Acinar adipose tissue infiltration in salivary gland biopsy is associated with kynurenines-interferon-γ pathway inflammation biomarkers

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ABSTRACT

Objective. *Assess if kynurenines metabolites are biomarkers of damage at labial salivary gland biopsy (LSGB).*

Methods. *This is a cross-sectional study including 99 patients with primary Sjögren's syndrome (AECG 2002 or ACR/EULAR 2017). Kynurenines were measured in plasma using liquid chromatography-tandem mass spectrometry.*

Results. *95.9% were females, 51±12 years. Most had focal lymphocytic sialadenitis with focus score ≥1 (73.7%, n=73/99). The majority had mild to severe acinar atrophy (70.4%, n=57/81) and adipose infiltration (51.2%, n=39/80). Individuals with adipose infiltration were older (53.49±12.33 vs. 47.51±11.29 years, p=0.016), showed higher frequency of glandular dysfunction and higher kynurenines levels. Schirmer's test ≤ 5 mm/5min was found in 69.2% of individuals with adipose infiltration compared to 41% without (p=0.012) and unstimulated whole salivary flow (UWSF) was found in 87.2% compared to 70% without adipose infiltration (p=0.063). Additionally, individuals with adipose infiltration showed higher kynurenines metabolites compared with those without: quinolinic acid (503.35±193.30 vs. 427.35±285.76 nmol/L, p=0.029), kynurenine (1.99±0.6, 54 vs. 1.61±0.46 µmol/L, p=0.006), kynurenine/tryptophan ratio (KTR) (0.030±0.09 vs. 0.025±0.01, p=0.031) and anthranilic acid (03±4.96 vs. 16.46±5.24 nmol/L, p=0.022).*

Conclusion. *Kynurenines are biomarkers of greater adipose infiltration in LSGB and glandular dysfunction suggesting that activation of interferon-γ pathway is involved in the salivary and lacrimal glands damage.*

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterised by lymphocytic infiltration of exocrine glands resulting in lacrimal and salivary dysfunction (1, 2). Additionally, extraglandular manifestations due to epithelitis or vasculitis are present in around 30–50% of patients (3). The prevalence pSS in the general population ranges from 0.01–0.72% and it predominantly affects middle-aged women (1).

Histopathological analysis of the minor salivary gland biopsy is an essential procedure in the diagnosis of pSS. The detection of focal lymphocytic sialadenitis (FLS), characterised by the presence of dense aggregates (foci) of ≥50 mononuclear cells (mostly lymphocytes) in a periductal or perivascular localisation adjacent to normal appearing acini, is a gold standard diagnostic test for pSS with high sensitivity (63.5 to 96.7%) and specificity (61.2 to 100%) (4, 5). Features of non-specific chronic sialadenitis (NSCS), such as acinar atrophy, duct dilation, fibrosis and/or adipose infiltration are common in the population and may thus coexist with pSS (6, 7).

More recently, it has also been demonstrated that the presence of germinal-centre *like* structures, focus score ≥3 and lymphoepithelial lesions (LESA) are associated with more systemic manifestations, glandular dysfunction and higher risk for developing lymphoma (8-10). Unfortunately, to date little attention has been dedicated to the presence of acinar atrophy and adipose infiltration in salivary glands in pSS. While several studies report association of adipose infiltration with aging, obesity and diabetes (11-13), few is known about their role in the inflammatory

process in pSS or other sialadenitis (14, 15). Considering the key role of adipocyte tissue in production of proinflammatory cytokines, such as IL-6 (14) and IL-17 (15), adipose infiltration in pSS might be an important contributor to the disease progression.

Patients with pSS have an activated type I interferon (IFN) system, including IFNα and IFNβ – the key immune mediators in activation of immune responses, which contribute to the aetiopathogenesis and clinical manifestations of the disease. The IFN type I signature is present in over half of pSS patients, associated with higher disease activity (16). Though IFN-α is considered as a predominant contributor to the pSS pathogenesis, the involvement of IFN-γ in pSS has been previously demonstrated in both humans and animal models (17, 18). New biomarkers associated with this pathway and salivary glands should be better explored (19).

IFN-γ is a major inducer of kynurenines metabolites by indoleamine 2,3 dioxygenase (20) activation in many cell types, including fibroblasts, endothelial cells, tumour cells, monocyte-derived macrophages, mesenchymal stromal cells and dendritic cells (21).

