

# Autoantibodies against Muscarinic Acetylcholine Receptor on Exocrine Glands in Sjögren Syndrome

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## Abstract

These investigations demonstrate that serum antibodies against muscarinic acetylcholine receptors (mAChR) in primary Sjögren syndrome (pSS) and associated Sjögren syndrome (aSS) bind and activate both cholinergic receptors of  $M_3$  in salivary gland and  $M_1$  in neonatal myocardium and in the cerebral frontal cortex area subtypes; triggering the production of the second messengers and proinflammatory mediators related to mAChR activation. In this way the cholinergic autoantibodies damages these receptors, which thus starts acting as an antigen. On this basis  $M_3$  and  $M_1$  mAChR IgG can be considered new markers of pSS/aSS allowing the differentiation between dry eye and mouth of autoimmune and non-autoimmune nature. Given that cholinergic autoantibodies also deregulate the parasympathetic system of the target organs, they can also be seen as a new factor contributing to the etiopathology of the syndrome.

**Keywords:** Autoantibodies; Anti- $M_3$ mAChR IgG; NO;  $PGE_2$

## Introduction

Sjögren syndrome (SS) is a devastating autoimmune illness with heterogeneous clinical expressions. These expressions reflect not only different etiologic factors, e.g. genetically and immunological abnormalities, but also the deregulation among them as well as the dysfunction of the parasympathetic system. They have the following cardinal clinical symptoms: xerostomia and xerophthalmia at the level of the exocrine glandular system [1], cognitive impairments such as perception, attention and executive function deficits [2] at the level of the cerebral frontal cortex, complete congenital heart block at the level of neonatal myocardium [3]. These alterations are reliable predictors of long term disabilities.

Numerous theories have been formulated and tested and continue to compete for supremacy as the essential explanation for why patients suffer from periodic episodes of altered exocrine secretion and remission and why these episodes typically result in same social and cognitive dysfunction in the chronic course of the SS. Relevant in this sense, are those theories shifting their attention to the organ specific immunological deregulation involved in the manifestations and the chronic course of the disease [4-7], and to the clinical features of an immunological or inflammatory disease [8,9].

In this line, anti-salivary/frontal antibodies have been shown to be involved in autoimmune disorders with cognitive manifestations in lupus [10] and in SS [11]. It should be noted that the main autoantibody involved in these disorders are the mAChR subtype  $M_3$  and  $M_1$  [11].

In this article we will examine the role of cholinergic autoantibodies subtypes  $M_3$  and  $M_1$  and its relationship with the signs and symptoms of SS and its pathological implications in SS.

## Autoimmune Basis of Sjögren Syndrome

Primary SS occurs in 0.1 to 3.0% of the population in general. The disease is more common among women (female/male ratio 9:1) aged 40 to 60 years old and is rarely seen in children and adolescents.

There are many factors to be considered in the etiology of the disease such as genetic factors in which the B cell [12] or the B-cell activation factor (BAFF) and the tumor necrosis factor (TNF) are implicated. Furthermore, it is presumed that the genetic predisposition leading to the increment of type I IFN may explain why this interferon is present in the salivary gland and peripheral blood in pSS patients. HLA-B8 of HLA-DW3, HLA-DR3 and DRW52 have also been

reported in pSS patients [13,14]. Other factors responsible for the development of pSS are viral infections -mainly, Epstein-Barr virus (EBV), cytomegalovirus (CMV) and Hepatitis C virus (HCV) [15]; neurohormonal disturbances in sex hormones [16]; environmental factors causing the disorganization of glandular epithelial cells, which provoke local innate immune response and activate the toll-like receptor (TLR) pathway involving cell apoptosis [5]; the stimulation of the dendritic cells responsible for the production of INF, which, in turn, leads to the proliferation and the differentiation of B cells and to the production of autoantibodies.

Nowadays autoimmunity is recognized as a very important factor responsible for the development of the disease [17,18]. In this line, oral and eye sicca symptoms provoking a decrease of the exocrine glandular function are taken as evidence of autoimmunity with the presence of autoantibodies to Ro/SS-A (Ro52, Ro60) or La/SS-B [19,20]. These autoantibodies are non organ specific and their role in the pathogenesis of SS was not been understood yet. Some explanations suggest that Ro60 and La autoantigen unlike Ro52 are involved in triggering and maintaining the tissue specific autoimmune response in pSS. This indicates that Ro60 and La autoantigens contribute to the antigen driven immune response and to the production of these autoantibodies. This idea is supported by a further research that documents the production of local anti Ro system and anti La autoantibody in the submandibular and parotid gland of pSS patients [21]. Subsequent studies [22] demonstrate that lymphocytes' infiltration in salivary gland is organized in the form of an ectopic germinal centre, in which anti-Ro60, anti-Ro52 and anti La autoantibodies are produced. They also show that these autoantibodies participate in the cell apoptosis [22]. The sustenance of these phenomena in time is responsible for the destruction of the gland and other extraglandular manifestations. However, glandular epithelial cells are also infiltrated by macrophages, plasma cells, T cells [23] and

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dendritic cells [24]. T cells are balanced toward Th1 and Th17, which release IL-17 [19] and promote the generation of pro-inflammatory cytokines as a main nitric oxide (NO) [25] and as prostaglandins (PG) [11]. This is why PGE<sub>2</sub> and NO together with IL-6 are seen as crucial factors of the maintenance of the inflammatory process and it's becoming chronic [26].

It is important to notice that, antinuclear antibodies (ANA) together with the rheumatoid factor (RF) and anti centromere antibody (ACA) are frequently found in patients with pSS in the early stages of the disease and at a younger age [20,27,28]. These antibodies are not specific to SS, but in a way, show a local response to autoantigens derived from salivary glands aggressors, which produce these antibodies at the level of the local system [29].

These immunological events together with histological studies of salivary lip glands showing a progressive focal infiltration of mononuclear lymphoid cells cause the replacement of the glandular epithelial cells and the subsequent reduction of saliva secretion [30,31]. In this vein, germinal centre like structures in the gland with elevated titres of RF, increased IgG levels and high focus score (FS) equal or more than one have been identified [32]. A positive biopsy is then given when FS has 50 inflammatory cells in a 4 mm<sup>2</sup> salivary lip glandular section [33,34].

### Action of Anti-muscarinic Acetylcholine Receptors Antibodies in Patients with SS

Experimental and clinical studies suggested the presence of cardiac anti-M<sub>1</sub>mAChR antibodies in mothers with SS whose children have congenital heart block [35-39] and the presence of IgG against M<sub>3</sub>mAChR in sera of patients with pSS that interact with rat exorbital lacrimal glands [1,25] and rat parotid gland [40]. The presence of IgG against M<sub>1</sub> and M<sub>3</sub>mAChR was also found in sera of patients with pSS interacting with rat cerebral frontal cortex [41].

Antibodies to neurotransmitter receptors have been described in idiopathic dilated cardiomyopathy [42] and in chagasicmyocardiopathy [43]. The latter is clinically characterized by a dysautonomic syndrome related to the progressive blockade of parasympathetic neurotransmitter receptors, with a denervation of both adrenergic and cholinergic branches of the autonomic nervous system [44]. We described the presence of antibodies against cholinergic receptors in cardiac, exocrine glands and cerebral frontal cortex in SS and proposed that the deposit of these autoantibodies could lead to a progressive blockade of these receptors behaving as a partial agonist and inducing desensitization and/or down-regulation [45,46].

