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# Role of Anti Muscarinic Acetylcholine IGA and Anti Autoantibodies in Whole Saliva from Primary Sjögren's Syndrome Patients

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## Abstract

Aims: The purpose of this report is to describe saliva IgA antibody against M3 muscarinic acetylcholine receptors (mAChRs) and anti-Ro autoantibodies in patients with primary Sjögren's syndrome (pSS). In addition we want to clarify if this antibody anti-Ro is or not related to the presence of anti-IgA M3 mAChRs autoantibodies in whole saliva of pSS patients.

**Methods:** Whole saliva samples were collected from healthy volunteers (n=30), patients with pSS anti-Ro positive (n=60) and patients with pSS anti-Ro negative (n=30). Saliva IgA patients and healthy subjects were tested by ELISA recognized the synthetic 25-mer peptide corresponding to the extracellular loop of the human M3 mAChRs. Also, concentration of nitrite/nitrate was determined by ELISA.

**Results:** Optical density values for saliva IgA from pSS anti-Ro positive are significantly higher than those from IgA anti-Ro negative patients and IgA from normal subjects. These molecular interactions between IgA and human M3 mAChR synthetic peptide increased in optical density values compared with IgA from pSS anti-Ro negative and healthy subjects when M3 mAChR synthetic peptide was used as coating antigen. The specificity of this reaction was assessed by the ability of the M3 synthetic peptide (1x10-5 M) to inhibit the action when whole saliva was incubated previously with the M3 synthetic peptide for 40 min at 37ºC and then added together in the microtiter plates. On the other hand, the concentration of nitrate/nitrite in whole saliva was significantly decreased in pSS anti-Ro positive patients in comparison with those from IgA anti-Ro negative patients and healthy subjects.

**Conclusions:** Patients presenting in saliva anti IgA anti-Ro positive are statistically significant in optical density values than those IgA from anti-Ro negative patients and healthy individuals. Also, the hypofunction of the salivary glands is associated with significant decrement of nitrate/nitrites levels in the saliva in pSS-Ro positive without any changes in pSS-Ro negative and healthy subjects.

**Key words:** Autoantibodies in Sjögren Syndrome, IGA, Saliva, Nitrites/Nitrates, Anti Iga Peptide Antibody

## Introduction

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease mainly affecting the salivary glands whose serologic hallmarck is the presence of anti-Ro/SSA antibodies [1]. Anti-Ro/SSA may found in isolation (70%) or concomitantly with the presence of anti-La/SSB antibodies (30%), whereas positivity for exclusively anti-La/SSB is rare [2, 3]. This autoimmune disorder is characterized by progressive lymphocytic infiltration and destruction of the salivary glands that leads to a marked reduction of saliva secretion [4].

Alter humoral autoimmune responses, B cell hyperactivity and many autoantibodies production that are non-organ specific, including RFs, ANAs, ACAs, Ro-SSA, La-SSB [3,5] and our studies and others have proved that the antibody against salivary gland mAChR M3 subtype was a very good in serum and saliva markers in pSS [6,7,8] and they are autoantibodies organ-specific.

Synthetic peptides corresponding to the second extracellular loop of the human salivary gland mAChR M3 subtype, have been used as antigens in ELISA procedure as a solid support to detect in the sera and in saliva these muscarinic cholinergic autoantibodies binding IgG and IgA in pSS respectively [4,8]. It is important to remark that the second extracellular loop of the mAChR M3 subtype are the most antigenic loop to detect autoantibodies in sera (IgG) and saliva (IgA) from pSS patients [9]; play a pathologic role in pSS and also play an important role in the activation of M3 salivary mAChR-intracellular signalling system by the production of different pro-inflammatory mediators induced by antibody-mAChR interaction on gland membranes [9, 10]. Moreover, autoantibodies against M3 mAChR are also present in more than 50% of patients with pSS and this molecule acts as an autoantigens in the pathogenesis of pSS [11].

Nitric oxide synthases (NOS) immunoreactive neurons and perivascular, periacinar and periductal nerve fibres have been demonstrated in submandibular salivary gland [12]. The neuronal localization of NOS suggests that nitric oxide (NO) could control exocrine function and blood flow in the submandibular gland [13]. NO is also involved in submandibular salivary response to stimulation of parasympathetic system innervation, enhancing the output of protein in saliva; acting presynaptically [14]. NO participates in host defence mechanisms and has been implicated in the pathogenesis of numerous inflammatory and autoimmune diseases [15]. Decreased resting levels of nitrate/nitrites and decreased whole saliva flow rates are found in patients with SS [16].

