notou-se que elas se encontravam em número aumentado nas GSm e reduzidas no sangue periférico circulante, sugerindo, assim, um papel importante na resposta imune inflamatória local[41].

As CDsub, também chamadas de CDs dérmicas, é um subtipo distinto de CDmi que, além de ativar células T específicas do antígeno, apresenta capacidade de estimular diretamente as células B *naïves* a se diferenciarem em plasmócitos *in vitro*[44]. Estas, podem ser avaliadas a partir da expressão fenotípica dos marcadores CD209 (DC-SIGN^{+}), um receptor de membrana tipo II, que apresenta um domínio de reconhecimento de carboidrato C-terminal, presente na superfície de macrófagos e CDs, o qual medeia interações intracelulares, reconhecimento e captura de patógenos[45]; e o fator XIIIa (FXIIIa), uma transglutaminase dependente de Ca^{2+}, gerada na cascata de coagulação sanguínea, que é expressado positivamente nas CDsub[44,45]. Um estudo ao avaliar a presença de CDsub DC-SIGN+ em tecido periodontal, verificou que estas eram similares às DC-SIGN+ encontradas na pele, contudo, o papel delas na gengiva humana ainda não eram bem definidos[45].

No contexto da SSp, o trabalho de Wildenberg et al.[46] avaliou as CDs por meio da expressão de CD208 (DC-LAMP) e CD209 (DC-SIGN) em biópsias de glândulas salivares labiais e parótidas em quatro pacientes com SSp; os autores encontraram que a expressão de CD209 foi identificada em todos os pacientes, na SLF e no parênquima glandular, enquanto o CD208+ foi expresso apenas na SLF[46]. Em outro estudo, ao avaliar a expressão de IL-7Rα em GSm na SSp e correlacionar com a inflamação glandular, foi possível identificar a associação entre o aumento dos níveis de IL-7Rα e número de CDmi que expressavam CD209 e CD208, sugerindo que estas células apresentavam um papel importante na intensificação e/ou ativação das células T e B *naïves* na inflamação glandular[43]. Porém, o mecanismo imunopatológico das CDsub na SSp ainda precisa ser melhor esclarecido.

1.3.2 Células dendríticas plasmocitoides (CDp)

As CDp são células derivadas de progenitores linfoides e altamente especializadas na produção e secreção de altos níveis de interferon tipo I (IFN-I), especialmente após ativação por agentes virais (resposta antiviral). Elas expressam constitutivamente receptores semelhantes a endossômicos *toll-like* (TLR), os quais atuam no reconhecimento de RNA e DNA virais e podem se ligar aos ácidos nucléicos endógenos[47,48]. Entre os receptores endossômicos, o TLR7 e TLR9 constituem vias cruciais pelas quais as CDp são ativadas em doenças autoimunes sistêmicas[48].

Um conjunto de evidências supõe que a secreção de IFN-I pelas CDp desempenha um papel importante na perpetuação do processo inflamatório na SSp, uma vez que estimulam os monócitos a produzir altos níveis de fator de ativação de células B (BAFF), um mediador da ativação de células B e da produção de autoanticorpos na SSp. Sugere-se, dessa forma, que as CDp podem estar relacionadas com aumento de atividade dessa doença[47,49].

Nesse contexto ao avaliar a presença das CDp em GSm de pacientes com SSp, através da expressão fenotípica dos anticorpos CD123[48], e CD303[35,48,50-53], os autores notaram que as CDp eram expressas em número aumentado nas GSm de pacientes com SSp, com distribuição nos focos linfocíticos e áreas ductais, quando comparado ao grupo controle (GSm saudáveis). Adicionalmente, ao correlacionar a presença dessas células na inflamação glandular e no sangue periférico da SSp, notou-se que, apesar de não haver uma correlação estatística, as CDp estavam aumentadas nas GSm e reduzidas no sangue periférico; sugerindo o envolvimento dessas na resposta autoimune inflamatória[35,46].

2 PROPOSIÇÃO

2.1 Objetivo Geral

Avaliar, por imunoistoquímica, a frequência de centros germinativos e a presença de células dendríticas mieloides e plasmocitoides em biópsias de glândulas salivares menores de pacientes com síndrome de Sjögren primária.

2.2 Objetivos Específicos

- Avaliar a frequência de centros germinativos em glândulas salivares menores de pacientes com SSp, através de imunomarcadores CD10, CD23 e Bcl-6;

- Caracterizar a presença de células dendríticas mieloides do tipo células de Langerhans em glândulas salivares menores de pacientes com SSp, através dos imunomarcadores CD1a e CD207;

- Caracterizar as células dendríticas plasmocitoides em glândulas salivares menores de pacientes com SSp, através dos imunomarcadores CD123 e CD303.

3 PUBLICAÇÕES

3.1 Publicação 1*

FOCAL LYMPHOCYTIC SIALADENITIS AND ECTOPIC GERMINAL CENTERS IN ORAL REACTIVE LESIONS AND PRIMARY SJÖGREN'S SYNDROME: A COMPARATIVE STUDY

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Keywords: Sjögren's syndrome, non-Sjögren syndrome, non-sicca control; oral reactive lesions; focal lymphocytic sialadenitis, ectopic germinal centers; immunohistochemistry.

Funding:

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 (Evânio Vilela Silva).

* O artigo segue as normas para publicação no periódico CLINICAL RHEUMATOLOGY (0770-3198)

Luciana Yamamoto Almeida (2016/02713-2), Heitor Albergoni Silveira (2018/12734-2), Karen Cristine Bortoletto (2016/14636-2), and Jorge Esquiche León (2016/11419-0) have received research Grants from State of São Paulo Research Foundation (FAPESP).

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NOTES

Compliance with Ethical Standards

Ethical Approval

This study was reviewed and approved by the Research Ethics Committee of the Ribeirão Preto Medical School, University of São Paulo (Protocol number: 60786216.8.0000.5440) and 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.

Conflicts of interest statement: The authors have no conflict of interest in the present manuscript.

Author contributions: All authors were involved in drafting the article or revising it critically for important intellectual content.

Data availability statement: The data underlying this article are available in the article and in its online supplementary material.

ABSTRACT

Objectives: Focal lymphocytic sialadenitis (FLS), an important diagnostic criterion for Sjögren's syndrome (SS) diagnosis, can also be observed when assessing minor salivary gland (mSG) biopsies from healthy asymptomatic individuals (non-SS patients). Some studies suggest that germinal centers (GCs) in FLS may be predictive for lymphoma development in SS patients.

Methods: Fifty cases of primary SS (pSS group) and 31 cases of oral reactive lesions (non-SS non-sicca, control group) containing also typical FLS features, were assessed by morphological and immunohistochemical (CD10, CD23 and Bcl-6) analysis, aiming at the detection of GCs.

Results: All pSS cases showed FLS with focus score (FS)≥1. In the control group, 12, 10 and 9 cases showed FLS with FS≥1, FLS with FS<1, and FLS associated with chronic sclerosing sialadenitis with FS<1, respectively. The morphological analysis revealed CGs in 20% (10/50) and 19% (6/31) of the pSS and control group, respectively. The FS and number of foci were significantly higher in pSS than control group with FS<1. Immunohistochemistry confirmed all morphological findings (GCs showing CD23 and Bcl-6 positivity, with variable CD10 expression) and additionally in 3 and 1 cases of the pSS and control group, respectively. Moreover, another 6 and 2 cases of the pSS and control group with FS≥1, respectively, showed positivity only for CD23.

Conclusion: FLS can also be observed when assessing oral reactive lesions, which showed similar frequency of GCs with those found in pSS patients. Further studies, including functional analysis of lymphocytic populations and GCs in FLS, are encouraged.

Key-points:

- FLS and GCs can also be observed when assessing intraoral reactive lesion biopsies.
- Similar frequency of GCs when assessing FLS (pSS) and oral reactive lesions with FLS-like features.
- GCs can be efficiently detected through Bcl-6 expression.
- Standardised criteria for evaluating GCs in pSS patients are recommended.

1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease that mainly affects the salivary and lacrimal glands, causing xerostomia and ceratoconjunctivitis sicca, respectively [1]. The SS prevalence in a general population varies from 0.5% to 3%, with an incidence of 4 cases per 100,000 per year. Moreover, the frequent reported male/female ratio of 1:9 seems to be more suitable in the range of 1:20. The SS is predominantly observed in middle-aged adults, and may manifest alone (primary SS [pSS]) or associated with other autoimmune disease (secondary SS [sSS]), especially rheumatoid arthritis and systemic lupus erythematosus [2]. Extraglandular manifestations such as myalgia, arthralgia, and fatigue are not uncommon findings (5% to 10% of pSS patients), and immunological disturbances such as hypergammaglobulinemia, rheumatoid factor, hypocomplementemia, cryoglobulinemia, and autoantibodies directed against SSA/Ro and SSB/La autoantigens are frequently detected [3].