Thus, Sjögren's autoantibodies appear to be reactive to the M<sub>1</sub> cholinergic receptor of neonatal heart, suggesting the multiplicity of the autoimmune responses in primary SS. Congenital heart block is thought to result from the transplacental passage of maternal autoantibodies that could cause an inflammatory reaction in the developing heart of the fetus resulting in severe defects of conduction [47,48]. In this regard the hypothesis that cholinergic autoantibodies can act as a "sensitizing" or "predisposing" condition present at a critical period during the development of the fetal electroconduction system seems reasonable. Being pSS one of the immune disorders strongly associated with congenital heart block, these findings suggest that M<sub>1</sub> muscarinic cholinergic autoantibodies are another factor that could be involved in the pathogenesis of congenital heart block associated with primary SS in addition to ribonucleoprotein antibodies (anti-Ro/SSA and anti-La/SSB).

We have already reported autoantibodies against rat salivary and

lacrimal glands M<sub>3</sub>mAChR, which trigger parasympathetic-receptor-mediated biological effects [25,39,49,50]. We have demonstrated that they are able to recognize a synthetic peptide corresponding in amino acid sequence to the second extracellular loop of the human M<sub>3</sub>mAChR. The distribution of the amino acid sequence between rat and human M<sub>3</sub> synthetic peptide has a great homology (84%). An isolated fraction from SSIgG enriched in anti-M<sub>3</sub> peptide antibodies could reproduce the effects of the corresponding whole immunoglobulins. This fact strongly suggests a prominent role of anti-M<sub>3</sub> peptide antibody in the mAChR-mediated effects of total SS IgG. In addition, the synthetic peptide involved selectively suppresses the biological effects of SS anti-M<sub>3</sub> peptide autoantibody and the corresponding total IgG. This supports the view that the second extracellular loop is not only the main immunogenic region of the receptor [51] but can be considered essential for the biological action of these autoantibodies. We also demonstrated that there is an association between the existence of circulating anti-M<sub>3</sub>mAChRIgG autoantibodies, the presence of ocular and mouth symptoms, gland surface alterations and a selected number of antibodies detected in SS. These findings point to these autoantibodies as a valuable marker for dry eye and mouth associated to SS. In addition, we have shown a good correlation between lacrimal function, serum IL-2 receptor, ANA, and RF in SS dry patients [52]. This process could lead to a progressive blockade of mAChR which, in turn, induces dry eyes and mouth, the classical signs of SS.

Further evidence is required to show persistent abnormal levels of IgGs in forebrain tissues of SS patients in order to understand the effect of these autoantibodies on the cognitive deficit in these patients. Towards this understanding we postulate on the basis of our results that the early agonistic-promoting activation of M<sub>1</sub> and M<sub>3</sub>mAChR initiated by antibodies bind to cerebral frontal cholinergic receptors persistently. Later, the agonistic activity displayed by these autoantibodies induces desensitization, internalization and/or intracellular degradation of the mAChR, leading to a progressive decrease of cerebral M<sub>1</sub> and M<sub>3</sub>mAChR expression and activity. Furthermore, IgG antibodies binding to mAChRs modify spare receptors' affinity, sensitivity and expression in brain tissue. Therefore, it could be hypothesized that the central nervous system manifestations, which are apparent during SS, might be induced by an impaired response to the cholinergic endogenous neurotransmitter's stimuli due to mAChR-antibody being fixed to its receptors [41,53-55].

Antibodies to muscarinic receptors, detected by functional methods or the use of synthetic peptides, have been described in SS patients. The functions of mAChR M<sub>1</sub> and M<sub>3</sub> subtypes are numerous, the most noteworthy being their function as the main receptors stimulated by acetylcholine released by postganglionic fibers in the parasympathetic nervous system [56]. It was demonstrated that the agonist-mediated stimulation of mAChR M<sub>1</sub> and M<sub>3</sub> subtypes in rat salivary, lacrimal glands leads to saliva and tears production [57]. Subsequent studies revealed that they are fundamental for the parasympathetic regulation of exocrine secretion [58]. Further functional studies led to the assumption that these mAChR M<sub>1</sub> and M<sub>3</sub> subtypes antibodies contribute to sicca manifestations, potentially via direct blockade of parasympathetic neurotransmission [17,59].

Fixation of IgG anti M<sub>3</sub>mAChR autoantibodies of patients with pSS has functional implications for the exocrine glands. This is because the antibody limits not only parasympathetic stimulation with decreased salivary and lacrimal function, but also the effectiveness of endogenous agonists [60]. It is well known that the activation of M<sub>3</sub>mAChR generates the secondary messenger's inositol phosphate (InsP<sub>3</sub>) and

prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). Each of these metabolites influences salivary and lacrimal secretion by mobilising calcium from intracellular stores and regulating the absorption of ions and water [61]. They also play a key role in the pathophysiology of chronic inflammation [62]. These facts have led us to think that the damage and inflammation in the exocrine glands commonly seen in SS patients might be a consequence of the production of pro-inflammatory mediators induced by antibody-mAChR interaction on gland membranes.

In the salivary glands, the basal lamina of the acini is connected to the cytoskeleton of acinar cells via integrins in the basal plasma membrane [63,64]. Matrix metalloproteinase-3 (MMP-3) degrades components of the basal lamina [64], and might be involved in the loosening of cell anchorage to the basal lamina. As a consequence of such changes, inhibition of the proliferation, differentiation and regeneration of epithelial cells [65], as well as activation of apoptosis [66,67], may account for salivary-gland damage. Patients with pSS show elevated levels of MMP-3 in their saliva [68].

Prostaglandins (PGs) have been implicated in normal cellular processes as well as in pathophysiological conditions such as inflammation [39,69]. Nitric oxide (NO) plays a key part in the pathophysiology of chronic inflammation and in the neurodegenerative process [38,70-72]. PGE<sub>2</sub> is synthesised by cyclooxygenase (COX) and prostaglandin E synthase (PGEs) *in vivo*; the two enzymes catalyse the reaction of transformation of arachidonic acid (AA) through PGH<sub>2</sub> into PGE<sub>2</sub>. The two isoforms of COX (COX-1 and COX-2) and PGEs [cytosolic (cPGEs) and membrane (mPGEs)] have been identified. In general, COX-1 and cPGEs are constitutively expressed in almost all tissues and have haemostatic effects, whereas COX-2 and mPGEs are inducible enzymes that are expressed in response to inflammation [73]. PGE<sub>2</sub> has been shown to be part of the signalling events involved in M<sub>3</sub>mAChR activation [49,71,74].

Previous studies analyzed the role of antibodies using microspectrofluorometry and surface the plasmon resonance-based optical biosensor system (BIAcore system). They showed that antibodies against the third extracellular domain of M<sub>3</sub>mAChR have an inhibitory activity against carbachol-induced calcium influx in human salivary gland cell lines [75].

