Standardization of a colorimetric technique for determination of enzymatic activity of diamine oxidase (DAO) and its application in patients with clinical diagnosis of histamine intolerance

Camila Beltrán-Ortiz, Teresa Peralta, Verónica Ramos, Magdalena Durán, Carolina Behrens, Daniella Maureira, Maria A. Guzmán, Carla Bastias and Pablo Ferrer*

ABSTRACT

Background: Diamine Oxidase (DAO) has an essential role for degradation of exogenous histamine in the intestine; thus, histamine intolerance (HI) mainly has been correlated to a low concentration and/or activity of this enzyme. The objective of the study was to standardize a colorimetric technique to measure the enzymatic activity (function) of hDAO to then apply it to a series of 22 patients with a clinical diagnosis of HI.

Methods: For the standardization variables such as volume and type of sample, incubation time, wavelength of maximum absorption, types of substrates, and concentration of oxidized ascorbate were evaluated. Then the activity and concentration of DAO was determined in 22 patients diagnosed with HI and 22 healthy subjects.

Results: The mean of serum DAO concentration in the 22 patients was of 9.268 ± 1.124 U/mL. The mean of serum DAO concentration in the 22 controls was of 20.710 ± 2.509 U/mL, being significantly higher (P value 0.0002) the mean of the samples. The mean of serum DAO activity of the patients was of 1.143 ± 0.085 U/L and the controls was 1.533 ± 0.119 U/L, significantly greater than the patients (P value 0.011). In addition, the sensitivity of both techniques was 0.63. In the measuring of DAO concentration the specificity was 0.9, constituting a good diagnostic test, especially to rule out the true negatives. The determination of DAO activity had a specificity of 0.68.

Conclusions: Although we used a small number of patients and controls and the absorbance values were lower than expected, statistically significant differences were found in the levels of concentration and DAO activity between the patients with histamine intolerance and the controls. Therefore, the measuring of DAO concentration and DAO activity is a good diagnostic strategy for
study suspect cases of HI. The simultaneous use of both assays allows to reduce positive and negative false results, for example, patients with normal DAO levels that could present a dysfunction in the activity of this enzyme.

**Keywords:** DAO, Diamine oxidase, Histamine intolerance, Histamine, Activity

**BACKGROUND**

Human diamine oxidase (hDAO) is codified by the AOC1 gene, also called ABP1 gene (≈ 10 kbp), that is located in chromosome 7q35. This homodimeric enzyme of 751 aminoacidic residues has one active site in each subunit; this active site contains a cooper ion and a topaquinone residue formed by the post-translational modification of a tyrosine. In each active site hDAO quickly performs oxidative deamination of the polyamine sperimidine, the diamines cadaverine and putrescine, and the monoamines: Histamine and \( \text{N-MeH} \). hDAO is expressed in various tissues and organs including the brain. However, it is highly expressed in placenta, kidney, and intestinal mucosa. hDAO plays an essential role in the intestine being crucial for degradation of exogenous histamine. Histamine intolerance (HI) is a pathology that occurs when there is a dysregulation between an excessive intake of histamine through food, or a deficit in its degradation by the detoxification system at the intestinal and hepatic level. Thus, HI mainly has been correlated to a low amount (concentration) and/or activity (function) of hDAO.

Currently, in Chile our laboratory is the only one that measures serum hDAO concentration as an analysis to be considered when establishing a diagnosis of HI, being a minimally invasive, inexpensive and easily accessible test. Since patients with normal hDAO levels could present a dysfunction in activity of this enzyme, it is recommended to correlate the concentration and hDAO activity with the clinical diagnosis of HI.

The objective of the study was to standardize a colorimetric technique to measure the enzymatic activity (function) of hDAO to then apply it to a series of 22 patients with a clinical diagnosis of HI. For this, variables such as volume and type of sample, incubation time, wavelength of maximum absorption, types of substrates, and concentration of oxidized ascorbate were evaluated.