In this study based on all these observations, we considered it of special relevance to investigate in whether salivary secretory IgA from pSS patients anti-Ro positive modify and could act on salivary gland M3 mAChRs and in same way are able to alter the levels of nitrate/nitrite in saliva, causing parasympathetic dysfunction.

## Materials and Methods

Patients: The subjects of this study were 55 pSS anti-Ro positive patients, 30 pSS anti-Ro negative patients and 30 healthy volunteers, all female, aged 35-54 years. They were selected from the metropolitan area of Buenos Aires (Table 1). The diagnosis fulfilled the criteria described by Vitali et al [17] and was given by means of a positive biopsy with score focus of 3.76 ± 0.07.

Saliva Collection: Participants were instructed to refrain from eating, drinking and practicing oral hygiene procedures 12 hours before saliva collection. Whole unstimulated saliva was collected from all patients by expectoration into sterile bulbs. Collected samples were placed immediately on ice and transported to the laboratory, where they were centrifuged at 5,000 g for 10 minutes, and the clear supernatants were stored in aliquots at –80ºC. The samples were thawed and the assays were performed within 2 months of collection. Total salivary IgA and its subtypes (IgA1, IgA2) and the values of the salivary flow were performed with commercial plates for radial immunodiffusion containing anti-IgA (Table 1).

Purification of saliva IgA: The IgA fraction of 55 patient's pSS anti-Ro positive, 30 patient's pSS anti-Ro negative and 30 healthy subjects were independently purified by standard DEAE-cellulose chromatography equilibrated with 10 mM phosphate buffer pH 8. Briefly, saliva samples were dialyzed against 10 mM pH 8 phosphate buffer for 18 hours and then applied DEAE-cellulose columns. The pass through IgG-rich fractions was discarded and IgA-rich fractions were eluted with 0.05 M NaCl in 0.01 M phosphate buffer pH 8.

The IgA concentration in the enriched fractions was determined by radial immunodiffusion after concentration by ultra filtration with PM-30 filtering membranes (Amicon, Beverly, MA, USA) [cut-off molecular weight 30,000 Da]. The concentration of IgA was 36.6 ± 13.7 mg/dl in the IgA-enriched fractions. IgA was also purified by affinity chromatography of different sera on Jacalin-agarose beads following recommendations of the supplier (ICN Pharmaceuticals, Irvine, CA, USA) and previously described methods [18].

Anti-Ro-SSA procedure: Saline-soluble extractable nuclear antigens (ENA) were obtained from human spleen in phosphate buffered saline (PBS) for anti-Ro. Patient sera were tested undiluted and diffusion was carried out at room temperature in a humidified chamber for 48 hours. Precipitin lines were identified by comparison with reference sera. ELISA for total anti-Ro (60kD and 52kD Ro-proteins) was performed with a commercial Kit based

**Table 1** Salivary flow, Lysozyme, IgA and protein secretion contents from patients and healthy subject

| Additions | Basal salivary flow | Lysozyme (µl/ml) | IgA (mg/dl) | Protein secretion (mg/g) |
|---|---|---|---|---|
| | | | | |
| pSS patients | 1.4 ± 0.2 | 3.8 ± 0.5 | 36.3 ± 12.1 | 40 ± 5 |
| | | | | |
| pSS patients Ro+ | 1.2 ± 0.2 | 4.1 ± 0.7 | 31.9 ± 10.7 | 36 ± 4 |
| | | | | |
| pSS patients Ro- | 1.8 ± 0.4 | 3.7 ± 0.3 | 39.2 ± 11.9 | 46 ± 7 |
| | | | | |
| Healthy subject | 11.2 ± 2.1 | 10.7 ± 1.6 | 40.3 ± 12.2 | 86 ± 8 |

**Note:** Values expressed the means ± SEM of 15 patients from pSS, pSS Ro+, pSS Ro-and healthy subject.

on purified antigens (Orgentec Diagnostika, Mainz, Germany) and the assays were carried out according to the manufacturer's protocols on an automated ELISA instrument (Radim, Pomezia RM, Italy). Values greater than 25 UI/ml were considered positive.