Other authors reported that IgG from patients with pSS reduced the expression level of M<sub>3</sub>mAChR in the membrane, inhibited carbachol-induced calcium transients in human salivary gland cells and decreased membrane clathrin expression. These results suggest that IgG from SS patients induce internalization of M<sub>3</sub>mAChR partly through a clathrin-mediated pathway. They also provide support to the notion that anti-mAChR M<sub>3</sub> antibodies cause salivary dysfunction in patients with SS via both a reduction of calcium influx and the down-regulation of M<sub>3</sub>mAChR molecules on epithelial cells of salivary glands [76].

All these results suggest a complex interplay between different factors involved in innate and adaptive immunity, glandular M<sub>3</sub>/M<sub>1</sub>mAChR and the corresponding release of second messengers provoked by the binding and activation of this receptor by the SS autoantibodies. These results could also provide a basis to understand the link between autoimmunity and exocrine parasympathetic dysfunction in SS. This link could be further explained by the early agonist-promoting activation of salivary and lacrimal gland M<sub>3</sub>mAChR initiated by autoantibodies binding to, and persistently activating cholinergic receptors. This result is accompanied by the production of large amounts of pro-inflammatory substances, contributing to inflammation. The agonist activity displayed by anti-M<sub>3</sub>mAChR peptide antibodies could subsequently

induce desensitisation, internalisation and/or intracellular degradation of glandular M<sub>3</sub>mAChR. This could lead to a progressive reduction in the surface expression and activity of glandular M<sub>3</sub>mAChR, resulting in xerostomy, xerophthalmia and other general and dysautonomic parasympathetic symptoms in SS patients.

## Influence of Anti-M<sub>3</sub> mAChR IgG on Submandibular Gland on the Activation and Expression of Nitric Oxide Synthase

### Methods

**Ethical approval of the study protocol:** The study protocol complied with the tenets of the Declaration of Helsinki and accomplished with the rules established by the Ethics Committee of the University of Buenos Aires (Buenos Aires, Argentina). All subjects provided written informed consent.

**Drugs:** 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 sequence of the second extracellular loop of the human M<sub>3</sub> mAChR was synthesized by Peptide Genetic Research Company (Livermore, CA, USA) as previously described [72]. Atropine, verapamil and trifluoroperazine (TFP) were obtained from Sigma-Aldrich (St. Louis, MO, USA); J104129, ODQ, U-73122, S-Methylisothiourides (S-Methy-U), L-NIO dehydrochloride (L-NIO), N-Propyl-L-arginine hydrochloride (N-PL) and L-NG-monomethyl arginine citrate (L-NMMA) were from Tocris Cookson (Ellisville, MO, USA). Stock solutions were freshly prepared in the appropriate buffers. The drugs were diluted in a water bath to achieve the final concentrations stated in the text.

**Patients:** The subjects of this study were 30 pSS patients' anti-Ro/SSA positive and 30 healthy volunteers all female, (age 39-54 years) selected from the metropolitan area of Buenos Aires. The diagnosis of pSS fulfilled the criteria described by Vitali et al. [34] and was given by means of a positive biopsy with a score focus of 3.8 ± 0.07.

**Purification of Human IgG:** The serum IgG fraction from patients with pSS and from normal individuals (control) was isolated using protein G affinity chromatography as described elsewhere [41]. Briefly, sera were loaded onto the protein G affinity column (Sigma-Aldrich, St Louis, MO, USA) equilibrated with 1 M Tris-HCl (pH 8.0) and the columns were washed with 10 volumes of the same buffer. The IgG fraction was eluted with 100 mM glycine-HCl, pH 3.0, and immediately neutralized. The concentration and purification of IgG were determined using a radial immune diffusion assay.

**Anti-M<sub>3</sub> peptide IgG procedure:** The IgG fraction from 30 patients with pSS and 30 healthy subjects was independently subjected to affinity chromatography on the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, CA, USA) as described by Reina et al. [49]. Briefly, the IgG fraction was loaded onto the affinity column equilibrated with phosphate buffered saline (PBS). 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 IgG concentration of non-anti-peptide antibodies and specific anti-muscarinic receptor peptide antibodies was determined by a radial immunodiffusion assay. Their immunological reactivity against muscarinic receptor peptides was evaluated by ELISA. The concentration of the affinity-purified anti-M<sub>3</sub> peptide IgG (1×10<sup>-8</sup> M) increased optical density (mean OD ± SEM, 2.4 ± 0.2).



The non-anti-M<sub>3</sub> peptide IgG fraction eluted from the column showed OD values (0.27 ± 0.06) similar to those of normal IgG from healthy individuals taken as control (0.26 ± 0.05). The normal IgG fraction purified by affinity column chromatography gave a negative result (0.30 ± 0.03). ELISA was performed as described previously [74].

**ELISA:** Fifty microliters of M<sub>3</sub> synthetic peptide solution (20 µg/ml) in 0.1 M Na<sub>2</sub>CO<sub>3</sub> buffer, pH 9.6, was used to coat microtiter plates (NUNC, Kastrup, Denmark) at 4°C overnight. After blocking the wells, diluted sera from pSS patients and healthy individuals were added in triplicate and allowed to react with the peptide for 2 hour at 37°C.

After the wells were thoroughly washed with 0.05% Tween 20 in a PBS, 100 µl of 1:6000 biotinylated goat anti-human IgG antibodies (Sigma Chemical Co., St. Louis, MO, USA) was added and incubated for 1 hour at 37°C. Then, a 1:6000 dilution of extravidin-alkaline phosphatase (Sigma) was allowed to react an extra 30 min at 37°C. After extensive washings, p-nitrophenylphosphate (1 mg/ml) was added as the substrate, and the reaction was stopped at 30 min. Finally, the plates were read at 405 nm and the results for each sample were expressed as the means ± SD of triplicate values.

**PGE<sub>2</sub> procedure:** Serum PGE<sub>2</sub> was measured by ELISA, carried out according to the manufacturer's protocols (Biotrack Enzyme Immune Assay System, Amersham Bioscience, Piscataway, NJ, USA).

The OD cutoff value of PGE<sub>2</sub> was 4.4 ± 0.33 ng/ml. All serum samples were frozen promptly after collection and kept at -80°C until used for PGE<sub>2</sub> determination. The result is expressed as ng/ml.

**Nitric oxide synthase (NOS) assay:** NOS activity was measured in rat submandibular gland tissue by production of [U-<sup>14</sup>C]-citrulline from [U-<sup>14</sup>C]-arginine according to the procedure described for brain slices [77]. Briefly, after 20-min preincubation in KRB solution, tissues were transferred to 500 ml of prewarmed KRB equilibrated with 5% CO<sub>2</sub> in O<sub>2</sub> in the presence of [U-<sup>14</sup>C]-arginine (0.5 mCi).

Drugs were added and the mixture incubated for 20 min under 5% CO<sub>2</sub> in O<sub>2</sub> at 37°C. Tissues were then homogenized with an Ultraturrax homogenizer in 1 ml of medium containing 20 mM HEPES (pH 7.4), 0.5 mM ethyleneglycol tetra-acetic acid (EGTA), 0.5 mM ethylenediamine tetra-acetic acid (EDTA), 1 mM dithiothreitol, 1 mM leupeptin and 0.2 mM phenylmethylsulphonyl fluoride (PMSF) at 4°C.

After centrifugation at 20,000×g for 10 min at 4°C, supernatants were applied to 2 ml columns of Dowex AG 50 WX-8 (sodium form). [<sup>14</sup>C]-citrulline was eluted with 3 ml of water and quantified by liquid scintillation counting. The results were expressed as picomol per gram tissue wet weight (pmol/g/tissue wet wt).

