

## RESEARCH ARTICLE

Salivary gland *LAMP3* mRNA expression is a possible predictive marker in the response to hydroxychloroquine in Sjögren's disease

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## Abstract

Hydroxychloroquine (HCQ) is a lysosomotropic agent that is commonly used for treating Sjögren's disease (SjD). However, its efficacy is controversial because of the divergent response to the drug among patients. In a subgroup of SjD patients, lysosome-associated membrane protein 3 (LAMP3) is elevated in expression in the salivary glands and promotes lysosomal dysregulation and lysosome-dependent apoptotic cell death. In this study, chloroquine (CQ) and its derivative HCQ were tested for their ability to prevent LAMP3-induced apoptosis, *in vitro* and on a mouse model of SjD. In addition, efficacy of HCQ treatment was retrospectively compared between high *LAMP3* mRNA expression in minor salivary glands and those with *LAMP3* mRNA levels comparable with healthy controls. Study results show that CQ treatment stabilized the lysosomal membrane in LAMP3-overexpressing cells via deactivation of cathepsin B, resulting in decreased apoptotic cell death. In mice with established SjD-like phenotype, HCQ treatment also significantly decreased apoptotic cell death and ameliorated salivary gland hypofunction. Retrospective analysis of SjD patients found that HCQ tended to be more effective in improving disease activity index, symptom severity and hypergammaglobulinemia in patients with high *LAMP3* expression compared those with normal *LAMP3* expression. Taken together, these findings suggested that by determining salivary gland *LAMP3* mRNA level, a patient's response to HCQ treatment could be predicted. This finding may provide a novel strategy for guiding the development of more personalized medicine for SjD.

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## Introduction

Sjögren’s disease (SjD) is a chronic exocrinopathy that leads to salivary and lacrimal gland hypofunction with resultant dry mouth and eye symptoms. SjD is considered to be a systemic autoimmune disease because of the presence of lymphocytic infiltration into affected tissues, serum autoantibodies, and hypergammaglobulinemia [1]. Increased apoptosis of salivary gland epithelial cells as a result of lysosomal dysfunction has been implicated in the development of salivary gland hypofunction and autoimmunity [2–6].

Chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) are lysosomotropic agents, which deactivate lysosomal function by alkalinizing the lysosomal pH. Specifically, they inhibit endolysosomal, autophagic, and endolysosomal toll-like receptor (TLR) pathways [7, 8]. HCQ is frequently prescribed to patients with SjD. Although its efficacy in SjD has been shown in several observational studies in the 1990s [9–12], two more recent randomized controlled trials in SjD patients did not substantiate the expected benefits [13, 14].

Transcriptome analysis of minor salivary glands of SjD patients identified increased expression of *LAMP3* mRNA, encoding lysosome-associated membrane protein 3 (LAMP3), and this expression was associated with some SjD-related clinical findings, such as lymphocytic infiltration, serum autoantibodies and hypergammaglobulinemia [2]. Unlike LAMP1 and LAMP2, which are distributed in many tissues and associated with general lysosomal functions, LAMP3 is expressed in limited several cell types, such as dendritic cells and type II pneumocytes, and involved in their specific functions, including antigen presentation via major histocompatibility complex class II and surfactant synthesis, secretion, and recycling [15]. Confocal immunofluorescent studies on SjD patients’ salivary glands revealed ectopic LAMP3 expression on both lysosomal membrane and plasma membrane of salivary gland epithelial cells as well as in infiltrating immune cells [2, 3]. Interestingly, non-obese diabetic mice, a spontaneous model of SjD, also have increased LAMP3 expression in their salivary glands, and mice in which LAMP3 overexpression was induced in their salivary glands develop an SjD-like phenotype with progressive salivary gland hypofunction, local lymphocytic infiltration, and the presence of serum anti-Ro/SSA and anti-La/SSB antibodies [4]. The underlying mechanism is thought to rely on promotion of apoptosis via induction of lysosomal membrane instability by LAMP3 [2–4].

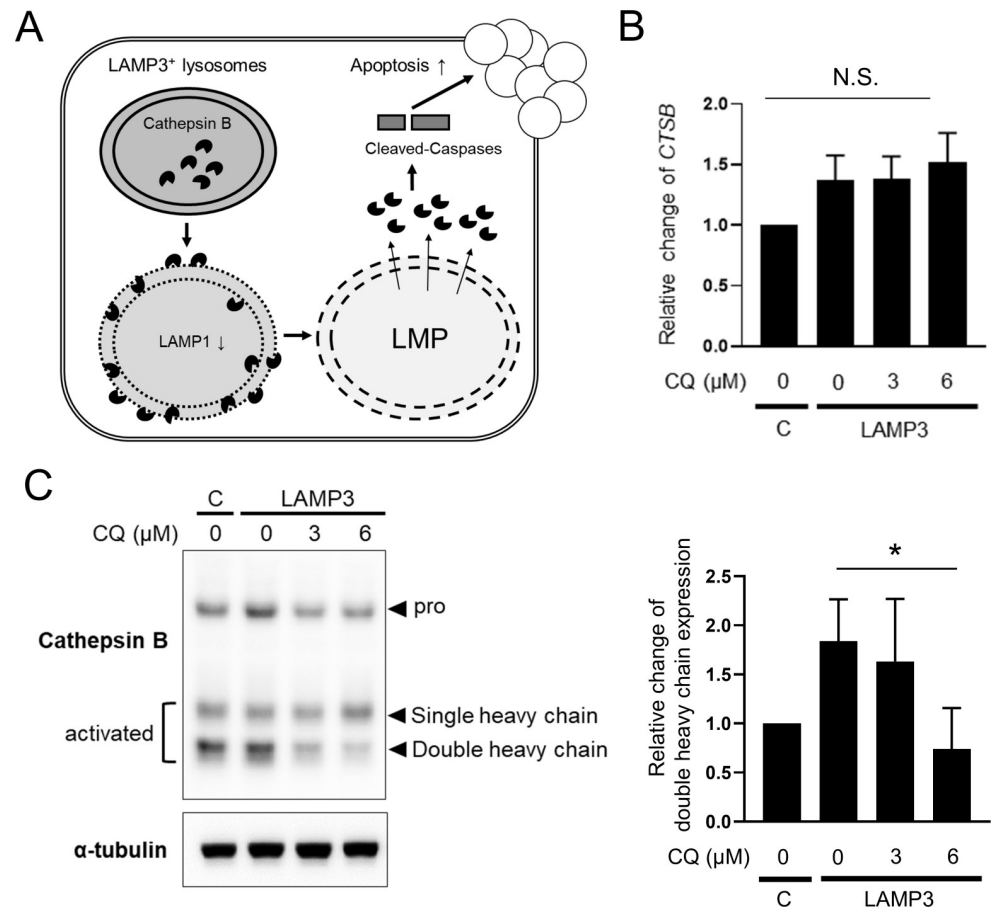
As patients with SjD present with heterogeneous symptoms, recent studies have focused on stratifying them into homogeneous subsets to develop a personalized treatment strategy [16, 17]. Considering the LAMP3-associated pathophysiology, correction of lysosomal function can be a promising therapeutic approach for SjD. In the current study, we utilized this information to test the hypothesis that targeting patients with elevated salivary gland *LAMP3* expression for HCQ therapy will improve overall clinical outcome of this drug by a retrospective cohort study in combination with *in vitro* and *in vivo* experiments.