M3 mAChR synthetic peptide: A 25-mer peptide K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-

S-N-P-A-V-T-F-G-T-A-I corresponding to the amino acid sequence of the extracellular loop of the human M3 mAChR was synthesised by F-moc-amino acids activated using 1-hydroxy-benzotriazole/ dicyclo-hexylcarbodiimide (Hob/DCC) strategy with an automatic peptide synthesiser (Model 431A, Applied Biosystems, Melo Park, CA, USA). The peptide was desalted and purified by high performance liquid chromatography (HPLC). It was then subjected to amino-terminal sequence analysis by automatic Edman degradation (470 A Sequencer, Applied Biosystems).

Purification of anti M3 peptide IgA by affinity chromatography: The IgA fractions obtained from 55 and 30 patients pSS anti-Ro positive and anti-Ro negative respectively and 30 healthy subjects were independently subjected to affinity chromatography on the synthesised peptide covalently linked to AffiGel (Bio-Rad, Richmond, CA, USA) as described [19]. Briefly, the IgA fraction was loaded onto the affinity column equilibrated with phosphate-buffered saline (PBS) solution. The non-peptide fraction was first eluted with the same buffer. Specific anti peptide antibodies were then eluted with 3 M KSCN and 1 M NaCl, followed by immediate extensive dialysis against PBS. The IgA concentration of non-anti peptide antibodies and specific anti M3 mAChR peptide antibodies were determined by a radial immunodiffusion assay. Their immunological reactivity against muscarinic receptor peptides was evaluated by enzyme-linked immunosorbent assay (ELISA). The concentration of the affinity purified anti M3

peptide IgA (1x10-7 M) that maximally increased optical density (OD: 2.4±0.2)

corresponded to a total IgA concentration of 1x10-6 M (OD, 2.2±0.2). The non anti M3 peptide IgA fraction eluted from the column showed OD values

(0.27±0.06) similar to those of normal IgA (OD, 0.25±0.04). The normal IgA fraction purified by affinity chromatography gave a negative result (OD, 0.25±0.04). ELISA assay was performed as described previously [20].

Ntitrate/Nitrite determination: The 20 µl saliva samples were mixed with an equal volume of 100 µl of Griess reagent (Ingredient A: 0.1% naphthalene diamine dihydrochloride at a final concentration of 5 mmol/l; Ingredient B: 1% sulfanilamide at a final concentration of 5 mmol/l in orthophosphoric acid) in a 96 well microtiter plate (NUNC, Roskilde, Denmark). Nitric oxide concentrations were determined by a nitrate/nitrite colorimetric assay kit (Cayman Chemical Co, Ann Arbor, MI, USA) and measured spectrophotometrically at 540 nm using a micro plate reader (Reader Model 230S; Organon Teknika, Boxtel, The Netherlands). The 20 µl saliva samples were mixed with an equal volume of 100µl of Griess reagent (Ingredient A: 0.1% naphthalene diamine dihydrochloride at a final concentration of 5 mmol/l; Ingredient B: 1% sulfanilamide at a final concentration of 5 mmol/l in orthophosphoric acid) in a 96 well microtiter plate (NUNC, Roskilde, Denmark). Nitric oxide concentrations were determined by a nitrate/nitrite colorimetric assay kit (Cayman Chemical Co, Ann Arbor, MI, USA) and measured spectrophotometrically at 540 nm using a micro plate reader (Reader Model 230S; Organon Teknika, Boxtel, The Netherlands). The nitrate and nitrites values are expressed as µM/ml.

Statistical analysis: The statistical significance of the difference between the groups was determined by Student's 2-tailed t-test for unpaired data. ANOVA test and Student-Newman-Keuls test were also performed when pair-wise multiple comparisons were necessary. In all cases, P values less than 0.05 were considered significant.