**Cyclic nucleotides assay (cGMP):** Rat submandibular gland (10 mg) was incubated in 1 ml Krebs Ringer Buffer (KRB) for 30 min, and the anti-M<sub>3</sub> mAChR peptide IgG was added in the last 15 min. When a blocker was used, it was added 25 min before the addition of the antibody. After incubation, submandibular gland tissue was homogenized in 2 mL of absolute ethanol and centrifuged (6000×g, 15 min, 4°C).

Pellets were then re-homogenized in ethanol-water (2:1) and re-centrifuged. The supernatant was collected and evaporated to dryness. Cyclic GMP in the residue was dissolved in 400 µl of 0.05 M sodium acetate buffer (pH 6.2). For the determination of nucleotide, we used ELISA employing the protocol for the production of cGMP from Amersham Biosciences (Piscataway, NJ, USA). Results are expressed as picomoles per milligram of wet weight of tissue (pmol/mg tissue wet weight).

**mRNA isolation and cDNA synthesis:** Total RNA was extracted from rat submandibular gland slices by homogenization using guanidiniumisothiocyanate method. As previously described [41], a 20-µl reaction mixture contained 2 ng of mRNA, 20 units of RNase inhibitor, 1 mM dNTPs and 50 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). First-strand cDNA was synthesized at 37°C for 60 min.

**PCR procedures:** NOS isoform-mRNA levels were determined by a method that involves simultaneous co-amplification of both the target cDNA and a reference template (MIMIC) with a single set of primers. MIMIC for eNOS, nNOS and glyceraldehyde-3-phosphate dehydrogenase (G<sub>3</sub>PDH) was constructed using a PCR MIMIC construction kit (Clontech Laboratories, Palo Alto, CA).

Each PCR MIMIC consists of a heterologous DNA fragment with 5' and 3'-end sequences that recognized by a pair of gene-specific primers. Sizes of PCR MIMIC were distinct from those of native targets. The sequence of oligonucleotide primer pairs used for construction of MIMIC and amplification of NOS isoforms and G<sub>3</sub>PDH mRNA is listed in Table 1.

Aliquots were taken from pooled first-strand cDNA from the same group and constituted one sample for PCR. A series of 10-fold dilutions of known concentrations of the MIMIC were added to PCR amplification reactions containing the first-strand cDNA. PCR MIMIC amplification was performed in 100 µl of a solution containing 1.5 mM MgCl<sub>2</sub>, 0.4 µM primer, dNTPs, 2.5 U Taq DNA polymerase and 0.056 µM Taq Start antibody (Clontech Laboratories). After initial denaturation at 94°C for 2 min, the cycle condition was 30 s of denaturation at 94°C, 30 s of annealing at 60°C and 45 s of enzymatic primer extension at 72°C for 45 cycles for NOS isoforms. The internal control was the mRNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G<sub>3</sub>PDH).

PCR amplification was performed with initial denaturation at 94°C for 2 min followed by 30 cycles of amplification. Each cycle consisted of 35 s at 94°C, 35 s at 58°C and 45 s at 72°C. Samples were incubated for an additional 8 min at 72°C before completion. PCR products were subjected to electrophoresis on ethidium bromide-stained gels. Band intensity was quantitated by densitometry using NIH Image software. Levels of mRNA were calculated from the point of equal density of the sample and MIMIC PCR products [41].

NOS isoforms mRNA levels were normalized with the levels of G<sub>3</sub>PDH mRNA present in each sample, which served to control for

Gene Product	Sense	Antisense	Predicted size (bp)
iNOS	5' GAT CAA TAACCT GAA GCC CG 3'	5' GCC CTT TTT TGC TCC ATA GG 3'	578
nNOS	5' GCGGA GCAGA GCGGC CTTAT 3'	5' TTTGGT GGGAG GACCG AGGG 3'	240
eNOS	5' CCGCA CTTCT GTGCC TTTGC TC3'	5'GCTCG GGTGG ATTTGC TGCTCT 3'	360
g <sub>3</sub> pdh	5' ACCAC AGTCCA TGCCAT CAC 3'	5' TCCAC CACCC TGTTG CTGTA 3'	452
Nitric oxide synthase (NOS) isoforms and glyceraldehydes-3-phosphate dehydrogenase (g <sub>3</sub> pdh)			

Table 1: Oligonucleotides of primers for PCR.

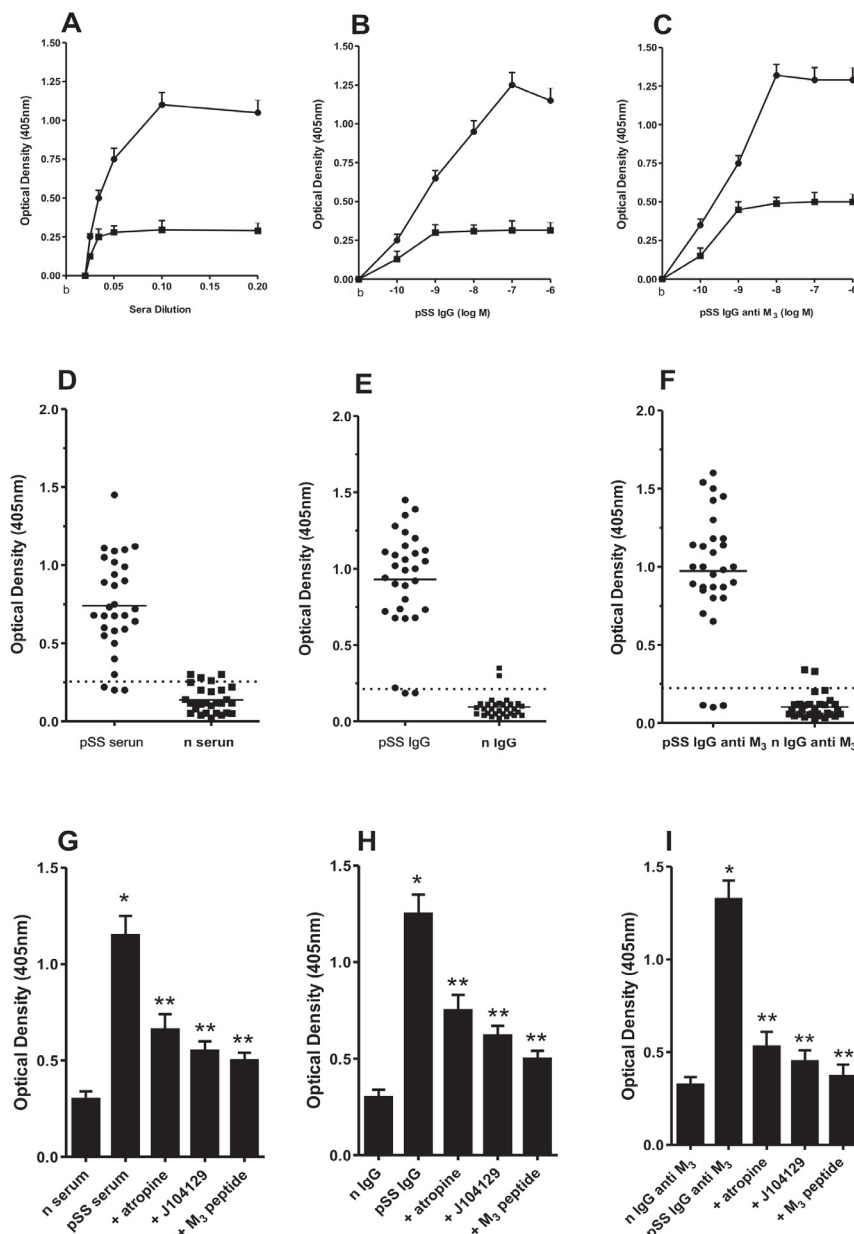
variations in RNA purification and cDNA synthesis. Relative mRNA expression of nNOS and eNOS were compared with those from the respective normal individuals and pSS patients reported as a percentage of normal.