Tryptophan is an essential amino acid catabolised by the enzyme indoleamine 2,3, dioxygenase (IDO) or by the enzyme tryptophan 2,3-dioxygenase (TDO) into N-formyl-kinurenine, which is then converted to kynurenine. Kynurenine is further metabolised into hydroxykynurenine and other biologically active derivatives collectively termed kynurenines (21, 22).

The main effect of kynurenines on the immune system is to induce regulatory T cells and thus to reduce the inflammatory response (23). It is generally accepted that IDO expression contributes to the ability of tumours to evade/escape the immune system and that IDO-mediated tryptophan catabolism is a mechanism for blocking the anti-tumour immune response. Increased plasma kynurenine levels and kynurenine-to-tryptophan ratio (KTR) have been found in patients with systemic inflammatory response syndromes, such as sepsis and septic shock, and in a few studies of autoimmune diseases, but the biological significance and prognostic value of these findings have remained uncertain (24, 25).

Adipose tissue infiltration is a late finding in the salivary gland histology of pSS. We hypothesised that there could be evidence of more severe disease mediated by the IFN-γ pathway. For this purpose, we assessed kynurenines, inflammatory biomarkers of the IFN-γ pathway, and their associations with adipose tissue infiltration in the minor salivary gland biopsy in patients with pSS.

Material and methods

This was a cross-sectional study including all patients with pSS from the Rheumatology Unit of the University Hospital of the Federal University of Espírito Santo (HUCAM/UFES/EBSERH), Brazil.

Inclusion criteria were patients above 18 years old, who underwent labial salivary gland biopsy (LSGB) and fulfilled the classification criteria for pSS, according to the American-European Consensus Group 2002 (AECG 2002) (26) and/or American College of Rheumatology 2012 (ACR 2012) (27) and/or American College of Rheumatology-European League Against Rheumatism 2016 (ACR-EULAR 2016) (28).

All recorded clinical information was collected from December 2016 to July 2017. The study was approved by the Ethics Committee on Research of the University Hospital of Espírito Santo, Brazil (protocol no. 1.834.602/2016).

Clinical parameters

Demographic data, autoantibody tests, and clinical manifestations were obtained through medical reports. Eular Sjögren's Syndrome Disease Activity Index (ESSDAI) (29, 30), Sjögren's Syndrome Patient Reported Index (ESSPRI) (29, 31), Syndrome Disease Damage Index (SSDDI) (32) were assessed. The autoantibodies evaluated were antinuclear antibody (ANA) using indirect immunofluorescence, RF measured by turbidimetry, and anti-Ro/SSA and anti-LA/SSB using haemagglutination.

Dryness symptoms and glandular dysfunction

Regarding the dryness symptoms, the data considered were the sensation of dry eye, use of ocular lubricant for symptoms of xerophthalmia, and the sensation of dry mouth, the increase in parotids and dysphagia for dry foods for symptoms of xerostomia.

Concurrently with biopsy, patients performed Schirmer's test (ST, without anaesthesia) and unstimulated whole salivary flow (UWSF). Glandular dysfunction was defined if ST was ≤5mm over 5 minutes using standardised sterilised test strips (Ophtalmos, São Paulo, Brazil) and/or UWSF was ≤1.5ml/min measured for 15 minutes. Patients were recommended to have breakfast without coffee or chocolate. All measures were done between 9 a.m. and 11 a.m.

Histopathological analysis

Histopathological patterns and assessment of focus score (FS) were made according to the Sjögren's International Clinical Collaborative Alliance (SICCA) protocol (6, 33). Just one pathologist experienced in SG (MCSSS) re-evaluated all histological specimens of LSGB. Inflammation was graded from 0-4 according to Chisholm & Mason (34). The presence of acinar atrophy, adipose infiltration, ductal dilatation was grade as mild, moderate, severe or absent according to the guidelines for assessing salivary gland biopsies published by Fisher *et al*. (25). For acinar and adipose infiltration, it was considered mild when their presence was as far as 1 to 10%; moderate 11 to 50%, and severe when was more than 50%. This method is used to score fatty infiltration in non-alcoholic steatohepatitis (35). The evaluation of ductal dilatation was based on the observer subjective experience.