**METHODS**

**Patients and controls**

In this study we employed 22 serum samples from patients (cases) older than 18 years, with a clinical history of HI. The patients were classified as HI according to that described by Rosell-Camps with slight modifications. We used 2 or more histamine-mediated symptoms, with at least 1 gastrointestinal symptom, with negative skin tests in which IgE-mediated food allergy was ruled out, by means of a clinical history associated with skin tests and/or specific IgE. As control we used 22 serum samples from healthy subjects, older than 18 years, without a clinical history of HI, without kinship with the subjects under study, without diseases associated with alterations of the immune system such as cancer and autoimmunity, chronic liver damage, severe burns, kidney damage, and non-pregnant women. In Table 1 we show the epidemiological and clinical characteristics of cases and controls. All study participants signed an informed consent.

**Determination of DAO concentration**

The concentration of DAO in serum was determined using the kit DAO ELISA K8500 from Inmunodiagnostik (Germany). Briefly, the
kit contains an anti-DAO antibody and a secondary antibody conjugated with streptavidin peroxidase and uses tetramethylbenzidine (TMB) as a substrate. The absorbance of the yellow chromogen produced was determined at 450 nm. The kit used has an analytical sensitivity indicated as limit of detection (LoD) or limit of quantification (LoQ) of 0.120 U/mL. Moreover, include for interpretation of results a reference range: levels <3U/mL are equivalent to a high probability of HI; levels between 3 and 10 U/mL at a probable HI and levels >10 U/mL at a low probability of HI.

Measurement of the DAO activity

To implement the measurement of the enzymatic activity of DAO, the technique published by Takagi et al (1994)\textsuperscript{8} was standardized using serum or plasma. To achieve the optimal conditions for the measurement of enzymatic activity different volumes of sample, concentrations of substrates, and reagents were used. To find the wavelength of greater absorption, readings were made at different wavelengths. Briefly, this technique is based on the measurement of DAO’s ability to degrade cadaverine, a process in which hydrogen peroxide is generated. Then chromogen DA-67 was used, which is quantitatively oxidized in the presence of hydrogen peroxide and peroxidase, to be transformed into methylene blue (Fig. 1). To eliminate the interference caused by the presence of ascorbic acid in the serum, an interfering agent of the peroxidase, we used the enzyme ascorbate oxidase. Finally the reaction was stopped with sodium diethyl-dithiocarbamate (stop solution), allowing the formation of methylene blue, which remained stable for 2 h, measured the absorbance with a maximum absorbance of 668 nm. Incubations at 37 °C were performed in a thermo regulated bath.

| Gastrointestinal Symptoms | Cases (n:22) | Controls (n:22) |
|---------------------------|-------------|----------------|
| Female (n)                | 20          | 14             |
| Age (years mean)          | 40.7 (21-62) | 35.2 (24-59)   |

| Skin Symptoms             | Cases (n:22) | Controls (n:22) |
|---------------------------|-------------|----------------|
| Flushing (%)              | 68          | 0              |
| Urticaria (%)             | 77          | 0              |
| Angioedema (%)            | 27          | 0              |
| Pruritus (%)              | 86          | 0              |

| Respiratory Symptoms      | Cases (n:22) | Controls (n:22) |
|---------------------------|-------------|----------------|
| Nasal Congestion (%)      | 36          | 0              |
| Airway obstruction (%)    | 23          | 0              |

| Other symptoms            | Cases (n:22) | Controls (n:22) |
|---------------------------|-------------|----------------|
| Headache (%)              | 72          | 0              |

Table 1. Epidemiological and clinical characteristics of patients and control population
Statistical analysis

For the statistical analysis between the averages of the different determinations, the unpaired student t-test was used, considering a confidence interval of 95%. In addition, to analyze the discriminative capacity of the techniques used to determine the amount and activity of DAO, we used the ROC (Receiver Operating Characteristic) curve.