**Statistical analyses:** The Student's "t" test for unpaired values was used to determine the level of significance. If multiple comparisons were necessary, after analysis of variance, the Student-Newman-Keuls

test was applied. Differences between means were considered significant if  $P < 0.05$ .

## Results

Figure 1 shows the immunoreactivity of sera (A), pSSIgG (B) and pSSIgG anti  $M_3$  synthetic peptide (pSSIgG anti  $M_3$ ) (C) of pSS patients and healthy individuals against  $M_3$  synthetic peptide.



**Figure 1:** Effect of serum, IgG and IgG anti  $M_3$  autoantibodies from patients with primary Sjögren syndrome (pSS) evaluated by enzyme immunoassay (ELISA). UPPER PANEL: Dose-response curve of different serum dilution (A), different concentration of pSS IgG (B) and different concentration of pSS IgG anti  $M_3$  (C) autoantibodies of pSS patients (●) against  $M_3$  AChR synthetic peptide. The values of normal sera and normal IgG and normal IgG anti  $M_3$  (■) is also shown. \* $P < 0.0001$  versus serum, IgG and IgG anti  $M_3$  of normal individuals. MIDDLE PANEL: Scattergram showing immunoreactivity of pSS serum (D), pSS IgG (E) and pSS IgG anti  $M_3$  (F) of SS patients (●) and normal individuals (■) against  $M_3$  synthetic peptide. The individual optical density (OD) values for each serum sample or pSS IgG or pSS IgG anti  $M_3$  were taken from 30 normal individuals and 30 patients with pSS. OD values more than 2 SD above the normal mean, were taken as positive. Cut off value of OD: D=  $0.25 \pm 0.05$ , E=  $0.24 \pm 0.06$  and F=  $0.18 \pm 0.03$  respectively. LOWER PANEL: Influence of  $1 \times 10^{-6}$  M atropine (unspecific mAChR antagonist),  $1 \times 10^{-8}$  M J104129 (specific  $M_3$  mAChR antagonist) and  $5 \times 10^{-7}$  M anti  $M_3$  synthetic peptide on the maximal dilution or concentration of serum (G), pSS IgG (H) and pSS IgG anti  $M_3$  (I) respectively. The values of normal sera and normal IgG and normal IgG anti  $M_3$  is also shown. Values are mean  $\pm$  SEM from a pool of 30 pSS patients and 30 normal individuals. \* $P < 0.0001$  versus normal (n) serum or IgG; \*\* $P < 0.001$  versus pSS serum or pSS IgG. All the experiments were performed by duplicates.

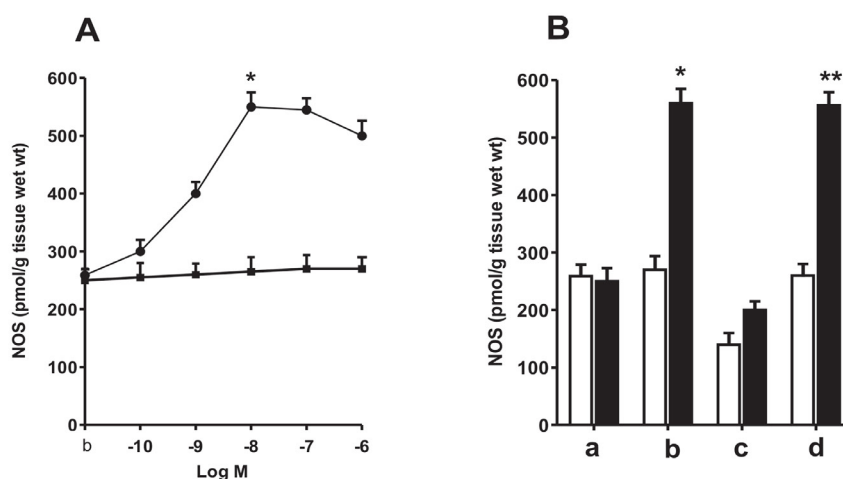
We can see that the optical density values (OD) of pSSIgG anti M<sub>3</sub> was significantly higher than those of pSSIgG and pSS serum. Serum, IgG and pSSIgG anti M<sub>3</sub> of healthy individuals showed similar OD values, which are significantly lower than those of SS patients.

Scatterogram (Figure 1) shows the immunoreactivity of pSS serum (D), pSSIgG (E) and pSSIgG anti M<sub>3</sub> (F) of pSS patients and normal individuals against M<sub>3</sub> synthetic peptide. The immunoreactivity of serum, pSSIgG and pSSIgG anti M<sub>3</sub> was significantly (P<0.0001) higher than that of normal individuals used as control. The specificity of the reaction was assessed by the ability of the M<sub>3</sub> synthetic peptide, atropine (unspecific muscarinic antagonist) and J104129 (specific M<sub>3</sub> muscarinic antagonist) to inhibit the reaction when serum or IgG was incubated with salivary gland membrane mAChR (Figure 1G-I).

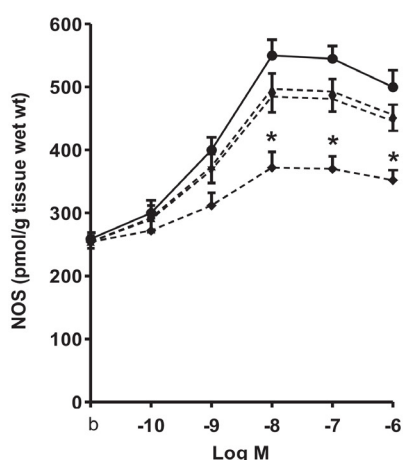
Figure 2A shows the ability of pSSIgG anti M3 to stimulate NOS

activity in a concentration-dependent manner. L-NMMA 5×10<sup>-5</sup> M blocked the action of pSSIgG anti M<sub>3</sub> on NOS activity. Figure 2B (black column) shows the maximal effect of 1×10<sup>-8</sup> M of pSSIgG anti M<sub>3</sub> alone (b) or in the presence of the 1×10<sup>-5</sup> M L-NMMA (c) or L-NMMA plus 1×10<sup>-4</sup> M L-arginine (d) (reverse experiment). Basal (control) values are also shown (a). The IgG anti M<sub>3</sub> of normal individuals (white column) used as a control, is shown (Figure 2Ba-d).