## Results

### CQ prevents LAMP3-induced apoptotic cell death via deactivation of cathepsin B

LAMP3 destabilizes the lysosomal membrane via degradation of LAMP1, which leads to lysosomal membrane permeabilization (LMP), and leakage of activated lysosomal enzymes into the cytoplasm. This in turn activates caspases, resulting in apoptosis. LAMP3-associated LAMP1 degradation and LMP are dependent on cathepsin B activity (Fig 1A) [3].

To investigate whether CQ can inhibit LAMP3-induced apoptotic cell death, we first treated stably LAMP3-overexpressing A253 cells with CQ for 24 hours and then analyzed cathepsin B



**Fig 1. Chloroquine deactivates cathepsin B in LAMP3-overexpressing cells.** Stably LAMP3-overexpressing A253 cells were treated with chloroquine (CQ) for 24 hours at increasing concentrations. (A) Molecular mechanism about LAMP3-induced apoptotic cell death. (B) Relative change in *CTSB* expression ( $n = 4$ ). C: control A253 cells. (C) Representative Western blots showing expression of cathepsin B and  $\alpha$ -tubulin (control). Bar chart shows relative change of cathepsin B expression normalized by  $\alpha$ -tubulin expression compared to control A253 cells ( $n = 3$ ). \* $P < 0.05$ , N.S. = not significant (Student's  $t$ -tests with Dunnett's correction).

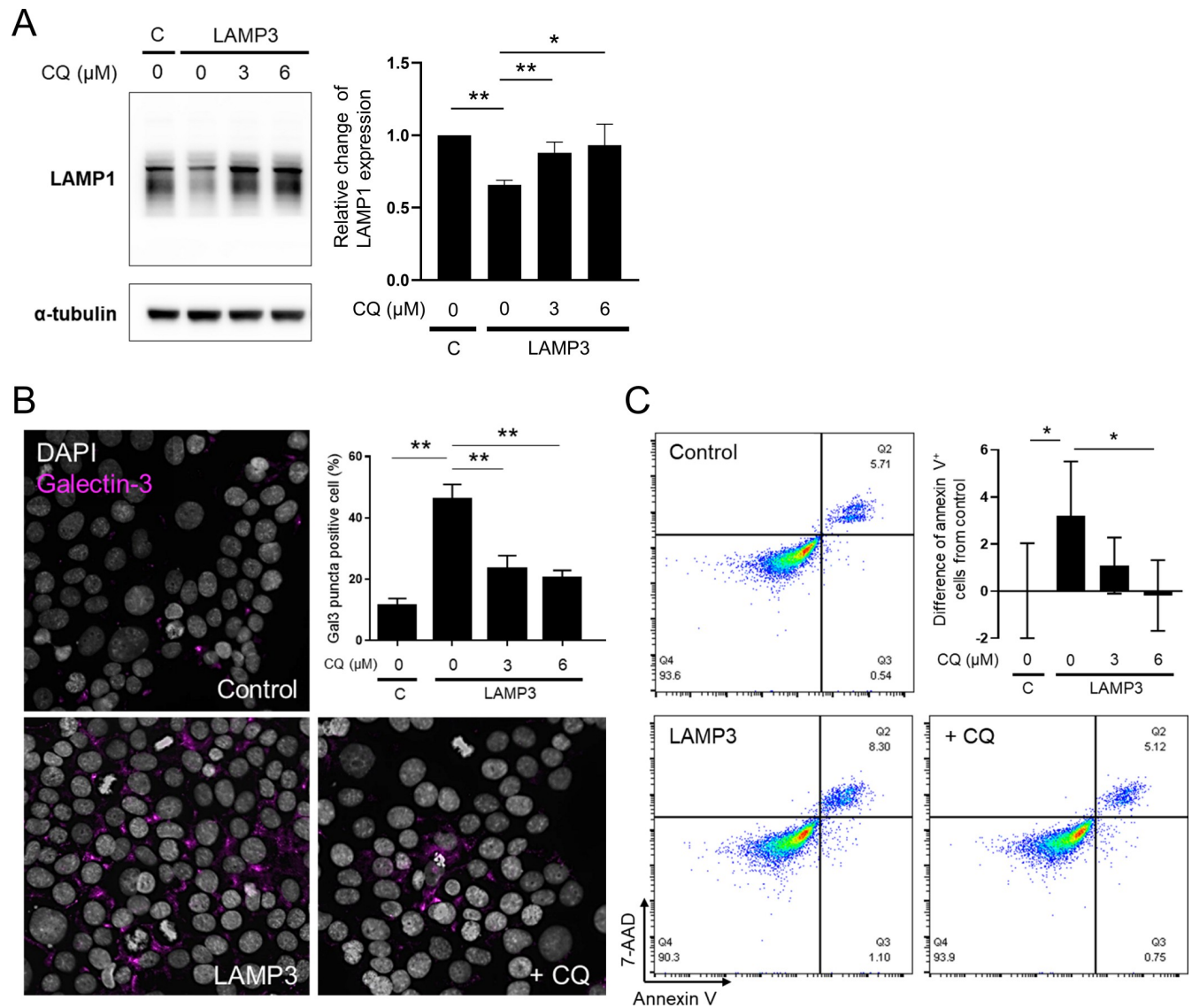
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expression, LAMP1 expression, galectin-3 puncta formation (a marker of LMP), and apoptotic cell death. Pro-cathepsin B, which is synthesized on the rough endoplasmic reticulum, matures into single and double heavy chains in the late endosome and lysosome, respectively [18]. Although CQ treatment did not change mRNA expression of *CTSB* (encoding cathepsin B) (Fig 1B), it did decrease the amount of the double heavy chain form of cathepsin B (Fig 1C), confirming that CQ, an alkalinizing agent, can inhibit the pH-dependent maturation process of cathepsin B in the lysosome [19].