RESULTS

Volume optimal of sample

To implement the determination of DAO activity in serum, the technique published by Takagi and collaborators in 1994 was used as a starting point. For the standardization, the volume of sample needed was first analyzed (Fig. 2). For this test a DAO standard of 25 U/L (Fig. 2A, B and 2C) and serum from a control subject (Fig. 2D and E) were employed. In Fig. 2A, the absorbance obtained was compared when using 100 and 200 µL as sample. Mean ± SD absorbance using 100 µL was of 0.4530 ± 0.0008, and using 200 µL was of 0.8120 ± 0.0016, being significantly higher (P < 0.0001). In Fig. 2B, the absorbance was compared when using 200 and 300 µL. The absorbance obtained using 200 µL was of 1.016 ± 0.0205, and using 300 µL was of 1.395 ± 0.0144, being significantly higher (P < 0.0001). For its part, comparison between 100, 200, and 300 µL of serum of a control subject in D showed that absorbance is not detectable when using 100 µL of serum; absorbance obtained using 200 µL was of 0.0107 ± 0.0079, and using 300 µL was of 0.0377 ± 0.0061, being significantly higher (P = 0.0189). Finally, comparisons between 200 µL and 400 µL of DAO standard of 25 U/L and serum of a control subject are shown in Fig. 2C and E, respectively. The absorbance obtained by using 200 µL of standard was of 0.7160 ± 0.0114 and with 400 µL was of 1.236 ± 0.0347, being significantly higher (P < 0.0001). The absorbance obtained by using 200 µL of serum of a control subject was
of 0.0015 ± 0.0012 and with 400 µL was of 0.0200 ± 0.0012, being significantly higher (P = 0.0001). These results show that the optimum sample quantity is 400 µL, maximum volume allowed by the physical conditions in which the technique was performed. Unlike Fig. 2A,B and D, the absorbance assays plotted in Fig. 2C and E were measured at 666 nm.

**Determination of optimal wavelength**

Although in the article published by Takagaki et al. a fixed wavelength of 668 nm was used to measure the absorbance of methylene blue, we considered that the absorbance values obtained for them were too much low. For this reason we scanned between 666 and 6672 nm to determine if there was another wavelength that gave absorbance values higher than obtained by Tekagi. We tested with various samples and determined that in spite of not having a significant difference, in all the assays the highest absorbance was recorded at 666 nm (data not shown).

**Time for incubation of the samples**

The incubation times for formation of hydrogen peroxide and methylene blue described by Takagaki et al. were also analyzed. However, increasing the incubation time increased the signal of the samples and the target; therefore, there was no real increase in absorbance (data not shown).

**Analysis of substrates for determination of DAO activity**

In addition, in the standardization we compared the results obtained when determining the activity of DAO in serum using the substrates cadaverine, histamine, and putrescine (Fig. 3). In Fig. 3A and B, 30 mM cadaverine, 30 mM Histamine, and 60 mM Histamine were compared using 300 µL of DAO standard 25 U/L or serum control, respectively. With DAO 25 U/L (Fig. 3A) Mean ± SD absorbance of 1.3953 ± 0.0144 was obtained using 30 mM Cadaverine, 0.3987 ± 0.0046 with 30 mM Histamine and 0.2670 ± 0.0168 with
60 mM histamine. Cadaverine demonstrated a significantly higher absorbance than 30 or 60 mM histamine (P < 0.0001). In addition, the absorbance of 30 mM histamine was significantly greater than 60 mM histamine (P = 0.0004).

Using serum of a control subject (3B), an absorbance of 0.0375 ± 0.0075 was obtained using 30 mM cadaverine, 0.0370 ± 0.001 with 30 mM histamine, and when 60 mM histamine was used, no signal was detected. Although 30 mM histamine and 30 mM cadaverine are significantly better than 60 mM histamine (P = 0.0007 and P = 0.0377 respectively), there is no significant difference between them.

In Fig. 3C-E, 30 mM concentration of cadaverine, histamine, and putrescine were used. In C, 400 μL of DAO standard 25 U/L were used; cadaverine 1.519 ± 0.071 and putrescine 1.291 ± 0.116 showed a significantly higher absorbance than histamine 0.3855 ± 0.0015 (P value 0.0039 and 0.0161, respectively). In Fig. 3D and E, 400 μL of serum of different control subjects were used; in both there was no significant difference between the different substrates. Due to these results, we maintained cadaverine as the substrate.