The particular NOS isoforms enzymes which participate in the generation of endogenous nitric oxide (NO), can be seen in Figure 3. The specific inhibition of iNOS with S-Methylisothioures (S-Methyl-U) prevented the stimulation of NOS activity by the pSSIgG anti M<sub>3</sub>. The inhibition of eNOS by L-NIO dehydrochloride (L-NIO) and nNOS by N-Propyl-L-arginine hydrochloride (N-PL) were without any effect being iNOS the only isoform able to impair the stimulation of NOS activity by the autoantibody. The table inserted in this figure, shows the



**Figure 2:** A: Concentration-response curve of pSS IgG anti M3 (●) and normal IgG anti M<sub>3</sub> (■) on nitric oxide synthase (NOS) activity from rat submandibular gland. Basal value (b) corresponds to NOS activity after 60 min of incubation [254 ± 19 pmol/g tissue wet wt]. Values are mean ± SEM of five experiments in each group performed in duplicate. \*P<0.0001 versus 5×10<sup>-5</sup> M L-NMMA + pSS IgG anti M<sub>3</sub>. B: Preparations [white columns: normal IgG anti M<sub>3</sub> (n IgG anti M3) and black columns pSS IgG anti M3] were incubated for 60 min with 1×10<sup>-8</sup> M pSS IgG anti M<sub>3</sub> in absence (b) or in presence (c) of 5×10<sup>-5</sup> M L-NMMA and L-NMMA plus 1×10<sup>-4</sup> M L-arginine (d). Basal values before the addition of any antibody or drugs (a). Values are mean ± SEM of five experiments in each group performed in duplicate. \*P<0.0001 versus n IgG anti M<sub>3</sub>, \*\*P<0.001 versus nIgG anti M<sub>3</sub>.



NOS activity in the presence of nIgG anti M<sub>3</sub>. Influence of different isoforms inhibitors

Additions	nIgG anti M <sub>3</sub>				
	1×10 <sup>-10</sup> M	1×10 <sup>-9</sup> M	1×10 <sup>-8</sup> M	1×10 <sup>-7</sup> M	1×10 <sup>-6</sup> M
None	255.2 ± 19	260.4 ± 20	265.2 ± 18	270.1 ± 19	270.2 ± 18
+ N-PL (5×10 <sup>-6</sup> M)	255.2 ± 20	255.8 ± 19	257.3 ± 15	258.8 ± 16	258.8 ± 15
+ L-NIO (5×10 <sup>-6</sup> M)	255.6 ± 16	258.6 ± 16	262.1 ± 16	265.8 ± 16	265.8 ± 14
+ S-Methyl-U (1×10 <sup>-5</sup> M)	255.8 ± 18	259.7 ± 15	264.5 ± 14	269.4 ± 14	269.4 ± 15

Values are mean ± SEM of five experiments in each case performed by duplicate.

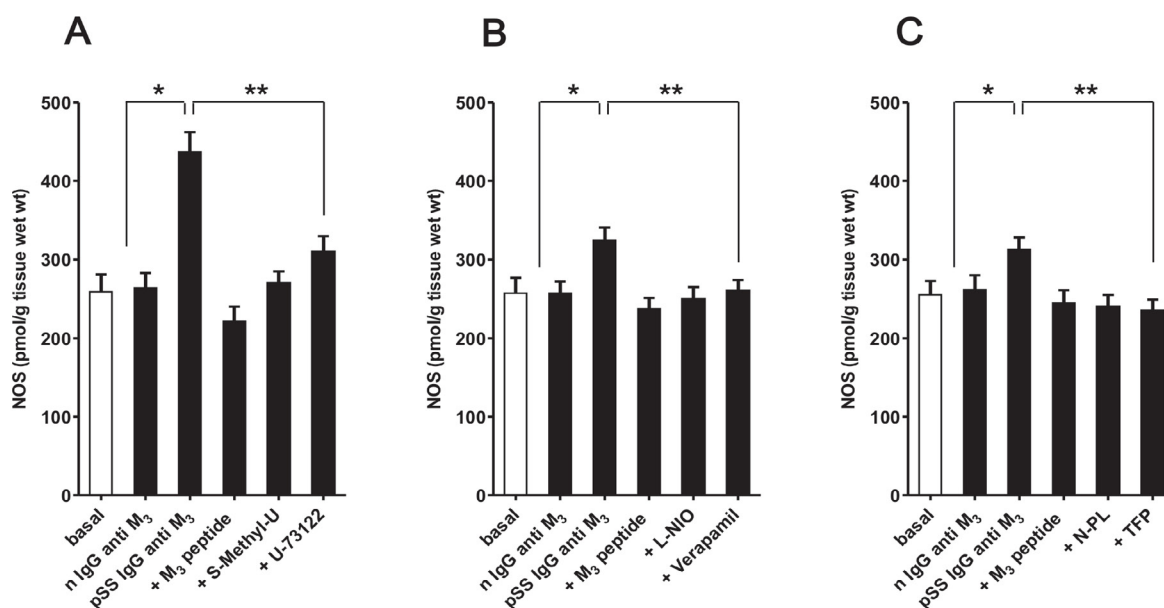
**Figure 3:** Upper panel: Effect of pSS IgG anti M<sub>3</sub> on NOS activity in salivary gland. Submandibular gland were incubated with increasing concentrations of pSS IgG anti M<sub>3</sub> alone (●) or in the presence of 1×10<sup>-5</sup> M (S) Methylisothioures (S-Methyl-U) (◆), 5×10<sup>-6</sup> M L-NIO dehydrochloride (L-NIO) (▲) or 5×10<sup>-6</sup> M N-Propyl-L-arginine hydrochloride (N-PL) (▼). \*P<0.0001 versus pSS IgG anti M<sub>3</sub>. Values are mean ± SEM of five experiments performed in duplicate. Lower panel: Table insert in Figure showed the effect of normal IgG anti M<sub>3</sub> (n IgG anti M<sub>3</sub>) alone or in the presence of different NOS isoforms inhibitors taken as control values.

values of NOS activity in the presence of normal IgG anti M<sub>3</sub> and the isoforms inhibitors taken as a control. These results indicate that the pSSIgG anti M<sub>3</sub> stimulated NOS activity is a result of the increment in NO with major participation of iNOS.

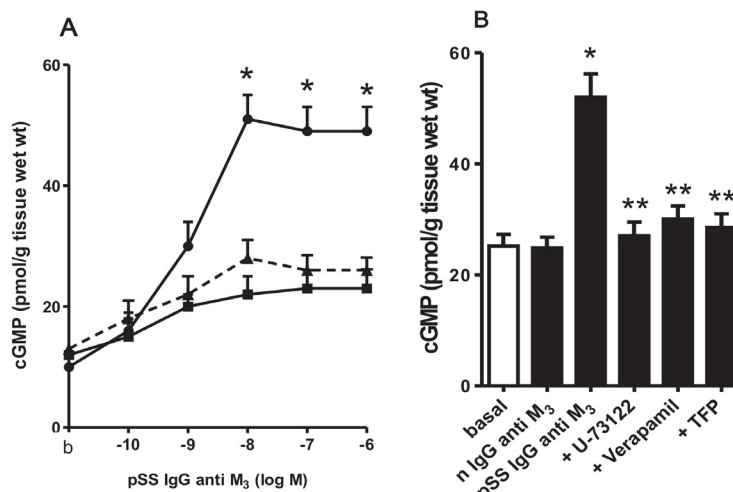
In order to discern, which endogenous mechanisms (second messengers) are implicated in pSSIgG anti M<sub>3</sub> NOS activation and the generation of NO production, several inhibitors of this enzymatic pathways were used. It can be seen in Figure 4 that the stimulation of NOS activity by the pSSIgG anti M<sub>3</sub> was inhibited by M<sub>3</sub> synthetic peptide (A, B, C), S-Methyl-U and U-73122 (A), L-NIO and verapamil

(B) and N-PL and TFP (C). These results indicate that pSSIgG anti M<sub>3</sub>-M<sub>3</sub>mAChR stimulation may trigger the production of NO synthesis in submandibular salivary glands by iNOS isoforms dependent on PLC activation. NO exerts its effects mainly by activating soluble guanylyl cyclases in the presence of the pSSIgG anti M<sub>3</sub> in a concentration dependent manner, increasing cGMP synthesis in rat submandibular gland (Figure 5).

