Photodiagnosis and Photodynamic Therapy 27 (2019) 85–91

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Surface-enhanced raman spectroscopy: A non invasive alternative procedure for early detection in childhood asthma biomarkers in saliva

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ARTICLE INFO

Keywords:
Asthma
saliva
SERS
Cytokines
Raman Spectroscopy

ABSTRACT

The early detection of bronchial inflammation in asthma, through a non-invasive, simple method and under a subclinical state, could lead to a more effective control of this condition. The aim of this study was to identify biomarkers of bronchial inflammation in the saliva of children with asthma through immunoassay and Surface Enhanced Raman Spectroscopy (SERS). We conducted an analytical cross-sectional study in 44 children ages 6–12; the diagnosis of asthma was made according to Global Initiative for Asthma (GINA) standards. The children's saliva was analyzed by immunoassay for the quantification of 37 cytokines, as well as SERS analysis in a confocal Raman microscope at 785 nm. We found a significant association between bronchial obstruction and IL-8 ($p = 0.004$), IL-10 ($p = 0.008$) and sCD163 ($p = 0.003$). The Raman spectra showed significant amplification in the region of 760 to 1750 cm^{-1}. The Principal Component Analysis and Linear Discriminant Analysis (PCA-LDA) method has a sensitivity of 85%, specificity of 82% and an accuracy of 84% for the diagnosis of asthma. These results demonstrate the presence of a subclinical inflammatory state, suggestive of bronchial remodeling in the population studied. The SERS method is a potential tool for identifying bronchial inflammation and its endotype, allowing for a highly sensitive and specific diagnosis.

1. Introduction

Asthma is evidenced by the result of forced spirometry, with post-bronchodilator reversibility > 12% forced expiratory volume in the first second (FEV_1). This condition, which affects more than 300 million people worldwide, is the leading cause of childhood morbidity in Latin America with a prevalence of 5.7–16% among 4–8 years [1,2]. This emphasizes the importance of making an opportune diagnosis to apply specific pharmacological therapy, as well as effective monitoring and control of this disease. The aim of the present work was to identify cytokines as potential biomarkers using a non-invasive method through SERS, in relation to cytokine profiles obtained by immunoassay for the early detection of bronchial inflammation.

The interactions between predisposing factors and signaling pathways of bronchial cells, especially in the immune system, explain the heterogeneous clinical expression, known as phenotype, which can occur in each patient. One or more endotypes are present within the phenotype when the inflammatory response is mediated by several cellular signaling pathways [2,3]. Likewise, this heterogeneity has allowed the identification of over 100 biomarkers for this condition, among which proinflammatory cytokines secreted by helper 1 and 2 T lymphocytes (Th1/Th2) play an important role in bronchial inflammation. These are proteins which participate in the differentiation and maturation of immune system cells [4,5] contributing to communication between them, and in some cases acting directly on those patients with severe asthma, difficult to control asthma and poor response to treatment. These cytokines are able to travel through the bloodstream and cross permeable biological barriers [6,7], thus making it possible to detect them in saliva and other biological fluids.

It has been shown that in patients with asthma the number of

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https://doi.org/10.1016/j.pdpdt.2019.05.009
Received 12 December 2018; Received in revised form 28 March 2019; Accepted 9 May 2019
Available online 10 May 2019
1572-1000/

neutrophils in the blood, interleukin 8 (IL-8) and metalloproteases that destroy the extracellular matrix and greatly contribute to bronchial remodeling, also increases. Likewise, IL-8 has been associated with poor response to inhaled corticosteroids, and severe difficult to control asthma [8]. In contrast, IL-10 is an immunomodulatory cytokine considered a Th2 cytokine with a central function and anti-inflammatory effects, since it inhibits the pro-inflammatory response of the immune system, preventing tissue damage, promoting the production of type G immunoglobulins (IgG), and protecting the body against allergic reactions [9]. Likewise, CD163 is a protein expressed on the surface of macrophages, promoting anti-inflammatory actions that lead to the increased synthesis of IL-10 and carbon monoxide, inhibiting the proliferation of T cell and regulating the in vitro expression of CD163, which is also regulated by IL-4, IL-6, IL-13, IL-10, and TNF-α, and promoting the shedding of CD163 free from macrophages M2 in its soluble form (sCD163) [10,11]. Thus, the detection of sCD163 has also been considered a marker of M2 macrophages associated with the eosinophil count, relevant in the inflammatory process of patients with asthma [12].