**Determination of optimal concentration of ASOD**

Given that previous results (Fig. 2) established a 4-fold increase in the volume of sample used for the determination of DAO activity in serum, we analyzed the increase the volume of 30 mM cadaverine solutions in PIPES buffer and color solution, described by Takagaki et al. Both proved to be in sufficient quantity and even decreased the absorbance when we increased the volume (data not shown).
Also, the amount of ascorbate oxidase (ASOD) needed was determined (Figure supplementary 1). Results obtained when using 5 or 20 U/mL of ASOD were compared. On the left is the comparison using 400 μL of standard DAO 25 U/L. In this assay, the absorbance obtained using 5 U/mL of ASOD was 1.236 ± 0.0425, and with 20 U/mL of ASOD was 1.225 ± 0.021. To the right, the comparison is shown using 400 μL of serum from a control subject; in this case the absorbance obtained using 5 U/mL of ASOD was 0.020 ± 0.002, and with 20 U/mL of ASOD was 0.033 ± 0.005. Both with the DAO 25 U/L standard and the serum of the control subject, there was no significant difference when comparing ASOD 5 U/mL and 20 U/mL (P = 0.8376 and P = 0.2116, respectively). Although using the serum of a control subject the absorbance using 20 U/L of ASOD is greater, it is not significant and numerically negligible. For this, we demonstrated that 5 U/mL of ASOD is enough.

Choosing plasma or serum for DAO activity assay

Finally, a comparison between serum and plasma (of heparinized tubes) of control subject was made for determination of enzymatic activity of diamine oxidase (Fig. 4). All the samples correspond to the same control subject. In addition, 5 mL of serum and plasma were ultracentrifuged and 1 mL from the bottom of the tube of each was collected and used to determine the utility of ultracentrifugation. Mean ± SD absorbance obtained using serum was of 0.06125 ± 0.00225, with plasma was 0.0625 ± 0.0020, using ultracentrifuged serum was 0.06125 ± 0.00825, and with ultracentrifuged plasma was 0.0635 ± 0.0130. There was no significant difference between serum, plasma, and both ultracentrifuged.

Concentration and DAO activity in patients and controls

With these results, the optimal conditions for the implementation of the DAO activity serum determination were standardized (Fig. 5). Briefly, 1.5 mL of substrate solution (25 mM PIPES buffer (pH 7.2), containing 30 mM cadaverine and 0.5% Triton X-100) must be incubated at 37 °C for 5 min. After adding 400 μL of the serum sample, it should be incubated at 37 °C for 30 min. Then, 1.5 mL of color solution (100 μM DA-67, 6 U/mL peroxidase and 5 U/mL ascorbate oxidase in 25 mM MES buffer (pH 5.4), containing 0.5% Triton X-100) must be added and incubated at 37 °C by 1 h. Finally, 50 μL of the stop solution (30 mM sodium dithyldithiocarbamate solution) is added, mixed, and the absorption must be measured at 666 nm against the blank solution. Porcine kidney DAO solution (5, 10, 15, 20 and 25 U/L, in 25 mM buffer (pH 7.2), containing 30 mM cadaverine and 0.5% Triton X-100, are used as standard.

As reference range was established, levels <0.6 U/L are equivalent to a high probability of HI; levels between 0.6 and 1.3 U/L at a probable HI and levels >1.3 U/L at a low probability of HI.

Having standardized the technique, we proceeded to determine the concentration and DAO activity in patients and controls (Fig. 6). The mean of serum DAO concentration in the 22 patients was of 9.268 ± 1.124 U/mL. While, the mean of serum DAO concentration in the 22 controls was of 20.710 ± 2.509 U/mL being significantly higher (P value 0.0002) the mean of the samples. The mean of serum DAO activity of the patients

![Fig. 4 Comparison between serum and plasma of control subject for determination of enzymatic activity of diamine oxidase. In this analysis, the technique described by Takagaki et al. was performed, using 400 μL of sample, and setting absorbance measurement to 666 nm. Serum and plasma (of heparinized tubes) of the same control subject was compared. In addition, 5 mL of serum and plasma were ultracentrifuged, and the lower volume of each was collected and used to determine the utility of ultracentrifugation. There was no significant difference between serum, plasma, and ultracentrifuged.](image-url)
was of 1.143 ± 0.085 U/L, and the controls was 1.533 ± 0.119 U/L, significantly greater than the patients (P value 0.011).

Additionally, we compared the average of concentration and DAO activity between female and male patients and control as indicators of Chilean people. The mean DAO concentration in men was of 22.760 ± 2.668 U/mL. The mean of DAO concentration in women was of 19.540 ± 3.674 U/mL. On the other hand, the mean of DAO activity of the men was of 1.388 ± 0.162 U/L, and the mean of DAO activity of the women was of 1.616 ± 0.162 U/L. In both comparisons no significant difference was observed.