In addition, CQ treatment restored LAMP1 expression (Fig 2A), reduced galectin-3 puncta formation (Fig 2B), and decreased apoptosis (Fig 2C) in LAMP3-overexpressing cells compared to vehicle-treated LAMP3-overexpressing cells. These results demonstrated that CQ decreases LAMP3-induced apoptotic cell death *in vitro* by deactivating cathepsin B.

### HCQ treatment decreases LAMP3-induced apoptotic cell death in mice

Next, to examine whether HCQ can decrease LAMP3-induced apoptotic cell death *in vivo*, we induced epithelial LAMP3 overexpression in the submandibular glands of female, 2-month-

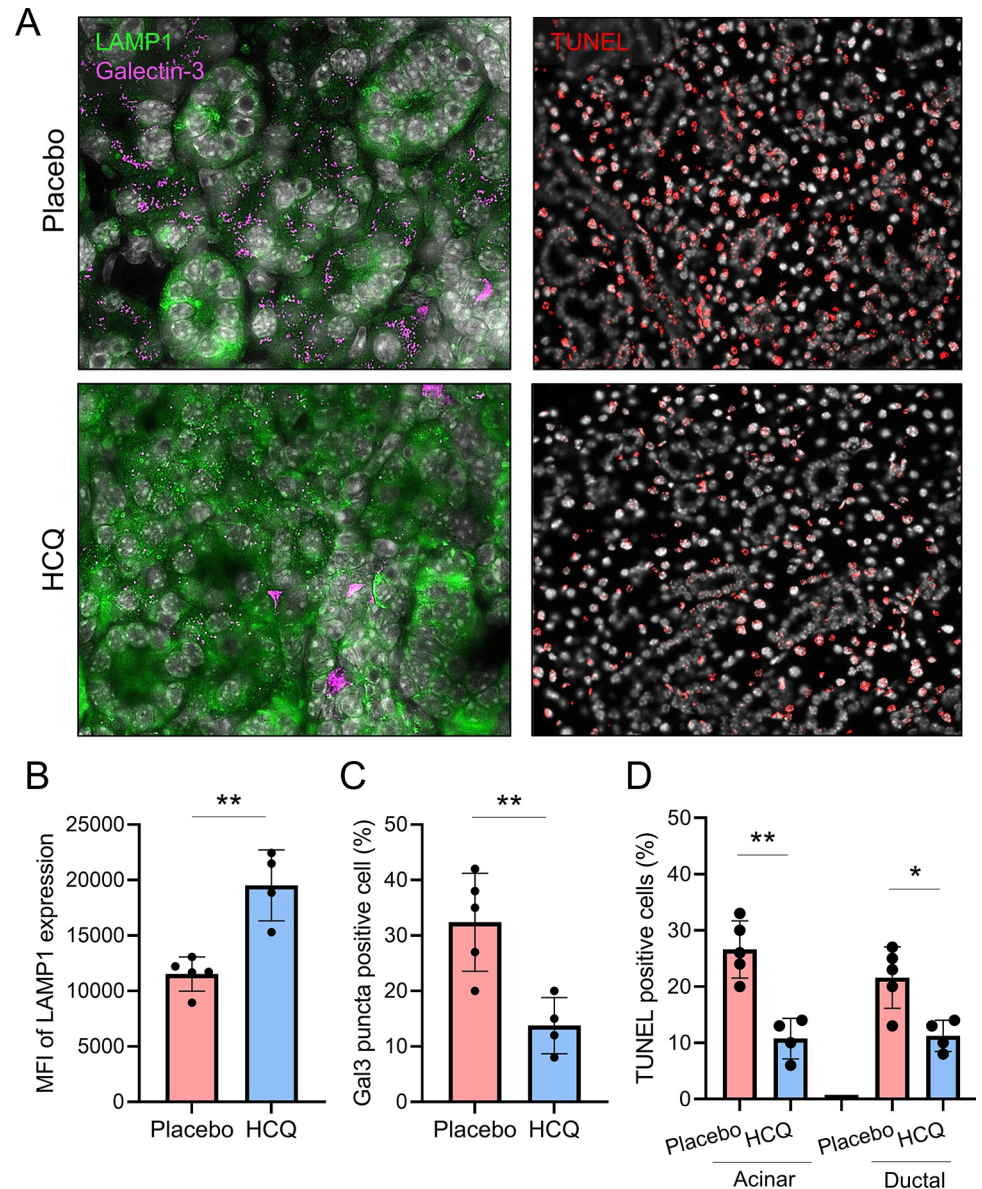


**Fig 2. Chloroquine prevents LAMP3-induced apoptotic cell death *in vitro*.** Stably LAMP3-overexpressing A253 cells were treated with chloroquine (CQ) for 24 hours at increasing concentrations. (A) Representative Western blots showing expression of LAMP1 and  $\alpha$ -tubulin (control). Bar chart shows relative change of LAMP1 expression normalized by  $\alpha$ -tubulin expression compared to control A253 cells ( $n = 3$ ). C: control A253 cells. (B) Representative immunofluorescent images showing staining for galectin-3 (magenta) (40 $\times$  magnification). Bar chart shows percentage of galectin-3 (Gal3) puncta-positive cells ( $n = 3$ ). (C) Number of apoptotic cells was determined by flow cytometry using APC Annexin V/7-AAD. Bar chart shows percentage of difference in Annexin V-positive cells from control ( $n = 5$ ). Values shown are mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , N.S. = not significant (Student's  $t$ -tests with Dunnett's correction).