Methods to determine the endotype and the degree of bronchial inflammation involve invasive techniques such as venous puncture and other potentially risky processes; for example bronchial aspiration, pulmonary function tests using Exhaled Nitric Oxide (FeNO) and spirometry, which require motor skills yet undeveloped in some children, elderly adults or people with disabilities. This has led to the exploration of new non-invasive identification or diagnostic techniques, which do not require laborious, economical, and highly sensitive sample preparation, such as immunoassays in saliva fluid.

In this regard, Raman spectroscopy is based on the Raman effect, discovered by Chandrasekhara Venkata Raman in 1928. The Raman scattering events occurs when incident photons in a sample illuminated with a laser light source, are scattered by molecular bond vibration. During this interaction, a small portion of photons (1 in 108) is inelastically scattered and transfers energy with molecular vibrations. The difference in energy between the incident and scattered photons is known as the Raman effect and corresponds to the energy of the molecular vibration. As mentioned, the Raman effect is a weak effect. Nevertheless, several techniques have been developed to address this shortcoming; the most widely used approach is surface enhanced Raman spectroscopy (SERS) which is a technique where by Raman signals of specific molecules can be amplified based on electromagnetic enhancement due to surface plasmon resonance of metallic nanostructures. One advantage is that Raman spectroscopy allows early detection of biomolecular changes. It is known that initially, asthma changes occur at molecular level. These changes are not predictable with routine laboratory tests [13,17]. Therefore, Raman spectroscopy could potentially be used for asthma diagnostic purposes.

One of the key advantages of Raman spectroscopy over other vibrational spectroscopy is that water produces weak Raman scattering, which means that water does not interfere with the spectrum under analysis. This characteristic is particularly relevant considering that saliva contains 98–99% water. Additionally, it has a high selectivity at low biomolecule concentrations; thus, not requiring a laborious sample preparation [14,15]. It has been shown that the integration of gold nanorods into biological samples amplifies the Raman spectrum. Some studies report SERS amplification factors of 10×10^4 to 10×10^{15} [16], facilitating the identification of these molecules at minimum concentrations, even down to a single molecule. This is due to the fact that the gold nanorods represents a large contact surface with the biomolecules present in saliva, forming a transfer of adsorption charges between the Raman dispersion molecules and the metallic surface, plus an electromagnetic mechanism that makes the greatest contribution SERS [17].

At present, only two studies has been reported on the use of Raman spectroscopy for the detection of inflammation in asthma. First study was performed on adult's population for the detection of Chitinase-3-like protein 1 (CHI3L1, also known as YKL-40) in serum, as a potential biomarker revealing significant differences in the levels of said biomarker, as well as in the Raman spectra of healthy versus asthma subjects [20]. Second study evaluate in serum and through Raman technique, the biological components in an asthmatic group between 29–47 years old reporting, they concluded the peaks assigned to β-carotene are either diminished or suppressed accompanied by other new Raman peaks demonstrating the clinical application of Raman spectroscopy in detection of biomarkers [51]. However, to this date no studies have been reported in children populations focused in the detection of specific biomarkers through Raman-SERS in asthma under non-invasive conditions, using saliva. The present work is a first approach that uses nanotechnology to identify biomarkers involved in the development and progression of different endotypes and stages of asthma.

2. Material and methods

2.1. Ethical aspects

This study was submitted for evaluation by the State Committee of Ethics in Health Services Research of San Luis Potosí (REG: SLP/005–2015) complying with the provisions of the National Bioethics Commission regulations for Mexico. (CNB) [45], Helsinki Declaration of the World Medical Association on ethical principles for medical research in humans [46], as well as International Ethical Guidelines for Biomedical Research involving Human Subjects from The Council for International Organizations of Medical Sciences (CIOMS) [47]. Participation of the children was voluntary. In this case, parents or guardians of the children signed a letter of informed consent, after requesting children's assent for the performance of diagnostic tests also.

2.2. Subject screening

We randomly recruited 180 children 6–12 years old, who attended a rural Mexican community elementary school, of which 43% were girls and 57% were boys. Clinical histories were collected for the detection of factors suggestive of asthma as well as quantification of FeNO and forced post-bronchodilator spirometry. Exclusion criteria were children who at the time of data collection had acute viral or bacterial respiratory disease, fever, periodontal disease, and showed up without fasting or who were under treatment with corticosteroids, bronchodilators, antihistamines, cognitive or motor disabilities and comorbidities that could be a factor affecting diagnosis.