Finally, we constructed the ROC curve for the techniques used for the determination of the concentration and DAO activity (Fig. 7). The sensitivity of the technique employed to measure the serum DAO concentration was 0.63 and the specificity 0.9. For this part, the standardized colorimetric technique for determination of

**Fig. 5 Summary of the optimal conditions for the determination of DAO activity.** 1.5 mL of substrate solution (25 mM PIPES buffer (pH 7.2), containing 30 mM cadaverine and 0.5% Triton X-100) must be incubated at 37 °C for 5 min. After adding 400 μL of the serum sample (or blank solution), it was incubated at 37 °C for 30 min. Then, 1.5 mL of color solution (100 μM DA-67, 6 U/mL peroxidase and 5 U/mL ascorbate oxidase in 25 mM MES buffer (pH 5.4), containing 0.5% Triton X-100) was added and incubated at 37 °C by 1 h. Finally, 50 μL of 30 mM sodium diethyldithiocarbamate solution was added, mixed, and the absorption was measured at 666 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
Serum DAO activity has a sensitivity of 0.63 and specificity of 0.68. It is important to mention that although the specificity is lower, the serum DAO activity (functional) analysis established that 3 of the 8 false negatives established for the determination of the DAO concentration (37.5%) had probability of HI.

**DISCUSSION**

Since patients with normal hDAO levels could present a dysfunction in activity of this enzyme, it is recommended to correlate the concentration and hDAO activity with the clinical diagnosis of HI. To implement the determination of DAO activity in serum of Chilean patients classified as intolerant to histamine, the technique published by Takagi et al in 1994\(^1\) was standardized. Although the conditions described in the original technique presented good values when using a DAO standard, these do not necessarily represent physiological conditions, this is reflected in that the signal was very low when using serum samples (in both, cases and controls). For this, the first standardization analysis that we established was of 400 μL as the optimum sample quantity (Fig. 2), maximum volume allowed by the physical conditions in which the technique was performed. Given the 4-fold increase in sample volume, it was shown that the amount used of reagents and incubation times are sufficient.

Also, a sweep test was carried out to various samples determining that in spite of not having a significant difference, in all the tests the highest absorbance was recorded at 666 nm (data not shown), thus, we established the absorbance at 666 nm.

The technique described by Takagi et al employs cadaverine as a substrate;\(^1\) however, DAO has been shown to have different affinity for the various amines on which it acts\(^1,2,4,5\).
In fact, the main interest of the technique is to establish the activity of DAO with respect to the oxidative deamination of histamine. Given this, in the standardization we compared the results obtained when determining the activity of DAO in serum using the substrates cadaverine, histamine, and putrescine (Fig. 3).

The use of serum samples did not show a significant difference between 30 mM cadaverine, 30 mM histamine, and 30 mM putrescine; this is probably due to the low absorbance obtained by these samples (Fig. 3B,D and E). Several analyzes were carried out to exclude the presence of interfering in the serum that would justify this low absorbance (data not shown), the results obtained rule out the presence of interferences and indicate that the low absorbance is probably related to a low DAO activity not only in people with HI but also in the general population.

By using the DAO 25 U/L standard, different absorbance to different substrates was shown, which is related to the variable activity described between them. Also, a significantly better absorbance with cadaverine and putrescine with respect to histamine (Fig. 3A and C) was shown, although kinetic studies reported in the biography describe a preference greater of DAO to histamine than to putrescine or cadaverine. This could be due to low stability of the histamine, which when we analyzed at double the concentration, considering having an equal quantity of available amines, resulted in a significantly lower absorbance when using DAO standard 25 U/L (Fig. 3A), and signal was not obtained when using control serum (Fig. 3B). For these results, although it would have been ideal to determine the activity using histamine as a substrate, we decided that the cadaverine was maintained.