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old C57BL/6 mice by retrodual cannulation with adeno-associated virus serotype 2 vectors encoding the gene for LAMP3 (AAV2-LAMP3), as previously reported [4]. After 4 months—when all mice have established SjD-like disease—mice were treated with weekly intraperitoneal injections of HCQ or placebo for 3 months. At the end of the study, the submandibular glands were collected and analyzed for changes in apoptosis and histology. Consistent with the above *in vitro* results, HCQ treatment restored LAMP1 expression (Fig 3A and 3B) and reduced galectin-3 puncta formation (Fig 3A and 3C) in salivary glands of LAMP3-overexpressing mice. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay



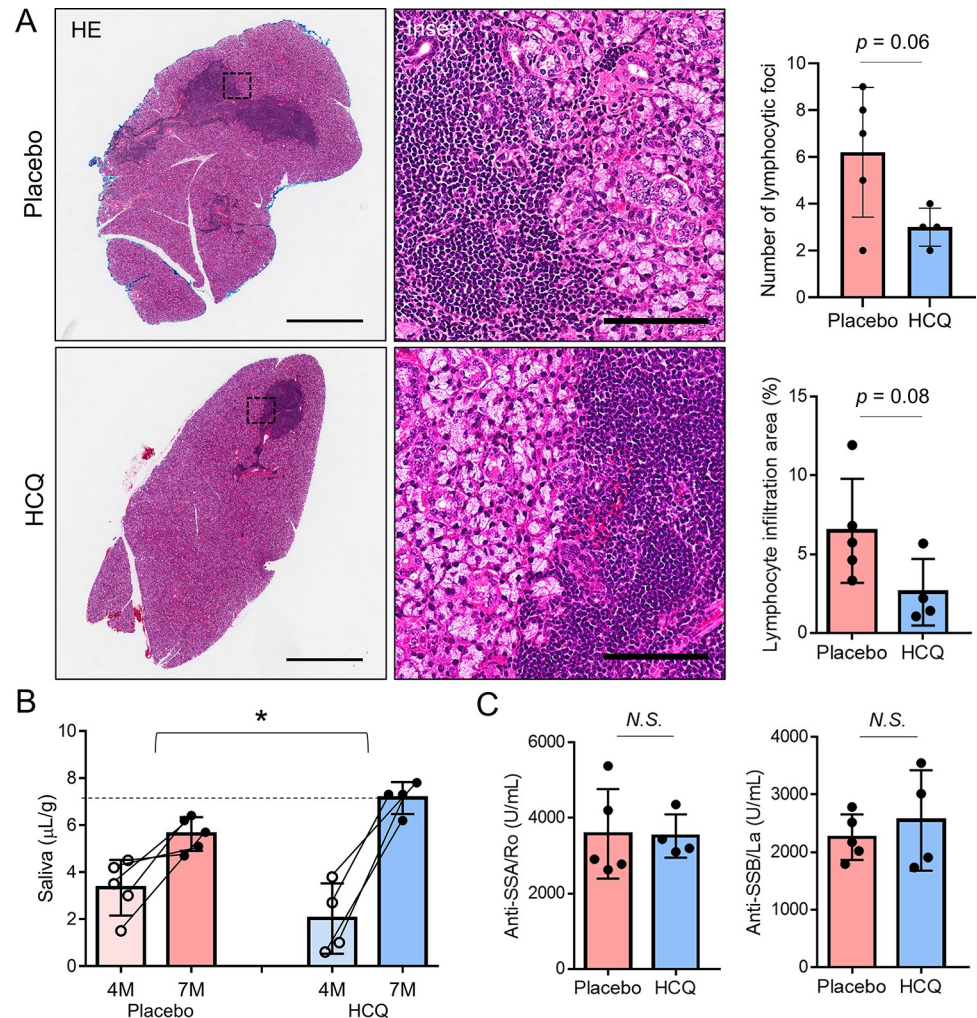


**Fig 3. Hydroxychloroquine treatment decreases LAMP3-induced cell death *in vivo*.** Submandibular glands of C57BL/6 mice were instilled with AAV2-LAMP3. After 4 months, mice were given weekly intraperitoneal injections of 60 mg/kg HCQ ( $n = 4$ ) or placebo ( $n = 5$ ) for 3 months. (A) Representative submandibular gland tissue sections showing staining for LAMP1 (green) and galectin-3 (magenta) (40 $\times$  magnification) or terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL, red) (20 $\times$  magnification). (B) Bar chart shows mean fluorescence intensity of LAMP1 expression. (C) Bar chart shows percentage of galectin-3 (Gal3) puncta-positive cells. (D) Bar chart shows percentage of TUNEL-positive epithelial cells. Values shown are mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , N.S. = not significant (Student's  $t$ -tests).