The increment in cGMP production is inhibited by the specific soluble guanylyl cyclases inhibitor (ODQ) and the IgG anti M<sub>3</sub> of normal individuals was without effect in our system (A). The NO-



**Figure 4:** Effect of pSS IgG anti M<sub>3</sub> (black column) on NOS activity in submandibular gland. The glands were incubated with 1x10<sup>-8</sup> M pSS IgG anti M<sub>3</sub> alone or in the presence of 1x10<sup>-4</sup> M synthetic M<sub>3</sub> peptide, 1x10<sup>-5</sup> M S-Methyl-U and 5x10<sup>-6</sup> M U-73122 (A), 1x10<sup>-4</sup> M synthetic M<sub>3</sub> peptide, 5x10<sup>-6</sup> M L-NIO and 5x10<sup>-6</sup> M verapamil (B) and 1x10<sup>-4</sup> M synthetic M<sub>3</sub> peptide, 5x10<sup>-6</sup> M N-PL and 5x10<sup>-6</sup> M TFP (C). Basal values (white column) without any additions and n IgG anti M<sub>3</sub> were also shown. Values are mean ± SEM of six experiments performed in duplicate. \*P<0.001 versus basal and n IgG anti M<sub>3</sub>, \*\*P<0.001 versus pSS IgG anti M<sub>3</sub> alone.



**Figure 5:** Effects of pSS IgG anti M<sub>3</sub> in salivary glands. (A) Submandibular glands were incubated with increasing concentration of pSS IgG anti M<sub>3</sub> alone (●) or in the presence of ODQ (1x10<sup>-7</sup> M) (▲) and n IgG anti M<sub>3</sub> (■). Values represent the mean ± SEM of five experiments performed in duplicate. \*P > 0.0001 versus pSS IgG anti M<sub>3</sub> + ODQ. (B) Action of pSS IgG anti M<sub>3</sub> on cyclic GMP (cGMP) accumulation rat submandibular gland were incubated with 1x10<sup>-8</sup> M of pSS IgG anti M<sub>3</sub> alone or in the presence of different inhibitors: U-73122 at 5x10<sup>-6</sup> M, verapamil at 5x10<sup>-5</sup> M and TFP at 5x10<sup>-6</sup> M. Results are mean ± SEM of four experiments performed in duplicate in each groups. \*P<0.0001 versus basal and n IgG anti M<sub>3</sub>, \*\*P<0.001 versus pSS IgG anti M<sub>3</sub>.

cGMP accumulation is mediated by the effect of PLC, calcium influx and calcium/calmodulin activation (B) since the NO-cGMP increment is blunted by U-73122, verapamil and TFP. To settle the role of NOS isoforms in the action of pSSIgG anti M<sub>3</sub> on rat submandibular gland M<sub>3</sub>mAChR activation, specific primers for iNOS (A), nNOS (B) and eNOS (C) were used.

Figure 6 shows RT-PCR products and semi-quantitative RT-PCR analysis demonstrating that the pSSIgG anti M<sub>3</sub> 1×10<sup>-8</sup> M stimulation for one hour increase NOS mRNA levels with no modification of NOS mRNA levels by normal IgG anti M<sub>3</sub> (control). The same figure shows that M<sub>3</sub> synthetic peptide, S-Methyl-U, L-NIO and N-PL attenuate the stimulatory effect of pSSIgG anti M<sub>3</sub> on NOS mRNA levels. The internal control for the mRNA of the housekeeping gene of glyceraldehyde-3-phosphate- dehydrogenase (g<sub>3</sub>pdh) is shown. Regarding mRNA expression these results demonstrate that pSSIgG anti M<sub>3</sub> acts as an inducer of iNOS mRNA without a significative action on nNOS and eNOS mRNA levels.

The sequence of oligonucleotide primers pairs used is listed in Table 1. The relative mRNA expression of iNOS, nNOS and eNOS in each group was compared to those of the corresponding normal group and reported as a percentage.

The fact that pSSIgG anti M<sub>3</sub> antibody induces iNOS activity and expression, tempts us to speculate that the antibody can have direct influence on rat submandibular gland through the production of a large amount of pro-inflammatory substances and cytotoxic NO by means of the activation of mAChR subtype M<sub>3</sub>. In turn, NO could be said to contribute to immune inflammation at the level of rat submandibular gland, regulating not only the degree of inflammation but also decreasing salivary secretion. All of these facts are the consequence of an abnormal glandular parasympathetic function (parasympathetic dysautonomia) with the participation of PLC (IP<sub>3</sub>), calcium influx and calcium/calmodulin activation.

## Conclusion

The present study suggests a complex interplay between different

factors involved in innate and adaptive immunity. The presence of anti-M<sub>3</sub>mAChR peptide IgG and the enhancement of NOS activity and its expression could provide a link between autoimmunity and parasympathetic system in Sjögren syndrome. Further, the early agonist-promoting activation of salivary gland M<sub>3</sub>mAChR initiated by cholinergic autoantibodies binds to and persistently activates cholinceptors, resulting in the production of large amounts of proinflammatory substances, contributing to inflammation.