For the diagnosis of asthma, we followed the guidelines of the Global Initiative for Asthma (GINA) and 2017 Mexican Guide for Asthma (GUIMA) [1,2]. Two pediatric pulmonologists determined the diagnosis and degree of asthma in 35 children from the total sample, randomly assigning 30 children as healthy controls. However, during the analysis of the samples with the SERS method and the statistical analysis, 9 samples of children with asthma and 12 samples of healthy infants were eliminated since no reproducible measurements were obtained and/or they represented non-reproducible observations, obtaining a final sample of 44, divided into children with asthma ($n = 26$) and healthy children ($n = 18$).

2.3. Saliva sample collection

The saliva was collected during fasting, between 8:00 a.m. and 11:00 a.m. Two ml of saliva were collected through a non-stimulated method by spitting into in a sterile tube; during transport to the laboratory it was sheltered at 4 °C. Once in the laboratory, the saliva samples were centrifuged at 1200 rpm for 10 min at 4 °C in Sorvall Biofuge Primo R (Thermo Scientific®) to remove oral impurities, oral mucosa epithelial cells and to extract 70 µl of the supernatant which was separated into two aliquots as follows: aliquot "A" (50 µl) for

immunoassay and aliquot "B" (20 µl) for SERS analysis. Finally, the saliva samples were stored at −20 °C immediately after centrifugation to reduce degradation [37,39].

2.4. Immunoassay

Aliquot "A" of saliva was analyzed by immunoassay in Bio-Plex® MAGPIX ™ equipment for the detection and quantification of a panel of 37 Th1 / Th2 cytokines. (IL-26, IL-27p28, IL-28A / IFN-λ2, IL-29 / IFN-λ1, IL-32, IL-34, IL-35, LIGHT / TNFSF14, MMP-1, MMP-2, MMP -3, Osteocalcin, Osteopontin, Pentraxin-3, sTNF-R1, sTNF-R2, TSLP, TWEAK / TNFSF12, APRIL / TNFSF13, BAFF / TNFSF13B, sCD30 / TNFRSF8, sCD163, Chitinase-3-like 1, gp130 / sIL- 6Rβ, IFN-α2, IFN-β, IFN-γ, IL-2, sIL-6Rα, IL-8, IL-10, IL-11, IL-12 p40, IL-12 p70, IL-19, IL-20.22). At this phase, aliquot (A) was added to a mixture of magnetic beads, coated with antibodies and color-coded, which bind to the analytes of the study forming an antigen-antibody sandwich to be detected in a double laser flow, where one magnet held the magnetic beads in a monolayer while a light emitting diode (LED) illuminated them and another diode determined the magnitude of the signal emitted by the beads.

2.5. Saliva analysis with SERS method

Gold nanorods were synthesized under the Turkevitch method [18,19]. Hydrogen tetrachloroaurate Gold (III) (HAuCl4, 99.99%) and hexadecyltrimethylammonium bromide (CTAB, BioXtra ≥ 99%) were purchased from Sigma Aldrich. Silver nitrate (AgNO3, 99.3%), sodium borohydride (NaBH4), hydrochloric acid (HCl) and ascorbic acid (ACS) were purchased from Fermont. Once the gold nanorods were synthesized, their absorption spectrum was obtained by ultraviolet-visible spectroscopy (UV /Vis) at 785 nm, with an average size of 36 × 56 nm and Z potential of 42 mV.

Aliquot "B" of saliva and the 15 µl of gold nanorods were mixed by shaking them in a mixing machine (Fisherbrand™) at 2000 rpm for 60 s. Once mixed, the sample was kept static for 2 h to allow for the integration of the gold nanorods with the biological components of the saliva. Ten µl were taken from this mixture and placed on an aluminum substrate with a drying time of 60 min at room temperature (20 °C) which favored the integration and interaction between the saliva matrix and the gold nanorods. Subsequently, these samples were analyzed in a confocal Raman spectroscope (XploRA Raman®) at a wavelength of 785 nm, Laser power on the sample was 2.5 mW. Triplicate measurements were obtained at an integration time of 60 s, considering the spectral region of 400 to 2000 cm^{-1}.