Notably, the histopathological analysis of the minor salivary glands (mSGs) showing focal lymphocytic sialadenitis (FLS) with focus score (FS) ≥1, is an important diagnostic criterion for defining SS [4]. Germinal centers (GCs), which are specialized microenvironments within lymphoid tissues, where B-cells undergo extensive proliferation, somatic hypermutation and antigen affinity selection, can be visualized when assessing mSG biopsies from SS patients [1,5–11]. Noteworthy, the presence of GCs have been linked to higher risk of lymphoma development [5,10–13], as well as higher FS, reduced saliva production, and higher levels of rheumatoid factor, anti-SSA antibodies, anti-SSB antibodies, and proinflammatory mediators [1,5–10,14,15]. However, other studies indicate that GCs are not predictive for lymphoma development in SS patients[3,16,17].

Previous studies, through hematoxylin and eosin (H&E) staining and some of them also by immunohistochemistry, have shown that GCs can be detected in approximately 25% of mSG biopsies obtained from pSS patients [5,9,10], ranging from 16.5% to 59%, respectively [11,18]. Additionally, other studies have also evaluated the presence of GCs in parotid gland biopsies [12,19–21], which presented similar [19,21] or high (76% and 100%) [12,20] proportion of GCs rather than mSGs in pSS. By immunohistochemistry, the GCs were assessed through CD21, CD23, CD35 and Bcl-6 markers [12,14,16,22–24], with a recent study [21] indicating Bcl-6 as a sensitive and

specific marker for unequivocal identification of GCs in mSGs and major salivary gland (MSG) biopsies obtained from pSS patients.

Interestingly, similar with previous studies [25–33] (Supplementary Table S1), we have observed typical microscopical features of FLS assessing intraoral biopsies from healthy asymptomatic, non-SS patients in our Oral Histopathology Laboratory. In fact, in the studies previously mentioned, 8 out of 54 (15%) mSGs of healthy volunteers [33] and 6 out of 40 (15%) labial salivary glands of coroner's autopsies with no clinical findings suggesting SS [29], were microscopically diagnosed as FLS with FS≥1. However, relevantly, the prevalence of GCs in these cases [25–33], which appears to have a prognostic impact in SS patients [5], is unknown.

Thus, the aim of the current study was to comparatively analyze mSG biopsies presenting FLS obtained from patients with SS and non-SS (healthy asymptomatic individuals), and to determine the frequency of GCs, aiming at a better understanding of the prognostic impact of these morphological finding.

2. Materials and methods

2.1 Patients and biopsy samples

This study was approved by the Research Ethics Committee of the Ribeirão Preto Medical School, University of São Paulo (protocol number: 68748117.0.0000.5440) (ANEXO A) and 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.

Informed consent of the patients was not obtained, because this study was done using excess tissue of biopsies used for diagnostic purposes. Private patient information was not accessed at any part of the study execution, including manuscript preparation, as recommended by the Resolution no. 466/12 of the National Health Council. We conducted a retrospective study including 81 cases, being 50 pSS cases (SS group) diagnosed according to previously established criteria [4] and 31 cases of healthy asymptomatic patients (non-SS non-sicca, control group). These 31 cases were selected after careful microscopical analysis of 792 oral biopsies (563 females [mean age, 55.4 years] and 229 males [mean age, 55.8 years]), clinically and

microscopically diagnosed mucocele, inflammatory fibrous hyperplasia or ranula, which also presented typical FLS features at the periphery of the specimen and away from the main lesion [29,33]. For non-SS non-sicca group, inclusion criteria were patients with available clinical data, as well as formalin-fixed paraffin-embedded (FFPE) tissue suitable for histopathological and immunohistochemical (IHC) analysis. Exclusion criteria were autoimmune diseases, endocrine disorders, immunodeficiency disorders, smokers and former smokers, ethilists and former ethilists, as well as use of antibiotics, corticosteroids or non-steroidal anti-inflammatory drugs.

2.2 Histopathological analysis

Sections of 5-µm were obtained from all cases and stained with H&E for diagnostic confirmation. All 81 cases presented mSGs with a diagnosis of FLS, in which the presence of GCs was assessed. The GC was defined as a well-circumscribed, round to oval shaped structure of at least 50 mononuclear cells, and presenting features indicative of lymphoid organization, such as a densely packed dark zone and a light zone within otherwise normal salivary gland epithelium [3,10]

2.3 Immunohistochemistry

For IHC analysis, consecutive (serial) histological sections of 3-µm were placed on organosilane coated slides (Sigma–Aldrich, St. Louis, MO). The deparaffinization was performed using xylene solution and the rehydration of the sections occurred through the passage in ethanol solution with serial concentrations. The antigen retrieval was prepared by immersion of the sections in citrate buffer (target retrieval solution, pH6). After, the sections were submitted to the immunohistochemistry technique by streptavidin-biotin-peroxidase method (Universal LSAB^{TM} + Kit/HRP, Dako, Carpinteria, CA, USA) to evaluate the presence of GCs using the primary antibodies: Bcl-6 (Clone LN22, dilution 1:500, Leica Biosystems, United Kingdom), CD10 (Clone 56C6, dilution 1:500, Leica Biosystems, United Kingdom) and CD23 (Clone 1B12, dilution 1:500, Leica Biosystems, United Kingdom).

The IHC staining was evaluated by three experienced pathologists (RC, JEL and ARS) blinded to the clinicopathological features, using a computerized system consisting of a light microscope (Leica DM500), adapted to a high resolution camera

(Leica ICC50) and a color video monitor. The images were obtained using the Leica IM50 Image Manager Program and the processing was done through the Leica QWin Image Processing and Analysis System. After evaluation of the slides at x100 magnification, areas presenting positive immunostaining were analyzed at x400 magnification (0.785 mm^2) for a detailed analysis and confirmation of the GC structures.

2.4 Statistical analysis

Statistical analysis was performed using the IBM SPSS 20.0 software. The Shapiro–Wilks test for assessing the normal distribution was used. The Student *t*-test and Pearson correlations were applied in samples with a normal distribution, whereas the Mann–Whitney *U* test and Spearman correlation were applied in samples with a non-normal distribution. For categorical data, the chi-square test was applied. A probability (p) value <0.05 was considered statistically significant.

3. Results

SS group

This group included 50 patients; 46 were women and 4 were men, with an age range from 12 to 81 years (mean age, 55 years). Only one pediatric case affecting a 12-year-old female patient was included in the current study. When assessing the lower lip mSG biopsies, all pSS cases showed FLS with FS≥1. The FS ranged from 1.2 to 10 (mean, 2.7) (Table 1 and Figure 1).

Non-SS non-sicca group (control group)

This group included 31 cases; 18 were women and 13 were men, with an age range from 9 to 69 years (mean age, 42.3 years). These cases were clinically and microscopically diagnosed as inflammatory fibrous hyperplasia (n=17), mucocele (n=13), and ranula (n=1). Of them, 12 cases (10 women, 2 men; mean age, 36.7 years) also showed FLS with FS≥1 (ranging from 1.1 to 2.7; mean, 1.4), 10 cases (6 women, 4 men; mean age, 30.1 years) also showed FLS with FS<1 (ranging from 0.1 to 0.6; mean 0.2) and 9 cases (6 women, 3 men; mean age, 56 years) also presented FLS

associated with focal areas of chronic sclerosing sialadenitis (CSS) (FLS/CSS) with FS<1 (ranging from 0.1 to 0.7; mean, 0.25) (Figure 2).

The FS values and number of foci were significantly higher in SS rather than control group with FS<1 ($p<0.05$).

Ectopic GC frequency

In the SS group, the morphological analysis revealed that 20% (10/50) of the cases presented GCs (ranging from 1 to 10; mean, 3.8). Moreover, in three cases, the presence of GCs (ranging from 1 to 3) was identified only by immunohistochemistry. In addition, there was a statistically significant correlation when comparing GCs with FS and number of foci ($p<0.05$).

In the control group, the morphological analysis revealed the presence of GCs (ranging from 1 to 6; mean, 1.8) in 5 out of 22 (23%) cases which presented FLS (4 with FS≥1 and 1 with FS<1), and in 1 out of 9 (11%) cases (3 GCs), which presented FLS/CSS with FS<1. Moreover, in one case, which presented FLS with FS≥1, the presence of 2 GCs was identified only by immunohistochemistry. There was no statistically significant correlation when comparing GCs with FS and number of foci.

All cases presenting GCs on morphological analysis, also exhibited GCs immunopositive for CD23 and Bcl-6. Of these cases, only 3 and 2 cases of the pSS and control group, respectively, showed CD10 positive GCs. All 3 and 1 cases of the pSS and control group with FS≥1, respectively (identified only by immunohistochemistry), were positive for CD23 and Bcl-6. In addition to these findings, another 6 and 2 cases of the pSS and control group with FS≥1, respectively, showed positivity only for CD23 (Figures 3 and 4).

4. Discussion

To the best of our knowledge, this is the first study which evaluates a large series of pSS and oral reactive lesions, both presenting FLS, and assessing in this sialadenitis type, by morphological and IHC analysis, the presence of GCs, which seems to have a prognostic impact in pSS patients [5]. Noteworthy, we have shown a similar frequency of GCs in these two populations, and the information available on the follow-up of patients with oral reactive lesions shows that in all of them there was complete resolution, without complications.