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for staining cells with damaged DNA and undergoing apoptosis showed that the number of apoptotic epithelial cells was significantly decreased in the HCQ-treated mice compared to placebo-treated mice (Fig 3A and 3D).

Histological analysis of the glands suggested a trend towards decreased lymphocytic infiltration in the HCQ-treated mice (Fig 4A). Treatment with HCQ resulted in a significant increase in salivary flow rate compared to placebo (Fig 4B), while serum anti-Ro/SSA and



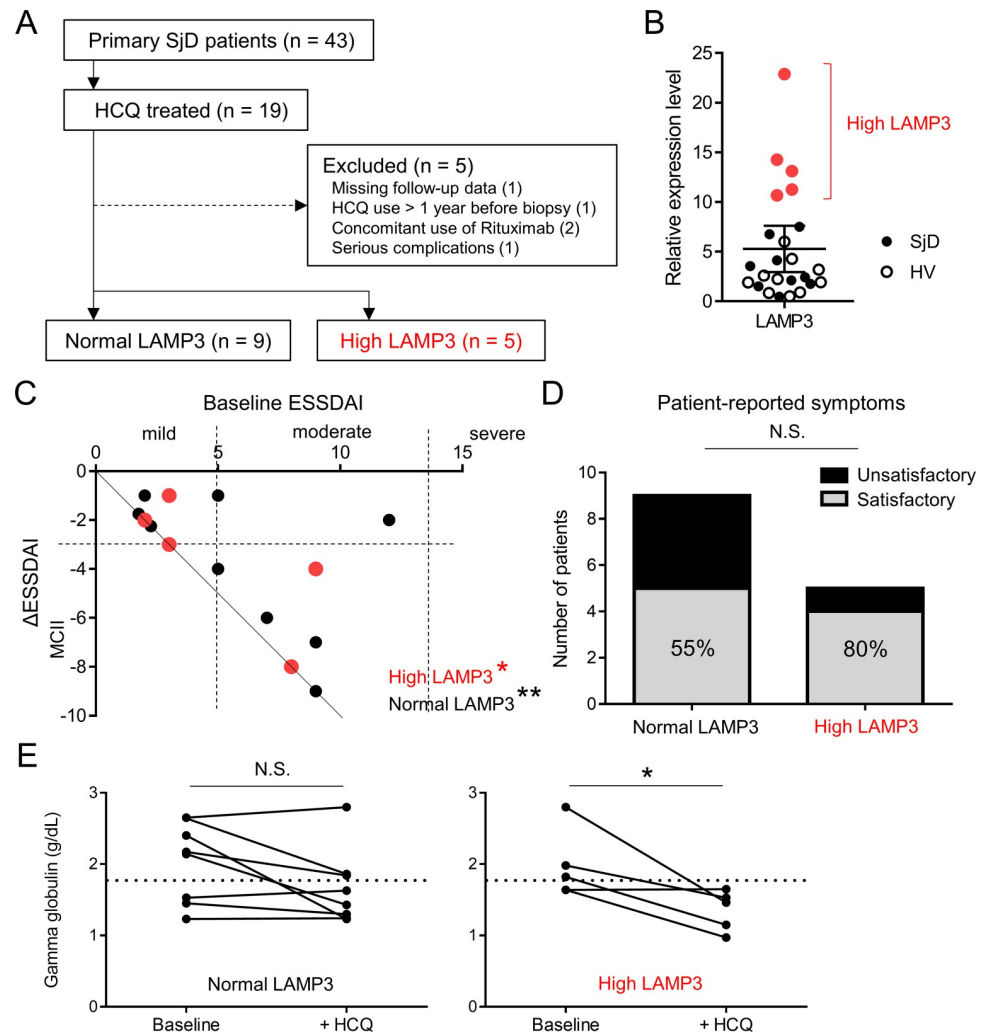
**Fig 4. Hydroxychloroquine treatment ameliorates LAMP3-associated sialadenitis *in vivo*.** Submandibular glands of C57BL/6 mice were instilled with AAV2-LAMP3. After 4 months, mice were given weekly intraperitoneal injections of 60 mg/kg HCQ ( $n = 4$ ) or placebo ( $n = 5$ ) for 3 months. (A) Representative submandibular gland tissue sections showing hematoxylin & eosin (HE) staining. Scale bars = 1 mm (inset: 100  $\mu$ m). Bar charts shows the number of lymphocytic foci and the average size of lymphocytic infiltration areas in both the submandibular glands of each mouse. (B) Graph showing pilocarpine-stimulated salivary flow rate per body weight in 20 minutes. The dashed line indicates a reference level of salivary flow rate derived from control mice (instilled with AAV2-GFP). (C) Bar charts showing serum anti-Ro/SSA or anti-La/SSB antibody levels. Values shown are mean  $\pm$  SD. \* $P < 0.05$ , N.S. = not significant (Student's *t*-tests).

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anti-La/SSB antibody levels were not affected (Fig 4C). In short, these results suggested that HCQ treatment decreased LAMP3-induced apoptotic cell death and restores salivary gland function in a mouse model of SjD.