Biomarkers

Concentrations in plasma of hydroxykynurenine, xanthurenic acid, quinolinic acid, kynurinine, kynurinemic acid, tryptophan, hydroxianthranilic acid, anthranilic acid were measured by liquid chromatography-tandem mass spectroscopy in the laboratory of Bevital AS (www.bevital.no) (36). Kynurenine:Tryptophan ratio (KTR) was calculated by dividing the concentration of kynurenine (nmol/L) by the

concentration of tryptophan (umol/L). If this was done correctly, numbers should be in the range of 20–30. To be noted that Kyn concentrations are given as umol/L from Bevital. C-reactive protein (CRP) was determined in serum by using an ultrasensitive immunoassay with the Behring nephelometer II system (Latex CRP mono; Behring Diagnostics).

Statistical analysis

Shapiro-Wilk, Student's t-test, Mann-Whitney tests were properly used for statistical analysis. Pearson or Spearman tests, Qui-square test were used to study association between histological analysis and clinical variables. A *p*-value ≤0.05 was considered statistically significant. The software IBM SPSS Statistics v. 20.0.0 was used to store data and to run statistical analysis.

Results

Our cross-sectional study included all of the pSS patients from the Rheumatology Unit of the University Hospital of the Federal University of Espírito Santo, Brazil (HUCAM/UFES/EBSERH). Overall, 104 pSS patients were evaluated between 2013 and 2015. Five patients lacking biopsy data were excluded from the analysis.

The baseline characteristics of the patients are summarised in Table I. Among 99 included patients, 95.9% were women, with mean age at diagnosis of 51 (SD±12) years. Histopathological findings of minor salivary glands showed focal lymphocytic sialadenitis with focus score ≥1 in 73.7% (n=73/99) of patients and, non-specific chronic sialadenitis in 25.3% (n=25 / 99) and only one histological examination was negative for sialoadenitis in 1% (n=1/99). Germinal centre-like structures were found in 17.9% (15/84). Granulomatous inflammation or marginal zone (MALT) lymphoma were absent. Acinar atrophy (70.4%, n=57/81) and/or ductal dilatation (86.4%, n=70/81) were present in the majority of samples. Adipose tissue infiltration was present in 48.8% (n=39/80) of samples (Table II). The mean biopsies area was 15 mm^2 and the mean focal score was 1.52.

Table I. Demographic and clinical characteristics of primary Sjögren's syndrome total sample.

Characteristics	n=104	Mean ± SD	%
Age (years)	104	51 ± 12	
Disease duration (months)	104	65 ± 42	
Female	99/104		95.2
Caucasian	37/104		35.6
Dry eye	96/104		92.3
Dry mouth	95/104		91.3
Lubricant eye drop	52/104		50.0
Dysphagia	41/104		39.4
Parotid enlargement	31/104		29.8
Schirmer's test ≤ 5mm/5min	59/98		60.2
UWSF ≤ 0.1ml/min	80/100		80.0
Anti-Ro/SSA	70/103		68
Anti-La/SSB	30/99		30.3
Antinuclear antibody	82/100		82
Rheumatoid factor	37/99		37.4
Focal lymphocitic sialoadenitis (focus-score ≥ 1)	71/97		73.2
BMI	104	28 ± 6	
ESSPRI	104	5.62 ± 2.56	
ESSDAI	104	3.63 ± 5.12	
SSDDI	104	1.77 ± 1.25	
IgG (mg/dL)	104	1,539 ± 699	
C3 (mg/dL)	104	104 ± 38	
C4 (mg/dL)	104	29.8 ± 12	

UWSF: unstimulated whole salivary flow; BMI: body mass index; ESSPRI: Sjögren's Syndrome Patient Reported Index; ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index; SSDDI: Sjögren's Syndrome Disease Damage Index; IgG: immunoglobulin G; C3: complement 3; C4: complement 4.

Patients with adipose tissue infiltration were older (53.49±12.33 years) than those without adipose tissue infiltration (47.51±11.29, *p*=0.016 years). The prevalence of positive anti-Ro was similar in patients with and without adipose infiltration (27/39, 69.2% vs. 28/41, 68.3%, *p*=0.343). The presence of positive anti-Ro/La was higher in patients with acinar adipose infiltration in salivary glands (13/39, 33.3% *vs*. 10/41, 24.4%, *p*=0.188), but it was not significantly different.

