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ORIGINAL ARTICLE

Immunolocalization of AQP5 in resting and stimulated normal labial glands and in Sjögren's syndrome

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OBJECTIVE: In our current work, *in vivo* examination of AQP5 distribution in labial salivary glands following stimulation of secretion has been carried out in normal individuals and in patients with Sjögren's syndrome.
SUBJECTS AND METHODS: For this study, we selected five patients with primary Sjögren's syndrome (mean age 62.4 ± 10.6 s.d. years) diagnosed in accordance with the European Cooperative Community classification criteria. There were five patients (mean age 27 ± 2.5 s.d. years) in the control group. The subcellular distribution of AQP5 in human labial gland biopsies was determined with light and immunoelectron microscopy before and 30 min after administration of oral pilocarpine.
RESULTS: In unstimulated control and Sjögren's labial glands, AQP5 is about 90% localized in the apical plasma membrane, with only rarely associated gold particles with intracellular membrane structures. We have found no evidence of pilocarpine-induced changes in localization of AQP5 in either healthy individuals or patients with Sjögren's syndrome.
CONCLUSIONS: Our studies indicate that neither Sjögren's syndrome itself, nor muscarinic cholinergic stimulation *in vivo* caused any significant changes in the distribution of AQP5 in the labial salivary gland cells.

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Keywords: aquaporin-5; oral pilocarpine; salivary secretion; Sjögren's syndrome

Introduction

Sjögren's syndrome (SS) is an autoimmune disease characterized by progressive infiltration of exocrine glands by mononuclear cells, mainly resulting in decreased secretions of salivary glands (xerostomia) and lacrimal glands (xerophthalmia) (Carpenter *et al*, 2000; Jonsson *et al*, 2001; Fox, 2005). In human minor and major salivary glands, AQP5 is abundantly localized to the apical plasma membrane domains of acinar cells and not detected in the duct cells (Gresz *et al*, 2001).

In the symptomatic treatment for xerostomia, oral pilocarpine has been used to increase salivary secretion (Fox *et al*, 1991, 2001; Rhodus, 1997; Vivino *et al*, 1999; Nyarady *et al*, 2006). At 30, 60, and 90 min after drug intake, salivary flow rate significantly increased with a peak salivary flow occurring approximately 60 min after drug intake. The main adverse effect was sweating. Five-milligram pilocarpine tablets/day improved symptoms significantly.

Examination of AQP5 distribution in intracellular structures and apical plasma membrane of the human labial salivary gland cells following stimulation of secretion have not previously been performed *in vivo*. Discrepancies in the literature regarding AQP5-localization in human salivary tissue were challenging to us (Ishikawa *et al*, 1998, 1999, 2000, 2002, 2004, 2005; Tada *et al*, 1999; Ishikawa and Ishida, 2000; Beroukas *et al*, 2001; Steinfeld *et al*, 2001, 2002; Steinfeld and Delporte, 2002; Waterman *et al*, 2002, 2003; Matsuzaki *et al*, 2003; Gresz *et al*, 2004; Li *et al*, 2004, 2006; Delporte and Steinfeld, 2006; Xiao *et al*, 2011). The aim of this study was to determine the effect of oral pilocarpine on the localization of AQP5 in human labial minor salivary glands of normal and patients with SS using light microscopy and immunogold electron microscopy with different types of anti-human AQP5 antibodies. To show the earliest events in salivary secretion, a stimulation time of 30 min was chosen. According to the data in the literature, in our current work, we chose a time point where the concentration of AQP5 is the highest at the place of its basic function, that is, at the place of water secretion, namely at the apical membrane. This was 30 min after stimulation with pilocarpine. At 90 min, a decrease in secretion has already been observed (Vivino *et al*, 1999).

Materials and methods

Diagnosis of patients with SS

In accordance with the World Medical Association Declaration of Helsinki (version 2002), the study was performed and all participants signed informed consent forms. The procedures were approved by the Semmelweis University Regional Committee of Science and Research Ethics (TU-KEB 153/2012). The trial was accomplished by the Good Clinical Practice norms. Symptoms were assessed by questionnaires. Every conditions where the use of pilocarpine would be contraindicated were excluded. Five patients with primary SS (mean age 62.4 ± 10.6 s.d. years, range 53–74 years) were selected and diagnosed according to the European Cooperative Community classification criteria (Vitali et al, 2002). Keratoconjunctivitis sicca and xerostomia were evaluated. After measuring whole-mouth salivary flow rates, labial salivary gland biopsies were taken. Less than 0.4 ml/min of unstimulated salivary flow rate was considered as salivary hypofunction and less or equal than 0.1 ml/min as xerostomia (Skopouli et al, 1989). The diagnosis of SS was confirmed by positive test results (Table 1).