2.6. Raman spectra processing

Before statistical analysis, all Raman spectra were pre-processed to remove background fluorescence using an iterative polynomial fitting algorithm, also known as the Vancouver Raman algorithm (VRA), [48] widely used for removal of fluorescence background in biomedical Raman spectra. VRA applies a smoothing to the Raman spectrum to reduce high frequency noise. Later, an iterative process is applied to adjust the polynomial function. The Raman spectrum sans fluorescence is computed by subtracting the adjusted polynomial from the original Raman spectrum. The algorithm takes into account the effects of noise level and Raman peak contribution [48]. VRA was performed using Vancouver Raman Algoritm® software (Version 1.1). Additionally, each Raman spectrum was normalized to ensure that the area under the curve was equal to 1. The mean Raman spectra of asthma and healthy groups were plotted using OriginLab® software (Version 8.6). Finally, the statistical analysis including linear regression, correlation, Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) was performed in R commander software (Version. 3.3.3).

Table 1
Analysis of Pearson's correlation coefficient of cytokines in saliva, FEV1 and FeNO.

	Mean (pg/ml)	FEV1 Correlation	FeNO Correlation
IL-26	465	0.453	0.440
IL-27p	342	0.312	0.290
IFN-λ2	534	0.654	0.600
IL-32	776	0.660	0.560
IL-34	345	0.469	0.460
IL-35	423	0.598	0.480
Chitinase-3-like 1	543	0.344	0.245
IL-19	256	0.276	0.202
IL-20	454	0.670	0.507
IL-22	89.9	0.330	0.303
Osteocalcin	43.1	0.219	0.201
Osteopontin	365	0.300	0.228
TNF	1006	0.6495	0.6486
sCD163	6263	**0.862***	**0.826***
sIL-6Rβ	7371	0.648	0.514
IL-2	60.8	0.146	0.627
IL-8	2724	**0.808***	**0.718***
IL-10	24.5	**0.796***	**0.741***
IL-12	2381	0.157	0.417
TNFSF14	3291	0.294	0.092

The rest of the non-listed cytokines obtained minimum values outside the detection range.
* Acceptable correlation.

3. Results

The results of the population study using spirometry showed that the male sex group 6–12-years old exhibited an obstructive pattern compatible with asthma (frequency of 46%). In addition, 65% of this group displayed a mild inflammation grade, 18% moderate inflammation, and 12% severe inflammation. Significant differences were identified in the groups; healthy control and group with asthma ($p = 0.001$) in relation to post bronchodilator bronchial obstruction in FEV_1 (Table 1).

A Pearson's correlation coefficient analysis was carried out where it was identified that IL-8, IL-10 and sCD163 showed correlation higher than 0.8 with FEV_1 and FeNO levels, unlike the rest of cytokines studied (Table 2). Under these results, a difference analysis of the mean concentration cytokines was carried out by Student's t test, obtaining significant differences between the groups; healthy control and asthma group relative to bronchial obstruction, mainly in the sCD163 levels (Table 1). Later, a linear regression analysis was accomplished integrating the study variables to the following model: (lm = FEV1 ~ sCD1630 + IL-8 + IL-10, data = data) obtaining significant differences between both groups ($p = 0.03$, $r^2 = 0.61$). It should be noted that cytokine levels relative to typical clinical symptoms like night cough, dyspnea with exercise and wheezing, showed no significant differences; however, for histories of dermatitis, IL-8 was significant ($p = 0.04$).

3.1. Raman Spectrum analysis

The measurements of saliva integrated with gold nanorods, showed high amplifications in the region of 760 to 1750 cm^{-1} (Fig. 1). Raman intensity increase in the group with asthma predominated in eleven bands: 760, 843,901, 1000, 1231, 1290, 1326, 1420, 1456, 1591, 1750 and region 1050–1127 cm^{-1}. The differences in Raman intensity between groups was analyzed by Student's *t*-test ($p \leq 0.05$), the major changes were in six bands: 843, 901, 1290, 1420, 1456, 1591 and 1750 cm^{-1} which correspond to biological components, such as nucleic acids, amides, lipids, proteins and triglycerides mainly [20–22 41]. However, in the region 1050–1127 a significant decrease in the Raman intensity of the asthma group in comparison to the healthy group was observed, corresponding to amide III, υ(C−O−C), Cytochrome C,

Table 2
Comparison of concentration means of cytokines in saliva, FEV1 and FeNO, between the healthy group and the asthma group.