Finally, the use of serum or plasma (heparinized tubes) from the same control subject with and without ultracentrifugation was compared to determine the DAO activity (Fig. 4); there was no significant difference between serum, plasma, and ultracentrifuged. As a result of this analysis, we can induce that, given the impossibility of working with serum, the plasma derived from heparinized tubes could be used for the determination of DAO activity as in other techniques, although we prioritized to maintain the sample originally described. Ultracentrifugation did not prove useful. Once we had the standardized technique (Fig. 5), we proceeded to determine the serum DAO activity and serum DAO concentration of controls and cases of HI (Fig. 6). In both, the average of controls was significantly higher than in the cases with a diagnosis of HI. Additionally, this is the first study that allowed characterization of DAO concentration and DAO activity in a Chilean population, being 20.71 ± 2.509 U/mL and 1.533 ± 0.1188 U/L, respectively. In the literature the occurrence of gender-related variability in DAO activity in healthy individuals has been previously reported.

Finally, the ROC curve for the techniques used for the determination of the serum concentration of DAO and the DAO serum activity were constructed (Fig. 7). The sensitivity of both techniques was 0.63. In the technique used to measure the concentration of DAO in serum, the specificity was 0.9, which constitutes a good diagnostic test, especially to rule out the true negatives. For other part, the standardized colorimetric technique for determination of serum DAO activity had a specificity of 0.68. Although the specificity is lower, the serum DAO activity (functional) analysis established that approximately one third of false negatives established by measuring the concentration of DAO in serum had probability of histamine intolerance, being a good complementary method in order to reduce false negatives, since patients with normal DAO levels could present a dysfunction in the activity of this enzyme for example due to single nucleotide polymorphisms.

By way of projection, we expect to evaluate these techniques by comparing the functional DAO data obtained by using a commercial KIT that uses Histamine as a substrate (D-HIT, Scietec, Austria), which has been used to correlate DAO activity in serum as a diagnostic test of HI.
Also, an uncomplicated pregnancy is believed to be dependent on the balance between DAO and histamine in the placenta,\(^{15}\) and low serum DAO Activity has been correlated to gastrointestinal toxicity and malnutrition due to anticancer drugs.\(^{16}\) For this reason, it would be worth evaluating the relevance of these techniques for monitoring the functioning of DAO in pregnancy and in oncologic treatments.

In conclusion, our results indicate that a colorimetric technique to measure the activity (function) of DAO present in human serum or plasma could be standardized. Although we used a small number of patients and controls and the absorbance values were lower than expected, statistically significant differences were found in the levels of concentration and DAO activity between the patients with histamine intolerance and the controls. Therefore, the measuring of DAO concentration and DAO activity is a good diagnostic strategy for study suspect cases of HI. The simultaneous use of both assays allows to reduce positive and negatives false results, for example, patients with normal DAO levels that could present a dysfunction in the activity of this enzyme.

Also, in Chilean people, serum DAO concentration and serum DAO activity in men and women do not present a significant difference.

Abbreviations
DAO: Diamine Oxidase; HI: Histamine Intolerance; ELISA: Enzyme-Linked Immuno Sorbent Assay; ROC: Receiver Operating Characteristic; hDAO: Human Diamine Oxidase; TMB: Tetramethylbenzidine; ASOD: Ascorbate Oxidase

Consent for publication
All authors approved the publication of this work.

Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author, Dr. Pablo Ferrer Campos (E-mail:pferrer40@gmail.com).

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Author’s contributions
Camila Beltrán-Ortiz, Biochemist, carried out the experiments of this work and revised the manuscript.

Dr Teresa Peralta designed the project, recruited the patients, and revised the manuscript.
Verónica Ramos, Biochemist, carried out the experiments of this work and revised the manuscript.
Magdalena Durán, Medical Technologist, carried out the experiments of this work and revised the manuscript.
Carolina Behrens, Biochemist, carried out the experiments of this work and revised the manuscript.
Daniella Maureira, Medical Technologist, carried out the experiments of this work and revised the manuscript.
Dr Maria A Guzmán designed the project, recruited the patients, and revised the manuscript.
Dr Carla Bastias designed the project, recruited the patients, and revised the manuscript.
Pablo Ferrer, Biochemist and Dr, PhD, designed the experiments, reviewed all the results, and carried out the final critical review of the manuscript.

Ethics approval and consent to participate
Prior to the study all the patients signed an informed consent approved for the Ethical Committee of the Hospital Clínico Universidad de Chile.

Declarations of competing interest
The authors declare that they have no competing interests.

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Appendix A Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2020.100457.

Author details
Section of Immunology, HIV and Allergy, Department of Medicine, Clinical Hospital University of Chile, Chile.

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