The control five patients (mean age 27 ± 2.5 s.d. years, range 24–31 years) were having treatment for mucocele (mucous cyst) inside the lower lip and had no evidence of SS.

Labial gland biopsy and salivary stimulation with oral pilocarpine

Labial gland biopsies were made as in Daniels for routine diagnosis of Sjögren's syndrome (Daniels, 1984, 1986). The biopsy samples were diagnosed by an expert in primary SS pathology in a blinded manner. A positive diagnosis of SS was made with focus scores >1 (Vitali et al, 2002). After unstimulated labial glands had been removed from both sets of patients, salivary secretion was induced by oral pilocarpine (5 mg tablet, Salagen, pilocarpinhydrochlorid, Novartis Pharma GmbH, 90327 Nürnberg, OGYI-T-5479) (Wynn, 1996; Salagen tablets, 1997) for 30 min, and then additional labial glands were excised. Finally, the wound was subsequently closed with single sutures (nonabsorbable polyamide monofilament 6.0, Braun AG, Germany). After uneventful healing, the stitches were removed at 4–7 days postoperative.

Table 1 Diagnostics of the patients with Sjögren's syndrome

	Gender	Age	Sialometry (unstimulated whole-mouth salivary flow rate ml/min)	Schirmer tear test (<7 mm/ 5 min)	Histology (focus score >1)	Anti-SS-A or anti-SS-B antibodies (at a titer of 1:160 or greater)
S1	F	74	0.01	0.5	3	+
S2	F	53	0.05	0.1	2	+
S3	F	55	0.26	3.25	1–2	+
S4	F	74	0.01	1.75	7	+
S5	F	56	0.05	2.75	1–2	+

SS, Sjögren's syndrome.

Antibodies

Previously characterized, affinity-purified polyclonal antibodies to human AQP5 were used in our immunohistochemical studies: One was provided by Professor Peter Agre (Johns Hopkins University, Baltimore, USA), and the other one was developed against the 20 amino acids of the C-terminal of human AQP5 (that is different from mouse and rat C-terminals).

Immunohistochemical procedure for light microscopy (Gresz et al, 2004)

Briefly biopsy samples of individual labial glands were transferred into cold 4% paraformaldehyde (in 0.1 M PBS, pH 7.4), stored for 24 h or more, dehydrated in ethanol and xylene, and embedded in paraffin. Two-micrometer-thick sections were dewaxed and rehydrated. Immunoperoxidase labeling was performed. After overnight incubation with primary antibody at 4°C, sections were rinsed and labeling was visualized with goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase. After washing in PBS, sections were counterstained in Mayers hematoxylin for 1 min, rinsed, dehydrated, and mounted. Photomicrographs were taken with an Eclipse E-600 (Nikon, Melville, NY, USA) TMS phase-contrast microscope.

Immunohistochemical procedure for immunoelectron microscopy (Gresz et al, 2004)

For immunoelectron microscopy, the samples were prepared and treated as in our previous studies described in details before (Nielsen et al, 1995, 1997; Yasui et al, 1999; Kwon et al, 2000; Gresz et al, 2004). Briefly, after freeze-substitution, equilibration over 3 days in 0.5% uranyl acetate–methanol, and rinsing in methanol, the samples were infiltrated with Lowicryl HM20 followed by UV polymerization. Ultrathin Lowicryl HM20 sections were pretreated, rinsed, and labeled with anti AQP-5 antibody diluted 1:200, and incubated overnight at 4°C. Sections were then rinsed and incubated at room temperature for an hour with goat anti-rabbit IgG conjugated to gold particles of 10 nm diameter. After staining with uranyl acetate and lead citrate, sections were examined in Philips CM100 electron microscope (Eindhoven, the Netherlands). Quantitation of the electron micrographs was performed. Labial glands from 5 to 5 patients from each group were investigated blindly. The number of gold particles associated with the apical membrane or with intracellular structures was determined per cell.