We have previously shown that inflammatory fibrous hyperplasia, a very common oral reactive lesion, presents oncocytic metaplasia areas and inflammatory lymphoid infiltrate which not uncommonly presents GCs [34]. Moreover, in our Oral Pathology Laboratory, we have had the opportunity to evaluate oral reactive lesions presenting mSGs, at the periphery of the specimen and away from the main lesion, presenting typical microscopical features of FLS. Noteworthy, FLS is an important criterion for SS diagnosis. After an extensive review of the literature, we have found previous studies emphasizing the FLS when evaluating mSGs, MSGs and lacrimal glands from healthy patients [26,27,29,33] and postmortem assessment [25,28,30–32]. These findings show that FLS can also be detected in non-SS non-sicca patients, such as shown in the present study. Moreover, these previous studies [25–33] (Supplementary Table S1) showed that the percentage of cases with FS≥1 varied between 8.3% and 32%, whereas in the current study 12 out of 31 (38%) patients presented FS≥1.

Another interesting finding in our study is the presence of CSS areas in the control group, which occurred in 9 out of 31 (29%) cases. These CSS areas represented less than 10% of the whole of the glandular surface area. Similarly, in the current pSS group, 5 out of 50 (10%) cases presented focal CSS areas, representing less than 7% of the whole of the glandular surface area. In fact, parenchymal atrophy and large area of fibrosis, alongside areas of FLS, can be detected in mSG biopsies obtained from pSS patients [35]. Moreover, from our results, it is possible that CSS areas in the control group may have the potential to reduce the FS, such as previously commented [35].

Such as above commented, GCs can be visualized when assessing mSG biopsies in SS patients [1,5–11], which have been linked to higher risk of lymphoma development [5,10–13], as well as higher FS, reduced saliva production, and higher levels of antibodies and proinflammatory mediators [1,5–10,14,15]. However, other studies have not supported this proposal indicating that GCs may not be predictive for lymphoma development [3,16,17]. These findings clearly show the need for standardisation of the detection method of GCs in salivary gland biopsies from pSS patients [16,35]. In fact, such as shown in Table 2, there are several studies assessing GCs in mainly mSG biopsies, through morphological (H&E stain) and IHC analysis. The percentage of cases presenting GCs on H&E-stained slides varied between 16.5% and 54.5% in mSGs, and between 50% and 75% in MSGs. The IHC analysis, in

general, showed an increase in these values (between 19% and 82% in mSGs, and 100% in a single study on MSGs). The immunomarkers also showed variability, since lymphocyte surface (CD3, CD20), follicular dendritic cell (FDC) (CD21, CD23, CD35), GC B cell (Bcl-6) and cell proliferation (Ki-67) markers were used to detect GCs (Table 2). Taken together, these data evidently show a notable variation in the frequency of CGs, possibly reflecting a lack of standardization in both morphological and IHC criteria, in pSS patients.

Notably, the IHC analysis is a powerful tool that complements the morphological analysis; however, it is important to carefully interpret its results. In this context, a recent study [21] has emphasized that positivity for CD21 (FDC marker) does not necessarily indicate the presence of GCs, proposing to use Bcl-6 as a sensitive and specific marker for unequivocal identification of GCs, when assessing mSG and MSG biopsies from pSS patients. Accordingly, in the current study, all GCs showed positivity for both CD23 and Bcl6, whereas 8 cases exhibited positivity only for CD23, which after evaluating consecutive serial sections (H&E, CD10-negative and Bcl-6-negative slides), providing rich details of cell morphology, absence of B-cell blasts was noticed.

The FDCs are an unique type of cell located within lymphoid follicles, containing and retaining immune complexes, and expressing molecules involved in the proliferation and differentiation of B-cells. FDC markers include antibodies to complement receptors such as CD21 and CD35, IgE FC receptor (CD23), and IgG FC receptor (CD32). In both primary (without GC) and secondary (with GC) follicles from reactive lymph nodes, FDCs are CD21 and CD35 positive. FDCs in GC light zone additionally upregulate CD23 and CD32. Notably, upregulation and downregulation of CD23 by FDCs appears to be related to B-cell centrocytic and centroblastic differentiation, respectively [36,37]. Such as shown in Table 2, eleven, four and two studies have assessed CD21, CD35 and Bcl-6, respectively, aiming to detect GCs in pSS. It is possible that primary follicles or FDC-networks have been interpreted as GCs [14,16,21]. Similar with previous study [18], we have used CD23, which seems to have apparent restriction to CGs [37], for comparative analysis with studies assessing CD21 and CD35, and we have found similar results.

The CD10 antigen is a cell surface zinc-dependent metalloprotease, which is expressed in several cell types, including hematolymphoid, epithelial, and mesenchymal origin. It is also known as “common ALL antigen (CALLA)”, due to its frequent expression in most cases of acute lymphoblastic leukemia. In normal lymph

node, CD10 expression is preferentially confined to GCs (i.e., secondary follicles). Stromal cells, scattered lymphocytes and granulocytes in the interfollicular areas are also CD10 positive [38]. Thus, it is due to the preferential expression in the secondary follicle that in the current study we decided to evaluate CD10. However, our results showed variable CD10 expression in GCs, being positive in only 3 out of 13 and 2 out of 7 cases of the pSS and control group, respectively.

The BCL6 gene encodes a 95-kD nuclear phosphoprotein, belonging to the BTB/POZ/ZincFinger (ZF) family of transcription factors, which is essential for GC B-cell and follicular helper T (Tfh) cell development [39]. In GC, unlike from centroblasts and centrocytes, Tfh cells are not organised in clusters and usually show a rounded shape [21,40]. Thus, it has been proposed that a cluster of ≥5 adjacent Bcl-6 positive cells within a focus be classified as a GC [16]. Similarly, in the current study, clusters of centroblasts were efficiently detected through Bcl-6 expression. Moreover, in our experience, the CD57 expression can be used to assist in the identification of Tfh cells (data not shown).

Such as previously commented, while some studies concluded that the presence of GCs in mSG biopsies from pSS patients was predictive of lymphoma development [10–12], other studies showed opposite results [3,16,17]. In this context, other findings such as FS≥3 [41,42], elevated FcRL4+ expression [43], higher pSTAT-3 expression [44], weak or absent A20 immunostaining [45], lymphoepithelial lesion [46], recurrent or permanent swelling of MSGs, lymphadenopathy, cryoglobulinemia, splenomegaly, low complement levels of C4 and C3, lymphopenia, skin vasculitis, M-component in serum or urine, peripheral neuropathy, glomerulonephritis, elevated beta2-microglobulin, and CD4 lymphocytopenia [47], have been proposed to identify SS patients with an increased risk for lymphoma development.

In summary, we have showed, for the first time, that FLS can also be detected when assessing intraoral reactive lesions, which exibited similar frequency of GCs (by both morphological and IHC analysis) when comparing with mSG biopsies obtained from pSS patients. All GCs detected on morphological analysis, were also CD23 and Bcl-6 positive, with variable expression of CD10, whereas 8 cases showed positivity only for CD23. Our results suggest standardised protocols for morphological and IHC analysis when assessing GCs in mSG biopsies from pSS patients. Because the similar frequency of GCs in oral reactive lesions and pSS, further studies including functional analysis of lymphocytic populations and GCs in FLS are encouraged.

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Legends:

Figure 1. Histopathological analysis on hematoxylin and eosin (H&E) staining showing focal lymphocytic sialadenitis in minor salivary gland biopsy specimens obtained from primary Sjögren's syndrome patients. In these cases, several foci were observed (white arrowheads), containing germinal center-like structures (black arrowheads) (A and B, ×2.5; C, ×5; D and E, ×10; and F, ×40).

Figure 2. Immunohistochemical analysis in consecutive (serial) sections of focal lymphocytic sialadenitis in minor salivary gland biopsy specimen obtained from primary Sjögren's syndrome patient. In this case, notice germinal center-like structures showing strong positivity for Bcl-6 (A and D), CD23 (B and E) and CD10 (C and F) (A, B and C, ×10; D, E and F, ×40). The D, E, and F photomicrographs represent higher magnification of A, B, and C photomicrographs, respectively.

Figure 3. Histopathological analysis on hematoxylin and eosin (H&E) staining showing focal lymphocytic sialadenitis in oral reactive lesions obtained from non-Sjögren's syndrome non-sicca patients. In these cases, several foci (white arrowheads) were observed, containing germinal center-like structures (black arrowheads) (A, B and C, ×4; D, E and F, ×40). The squares in A, B, and C, are shown in increasing magnification in D, E and F, respectively.

Figure 4. Immunohistochemical analysis in consecutive (serial) sections of focal lymphocytic sialadenitis in oral reactive lesion obtained from non-Sjögren's syndrome non-sicca patient. In this case, the germinal center-like structures showed positivity for Bcl-6 (A and D), CD23 (B and E) and CD10 (C and F) (A, B and C, ×10; D, E and F, ×40). The D, E, and F photomicrographs represent higher magnification of A, B, and C photomicrographs, respectively.