## Results

The distribution of anti M3 mAChR IgA peptide anti-Ro positive, anti-Ro negative and healthy individuals was determined by ELISA. It can be seen in the scatter grams of Figure 1 that the

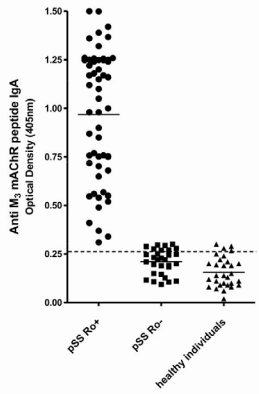


**Figure 1:** Immune reactivity of anti M3 peptide IgA antibody of saliva from pSS 30 Ro+ patients, 20 pSS Ro- patients and 20 healthy subjects. Saliva (1/30 dilution) was assayed on sensitized micro plates with 1x10-5 M synthetic M3 peptide. Dotted dashed line, cutoff value of 0.26 optical density ± 3 S.D. for healthy individuals (black triangles), whole black line: median optical density values. P < 0.0001 between pSS Ro + patients and pSS Ro - patients and healthy individuals.

anti M3 peptide IgA anti-Ro positive was significantly higher than those values of optical density from anti-Ro negative patients and healthy subjects (P < 0.0001). Also, the values of the autoantibodies against M3 mAChR were similar in both groups before cited.

The optical density values of whole saliva from pSS anti-Ro positive were always at least three standard deviation from that of saliva from pSS anti-Ro negative patients and healthy individuals.

In Figure 2 we can see that both saliva and IgA from pSS anti-Ro positive patients but not from pSS anti-Ro negative patients and healthy individuals gave a concentration-dependent increase in optical density values when the M3 synthetic peptide was used as a coating antigen.

The specificity of this reaction was assessed by the ability of the synthetic M3 peptide (1x10-5 M) to inhibit the reaction when

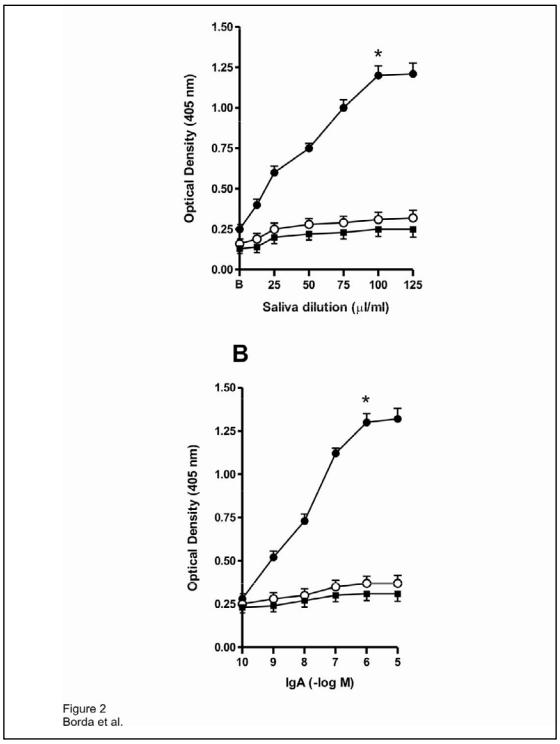


**Figure 2:** Immune reactivity of anti M3 mAChR antibodies of pSS patient saliva directed the second extracellular loop of M3 mAChR peptide by ELISA assay. Effect of increasing concentrations of saliva (A) and anti M3 peptide IgA (B) from pSS Ro+ patients(), pSS Ro-patients (○) and healthy (control) individuals (■). b: non-antigen paired control wells subtracted from the antigen containing wells. Results are mean ± SEM of 20 independent pSS patients Ro+ and Ro- and healthy individuals performed in duplicate. $P < 0.0001$ different from pSS Ro+ patients and healthy individuals.

saliva or IgA was incubated with the synthetic M3 peptide for 40 min at 37ºC and then added together in the microtiter plates (Figure 3).

Moreover, salivary tests from pSS anti-Ro positive, pSS anti-Ro negative and healthy subjects were performed and showed that basal salivary flow was decreased, accompanied by decreased lysozyme. Total protein in pSS patient anti-Ro positive or negative and in healthy subjects and total salivary IgA concentrations, showed no differences (Table 1).