### Clinical response to HCQ in SjD patients is associated with salivary gland LAMP3 mRNA expression level

Our *in vitro* and *in vivo* experiments showed that CQ/HCQ can reduce LAMP3-induced apoptotic cell death. Finally, to confirm HCQ's effect on LAMP3-induced changes in SjD patients, we retrospectively analyzed the efficacy of HCQ treatment in a cohort of SjD patients seen at the Clinical Hospital of the Medical School of Ribeirão Preto in São Paulo, Brazil. For this



**Fig 5. Clinical response to hydroxychloroquine in SjD patients is associated with salivary gland *LAMP3* mRNA expression level.** (A) Flow diagram showing SjD patient selection. (B) Dot plot showing relative expression of *LAMP3* with mean and 95% confidence interval in labial minor salivary glands from SjD patients ( $n = 14$ ) and healthy volunteers (HV,  $n = 10$ ). (C) Dot plot showing baseline ESSDAI and change in ESSDAI after HCQ treatment.  $\Delta$ ESSDAI  $\geq 3$  is considered as minimal clinically important improvement (MCI). Continuous line represents complete response (ESSDAI = 0) to HCQ treatment. (D) Bar chart showing patient-reported symptoms after HCQ treatment. ESSPRI  $< 5$  is considered as satisfactory control of SjD symptoms. (E) Graphs showing serum gamma globulin levels before and after HCQ treatment. Dotted lines show upper limit of normal range. \* $P < 0.05$ , \*\* $P < 0.01$ , N.S. = not significant (paired Student's t-test for quantitative variables or Chi-squared test for categorical variables).

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analysis, a cohort of 43 SjD patients were selected. Of the 43 patients, 19 had been treated with HCQ. We excluded 5 patients because of missing follow-up data, HCQ use more than 1 year before labial minor salivary gland biopsy, concomitant use of rituximab, or serious complications, such as new diagnosis of cancer (Fig 5A). For comparison, a group of 10 age-matched healthy volunteers were recruited from the same hospital.

RNA-sequencing data showed that 5 SjD patients had a high *LAMP3* level (above mean and 95% confidence interval) and 9 showed a normal *LAMP3* level, comparable with that of the healthy volunteers (Fig 5B). At baseline (before HCQ treatment), there were no significant differences in patient characteristics between the high-*LAMP3* and normal-*LAMP3* groups. But, the proportion of patients with a high lymphocytic focus score ( $\geq 3$ ) was higher in the high-



**Table 1. Baseline profile of SjD patients treated with hydroxychloroquine, stratified by minor salivary gland *LAMP3* mRNA expression.**

<i>LAMP3</i> mRNA expression	High ( <i>n</i> = 5)	Normal ( <i>n</i> = 9)	<i>P</i> value
Age, years	47 ± 13	49 ± 12	0.81
Female	5 (100%)	9 (100%)	-
Anti-SSA/SSB antibody positivity	5 (100%)	8 (89%)	0.44
Focus score ≥ 3	4 (80%)	4 (44%)	0.20
Salivary flow rate ≤ 0.1 mL/min	1 (20%)	5 (56%)	0.20
Schirmer test ≤ 5mm/5min	3 (60%)	6 (67%)	0.80
Extraglandular manifestations	4 (80%)	6 (67%)	0.60
Fever/weight loss	0	1	
Arthritis/arthritis	3	4	
Lymphadenopathy	2	0	
Purpura	0	2	
Interstitial lung disease	0	1	
ESSDAI	5.0 ± 2.9	5.9 ± 3.4	0.66
Gamma globulin, g/dL	1.98 ± 0.48	1.99 ± 0.50	0.65
Concomitant treatment	4 (80%)	7 (78%)	0.92
Corticosteroid	2	6	
Methotrexate	3	4	

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*LAMP3* group (80% vs. 44%,  $P = 0.20$ ), while the proportion of patients with a decreased salivary flow rate ( $\leq 0.1$  mL/min) was higher in the normal-*LAMP3* group (20% vs. 56%,  $P = 0.20$ ). Most of the patients were concomitantly treated with low-dose corticosteroids (prednisolone-equivalent:  $< 10$  mg/day) and/or methotrexate (Table 1).

The median duration of HCQ treatment was 2 years in the high-*LAMP3* group and 4 years in the normal-*LAMP3* group. The European League against Rheumatism Sjögren's syndrome disease activity index (ESSDAI) was significantly decreased after HCQ treatment in both the high-*LAMP3* group ( $5.0 \pm 2.9$  to  $1.4 \pm 2.2$ ,  $P < 0.05$ ) and normal-*LAMP3* group ( $5.9 \pm 3.4$  to  $2.2 \pm 3.2$ ,  $P < 0.01$ ). However, of the 8 patients with moderate disease activity (ESSDAI: 5–13 [20]) at baseline, 2/2 patients (100%) in the high-*LAMP3* group and 4/6 patients (67%) in the normal-*LAMP3* group showed minimal clinically important improvement (i.e., change in  $\Delta$ ESSDAI  $\geq 3$  [21]). Furthermore, 3/5 patients (60%) patients in the high-*LAMP3* group achieved an ESSDAI of 0 after HCQ treatment, while the same was true for 3/9 patients (33%) in the normal-*LAMP3* group (Fig 5C). After HCQ treatment, 4/5 patients (80%) in the high-*LAMP3* group and 5/9 patients (55%) in the normal-*LAMP3* group reported satisfactory control of SjD symptoms such as dryness, pain, and fatigue (i.e., the European League against Rheumatism Sjögren's syndrome patient-reported index (ESSPRI)  $< 5$  [21]) (Fig 5D). Analysis of clinical laboratory data showed that HCQ treatment significantly decreased serum gamma globulin levels in the high-*LAMP3* group ( $1.98 \pm 0.48$  to  $1.35 \pm 0.28$ ,  $P < 0.05$ ) but not in the normal-*LAMP3* group ( $1.99 \pm 0.50$  to  $1.67 \pm 0.52$ ,  $P = 0.07$ ). Hypergammaglobulinemia (i.e., serum gamma globulin level  $> 1.7$  g/dL) was improved in 3/3 patients (100%) in the high-*LAMP3* group and 2/5 (40%) in the normal-*LAMP3* group after HCQ treatment (Fig 5E). Severe adverse events related to HCQ use were not reported during the observation period.