Table 1. Clinicopathological features of the cases included in the current study.

Clinicopathological features	**pSS group (nº of patients = 50)**	**Control group (nº of patients = 31)**
Mean age / range (years)	55 / 12 – 81	42.3 / 9 – 69
Gender		
Female	46	18
Male	4	13
Diagnosis		
pSS	50	-
Inflammatory fibrous hyperplasia	-	17
Mucocele	-	13
Ranula	-	1
Histopathological findings		
FLS with FS≥1	50	12
FLS with FS≤1	-	10
FLS/CSS with FS≤1	-	9
GC based on H&E, nº (%)	10/50 (20)	6/31 (19)
Range of GC (mean)	1 – 10 (3.8)	1 – 6 (1.8)
GC based on IHC analysis, nº (%)*	13/50 (26)	7/31 (22)

Abbreviations: CSS, chronic sclerosing sialadenitis; FLS, focal lymphocytic sialadenitis; FS, focus score; GC, germinal centers; H&E, hematoxylin and eosin stain; IHC, immunohistochemical analysis; pSS, primary Sjögren's syndrome.

***** IHC analysis included CD10, CD23 and Bcl-6 markers. All GCs on morphological analysis were also CD23 and Bcl-6 positive, with variable CD10 expression.

Table 2. List of studies showing clinicopathological features and frequency of germinal centers in primary Sjögren syndrome patients.

No	Author [ref.]	pSS cases/ female (n)	Age at diagnosis (mean±SD, median, range) years	FS ≥ 1 (mean±SD, range, %)	SG biopsy #	GCs (H&E) (%)	GCs (IHC)* (%)	Immunomarkers used
01	Szodoray et al., 2005 [48]	19/19	51±15	3.75±2.52 GC+ 4.81±3.06 GC-	Minor	8/19 (42.1)	ND	-
02	Jonsson et al., 2005 (a) [1]	130/NS	51±13	4.1 GC+ 3.6 GC-	Minor	33/130 (25.4)	10/18 (55.5)	CD35, CD20, Ki-67
03	Jonsson et al., 2005 (b) [49]	22/22	50±3	4.0±1.0	Minor	12/22 (54.5)	ND	-
04	Daridon et al., 2006 [22]	18/15	range 32-77	NS	Minor	NS	7/18 (39)	CD21, CD35, Ki-67
05a	Bombardieri et al., 2007 [12]	19/NS	53; range 21-72	NS	Minor	NS	8/19 (42.1)	CD3/CD20, CD21
05b	Bombardieri et al., 2007 [12]	5/4	52±4	NS	Major	NS	5/5 (100)	CD3/CD20, CD21
06	Jonsson et al., 2007 [6]	169/160	54; range 45-62	2.0±1.0	Minor	47/169 (28)	9/11 (82)	CD21
07	Manoussakis et al., 2007 [50]	21/19	49; range 28-80	range 1.0-12.0	Minor	NS	4/21 (19)	CD3, CD20, Ki-67
08	Pijpe et al., 2007 [19]	15/NS	NS	87%	Major	6/12 (50)	ND	-
09	Jonsson and Skarstein, 2008 [14]	60/58	55±2	2.6±0.24	Minor	11/60 (18)	12/60 (20)	CD21, CD23, CD35
10	Gatumu et al., 2009 [18]	17/16	51±15	range 1.0-7.0	Minor	NS	10/17 (59)	CD21
11	Le Pottier et al., 2009 [23]	40/40	range 31-72	NS	Minor	NS	11/40 (27.5)	CD21, CD35
12	Reksten et al., 2009 [7]	115/NS	51±1	2.42±0.16	Minor	27/115 (23.5)	ND	-
13	Christodoulou et al., 2010 [13]	39/NS	55; range 24-70	3.38; range 1.0-8.9	Minor	8/39 (20.5)	ND	-
14	Szyszko et al., 2011 [15]	21/19	55±9	range 1.0-12.0	Minor	7/21 (33.3)	ND	-
15	Theander et al., 2011 [10]	175/161	51±13	78%	Minor	43/175 (25)	ND	-
16	Fei et al., 2014 [24]	103/100	44±11	89.5%	Minor	NS	22/103 (21.1)	CD21
17	Johnsen et al., 2014 [3]	49/ 42	51; range 29-85	2.0; range 0–10	Minor	17/40 (43)	27/40 (68)	CD21
18	Delli et al., 2016 [20]	25/NS	NS	1.6; range 0.8-3.3	Major	19/25 (76)	ND	-

19	Lee et al., 2016 [8]	93/91	44±11	2.71±0.66 GC+ 1.65±0.97 GC-	Minor	28/93 (30.1)	28/93 (30.1)	CD21
20a	Haacke et al., 2017 [16]	11/11[Δ]	47±14	1.8; range 0.8-4.0	Minor	2/11 (18)	3/11 (27)	Bcl-6
20b	Haacke et al., 2017 [16]	22/22 [↕]	48±17	2.7; range 1.4-3.5	Minor	4/22 (18)	5/22 (23)	Bcl-6
21	He et al., 2017 [9]	126/124	49±11	2.02±0.71 GC+ 1.78±0.78 GC-	Minor	36/126 (28.6)	36/126 (28.6)	CD21
22	Sène et al., 2018 [11]	115/100	53; range 50-56	76.5%	Minor	19/115 (16.5)	ND	-
23a	Nakshbandi et al., 2020 [21]	36/NS	NS	100%	Minor	3/210 foci	50/210 CD21+ 9/210 Bcl-6+	CD21, Bcl-6
23b	Nakshbandi et al., 2020 [21]	31/NS	NS	100%	Major	9/141 foci	69/141 CD21+ 22/141 Bcl-6+	CD21, Bcl-6

Abbreviations: FS, focus score; GCs, germinal centers; H&E, hematoxylin and eosin stain; IHC, immunohistochemical analysis; NS, not specified; ND, not done; pSS, primary Sjögren's syndrome; SD; standard deviation; SG, salivary gland. [#] All biopsies were obtained from lower lip (minor SG) and parotid gland (major SG). [*] Only in #4 and #11 studies, the GCs were assessed by immunofluorescence. [Δ] pSS patients diagnosed with parotid MALT lymphoma. [↕] pSS patients without lymphoma.

Supplementary Table S1. Studies evaluating focal lymphocytic infiltration in healthy, non-Sjögren syndrome patients.

No.	Author [reference]	No. patients	Mean age, range (years)	SG biopsy	FS ≥ 1 (%)
1	Scott, 1980 [25]	70, postmortem	range, 18 - 90	Minor	25
2	Syrjänen, 1984 [26]	78, healthy	48.5	Minor	32
3	De Wilde et al., 1986 [27]	68, healthy	range, 10 – 79	Minor	9
4	Kurashima and Hirokawa, 1986 [28]	207, postmortem	range, 40 - 98	Major	30.6
5	Segerberg-Konttinen et al., 1986 [29]	40, healthy	NS	Minor	15
6	Takeda and Komori, 1986 [30]	190, postmortem	range, 19 - 80	Minor	26
7a	Segerberg-Konttinen, 1989 [31]	102, postmortem	range, 17 - 96	Minor	4.9
7b	Segerberg-konttinen, 1989 [31]	102, postmortem	range, 17 - 96	Major	3.9
7c	Segerberg-Konttinen, 1989 [31]	102, postmortem	range, 17 - 96	Lacrimal	12.7
8	Vered et al., 2001 [32]	120, postmortem	<30, 30-60, >60	Minor	8.3
9	Radfar et al., 2002 [33]	54, healthy	31.1	Minor	15

Abbreviations: FS, focus score; SG, salivary gland. All biopsies were obtained from lip (minor SG) and submandibular (major SG).