Table 2 described the clinical manifestations of organ/systems

**Table 2** Clinical manifestation related to anti M3 IgA, from pSS patients and healthy subject

| | pSS Ro+ | pSS Ro- | Healthy subject |
|---|---|---|---|
| | | | |
| Xerostomia, n (%) | 55 (100%) | 30 (100%) | N/A |
| | | | |
| Xerophthalmia, n (%) | 55 (100%) | 30 (100%) | N/A |
| | | | |
| Xerosis, n (%) | 33 (60%) | 27 (90%) | N/A |
| | | | |
| Fatigue, n (%) | 39 (71%) | 24 (80%) | N/A |
| | | | |

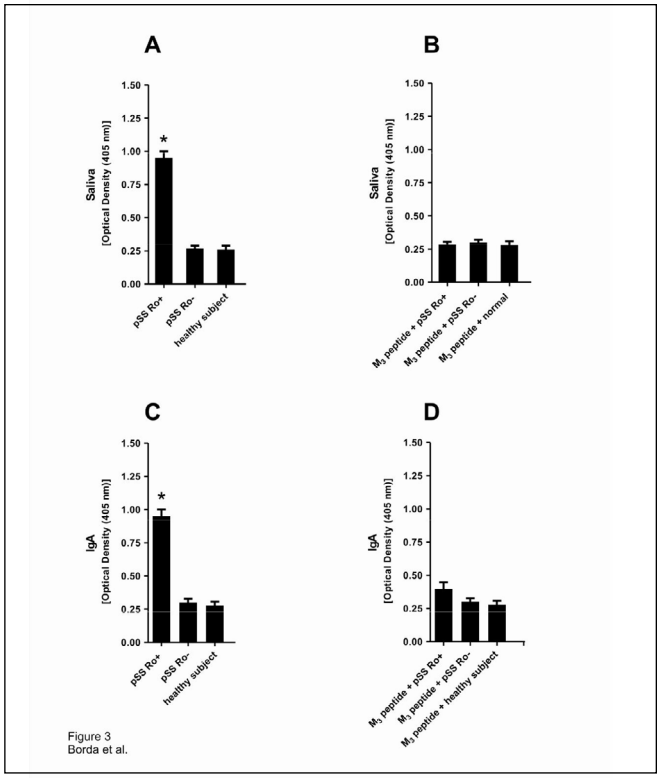


**Figure 3:** Immune reactivity of anti M3 mAChR antibodies from pSS patients against the second extracellular loop of M3 mAChR peptide by ELISA assay. Histograms shows: (A) 100 µl/ml whole saliva from pSS Ro+ patients or pSS Ro- patients or healthy individuals; (B) 100 µl/ml whole saliva from pSS Ro+ patients or pSS Ro- patients of healthy individuals previously treated with 1x10-5 M synthetic M3 peptide. P<0.001 from pSS Ro- and healthy individuals; (C) 1x10-6 M anti M3 IgA peptide from pSS Ro+ patients or pSS Ro- patients or healthy subjects; (D) 1x10-6 M anti M3 IgA peptide treated with 1x10-5 M synthetic M3 peptide. P<0.001 from pSS Ro- and healthy subjects.

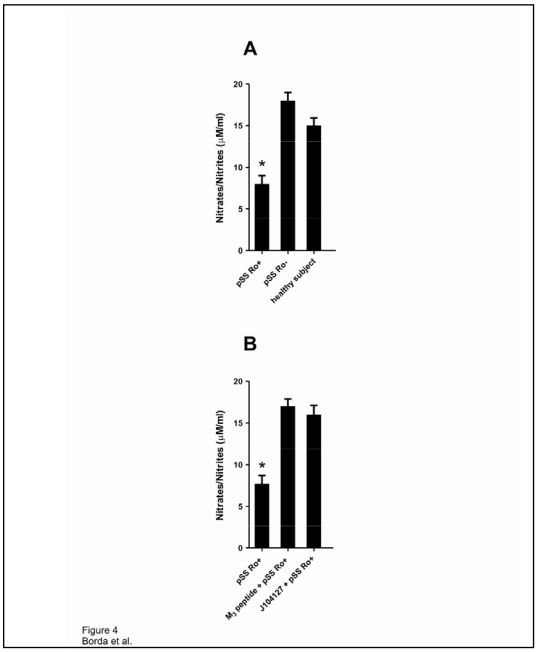


**Figure 4:** Decrease of nitrates/nitrites by (A) whole saliva from pSS Ro+patients and pSS Ro- patients alone or (B) in the presence of 1x10-5 M synthetic M3 peptide or 1x10-8 M J104127, a specific M3 mAChR antagonist. Results are the mean ± SEM of 20 independent pSS patients in each case performed in duplicate. Healthy subjects were also tested as control. P<0.001 significantly different from pSS Ro+ and healthy subjects (A) and P<0.001 from pSS Ro+ patients and pSS Ro- patients (B).

involved in pSS anti-Ro positive or anti-Ro negative patients and healthy individuals respectively, related to anti M3 mAChR IgA in all the patients studied in the present work. Concentration of nitrate/nitrite in whole saliva from healthy volunteers and pSS patients were measured. Figure 4 A shows that the total amount of nitrate/nitrite in whole saliva was lower in pSS anti-Ro positive patients than the concentrations determined in pSS anti-Ro negative and healthy individuals.