Results

Normal subjects

Immunoperoxidase labeling with human AQP5 antibodies was performed on paraffin sections of human labial salivary glands. They did not cross-react with rat salivary gland tissue (data not shown) and they gave the same labeling pattern (Figure 1). AQP5 labeling was observed apically in the acinar cells including the membranes of the secretory canaliculi in a punctate appearance (Figure 1a). No AQP5 labeling was present in the ducts of the labial glands (e.g., D in Figure 1a).

The subcellular localization of AQP5 in human labial salivary glands was further investigated by immunoelectron
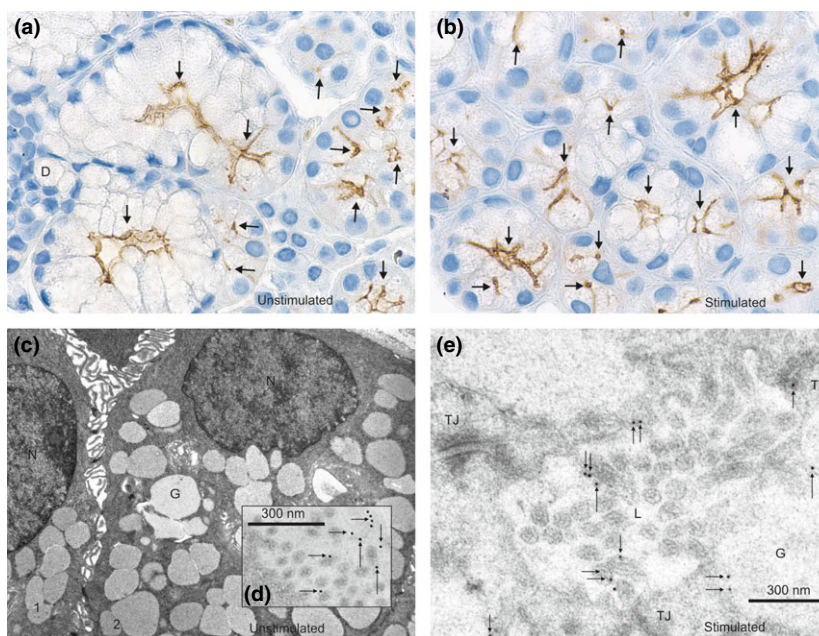
Figure 1 AQP5 distribution in human labial salivary glands of normal subjects by immunohistochemistry. Immunoperoxidase light microscopical and immunogold electron microscopical localization of AQP5 under unstimulated (**a**, **c**, **d**) and stimulated (**b**, **e**) conditions of salivary secretion. (**a**) In unstimulated normal human labial glands, AQP5 labeling is associated exclusively with the apical membrane of acinar cells (arrows), and it is absent in the ducts (D). (**c**) Low magnification shows two adjacent cells of a human labial gland acinus. (**d**) Gold particles are associated with microvilli of the apical membrane and of intercellular secretory canaliculi (arrows) in the acini. (**b**) Immunolocalization of AQP5 30 min after administration of oral pilocarpine. The localization pattern is similar to that in normal tissues (panel a). (**e**) Immunogold electron microscopic localization of AQP5 after 30-min oral pilocarpine administration. Compared with control tissues (Panels **c**, **d**), the subcellular localization of AQP5 has not changed. Immunogold labeling can be observed on microvilli of the acinar lumen (arrows). Cells labeled with 1 and 2 are two adjacent cells; G, secretory granule; L, lumen; N, nucleus; TJ, tight junction. Magnifications: ×200 (**a**); ×260 (**b**); ×8.200 (**c**)

microscopy. A quantitative assessment of immunogold labeling on sections from five different patients showed that gold particles were few in number in intracellular structures [$9.9 \pm 0.8\%$ (mean $\pm$ s.e.m.)], they were mainly associated with the apical plasma membranes (Figure 1c, d), especially with the microvilli [$90.1 \pm 0.8\%$ (mean $\pm$ s.e.m.)].