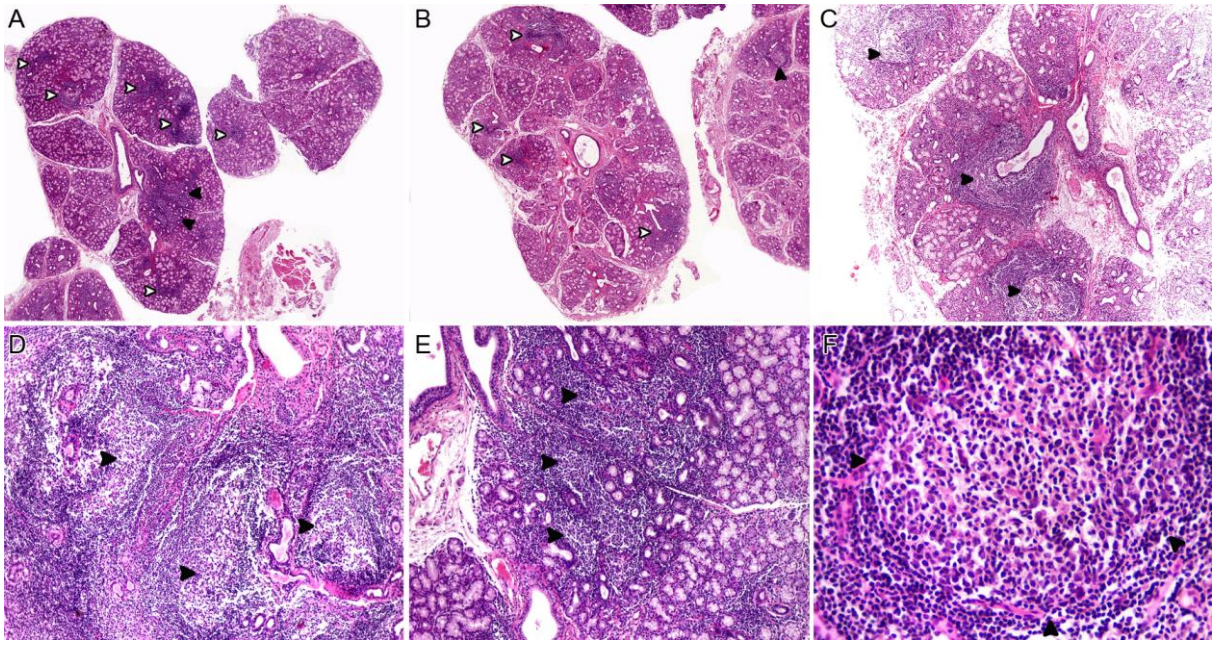

Figure 1. Histopathological analysis on hematoxylin and eosin (H&E) staining showing focal lymphocytic sialadenitis in minor salivary gland biopsy specimens obtained from primary Sjögren's syndrome patients. In these cases, several foci were observed (white arrowheads), containing germinal center-like structures (black arrowheads) (A and B, ×2.5; C, ×5; D and E, ×10; and F, ×40).

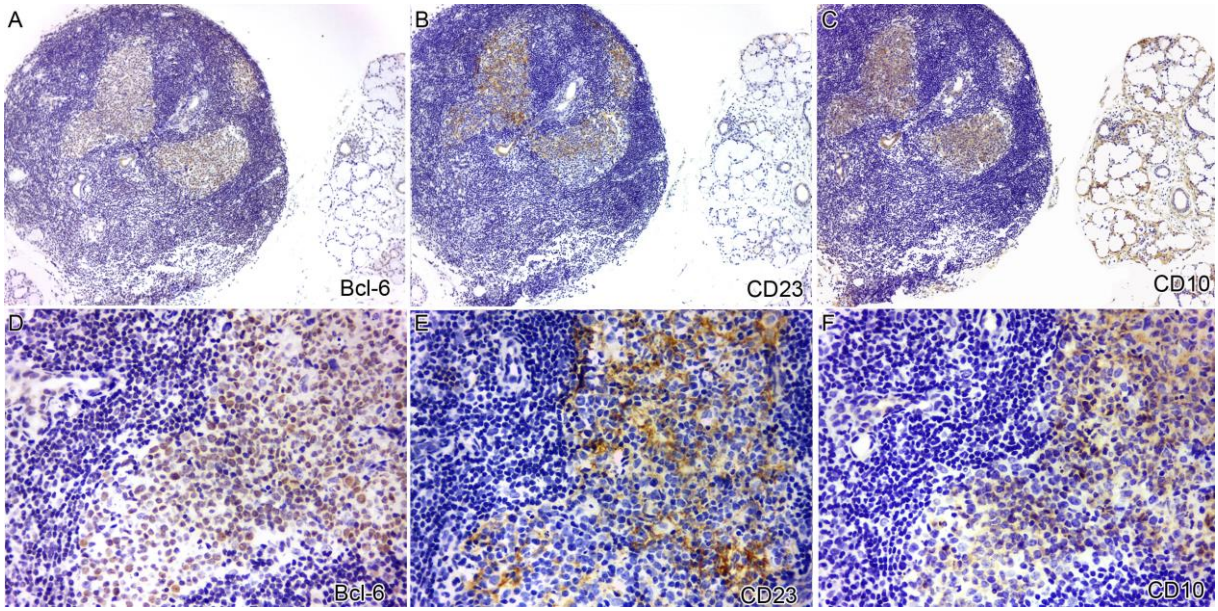

Figure 2. Immunohistochemical analysis in consecutive (serial) sections of focal lymphocytic sialadenitis in minor salivary gland biopsy specimen obtained from primary Sjögren's syndrome patient. In this case, notice germinal center-like structures showing strong positivity for Bcl-6 (A and D), CD23 (B and E) and CD10 (C and F) (A, B and C, ×10; D, E and F, ×40). The D, E, and F photomicrographs represent higher magnification of A, B, and C photomicrographs, respectively.

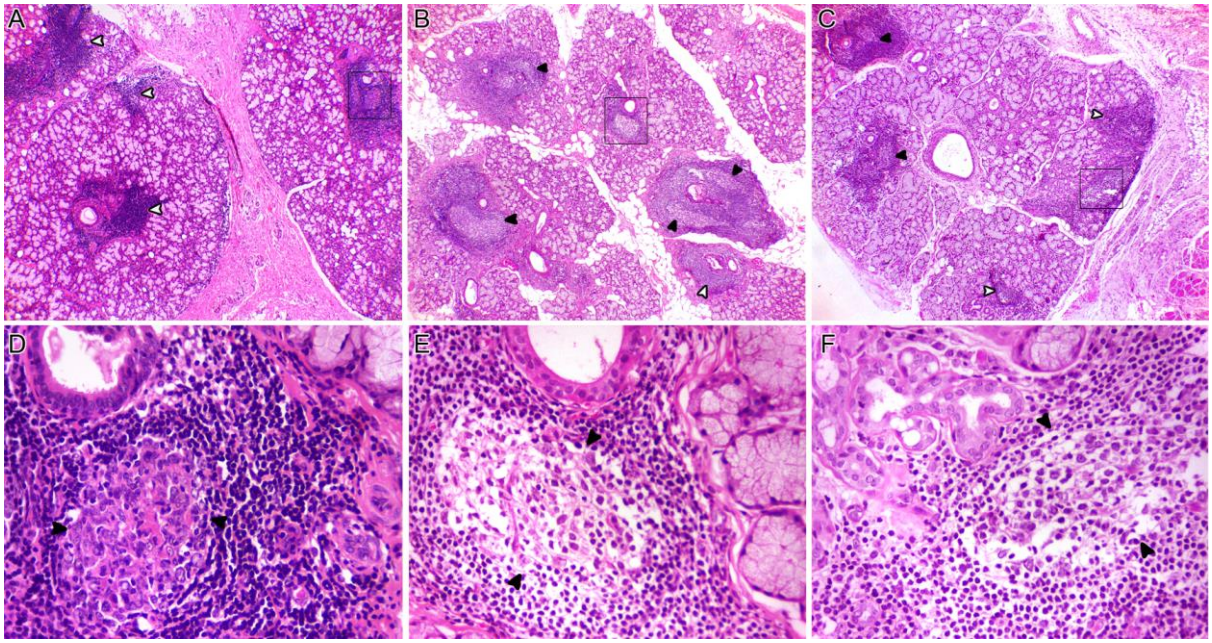

Figure 3. Histopathological analysis on hematoxylin and eosin (H&E) staining showing focal lymphocytic sialadenitis in oral reactive lesions obtained from non-Sjögren's syndrome non-sicca patients. In these cases, several foci (white arrowheads) were observed, containing germinal center-like structures (black arrowheads) (A, B and C, ×4; D, E and F, ×40). The squares in A, B, and C, are shown in increasing magnification in D, E and F, respectively.

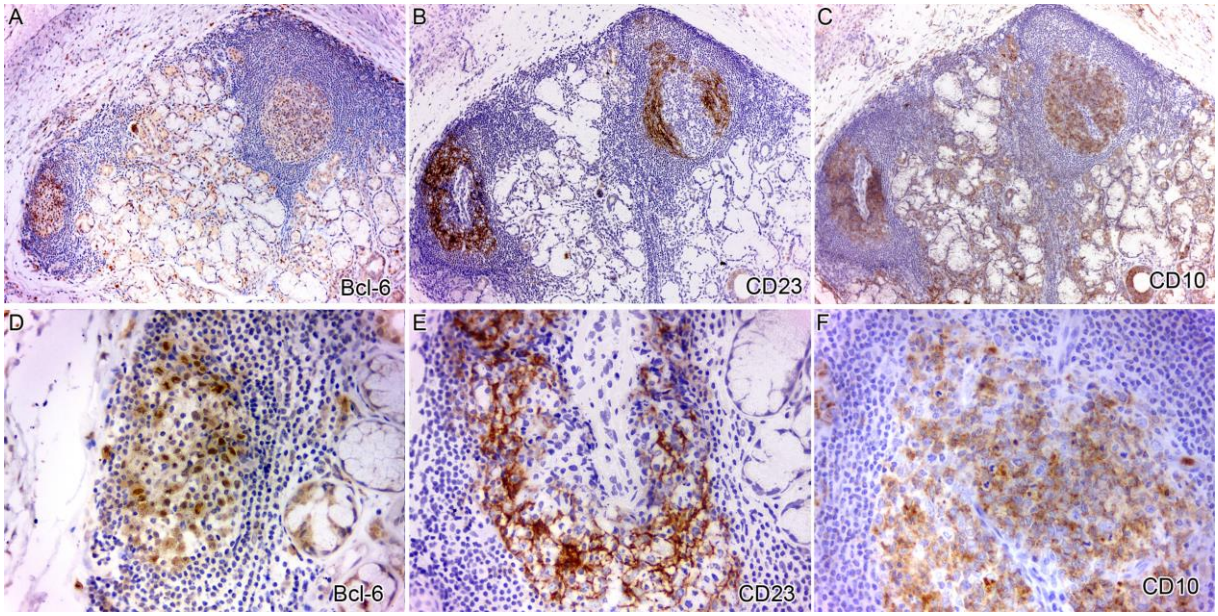

Figure 4. Immunohistochemical analysis in consecutive (serial) sections of focal lymphocytic sialadenitis in oral reactive lesion obtained from non-Sjögren's syndrome non-sicca patient. In this case, the germinal center-like structures showed positivity for Bcl-6 (A and D), CD23 (B and E) and CD10 (C and F) (A, B and C, ×10; D, E and F, ×40). The D, E, and F photomicrographs represent higher magnification of A, B, and C photomicrographs, respectively.