Variables/Group	Asthma group	Healthy group	IC (95%)	*t*	*p*
FEV_1 Reversibility (%)	17.42	4.25	9.73-16.60	8.07	0.001*
FeNO (ppb)	23.26	9.35	9.80-18.01	6.93	0.001*
IL- 10 (pg/ml)	24.60	15.82	2.45-15.11	2.88	0.008*
IL8 (pg/ml)	3056.9	1070.87	1002.19-2969.89	0.66	0.004*
sCD163(pg/ml)	1852.08	659.56	319.7-2064.9	2.82	0.003*

FEV_1: Forced Expiratory Volume in the first second.
FeNO: Exhaled Nitric Oxide.
IC: Confidence interval.
* $p \leq 0.05$.

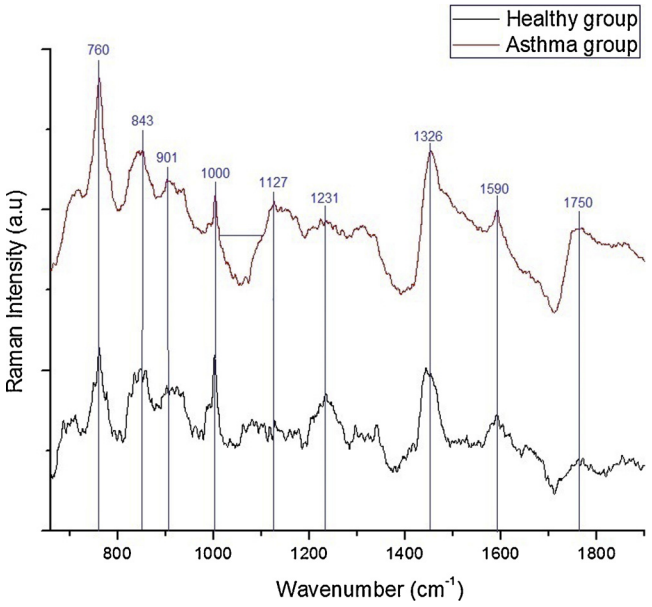

Fig. 1. SERS spectrums display a comparison between the normalized mean of the healthy group (Black line, $n = 18$) and asthma group (Red line, $n = 26$).

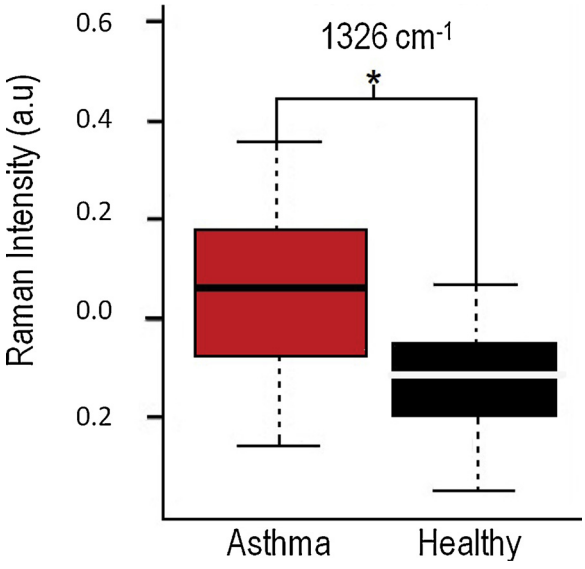

Fig. 2. Comparison between Raman Intensity (a.u) score values from asthma (red box) and healthy (black box) groups. Difference between groups was analyzed by *t*-test. Raman Intensity values from asthma samples are different from those found in healthy group. The region 1326 cm^{-1} corresponds to IL-8. Differences were statistically significant ($p < 0.001$).

hydrocarbon chain of lipid, triglycerides (Table 3). These Raman peaks were assigned in accordance to several references related to the specifications of Raman Spectroscopy or bio-molecules detected in body fluids by SERS. Peaks with wavenumber difference within 10 cm^{-1} were included as the same peak (Table 3).

IL-8 stands out with a characteristic peak at 1326 cm^{-1}, this being the only cytokine in the panel studied, which has been previously characterized under the SERS method [23,24], showing significant differences in the mean intensity between groups ($p = < 0.001$), 1326 cm^{-1} (Fig. 2).

3.2. Multivariate analysis

Principal Components Analysis (PCA) allows the analysis of high

Table 3
Assignments of SERS peaks and the *t*-test of average intensity between healthy and asthma groups.