Furthermore, adipose tissue infiltration was associated with lacrimal dysfunction (Schirmer's test ≤5 mm = 69.2% *vs*. 41%, *p*=0.012; Fig. 1) and kynurenine (1.99 ± 0.54 *vs*. 1.61 ± 0.46 µmol/L, *p*=0.006), kynurenine/tryptophan ratio (KTR) (0.030±0.09 *vs*. 0.025±0.01, *p*=0.031) higher quinolinic acid (503.35±193.30 *vs*. 427.35±285.76 nmol/L, *p*=0.029), and anthranilic acid (19.03 ± 4.96 *vs*. 16.46±5.24 nmol/L, *p*=0.022), (Table III).

Patients with focus score ≥1 had higher level of CRP than those with non-focal sialoadenitis (2.0±5.45 *vs*. 0.43±0.88 mg/dL, *p*=0.04). CRP showed a posi-

Table II. Histopathological findings in the labial salivary glands.

Characteristics	n	%
Degree of inflammation		
Grade 0	1/97	1.1
Grade 1	6/97	6.2
Grade 2	19/97	19.3
Grade 3 (focus score = 1)	14/97	14.4
Grade 4 (focus score >1)	57/97	58.8
No information	2	-
Germinal centre-like		
Present	15/84	17.9
No information	15	-
Acinar atrophy		
No	24/81	29.6
Mild	43/81	53.1
Moderate	12/81	14.8
Severe	2/81	2.5
No information	18	-
Ductal dilatation		
No	11/81	13.6
Mild	49/81	60.5
Moderate	21/81	25.9
No information	18	-
Adipose tissue infiltration		
No	41/80	51.2
Mild	30/80	37.5
Moderate	7/80	8.8
Severe	2/80	2.5
No information	19	-
Total	**99**	**100.0**

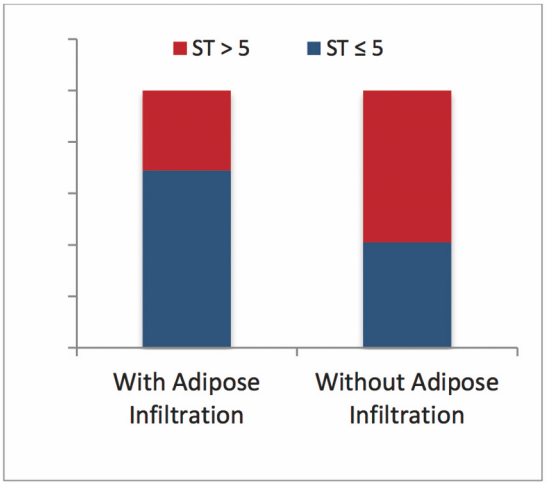

Fig. 1. Association of adipose tissue infiltration with glandular dysfunction ($p \leq 0.05$). ST: Schirmer's test.

tive correlation (r=0.391, p=0.009) with focus score and no association with total or each domain of the Sjögren's Syndrome Disease Damage Index (SSDDI). On the other hand, SSDDI (r=0.256, p=0.036) was correlated with kynurinemic acid.

Discussion

Primary Sjögren's syndrome is a systemic autoimmune disease that mainly affects the exocrine glands. Histopathological abnormalities in LSGB with focal lymphocytic sialadenitis and focus score of ≥1 are widely accepted as pathological findings, confirming the salivary gland (SG) component of pSS (33). Acinar atrophy, fatty replacement and non-specific chronic inflammation have been also described in SG in pSS (6). Adipocytes can occupy a large SG area in pSS and it is more common in pSS patients compared to sicca syndrome individuals (14). Indeed, our study showed that fatty replacement was found in 49% of patients with pSS and it was associated with greater glandular dysfunction and higher kynurenines metabolites suggesting that activation of interferon-γ pathway is involved in the salivary and lacrimal gland damage.

Adipose tissue and inflammation in SG in pSS

There is an increased interest to understand the significance of adipose tissue and its evolved mechanisms in salivary and lacrimal damage.

It was observed that adipose infiltration in salivary gland biopsy was present in patients who also had evidence of dry eye obtained by the Schirmer test as seen in Figure 1. Considering that pSS is a systemic disease and understanding that adipose infiltration is involved in around an inflammatory process, we

Table III. Association between adipose tissue infiltration with age, disease duration, anti-Ro/SSA, glandular dysfunction, smoking, EULAR Sjögren's Syndrome Disease Activity Index and Sjögren's Syndrome Patient Reported Index, and metabolites of the inflammatory kynurenine pathway.