3.2 Publicação 2*

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF DENDRITIC CELL SUBGROUPS IN PRIMARY SJÖGREN'S SYNDROME: A comparative study.

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Article type: Short communication

Keywords: Sjögren's syndrome, non-Sjögren syndrome, non-sicca control; focal lymphocytic sialadenitis, dendritic cells; Langerhans cells; immunohistochemistry.

Funding:

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 (Evânio Vilela Silva).

* O artigo segue as normas para publicação no periódico ORAL DISEASES (1354-523X).

Luciana Yamamoto Almeida (2016/02713-2), Heitor Albergoni Silveira (2018/12734-2), and Jorge Esquiche León (2016/11419-0) have received research Grants from State of São Paulo Research Foundation (FAPESP).

NOTES

Compliance with Ethical Standards

Ethical Approval

This study was reviewed and approved by the Research Ethics Committee of the Ribeirão Preto Medical School, University of São Paulo (Protocol number: 60786216.8.0000.5440) and 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.

Conflicts of interest statement: The authors have no conflict of interest in the present manuscript.

Author contributions: All authors were involved in drafting the article or revising it critically for important intellectual content.

Data availability statement: The data underlying this article are available in the article and in its online supplementary material.

Primary Sjögren's syndrome (pSS) is a chronic autoimmune diseases primarily targeting salivary and lacrimal glands, causing to symptoms of oral and ocular dryness (xerostomia and keratoconjunctivitis sicca, respectively). Sometimes serious extraglandular manifestations occur, and the development of B-cell non-Hodgkin lymphoma is increased (Risselada et al., 2013). Microscopic evaluation of salivary glands of pSS patients reveals characteristic focal lymphocytic sialadenitis (FLS), which mainly consist of T- and B-lymphocytes, as well as a variety of nonlymphoid cells, including dendritic cells (DCs) and macrophages (Nakshabandi et al., 2020). DCs are antigen presenting cells and represent immune sentinels that operate an immune response against tumor cells and assist in the activation and function of T-lymphocytes. DCs are candidate key players in the activation of T- and B-cells in pSS, however, their contribution needs to be better clarified (Hillen et al., 2014).

Langerhans cells (LCs) are myeloid DCs (mDCs) found in stratified squamous epithelium and are characterized by the expression of S100, CD1a, CD45 and the C-type lectin Langerin (CD207) (Hillen et al., 2014). Previous studies evaluating mDCs in biopsies of minor salivary glands (mSGs) obtained from patients with pSS, have observed the presence of these cells in periacinar and periductal áreas around lymphocytic infiltrates, correlating with the perpetuation of the inflammatory process (Van Blokland et al., 2000; Xanthou et al., 1999). However, its adequate immunotyping, considering the heterogeneity of DCs, requires a detailed analysis. Plasmocytoid DCs (pDCs) are highly specialized cells in the production and secretion of high levels of type I interferon (IFN-I) after a antiviral response. Studies suggest that IFN-I secretion by pDCs are related to increased inflammatory activity in pSS, since they stimulate monocytes to produce high levels of B cell activation factor (BAFF), a mediator of B cell activation and the production of autoantibodies in this disease (Hillen et al., 2019; Ainola et al., 2018).Notably, in healthy human salivary glands, DCs are present in substantial numbers (comprising to 10% of the striated and excretory duct walls) mainly located between epithelial cells and interstitial tissues, suggesting that the salivary gland DCs play a significant role in the normal function of these organs; however, further studies are needed to better characterize its function under normal and pathological conditions (Le et al., 2011). Hence, the aim of this study was to characterize, through immunohistochemistry, the presence of LCs and pDCs in mSGs biopsies of pSS patients and oral reactive lesions presenting also FLS-like features.

In this study, 32 cases were evaluated, of which 20 cases were patients with pSS, FLS≥1 (SS group) classified according to American College of Rheumatology (ACR) - European League Against Rheumatism (EULAR) classification criteria (Shiboski et al., 2017), and 12 cases of oral biopsies from asymptomatic healthy patients (control group) that were clinically and histologically diagnosed as inflammatory fibrous hyperplasia, mucocele or ranula, which also presented typical FLS features at the periphery of the specimen and away from the main lesion (Table 1). The CD1a and CD207 immunomarkers were used to assess the presence of LCs, while pDCs were evaluated using the CD123 and CD303 immunomarkers. Ethical approval information, staining characteristics and statistical analysis are shown in Supplementary material.

In the SS group, a heterogeneous population of CD1a+ and CD207+ LCs were visualized, being preferably in a periductal pattern and around lymphocytic infiltrates. A significant greater number of CD207+ cells than CD1a+ cells was observed (mean±SD, 17.8±6.1 *versus* 6.5±3.9 respectively; $p<0.05$). In addition, the CD123+ and CD303+ pDCs showed an ovoid and circular morphology in a perivascular pattern forming cellular aggregates and commonly located at the periphery of the lymphocytic infiltrates. All cases showed expression of CD123+ cells and CD303+ cells (mean±SD, 15±8.7 *versus* 09±10.43 respectively; $p>0.05$). There were no statistical differences when comparing LCs and pDC in pSS.

In the control group, the CD1a+ and CD207+ LCs were visualized in a smaller number with location in a periductal pattern (mean±SD, 3.18±3.0 *versus* 2.85±4.31 respectively). The frequency of LCs was significantly higher in pSS than control group ($p<0.05$). The pDC were seen mainly in areas of lymphocytic infiltrate. These cells were positive in a smaller number (mean±SD, 6.20±8.40 *versus* 0.68±1.84, CD123+ cells *versus* CD303+ cells, respectively) when compared to the SS group; however, there was no statistically significant difference.

Few studies have evaluated the presence of mDCs in mSG biopsies of patients with pSS, these showed increased immunophenotypic expression of CD11c marker (Ozaki et al., 2010). Moreover, fascin+ cells (Ozaki et al., 2010), S100+ cells (Manoussakis et al., 2007) and CD1a+ cells, located preferably juxtaposed to ductal structures close to inflammatory infiltrates (Bikker et al., 2012; Van Blokland et al., 2000), were also observed. Though Fascin and S100 are not DC-specific markers, the

results are supported by providing additional data on other DC properties such as morphology and HLA-DR expression (Hillen et al., 2014). Interestingly, when evaluating the presence of these cells in mSG and peripheral blood (PB) of patients with pSS, it was noted that they were found in an increased number in mSG and decreased in PB, suggesting an important role in the local inflammatory immune response (Ozaki et al., 2010). In the present study, there was a high prevalence of CD207+ cells in when compared to CD1a+ cells in SS samples. The increase in these cells, as well as their periductal distribution, seems to show that these CD207+ LCs interact first with the ductal epithelial cells, probably capture antigens from these cells, process them and present them to the cells around the multifocal lymphocytic infiltrates in the pSS samples.

Our study also showed a predominance of CD123+ cells and CD303+ cells in pSS, compared to the control group, suggesting that they have an involvement in the modulation of the pathogenesis of pSS. These findings corroborate with previous studies, which documented the presence of an increased number of pDCs, main IFN-I producers, in mSG in pSS and found in reduced levels in BP (Vogelsang et al., 2010; Gottenberg et al., 2006). Notably, increased levels of IFN-I have been observed in biopsies from patients with pSS. IFN-I was present in the epithelial cells of the ducts and in the lymphocytic foci. Serum IFN-I levels were increased in only a few patients with pSS, and INF-I mRNA levels were only slightly elevated in BP cells compared to controls (Zheng et al., 2009). Also, a recent study identified the expression of the *toll-like* endosomal receptor, TLR7, distributed juxtaposed to ducts of mGSs, correlating with the activation and presence of pDCs (Shimizu et al., 2019).