Salivary secretion was stimulated by administration of a pilocarpine tablet (5 mg) for 30 min. Light microscope immunohistochemistry (Figure 1b) and immunoelectron microscopy (Figure 1e) revealed that there were no significant changes in the distribution of AQP5. On average, the level of intracellular labeling remained almost the same ($9.6 \pm 0.8\%$), and the other gold particles ($90.4 \pm 0.8\%$) were associated with the apical plasma membrane in a punctate appearance that can be a result of clustering of AQP5, around the area of the microvilli. Thus, in control human labial salivary glands, no indication of changes can be observed in distribution of AQP5 after stimulation with oral pilocarpine.

Patients with SS
Distribution and localization of AQP5 in human labial minor salivary glands were the same in patients with SS as in the controls. We have used the same human AQP5 antibodies as on normal subjects and they gave the same labeling pattern also in patients with SS (Figure 2). AQP5 was definitely localized in the apical plasma membranes of acinar cells (Figure 2a). Thus, in patients with Sjögren's syndrome, almost the same subcellular localization pattern

of AQP5 was found: $91.8 \pm 1.4\%$ (mean $\pm$ s.e.m.) of the gold particles were present in the apical plasma membrane, and only $8.2 \pm 1.4\%$ (mean $\pm$ s.e.m.) at the intracellular structures (Figure 2c).

The muscarinic stimulation for 30 min significantly increased saliva production, as it was nearly undetectable without stimulation (data not shown). Immunohistochemistry demonstrated that even after pilocarpine treatment, the AQP5 labeling remained localized to the apical plasma membrane of the acinar cells (Figure 2b). It was confirmed by immunoelectron microscopy: $91.9 \pm 0.5\%$ (mean $\pm$ s.e.m.) of gold particles were present in the apical plasma membrane, including the microvilli, and $8.1 \pm 0.5\%$ (mean $\pm$ s.e.m.) were present in the intracellular compartments (Figure 2d).

Discussion

In this study, we have tested the effect of oral pilocarpine on AQP5 distribution in human labial salivary gland biopsies of control and patients with Sjögren's syndrome before dosing and 30 min after drug exposure with light microscopy and immunogold electron microscopy.

Corresponding with our previous results on human salivary glands (Gresz *et al*, 2001), AQP5 labeling was clearly apical at the acinar membrane domains, and no AQP5 labeling was present in the ducts of the labial glands (e.g., D in Figure 1a). In line with the results of Beroukas *et al*, (2001) (Waterman *et al*, 2002, 2003), AQP5 was localized apically in the acinar cells of patients
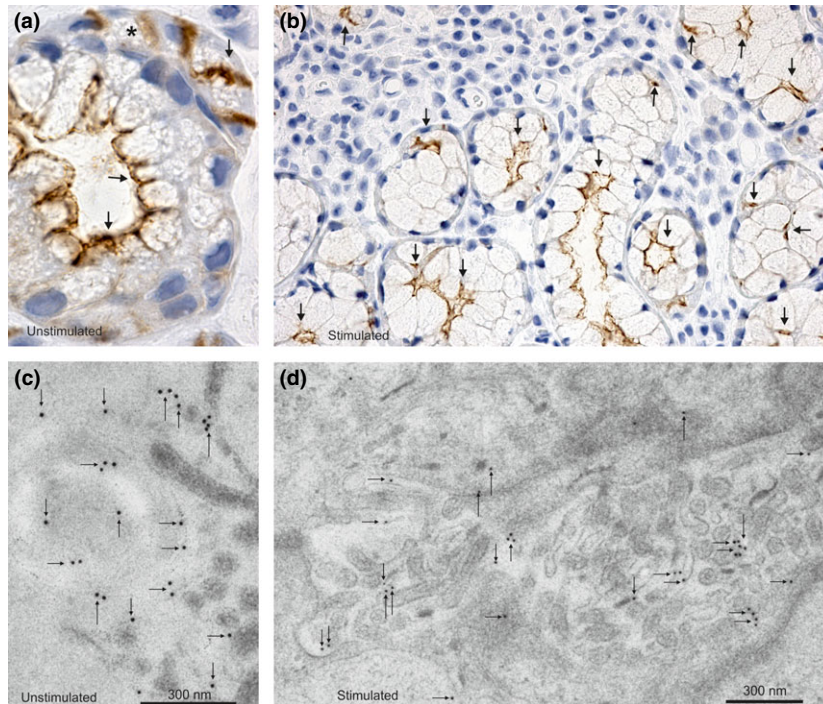
Figure 2 AQP5 distribution in human labial salivary glands of Sjögren's syndrome (SS) patients by immunohistochemistry. Immunoperoxidase light microscopical and immunogold electron microscopical localization of AQP5 under unstimulated (**a**, **c**) and stimulated (**b**, **d**) conditions of salivary secretion. (**a**) Large magnification of a mucous acinus with a serous demilune (marked with *) of a SS patient under resting conditions. AQP5 is associated with the apical membrane domains (arrows) and is similar to control patients (Figure 1). (**c**) Electron microscopy shows extensive immunogold labeling in the apical plasma membrane, especially on microvilli of unstimulated Sjögren's labial glands. (**b**, **d**) Immunolocalization of AQP5 30 min after oral pilocarpine administration. The subcellular localization of AQP5 has not changed as it can be detected by light (**b**) or immunogold electron microscopy (**d**). AQP5 is localized basically in the apical membrane, especially on the microvilli of the acinar lumen (arrows). Magnifications: ×400 (**a**); ×150 (**b**)