Taken together, these results suggested that HCQ treatment can decrease the severity of SjD symptoms associated with high salivary gland *LAMP3* expression in agreement with those of our *in vitro* and *in vivo* experiments.



## Discussion

HCQ has been used for many years to treat various autoimmune diseases, especially systemic lupus erythematosus and rheumatoid arthritis [22]. In this context, HCQ has been considered as an immunomodulator that regulates immune cells by impairing endolysosomal TLR pathways [23, 24]. Ours is the first study that focused on the effect of HCQ on salivary gland epithelial cells specifically in autoimmune sialadenitis. In this study, we demonstrated that HCQ decreased apoptotic cell death induced by *LAMP3* overexpression in salivary gland epithelial cells. In our mouse experiment, administration of HCQ ameliorated the SjD-like phenotype induced by *LAMP3* overexpression. Furthermore, in our cohort of SjD patients, HCQ treatment showed a larger decrease in the ESSDAI, ESSPRI, and serum gamma globulin levels in the patients with high *LAMP3* expression compared to those with normal *LAMP3* expression.

Our results support the need for better molecular characterization of SjD to understand the etiology of the disease and to better personalize treatment options for patients. Recently, various methods for clustering or subgrouping SjD patients have been reported [16, 17]; however, it is still unknown which method is appropriate and meaningful in real-world clinical practice. Compared to existing methods, our subgrouping based on salivary gland *LAMP3* mRNA expression is practical because this level can be easily evaluated by real-time reverse transcription PCR analysis of labial minor salivary gland biopsies, which are commonly performed during diagnostic evaluation for SjD. The determination of *LAMP3* protein expression by immunostaining of salivary gland biopsies may provide more direct evidence, although the staining and quantification methods need to be standardized.

HCQ is known as a toxic agent that inhibits the physiological lysosomal trafficking and degradation system and induces apoptotic cell death in a dose-dependent manner [25, 26]. Although the safe dosage and administration of this old drug have been established in a long clinical history, rare but serious risks have been associated with HCQ use, including cardiac and retinal toxicity [27]. Interestingly, our results indicated that HCQ paradoxically prevented apoptosis in *LAMP3*-overexpressing epithelial cells. Presumably, HCQ balances the lysosomal dysfunction in *LAMP3*-overexpressing cells, while impairing the lysosomal system in normal cells, making it a good candidate for a personalized strategy in SjD patients with high *LAMP3* expression.

Although both the lacrimal and salivary glands are targeted in SjD, the effect of HCQ on lacrimal glands was not investigated in this study. A recent study showed that *LAMP3* expression was upregulated in conjunctiva of SjD patients, and the expression levels were significantly correlated with the severity of dry eye symptoms [28], suggesting that *LAMP3* overexpression is associated with the development of dacryoadenitis as well as sialadenitis. Future studies need to clarify if HCQ can ameliorate *LAMP3*-associated dacryoadenitis.

Limitations of this study are limited statistical analysis due to the small sample size of our patient cohort and the potential for bias in selection and reporting because of the retrospective design. We acknowledge the absence of statistically significant differences in ESSDAI and ESSPRI changes by HCQ treatment between the high-*LAMP3* and normal-*LAMP3* group. The study also lacked the evaluation of salivary flow rate in patients to validate the finding that HCQ treatment restored the salivary flow rate in mice. In addition, we still have no direct evidence on association between *LAMP3* expression and salivary gland hypofunction in SjD patients beyond the mouse data. Previous studies suggest SjD patient's salivary flow rate is a response to various conditions, including lymphocytic infiltration, atrophy and fibrosis of the glands [29, 30]. Further research in a larger set of SjD patients is required to statistically verify our findings with corrections for confounding factors, such as baseline disease activity and concomitant medications.

In conclusion, we have shown that HCQ can ameliorate *LAMP3*-associated salivary gland hypofunction and our pilot analysis on the patients' data suggested that high *LAMP3* expression could be used as a biomarker to stratify SjD patients who respond adequately to HCQ treatment. Determining the salivary gland *LAMP3* level prior to initiating HCQ treatment may therefore provide a novel strategy for guiding personalized medicine for SjD.

## Materials and methods

### Human subjects

We included the consecutive patients who visited the Clinical Hospital of the Medical School of Ribeirão Preto of the University of São Paulo, underwent labial minor salivary gland biopsies between 2013 and 2016, and met the 2002 American-European Consensus Group criteria [31] and age-matched healthy volunteers. Labial minor salivary gland biopsies were surgically excised from all subjects for bulk RNA sequencing and histological evaluation to determine lymphocytic focus score. RNA sequencing libraries were prepared as described previously [4], and the raw data files were deposited in the National Center of Biotechnology Information Gene Expression Omnibus database (GSE154926). Clinical symptom data were collected using standardized questionnaires and physical examinations at each visit. Data regarding laboratory measures, such as serum anti-Ro/SSA antibody, anti-La/SSB antibody and gamma globulin levels, were obtained from clinically ordered tests.

All clinical investigations were conducted in accordance with the Declaration of Helsinki principles and approved by the Brazilian Committee of Ethics in Research (37688914.2.0000.5440). All subjects provided written informed consent prior to the initiation of any study procedure.

### Animals

*LAMP3*-overexpressing mice were established as described previously [4]. Briefly, AAV2-*LAMP3* were delivered to both submandibular glands ( $10^{11}$  particles/mouse in 100  $\mu$ L) of female, 2-month-old C57BL/6 mice (Charles River Laboratories, USA) by retrograde ductal instillation through a thin cannula. After 4 months, mice were randomly assigned to weekly intraperitoneal HCQ injections (Plaquenil® tablet (Sanofi, USA) in a vehicle of phosphate buffered saline (PBS) at 60 mg/kg or placebo (PBS only) for 3 months, based on a previous report [32]. Pilocarpine-stimulated salivary flow rate in 20 minutes was determined immediately prior to initiation of HCQ treatment and after 3 months.