In conclusion, our results suggest the participation of LCs and pDC in the pathogenesis of SS. Further studies, including functional studies on DC populations, are important to elucidate the innate immunological pathways and, eventually, contribute to the optimization of immunotherapeutic drug targets.

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Table 1. Clinicopathological features of the cases included in the current study.

Clinicopathological features	**pSS group (n = 20)**	**Control group (n = 12)**
Mean age / range (years)	49 / 12 – 81	45/ 10 – 88
Gender		
Female	19	11
Male	1	1
Diagnosis		
pSS	50	-
Inflammatory fibrous hyperplasia	-	8
Mucocele	-	3
Ranula	-	1
Histopathological findings		
FLS with FS≥1	20	5
FLS with FS≤1	-	6
FLS/CSS with FS≤1	-	1

Abbreviations: CSS, chronic sclerosing sialadenitis; FLS, focal lymphocytic sialadenitis; FS, focus score; pSS, primary Sjögren's syndrome.

Figures:

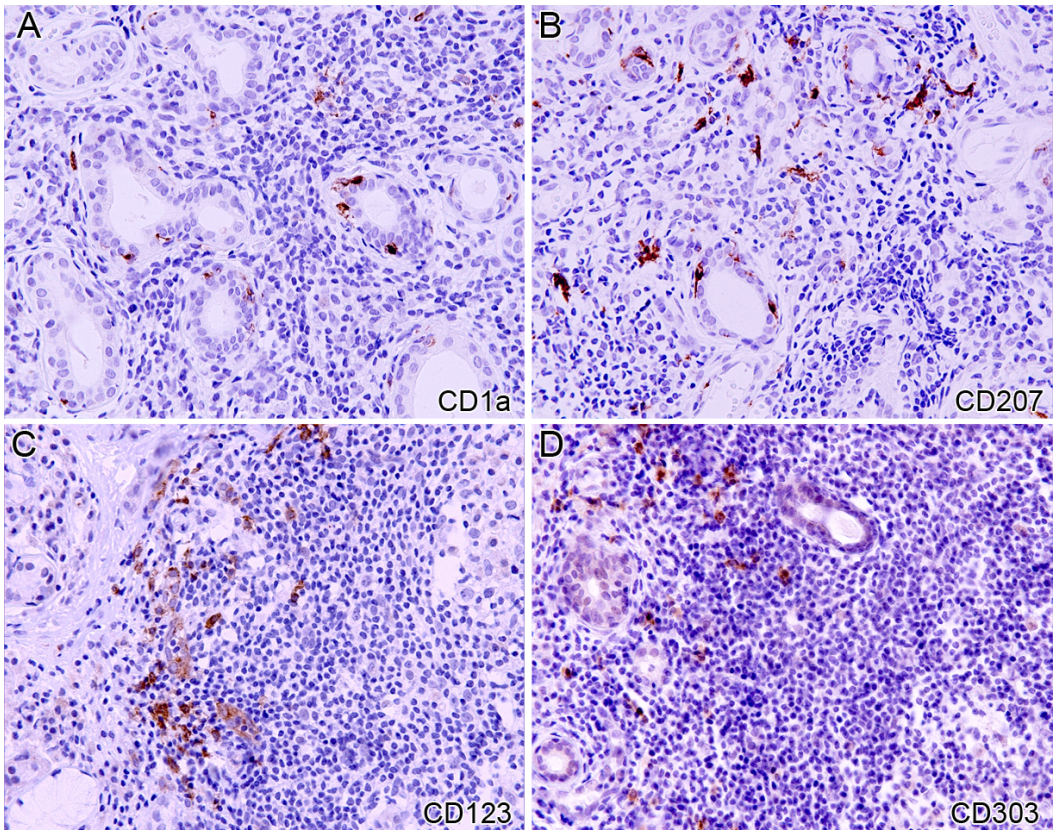

Figure 1. Immunohistochemical analysis of mSG biopsies from patients in the SS group showing the presence of LCs positive for CD1a (A) and CD207 (B) with periductal distribution; and pDCs with positive expression for CD123 and CD303 (C and D respectively). (x400).

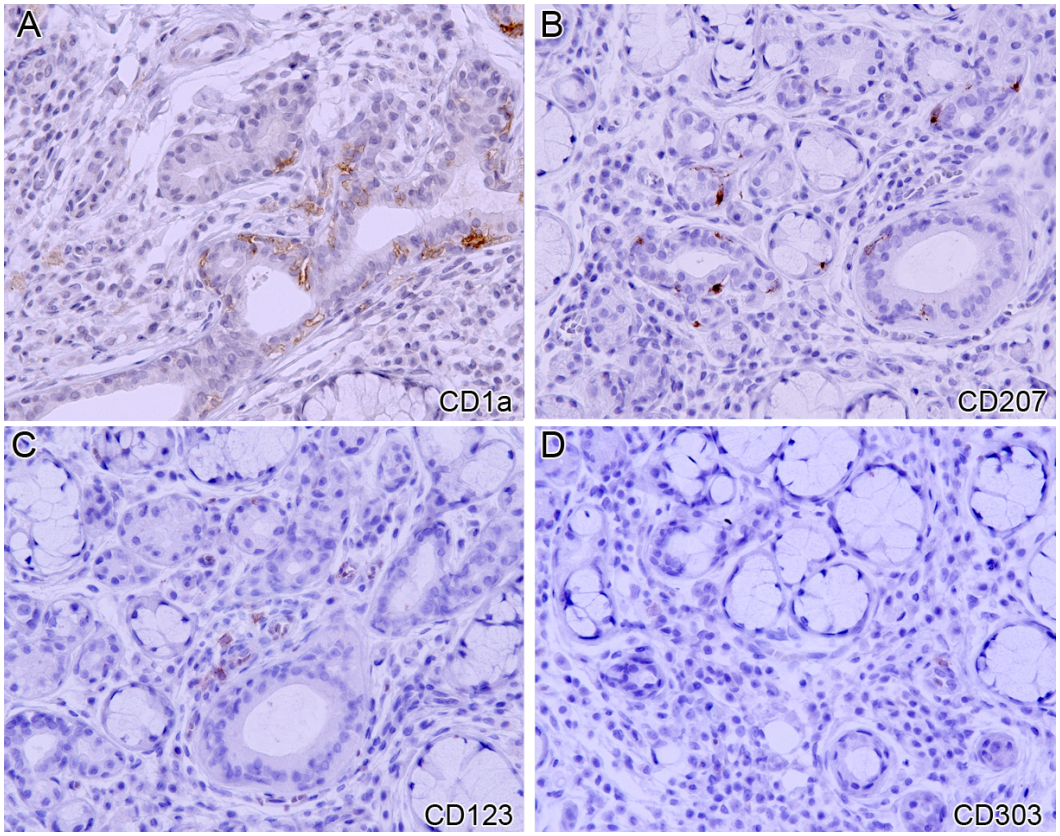

Figure 2. Immunohistochemical analysis of biopsies of reactive oral lesions (control group) for LCs, showing positivity for CD1a (A) and CD207 (B), located preferentially in ectatic areas and in smaller numbers when compared to the control group; and for pDCs with low expression for CD123 and CD303 (C and D respectively). (x400)

Supplementary material

Ethical approval information: This study was approved by the Research Ethics Committee of the Ribeirão Preto Medical School, University of São Paulo (protocol number: 68748117.0.0000.5440) and 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.

Histopathological assessment: Sections of 5-µm were obtained from all cases and stained with H&E for diagnostic confirmation. FLS was evaluated according to the presence of one or more dense aggregates with 50 or more lymphocytes in a 4mm² glandular area, defined by of the American College Rheumatology - European League Against Rheumatism classification criteria (Shiboski et al., 2017).

Immunohistochemical staining: For IHC analysis, consecutive (serial) histological sections of 3-µm were placed on organosilane coated slides (Sigma–Aldrich, St. Louis, MO). The deparaffinization was performed using xylene solution and the rehydration of the sections occurred through the passage in ethanol solution with serial concentrations. The antigen retrieval was prepared by immersion of the sections in citrate buffer (target retrieval solution, pH6). After, the sections were submitted to the immunohistochemistry technique by streptavidin-biotin-peroxidase method (Universal LSAB^{TM} + Kit/HRP, Dako, Carpinteria, CA, USA). The primary antibodies used are shown supplementary table. Stained specimens were examined using a computerized system consisting of a light microscope (LeicaDM500) connected adapted to a high-resolution camera (Leica ICC50) and a colour video monitor. Images were obtained using the Leica IM50 Image Manager program, and the processing was performed using the Leica QWin Image Processing and Analysis System. After evaluation of the slides at 100x magnification, areas with a higher density of immunostaining were selected,and 5 images were recorded at 400x; the examined area was 0.785 mm2 (positive cells/mm2).

Supplementary table. List of primary antibodies used.