with Sjögren's syndrome. However, our finding for the distribution and density of AQP5 in human labial minor salivary glands was contrast to the results in other papers (Steinfeld *et al*, 2001; Steinfeld *et al*, 2002; Steinfeld and Delporte, 2002; Xiao *et al*, 2011) They claim that AQP5 labeling was present also at the basolateral membrane of acinar cells in patients with SS (Steinfeld *et al*, 2001, 2002), not to say that a more recent study reported about surprising localizations of AQP5 in human labial glands (Xiao *et al*, 2011), where an abundance of staining at nearly every structure of human labial glands including different types of ducts was shown in controls. However, the possibility of non-specific immunohistochemical bindings arises, for their immunohistochemical results particularly claiming that there is a change in distribution of AQP5 in the labial glands of patients with SS.

Reacting on the contradictions, Beroukas *et al*, and Waterman *et al*, published a paper (Waterman *et al*, 2002) and then a longer letter (Waterman *et al*, 2003) and confirmed our results (Gresz *et al*, 2001). They concluded that basolateral staining of AQP5 in salivary acini is non-specific background staining, and they were unable to demonstrate any altered AQP5 expression in Sjögren's salivary glands.

The contradictory data may have been caused by the use of different AQP5 antibodies, because anti-rat AQP5 antibody does not cross-react with the human AQP5 protein (Steinfeld *et al*, 2002; Delporte and Steinfeld, 2006). We have tested different human antibodies, and our results

have confirmed an exclusive apical labeling of AQP5 in Sjögren's labial minor salivary glands.

Another controversial issue is the possibility that the distribution of AQP5 is altered by trafficking to the apical membrane. *In vitro* experiments using gland slices and cultured cells supported this idea (Ishikawa *et al*, 1998, 1999, 2000, 2002, 2004, 2005; Tada *et al*, 1999; Ishikawa and Ishida, 2000; Li *et al*, 2004, 2006). We tested this hypothesis *in vivo* in rat salivary glands, following stimulation or inhibition of salivary secretion in a previous study: we have not found evidence of AQP5 translocation, AQP5 was mainly localized in the apical membrane structures of the acini and of the intercalated ducts of the rat submandibular gland, and there was no difference between controls and treated subjects (Gresz *et al*, 2004). The only difference was the clustering of AQP5 at the apical membrane, especially at the microvilli in stimulated salivary secretion. It could be explained by the intensive exocytosis, where clustering seems to be an important step for apical sorting of proteins (Hannan *et al*, 1993; Weisz and Rodriguez-Boulan, 2009). Matsuzaki *et al*, (2003, 2006, 2012) have also performed immunoelectron microscopy in rodent salivary glands to clarify whether AQP5 is localized intracellularly; however, they were not able to detect apparent labeling in the cytoplasm, so their results also suggested that AQP5 translocation is unlikely to occur.