	With adipose infiltration		Without adipose infiltration		*p*-value
	n	Mean ± SD or %	n	Mean ± SD or %	
Age (average in years)	39	53 ± 12	41	47 ± 11	0.016[a]
Duration of symptoms (average in months)	37	99 ± 49	39	109 ± 63	0.731[a]
Duration of diagnosis (average in months)	38	62 ± 37	40	63 ± 46	0.745[a]
Anti-Ro/SSA positive	27	71	28	68	0.790[b]
UWSF ≤1.5ml/15min	34	87	28	70	0.063[b]
Schirmer's test RE ≤5mm/5min	25	64	16	41	0.041[b]
Schirmer's test LE ≤5mm/5min	27	69	16	41	0.012[b]
Smoking	5	13	2	5	0.258[c]
ESSPRI Dryness ≥5	29	81	23	66	0.060[b]
ESSPRI Pain ≥5	22	61	25	66	0.676[b]
ESSPRI Total ≥5	23	62	27	68	0.624[b]
ESSDAI moderate/high activity >5	9	23	11	27	0.698[b]
CRP (mg/dL)	16	0.31 ± 0.40	18	2.04 ± 5.93	0.384[a]
Hydroxykynurenine (nmol/L)	26	61 ± 57	26	55 ± 75	0.066[a]
Xanthurenic acid (nmol/L)	26	14 ± 12	26	10 ± 4	0.448[a]
Quinolinic acid (nmol/L)	26	503 ± 193	26	427 ± 285	0.029[a]
Kynurinine (μmol/L)	26	1.99 ± 0.54	26	1.61 ± 0.46	0.006[a]
KTR	26	30 ± 9	26	25 ± 10	0.031[a]
Kynurinemic acid (nmol/L)	26	38 ± 17	26	37 ± 15	0.848[a]
Tryptophan (nmol/L)	26	68 ± 11	26	66 ± 9	0.615[a]
Hydroxianthranilic acid (nmol/L)	26	38 ± 20	26	33 ± 9	0.390[a]
Anthranilic acid (nmol/L)	26	19.03 ± 4.96	26	16.46 ± 5.24	0.022[a]

[a]Mann-Whitney U; [b]chi-square test; [c]Fisher's exact test. (p<0.05).
SD: standard deviation; UWSF: unstimulated whole salivary flow; RE: right eye; LE: left eye; ESSPRI: Sjögren's Syndrome Patient Reported Index; ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index; CRP: C-reactive protein; KTR: kynurenine/tryptophan ratio.

assume that this inflammation also occurs in other glands and determines its dysfunction.

The prevalence of anti-Ro/La was higher in patients with acinar adipose infiltration in salivary glands but it was not significantly different. We have previously published that fatty replacement was evident in all pSS patients possessing autoantibodies (Anti-Ro/SSA and/or Anti-La/SSB) as well as a positive SG biopsy (focus score ≥1). Additionally, 62% of pSS patients having autoantibodies but a negative biopsy showed adipose infiltration while non-pSS controls demonstrated fatty replacement in only 32% of the cases (14).

Subsequently, we have demonstrated that adipocytes of the minor salivary glands in pSS were located in areas rich in IL-6 suggesting that adipocytes are involved in immune reactions (14). Another study has shown the expression of an adipocyte-derived factor - adiponectin - in patients with pSS, a hormone that has immunoregulatory function, and found that there is a greater production of adiponectin in the salivary glands of pSS patients than in healthy controls (37).

Among the 97 biopsies of the minor salivary gland evaluated, 80 biopsies presented material with the possibility of quantifying adipose infiltration, ductal dilatation and acinar atrophy. The other 17 biopsies were only possible to detect the focal score, but this did not affect the statistical analysis. We found adipose infiltration in 48.8% of our samples, similarly to Skarstein *et al*. (14). On the other hand, other authors found no differences between the presence of adipose infiltration in patients with pSS and healthy controls (11-13). They concluded that adipose infiltration is an age-associated phenomenon and not a selective feature of pSS. However, they only compared the presence of adipose infiltration, but not if activity of cytokines could be involved. Aging has previously been related to acinar atrophy (38) and adipose infiltration (39, 40) for many years, but this does not reduce the hypothesis that these changes are also involved with glandular and extra-glandular inflammatory processes. Our current study demonstrated that adipose infiltration is associated not only with aging, but also with glandular dysfunction and the kinurinine inflammation biomarkers of IFN-γ pathway, corroborating Skarstein findings that adipose infiltration could be involved in the inflammatory process and glandular damage.