Raman Shift	t-Test Average Intensity			
cm^{-1}	**t**	***p* value**	Assignments	Ref.
760	3.0253	< 0.001*	Triptophan Aliphatic υ(C-S), υ(C-CI)	[30,39,43]
843	2.565	0.124	nucleic acid,diester B-DNA υ(O = O) Tryptophan	[39,43,44,41]
901	1.288	0.160	υ(O = O), υ(C-O-C)	[43]
1000	1.568	< 0.001*	Phenylalanine, Lysine, Triptophan	[42]
1050-1127	−3.2706	< 0.001*	Amide III υ(C-O-C) Cytochrome C Hydrocarbon chain of lipid, Triglycerides	[44,43,40,30]
1231	−2.9171	< 0.001*	Protein, υ (C-N) Hydrocarbon chain of lipid Amide III	[30,41,44]
1290	−2.58	0.061	Phospholipid, amide III, preoteins, lipid.	[36,40]
1326	−4.825	< 0.001*	IL-8	[23,24]
1420	−2.668	0.091	df(CH_2); B- DNA	[44,40]
1456	−2.9368	< 0.001*	Hydrocarbon chain of lipid, Triglycerides CH_3 Deformation of lipids	[44,41]
1591	2.342	0.124	Amide I, C = O Adenine, Guanina	[44,30]
1750	−2.9171	< 0.001*	Lipids, υ(C = C), υ(C = O) Cholesteryl esters, Tiacilglycerol	[44,38,41]

υ= Stretching, df = deformation. Peaks were regarded as the same Raman peaks with a wavenumber difference of less than 10 cm^{-1}.
* $p \leq 0.05$.

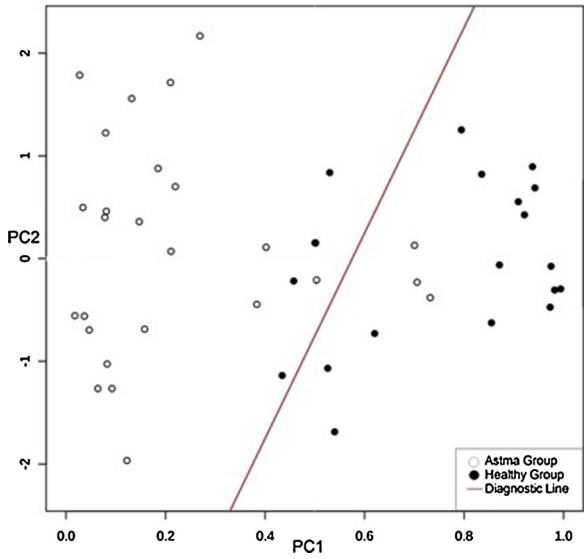

Fig. 3. Plot of the first two principal components (PC1 and PC2) using Linear discriminant analysis, between asthma (white circles) and healthy group (black circles). The red line represents the diagnostic line, which allows discriminate between the asthma and healthy group.

dimensional data, in order to reduce its size and describe the variation between them, through a new set of orthogonal components, which facilitate the identification of the characteristics that represent the variance in total data [25]. For this study, it was found that 89% of the total variance could be explained by the first 4 principal components, finding a greater explanation of the variance in the first two principal components (PCI and PC2).

To verify diagnostic ability, the Linear Discriminant Analysis (LDA) was used in PC1 and PC2 scores (PCA-LDA), which provided data classification based on an optimized criterion aimed at a better classification of healthy control groups and groups with asthma, (Diagnostic line) by eliminating noise from the data and concentrating on the important variables (Fig. 3). The leave- one-out cross-validation (LOOCV) model was performed to validate the classifier. Sensitivity obtained was 85%, with a specificity of 82% and accuracy 84% (Table 4). The classification of healthy and sick subjects through the use of SERS showed statistical effectiveness, showing an acceptable discrimination of healthy group and group with asthma, suggesting a potential tool in the diagnosis of this disease.

4. Discussion

This study reveals an expected frequency of asthma in line with literature reports for Mexico [1,2]. Due to the increase of FeNO in the group with asthma as well as suggestive clinical history, we can elucidate the existence of an eosinophilic endotype recognizing that currently the eosinophilic type Th2 inflammation occurs with a frequency of 70–80% in population with asthma [26].

In addition, this work shows the relevance as well as the feasibility of the measurement of pro-inflammatory cytokines in saliva, as a practical and non-invasive method. Likewise, Williamson et al, demonstrated in a study with 50 healthy volunteers, that there are no significant differences in the levels of cytokines detected in saliva in relation to those detected in serum using Bio-Plex technology [6]. However, the high cost of the latter is a limitation for this method, so detection with SERS could mitigate this disadvantage.