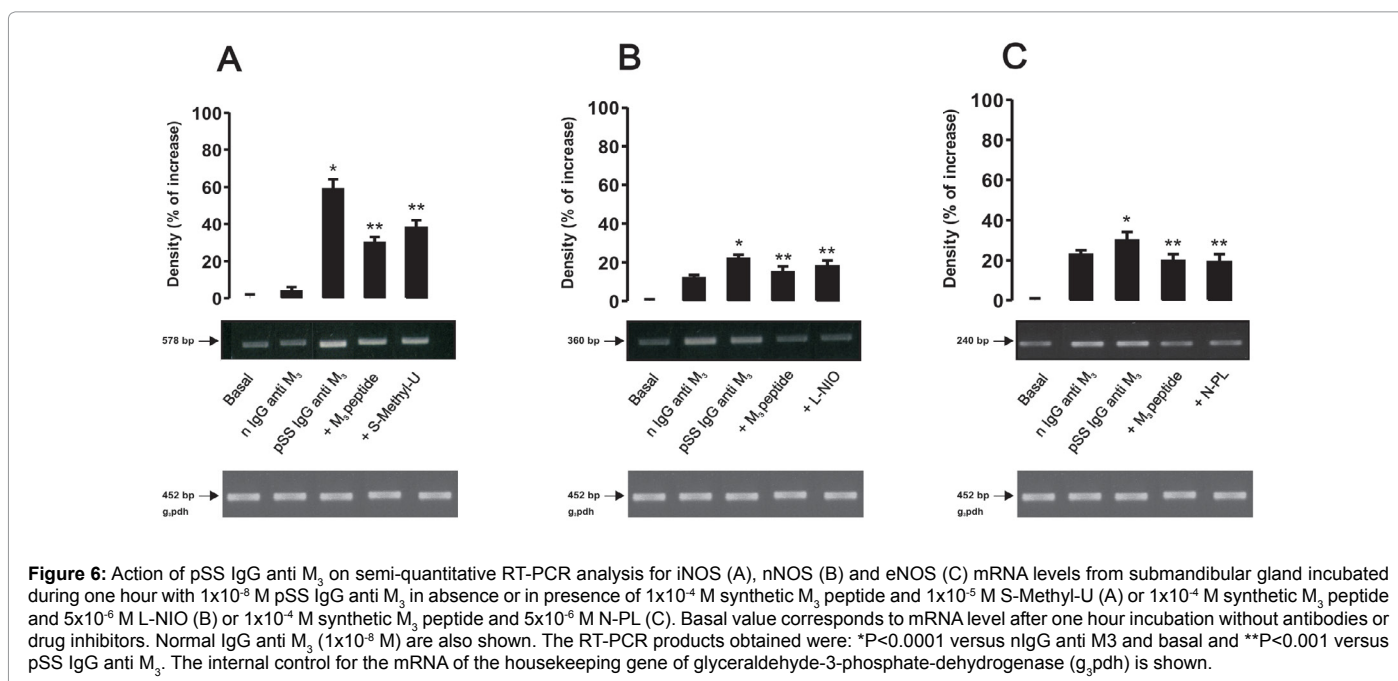
The cholinergic agonistic activity displayed by anti-M<sub>3</sub>mAChR peptide IgG could subsequently induce desensitisation, internalisation and/or intracellular degradation of glandular M<sub>3</sub>mAChR. This would lead to a progressive reduction in the surface expression and activity of glandular M<sub>3</sub>mAChR, resulting in xerostomy, xerophthalmia and other general parasympathetic symptoms in SS patients.

In this paper we propose a model to explain the mechanism whereby pSSIgG stimulate rat submandibular gland mAChR subtype M<sub>1</sub> and M<sub>3</sub>. According to our model pSSIgG acting on frontal cerebral cortex and submandibular gland mAChR subtype M<sub>1</sub> and M<sub>3</sub> activate the Gβγ subunit protein.

The activation of Gβγ leads to the activation of caspase-8 and the Gas/q subunit provoking JNK phosphorylation and increasing MMP-3 production, which contributes to increase PGE<sub>2</sub> levels. Gas/q subunit itself also activates PLC with the production of IP<sub>3</sub> (that in turn increase intracellular calcium concentration) and DAG (that in turn activating PKC activity) and provokes the decrement of the salivary mucin leading to the reduction in the protection of the oral tissues.

Activation of adenylatecyclase leads to cAMP accumulation with an increase in the efflux of extracellular calcium. This, in turn, increases intracellular calcium concentrations; and induces their binding to the calcium/calmodulin complex (CaM). The CaM complex increases nitric oxide synthase activity through the inducible isoform (iNOS) that, in turn, increases Nitric Oxide (NO) production, triggering cyclic GMP (cGMP) accumulation.

The overproduction of NO also triggers the induction of iNOS





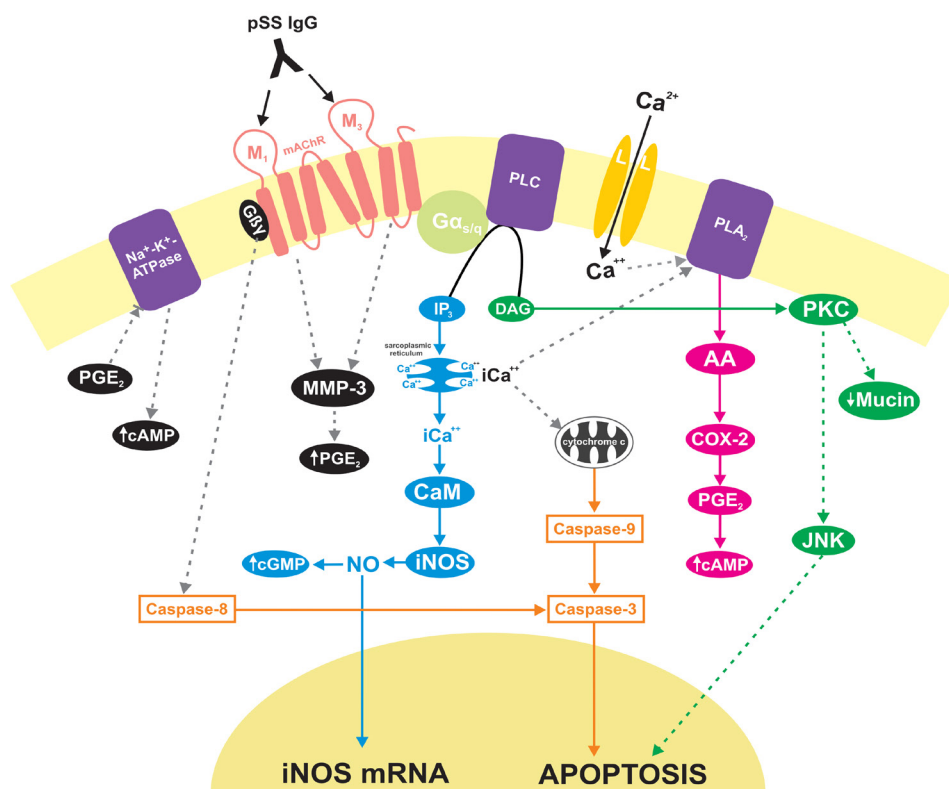


Figure 7: The previously related facts (findings and shadings) indicating direct mechanisms (filled arrows) and indirect mechanisms (dotted arrows).

mRNA levels. The rise in cytosolic calcium activates phospholipase A<sub>2</sub> (PLA<sub>2</sub>) with activation of COX-2, which results in PGE<sub>2</sub> generation with an increased production of cAMP levels. The generation of PGE<sub>2</sub> induces the inhibition of membrane Na<sup>+</sup>-K<sup>+</sup>-ATPase activity accompanied by an increment of cAMP accumulation. The activation of caspases-8 and -9 activates caspase-3, leading to apoptosis. Figure 7 below depicts the previously related facts (findings and shadings) indicates direct mechanisms (filled arrows) and indirect mechanisms (dotted arrows).

## Perspective

The activation of glandular M<sub>3</sub>mAChR by the serum autoantibody present in patient with SS induces changes in the production of the second messengers. These changes are generated by the activation and binding of the glandular cholinceptor. A synthetic M<sub>3</sub> peptide is able to block all actions generated by the antibody on glandular cholinceptors. This being the case, the synthetic M<sub>3</sub> peptide could be used as a therapeutic mean. Such a therapeutic mean, would capture the circulating mAChR autoantibodies thus reducing the destruction of the exocrine glands, the subsequent inflammatory process and indirectly the sicca symptoms of SS.

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