SG of pSS patients expresses genes promoting adipose tissue development and reduced mitochondrial fatty acid beta-oxidation (ARID5B, OXCT1, BDH1, SOX8, HMGCS2, FTO, ECHS1, PCCA, ACADL and ACADVL), inflammatory responses (IL1R1, IL7R, IL10RA, IL15, IL18RAP, CCL2, CCL5, CCL22, CXCR6, CD14, and CD48), and lymphoma development via JAK-STAT signalling (STAT2, TYK2, EBI3, FAS, TNFRSF1B, MAP3K8, HMOX1, LTB, TNF, STAT1, and BAK1). Genes involved in interferon production and signalling were also detected (IRF1, IRF9, and IRF7), in addition to IL6, IL10, and IL17 (15).

Kynurenine-interferon-γ pathway activation in pSS: is it a cause or consequence of adipose infiltration?

Both type α and γ interferons (IFNs) have been implicated in the pathogenesis of pSS. The tryptophan/kynurenine pathway can be stimulated by IFN-γ and other cytokines, activating indoleamine-pyrrole 2,3-dioxygenase (IDO). Few recent studies have been published about IDO activity and kynurinine pathway in pSS (41, 42).

In the previous studies, saliva IFN-γ levels were found to be elevated and an IFN-γ signature was found to be present in patients with pSS (13, 14). Kang *et al*. (13) reported increased levels of saliva IFN-γ, IL-1, IL6, and IL-10 levels in patients with pSS compared to patients having only sicca symptoms (43, 44).

The frequency of IDO-expressing antigen-presenting cells, as well as intracellular IDO content in T cells was higher in peripheral blood cells of pSS compared to age and sex matched controls (45). Higher IDO activity was observed in IFN-γ positive pSS patients, with higher levels of IDO mRNa and IFN-γ mRNA in circulating monocytes, and those observations were associated with tryptophan/kynurenine pathway (42) in pSS.

The levels of the serum tryptophan are lower and kynurenine and the ratio between kynurenine/tryptophan are higher in pSS than in healthy women and sicca non-pSS patients (41). Kynurenine:tryptophan ratio was positively correlated with other features of active disease, such as the levels of rheumatoid factor (RF), IgG, IgA, IgM and CRP. The ESSDAI scores were significantly increased in pSS patients positive for IFN compared to those negative for IFN (42).

It thus seems that interferon-γ and kynurenines pathway are part of the disease mechanisms, but little is known about clinical associations with kynurenines. We have found association of adipose infiltration with many metabolites of kynurenine pathway in pSS. Adipose infiltration could be consequence or could contribute to IFN-γ activation.

In an animal model, the ductal ligation of local salivary gland tissue causes an increase in the systemic level of kynurenine, indicating that exocrine gland dysfunction is capable of affecting the tryptophan metabolic pathway with systemic repercussions (46).

It has also been observed that kynurenines may be associated with immune dysfunction and nervous system disorders (22). These metabolites modulate the function of endothelial cells, recruit leukocytes to the sites of inflammation and stimulate the production of autoantibodies (24).

The IDO enzyme regulates both innate and adaptive immune responses through degradation of the essential amino acid tryptophan into kynurenine and other metabolites, which suppress effector T-cell function and promote the differentiation of regulatory T cells (23). We found that CRP showed a positive correlation with focus score but no association with total or each domain of the SSDDI. On the other hand, fatty infiltration was associated with glandular dysfunction, SSDDI and kynurinine metabolites.

Taking these results together it suggests that fatty infiltration is a consequence of IFN-γ activation mediated by kynurinine pathway. To block IFN-γ

can be an interesting target to control disease activity and also to reduce the progress of glandular damage.

In conclusion, our results suggest that kynurenines are biomarkers of greater adipose infiltration in LSGB and glandular dysfunction suggesting that activation of interferon-γ pathway is involved in the salivary and lacrimal glands damage.

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