Similar to our findings in rat submandibular gland (Gresz *et al*, 2004), following pilocarpine administration,

the subcellular distribution of AQP5 did not change in human labial minor salivary glands, either. Our immuno-electron microscopic findings supported our light micro-scopical results showing that the density of gold particles remained very sparse in intracellular structures both in normal and in Sjögren's groups. There was no evidence of AQP5 trafficking after 30-min simulation of salivary secretion with oral pilocarpine. This suggests that during stimulated secretion, the accelerated turnover of the apical membrane happens without movement of AQP5; thus, an anchoring mechanism is responsible for retaining AQP5 in the apical membrane. This could cause clustering of AQP5 labeling after stimulation with pilocarpine, especially at the microvilli.

In a study, Nielsen *et al,* suggested that AQP5 is regulated differently in the acinar and interlobular ducts (Ishikawa *et al*, 2005). They proposed that AQP5 translocation to the apical membrane and dissociation from lipid rafts takes place at the apical plasma membrane of the interlobular ducts of the rat parotid glands as it arrived there. Lipid rafts are small, dynamic membrane microdomains that compartmentalize cellular processes, influencing membrane protein and receptor trafficking.

The different authors focus mainly on the distribution and translocation of AQP5 in unstimulated and stimulated salivary glands and in Sjögren's syndrome and diabetes, as well. In the past 10 years, there have been many publications about the role of AQP5 in salivary secretion, relating to its distribution and translocation. We consider the contradictory data originated from the diversity of the experiments, which were based mainly on rats and mice, and relatively few of them were carried out on humans. The contradictions are multiplied by the species differences, by the type of the salivary glands investigated, by the different cell types within the glands, and by the differences in unstimulated and stimulated conditions. Not surprisingly Peter Agre, the Nobel Prize winner and father of AQP-research emphasized 'Man Is not a rodent: . . .' in a title of a paper (King and Agre, 2001).

It is generally accepted that AQP5 is essential for the movement of water into secretion (Ma *et al*, 1999). We know almost nothing about the mechanism of secretion of AQP5 protein itself into the saliva. The correlation between mRNA and protein levels of AQP5 is not necessarily parallel. In the parotid glands of diabetic rats, an increase in AQP5 mRNA and a decrease in AQP5 protein levels were measured (Wang *et al*, 2011). The cellular distribution of AQP5 has been investigated by many workers, but there are little data about expression, degradation, and trafficking of AQP5 or any connection between them (Hoffert *et al*, 2000; Azlina *et al*, 2010). One wonders whether the channels are always open and working. As there are normal amounts in SS glands, it seems that the rate of secretion is not dependent on its presence. (Do not forget that most of all salivary secretion is dependent on nerve impulses!). A recent study about AQP5 trafficking in Sjögren's syndrome clearly indicated that autoantibodies against M3 muscarinic receptors inhibit AQP5 translocation from intracellular compartments to the apical membrane and may lead to decreased salivary secretion (Lee *et al*, 2013).

In summary, our studies indicate that AQP5 labeling is basically associated with the microvilli and the intercellular secretory canaliculi of the acini in human labial minor salivary glands, including resting conditions. Neither SS itself, nor muscarinic cholinergic stimulation resulted in any significant changes in distribution of AQP5 after 30-min stimulation of salivary secretion. The present AQP5 research leads us to believe that in a few seconds after stimulation, the possible changes in AQP5-distribution is not a result of higher level protein synthesis or translocation, but a result of a cytoskeletal reorganization and retention of AQP5 in the apical membrane of the acini. The mechanism is so far undefined, but most likely it is a critical step in the huge acinar water transport during stimulated salivary secretion. The poor flow of saliva in SS is not caused by reduced presence of AQP5.

Author contributions

Veronika Gresz (Department of Oral Diagnostics, Semmelweis University) is the corresponding author, and she did the immuno-histochemical experiments, light microscopy, and electron microscopy. Istvan Gera and Attila Horvath from the Department of Periodontology, Semmelweis University, presented the patients, and they were responsible for the diagnosis of Sjögren's syndrome, they have made sialometry, Schirmer test, and labial gland biopsy. Soren Nielsen, the head of The Water and Salt Research Center and Institute of Anatomy, University of Aarhus, was the leader and supervisor of the immunohistochemical experiments, light microscopy and electron microscopy. Tivadar Zelles (Department of Oral Biology, Semmelweis University) has updated knowledge in the field and in the relevant literature, and he was advising and editing the manuscript.

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