When 1x10-5 M synthetic M3 peptide and 1x10-8 M J104127, an specific antagonist of salivary M3 mAChR, both are able to prevent the decrease in nitrate/nitrite concentrations in pSS anti-Ro positive; indicating the specificity of the reaction between anti M3 peptide IgA with the second loop of the salivary M3 mAChR in the first and in the second the direct binding and activation of the autoantibody with salivary M3 mAChR respectively (Figure 4 B).

## Discussion

Previous studies have confirmed the presence of anti M3 mAChR peptide IgA autoantibodies together with anti M3 mAChR peptide IgG in pSS patients and have shown that these autoantibodies may be involved in the pathogenesis of pSS [8, 10, 21-25]. Also, the presence of anti-Ro SS/A autoantibodies are about 70% of pSS patients and these autoantibodies are part of the current classification criteria [26].

In the present study, we analyzed if the M3 mAChR IgA from whole saliva are in relationship with the anti-Ro positive or anti-Ro negative in pSS patients and the ability of this autoantibody to modify the concentration of total nitrate/nitrites in whole saliva.

Our results show that pSS patients anti-Ro positive present in their whole saliva an IgA autoantibody against salivary M3 mAChR significantly higher than in pSS anti-Ro negative patients. Moreover, the presence of this antibody in pSS anti-Ro negative is similar to that determined in saliva of healthy individuals.

The fact that synthetic M3 peptide corresponding to the sequence of the second extracellular loop of human mAChR of salivary gland recognized this autoantibody, demonstrates the immunological specificity of the reaction and also, the binding, fixation and activation of the mAChR M3 subtype of the salivary (parotid an submandibular) glands by this autoantibody, triggering a parasympathetic receptor-mediated biological effect [21, 24], corroborates that all this events resulted in a primary organ-specific parasympathetic dysfunction. Therefore, the SS IgA associated with sicca symptoms proof that this autoantibody in saliva is a valuable marker for pSS anti-Ro positive patients.

It is interesting to note, that anti M3 peptide IgA interacting chronically with the membrane M3 mAChR of the salivary gland provoke a blockade of these receptors, eliciting not only a loss of saliva but also, it causes real damage to the salivary gland with subsequent dysautonomia at the level of glandular parasympathetic system.

The function, if any, of nitrate/nitrites in saliva is still unknown. Previously, different authors [27-29], reported that the formation of nitrite in whole saliva is caused by bacterial reduction of the nitrate in saliva and other author [30] suggested that the formation of nitrite from salivary nitrate might contribute to the host defence against ingested pathogens.

The fact that our results show that the total amount of nitrate/nitrite are low in pSS anti-Ro positive patients may be suggested same mechanism for certain increased susceptibility to pathogenic infection in these patients compared with those from pSS anti-Ro negative and healthy individuals.

On the other hand, the result of this study showed that the total amount of nitrate/nitrite was markedly decreased in pSS anti-Ro positive, could be in relationship with the level of destruction of salivary gland by lymphocytic infiltration, that contribute to the decreased in the total amount of nitrite/nitrate in saliva.

## Conclusion

This work shows a complex interplay between different factors involved in adaptative autoimmunity in pSS patients at the level of exocrine glands. The presence of pSS IgA anti M3 peptide in anti-Ro positive patients but without action of anti-Ro negative patients and the fact that this autoantibodies can be abolished by synthetic M3 peptide and M3 mAChR specific antagonist, could provide a link between autoimmunity and the glandular parasympathetic system. The chronic fixation of this pSS IgA to the glandular M3 mAChR displaying the cholinergic agonist at the level of this receptor, could induce desensitization, internalization and intracellular degradation of the glandular M3 mAChR of the glands, resulting in xerostomy, xerophthalmia, xerosis and other parasympathetic symptoms observed in pSS.

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