IL-8, IL-10 and sCD163 levels could be used as potential biomarkers of bronchial inflammation in asthma, finding patterns of cellular expression or endotype suggestive of sub-clinical inflammation in this population, because no significant statistical association was found with typical symptoms of the condition, rather a strong association between the degree of obstruction and elevated levels of FeNO. The elevation of IL-8 has been related to the process of bronchial remodeling, since it has been shown to be present in the inflammation environment as one of the growth factors produced by the activation and transformation of the perivascular fibroblast in myofibroblast and myocyte capable of producing sub epithelial collagen and thickening of the reticular lamina, generating different histopathological changes [27], which have been related to a greater severity of the disease [10,28].

On the other hand, Lamotte V. et al, showed through induced sputum samples that the concentration of Th2 cytokines (IL-4 and IL-5) and Th1 (IFN gamma, IL-2 and IL-12p70) is higher in atopic asthmatics than in healthy and non-atopic asthmatics ($p = 0.0001$). Likewise, IL-8 was higher in children with asthma than in controls ($p = 0.0001$) and IL-10 was higher in controls than in children with asthma ($p = 0.03$) [5].

Another study on the identification of pro inflammatory mediators and correlation of clinical manifestations of asthma in saliva reported that there are strong correlations between the use of inhaled corticosteroids and IL-8 in the pediatric population in general [29]. In the present work, the findings in the detection of IL-8 are in line with results obtained by Kamińska A. et al, who characterized the Raman spectrum for IL-8 at 1326 cm^{-1} in a serum matrix using SERS [16,17]. In this study it has been possible to identify increased intensity in the characteristic peaks of this cytokine in the spectra of the group with asthma, unlike in the healthy group.

Ladiwala U. et al, characterized a series of cytokines in neural stem cells, including IL-10 identifying characteristic peaks in the region of 775–875 cm^{-1} [30], where, lipids, proteins and nucleic acids have been reported [41,43,44], and that are shown with greater intensity and association to asthma group that allows the possibility of suggesting detection of this anti-inflammatory cytokine which in turn is closely linked to the expression of sCD163.

sCD163 is particularly interesting in the present study, since the role of this protein has been shown to be an important mediator for the development and maintenance of bronchial inflammation, as well as remodeling of the airway in the patient with asthma. Likewise, the correlation of this protein with the elevation of IL-6, IL-8 and IL-10 has been detected, and has been associated with insulin resistance, poor corticosteroids response in asthma, cardiovascular disease, and obesity [10]. It should be noted that the population studied shows an overweight prevalence of over 30% and an increase of the waist-hip indexes

Table 4
Diagnostic results PCA–LDA on the diagnosed groups through the gold standard and SERS of saliva.

	Diagnosis SERS					
Diagnosis by gold standard	Asthma group	Healthy group	Total	Accuracy	Sensitivity	Specificity
Asthma group	23	3	26	84%	85%	82%
Healthy group	4	14	18			

Sensitivity = number of true positives/number of true positives + number of false negatives.
Specificity = number of true negatives/ number of true negatives + number of false positives.
Accuracy = number of true negatives + number of true positives/number of total samples.

[31], a fact that currently also constitutes one of the main health problems in Latin America, mainly Mexico, showing high correlation with the presence of asthma and its poor control [32,33]. On the other hand Kowal K. et al, has studied the effect of inhaled corticosteroids on the concentration of sCD163 in induced sputum of allergic asthma. They reported that a change in sputum sCD163 concentration inversely correlated with changes in sputum eosinophilia or FeNO concentration and they concluded that inhaled corticosteroids therapy leads to local upregulation of sCD163 expression, which in turn may participate in the anti-inflammatory effects of corticosteroids therapy [34].

CD163 is a protein that is expressed on the surface of macrophages promoting anti-inflammatory actions that lead to the increased synthesis of IL-10 and regulate the in vitro expression of CD163, which is also regulated by IL-4, IL-6, IL-13, IL-10, and TNF-α, and promote the shedding of CD163 to free macrophages M2 in its soluble form (sCD163) [10,11]. Its expression is conditioned by inflammation process, lipid elevation and obesity. In the present study we observed that the increase in the intensity bands: 760, 1231, 1591, 1456, 1750 cm^{-1} correspond to lipids, cholesteryl esters, triacylglycerol, proteins and amino acids mainly [36,38,40,41,44]. Several studies show the relationship of lipids with asthma, one of the most important is a meta-analysis that included twenty studies with a total of 32,604 patients (3458 in the asthma group and 2914 in the healthy group), finding significant differences in the average of Low Density Lipoproteins (LDL) and total serum cholesterol, this finding could support the results obtained in this study on the increase of body mass index and significant presence of biomolecules corresponds to lipids in the Raman spectrum of the asthma group [50].