At the end of the study, mice were sacrificed and blood and whole submandibular glands were collected. Serum was separated by centrifugation and then stored at  $-80^{\circ}\text{C}$ . Presence of serum anti-Ro/SSA and anti-La/SSB antibodies were tested using the Mouse Anti-SSA/Ro60 Ig's (total) ELISA Kit (#5710) and Mouse Anti-SSB Ig's (total (A+G+M) ELISA Kit (#5810), respectively, according to the manufacturer's instructions (both purchased from Alpha Diagnostic International, USA). Lymphocytic infiltration in both the submandibular glands were investigated using hematoxylin & eosin staining. The outcomes were evaluated by unblinded researchers.

All procedures involving live animals were approved based on institutional guidelines and standard operating procedures following the NIH Guide for the Care and Use of Laboratory Animals (approval number: 18–863).

### Cells

A253 cells (ATCC, USA)—a human salivary gland cell line—were cultured in McCoy's 5A Medium (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum. Stably

LAMP3-overexpressing and control A253 cells were established as described previously [3]. All cells were incubated at 37°C with humidity and 5% CO<sub>2</sub>.

### Quantitative real-time reverse transcription PCR

Total RNA was extracted from A253 cells using the RNeasy Mini Kit (QIAGEN, USA), treated with RNase-Free DNase Set (QIAGEN), and reverse transcribed into cDNA using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). To calculate transcript expression levels, TaqMan gene expression assays for *CTSB* (Hs00947439\_m1) and *ACTB* (Hs01060665\_g1) were used (Thermo Fisher Scientific). Gene expression relative to *ACTB* was calculated using the  $\Delta\Delta C_t$  method.

PCR cycles were performed using the Quantstudio3 Real-Time PCR System (Life Technologies, USA) with the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, 50 cycles of 15 seconds at 95°C, and 1 minute at 60°C.

### Western blotting

A253 cells were lysed in RIPA Lysis and Extraction Buffer with protease and phosphatase inhibitors (all from Thermo Fisher Scientific) and cleared by centrifugation at 17,000 g at 4°C for 25 minutes. Supernatants were heated at 97°C in NuPAGE LDS Sample Buffer for 10 minutes, resolved by SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride membranes (all from Thermo Fisher Scientific). Membranes were blocked with 2% non-fat dried milk at 25°C for 1 hour, and then incubated at 4°C overnight with one of the following primary antibodies: anti-cathepsin B (#AF953, R&D system, USA), anti-LAMP1 (#21997-1-AP, Proteintech, USA) or anti- $\alpha$ -tubulin (#T6199, Sigma-Aldrich, USA). After washing three times, membranes were incubated with rabbit or mouse IgG horseradish peroxidase-linked whole antibody (Sigma-Aldrich) at 25°C for 1 hour. Signals were visualized using Super Signal West Pico Chemiluminescent Substrate or Super Signal West Pico PLUS Chemiluminescent Substrate (both from Thermo Fisher Scientific).

### Galectin-3 puncta assay

A253 cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized using 0.1% Triton-X-100 (Sigma-Aldrich) for 10 minutes and blocked with 2% bovine serum albumin (BSA) for 30 minutes, all at room temperature. Then, cells were incubated with a mixture of 10  $\mu$ g/mL mouse anti-galectin-3 antibody (#ab2785, Abcam, USA) in 2% BSA at 4°C overnight. The next day, cells were incubated with a mixture of 10  $\mu$ g/mL Alexa Fluor-647 anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., USA) in 2% BSA at room temperature for 1 hour.

Formalin-fixed paraffin embedded sections of murine submandibular glands were deparaffinized, rehydrated and subjected to citric acid microwave antigen retrieval. Slides were blocked with 2% BSA (Sigma-Aldrich) and permeabilized by 0.1% Triton-X-100 (Sigma-Aldrich) for 30 minutes at 25°C. Slides were incubated with mouse anti-galectin-3 (#ab2785, Abcam) and rabbit anti-LAMP1 (#21997-1-AP, Proteintech) antibodies at 4°C overnight, followed by incubation with Alexa Fluor-647 anti-mouse IgG and Alexa Fluor-488 anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 1 hour.

Sections were subsequently counterstained with DAPI (#ab104139, Abcam). Images were acquired using a fluorescent microscope (Nikon, Japan) and analyzed using ImageJ software (public domain, source: National Institutes of Health, USA).

## Apoptosis assay

To detect apoptosis *in vitro*, A253 cells were stained using the APC Annexin V Apoptosis Detection Kit with 7-AAD (#640930, BioLegend, USA), and apoptotic cells were detected using the BD Accuri Flow Cytometer (BD Biosciences, USA).

Apoptosis *in vivo* was visualized using a TUNEL assay (#ab66110, Abcam) on formalin-fixed, paraffin-embedded murine submandibular gland sections, according to the manufacturer's instructions. After counterstaining with DAPI mounting medium (Abcam), images were acquired with a fluorescent microscope (Nikon) and analyzed using ImageJ software.

## Statistical analysis

Data are presented as mean  $\pm$  standard deviation (SD). Quantitative variables were compared using a two-tailed paired or unpaired Student's *t*-test. When appropriate, Dunnett's correction was used to correct for multiple hypothesis testing. Categorical variables were compared using the Chi-squared test. *P* values  $< 0.05$  were considered statistically significant. All analyses were performed using GraphPad Prism 8.0 software.

## Supporting information

**S1 File.**  
(PDF)

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