Antibodies	Clone	Dilution	Manufacturer
CD1a	010	1:400	DakoCytomation, Glostrup, Denamark
CD123	BR4MS	1:200	Leica Biosystems Newcastle, UK
CD207	12D6	1:200	Monosan, Uden, the Netherlands
CD303	124B3.13	1:500	Dendritics, Lyon, France

Statistical analysis: Statistical analysis was performed using the IBM SPSS 20.0 software. The Shapiro–Wilks test for assessing the normal distribution was used. The Student *t*-test and Pearson correlations were applied in samples with a normal distribution, whereas the Mann–Whitney *U* test and Spearman correlation were applied in samples with a non-normal distribution. For categorical data, the chi-square test was applied. A probability (p) value <0.05 was considered statistically significant.

4 CONCLUSÃO

A SLF também pode ser observada quando avaliando lesões orais reativas, as quais apresentaram uma frequência de CGs semelhante às encontradas em biópsias de GSm de pacientes com SSp.

Nossos resultados, também, sugerem protocolos padronizados para análise morfológica e imunoistoquímica ao avaliar CGs em biópsias de GSm de pacientes SSp.

Devido à frequência semelhante de CGs em lesões orais reativas e SSp, estudos adicionais, incluindo análise funcional de populações linfocíticas e CGs em SLF, são encorajados.

A expressão de imunomarcadores de células dendríticas mieloides e plasmocitoides em biópsias de pacientes com SSp, pode sugerir a participação dessas células na patogênese dos eventos imunoinflamatórios na SS. Estudos futuros analisando funcionalmente subconjuntos de CDs são essenciais para melhor compreensão da patogênese da SS, visando alvos imunoterapêuticos.

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ANEXO A – Parecer do Comitê de Ética em Pesquisa

USP - HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DE RIBEIRÃO PRETO DA USP -

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: FREQUÊNCIA DE CENTROS GERMINATIVOS E CARACTERIZAÇÃO DE CÉLULAS DENDRÍTICAS MIELOIDES E PLASMOCITOIDES NA SÍNDROME DE SJOGREN

Pesquisador: Jorge Esquiche León

Área Temática:

Versão: 1

CAAE: 22306619.6.0000.5440

Instituição Proponente: UNIVERSIDADE DE SAO PAULO

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.624.809

Apresentação do Projeto:

A síndrome de Sjögren (SS) é uma doença crônica autoimune, frequentemente afetando mulheres (9:1) e caracterizada por apresentar xerostomia e xeroftalmia. Microscopicamente, a identificação de sialadenite linfocítica focal (SLF) com escore focal (EF) 1 nas glândulas salivares menores (GSMs) labiais é um critério diagnóstico importante. Notavelmente, a identificação de centros germinativos (CGs) na SLF parece ter um impacto prognóstico significativo, enquanto que a participação de células dendríticas (CDs) na patogênese da SS precisa ser mais bem definida. Assim, o objetivo desse trabalho será avaliar a frequência de CGs e caracterizar às CDs mieloides (CDmi) e plasmocitoides (CDp) em biópsias de GSMs de pacientes com SS primária (SSp). Será realizado um estudo retrospectivo, para o qual serão selecionados 100 casos divididos em três grupos, Grupo A: 50 casos de SSp; Grupo B: 25 casos de sialadenite crônica inespecífica; Grupo C: 25 casos controle. Na análise histopatológica serão realizados cortes com 5 µm de espessura, os quais serão corados com hematoxilina-eosina, e posteriormente analisados para descrição morfológica, confirmação do diagnóstico e frequência de CGs. Na análise imunoistoquímica serão realizados cortes com 3 m de espessura, os quais serão colocados sobre lâminas devidamente revestidas com organo-silano, para avaliar os anticorpos individuais, visando identificar CGs (Bcl6, CD10, CD23), células de Langerhans (S100, CD1a e CD207), CDs submucosas (CDsub, CD209 e FXIIIA) e CDp (CD123 e CD303). Posteriormente, será realizada uma análise descritiva dos marcadores, identificando a

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frequência absoluta e relativa dos achados obtidos. Depois será comparada a expressão dos marcadores entre as lesões (teste exato de Fisher) e a correlação entre os mesmos (coeficiente de correlação de Spearman), adotando significância de 5%, por meio do software SAS (Cary, Carolina do Norte, EUA).

Objetivo da Pesquisa:

Avaliar, por imunoistoquímica, a frequência de centros germinativos e a presença de células dendríticas mieloides e plasmocitoides em biópsias de glândulas salivares menores de pacientes com síndrome de Sjögren primária.

Avaliação dos Riscos e Benefícios:

Riscos:O estudo será realizado em blocos parafinados de amostras que já foram excisadas como parte do tratamento realizado no paciente. Os espécimes serão devidamente armazenados em local adequado identificados por códigos, garantindo a preservação do material e seu anonimato visando diminuir minimizar os riscos. O material ficará sob a guarda do Prof. Dr. Jorge Esquiche León e do Prof. Dr. Alfredo Ribeiro da Silva, os quais ficarão responsáveis pela autorização do uso do material.

Benefícios: Estudos desta natureza são importantes porque as associações clinico-patológicas que serão realizadas podem esclarecer o papel e participação do sistema imune (células dendríticas) na patogênese destes tumores, focando fatores terapêuticos e prognósticos para o paciente.

Comentários e Considerações sobre a Pesquisa:

Será realizado um estudo retrospectivo, cujos dados clínicos serão obtidos dos formulários clínicos de encaminhamento das biópsias das GSMs. Para o estudo, serão selecionados 100 casos divididos em três grupos: Grupo A: 50 casos de GSMs diagnosticados como SSp; Grupo B: 50 casos diagnosticados como sialadenite crônica inespecífica (hiperplasia fibrosa, trauma por prótese);Grupo C: 50 casos controle (GSMs com diagnóstico de mucocele).Análise histopatológica: Serão realizados cortes com 5 µm de espessura, os quais serão corados com hematoxilina eosina (H&E), e posteriormente analisados para descrição morfológica e confirmação do diagnóstico, bem como a identificação de CGs.

Análise imunoistoquímica: Serão realizados cortes com 3 m de espessura, os quais serão colocados sobre lâminas devidamente revestidas com organo-silano (Sigma-Aldrich, St Louis, MO, EUA). Os cortes obtidos serão submetidos à técnica imunoistoquímica pelo método da estreptavidinabiotina peroxidase (Universal LSAB™+ Kit/HRP) para avaliar os anticorpos individuais para frequência de CGs (Bcl-6, CD10, CD23), CLs (S100, CD1a e CD207), CDsub (CD 209 e FXIIIA)

e CDp(CD123 e CD303). Após a avaliação das lâminas, 10 áreas com maior densidade de imunomarcação serão selecionadas e a densidade e porcentual de células positivas serão registradas em um campo microscópico de 400x (0,785mm2) (células positivas/mm2). A contagem de células positivas será realizada através do software Image J (National Institutes of Health Bethesda, MD) e os dados serão compilados em uma panilha em Excel.

Considerações sobre os Termos de apresentação obrigatória:

Documentos devidamente apresentados. Propõe dispensa do TCLE, sendo um estudo retrospectivo e baseado na análise de espécimes de arquivo, não haverá contato direto com os pacientes.

Recomendações:

não se aplica

Conclusões ou Pendências e Lista de Inadequações:

Diante do exposto e à luz da Resolução CNS 466/2012, o projeto de pesquisa, assim como a solicitação de dispensa de aplicação do Termo de Consentimento Livre e Esclarecido, podem ser enquadrados na categoria APROVADO.

Considerações Finais a critério do CEP:

Projeto Aprovado: Tendo em vista a legislação vigente, devem ser encaminhados ao CEP, relatórios parciais anuais referentes ao andamento da pesquisa e relatório final ao término do trabalho. Qualquer modificação do projeto original deve ser apresentada a este CEP em nova versão, de forma objetiva e com justificativas, para nova apreciação.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1436397.pdf	27/09/2019 14:09:22		Aceito
Outros	Formulario_SAM.pdf	27/09/2019 14:07:22	EVANIO VILELA DA SILVA	Aceito
Outros	autorizacao_laboratorio.pdf	26/09/2019 12:58:33	EVANIO VILELA DA SILVA	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO_DE_PESQUISA_CEP.pdf	24/09/2019 11:52:05	EVANIO VILELA DA SILVA	Aceito
Folha de Rosto	folha_de_rosto.pdf	20/09/2019 17:07:23	EVANIO VILELA DA SILVA	Aceito
Outros	upc_aprovacao.pdf	20/09/2019	EVANIO VILELA DA	Aceito

Outros	upc_aprovacao.pdf	14:02:10	SILVA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	solicitacao_isencao_tcle.pdf	16/09/2019 17:55:33	EVANIO VILELA DA SILVA	Aceito
Orçamento	orcamento_detalhado.pdf	16/09/2019 15:41:42	EVANIO VILELA DA SILVA	Aceito
Cronograma	Cronograma.pdf	16/09/2019 15:35:59	EVANIO VILELA DA SILVA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RIBEIRAO PRETO, 07 de Outubro de 2019

Assinado por:
Marcelo Riberto
(Coordenador(a))

Não autorizo a publicação deste trabalho até dia 05 de março de 2023

Araraquara, 05 de março de 2021.

Evânio Vilela da Silva