This observation was evident in the asthma group where some specific biomolecules were different in comparison to the healthy group, a finding that needs to explore in more detail in future studies in order to establish asthma diagnostic biomarkers in children.

On the other hand, the decrements of Raman intensity in the region 1050–1127 cm^{-1} in the group with asthma, but no healthy group correspond to the molecules: cytochrome C, amide III, hydrocarbon chain of lipids and triglycerides could suggest an auto modulation of the inflammatory process. The role of cytochrome C has not been clearly described in this disease, however, since it is a protein that participates in very important biological processes such as mitochondrial electron transporter in the respiratory chain and activator of the protein complex called "apoptosome", triggering the last phases of the disease.

Apoptosis derived from oxidative stress among other processes [49]. In asthma, oxidative stress can be conditioned by the increase in the generation of reactive oxygen species, which overcome the antioxidant misbalance mechanisms in the airway environment of the asthmatic, process, that in turn promotes the generation of free radicals, altering at cellular, biochemical, tissue the respiratory tract physiology, as well modifying the organic homeostasis even exists evidences about the role of cytochrome C in asthma disease, today, there is not reports that display the identification of cytochrome C by SERS in asthmatic groups, however this molecule play an important pivotal role of this disease. In the present study, the group with asthma but no in the healthy group, exhibited a decrease in the region corresponding to cytochrome C, this could be involved in the processes of self-modulation of inflammation, nevertheless to corroborate this hypothesis it is necessary to generate more related evidence.

In order to support these findings, recently has been published a study in which evaluate in serum and through Raman technique, the biological components in an asthmatic group between 29–47 years old [20,51], reporting a similar pattern of Raman intensity than those included in the saliva, this observation can reinforce our findings, suggesting that the saliva fluid could represent a reliable matrix, that reflects the components containing in sera with the advantage that this effort represents a non-invasive method.

To verify this fact, SERS characterization, of the cytokines that participate in the inflammatory and anti-inflammatory process of asthma, and the specific roles of other proteins and molecules such as sCD163, cytochrome C, amino acids and lipids is necessary, considering these results as a first approach to understanding the mechanisms of inflammation through the use of SERS.

Finally, several studies have been conducted in recent years for the non-invasive detection of several biomarkers, mostly those related to cancer, of which a detection sensitivity and specificity greater than 80% has been reported by using the SERS method [35–37], similar to that obtained in this study, showing the wide range of possibilities of the application of SERS in the detection of biomarkers, including asthma.

5. Conclusions

The study population showed an eosinophilic inflammatory endotype, explained by the increase in FeNO, as well as a probable neutrophilic component by the expression of IL-8, coexisting with a compensatory mechanism of IL-10 and CD163 as anti-inflammatory cytokines [10], which suggests a process of bronchial remodeling as well. This process could be represented by the increase in the variation of intensities of the Raman spectrum in the asthma vs healthy groups, particularly in the region of 1050–1127 cm^{-1}.

The increase of sCD163 in asthma vs healthy groups, added to the epidemiological characteristics and to the significant difference of Raman intensities between groups in bands 1231, 1290, 1456, and 1750 cm^{-1}, where lipids such as cholesterol and triglycerides are detected, could determine an endotype of inflammatory characteristics for this population which could in turn be influenced by the pro-inflammatory state that overweight and obesity represents. The above is part of the study perspectives, as well as the identification of the Raman spectrum that characterizes sCD163 [21,22,34].

In conclusion, SERS methodology offers the advantage of achieving identification of the endotype of bronchial inflammation in a non-invasive and simple way, even when the patient does not present any signs or evident clinical symptoms, which are crucial for diagnosis and control. This methodology could facilitating the monitoring of disease control as well as the direction of pharmacological treatment, since due to the heterogeneity of the disease, there is a wide range of drugs directed to specific endotypes, such as corticosteroids, antileukotrienes, immune modulators etc., fact that also leads to a timely prevention of bronchial remodeling and fixed airflow limitation.

Declaration of non-conflict of interests

The authors have no conflicts of interest. This research was financed by the Mixed Fund CONACYT- Government of San Luis Potosi, Mexico. (FMSLP-2014 – 02251723)

Acknowledgments

Robert Camargo Angeles, MD. Head of the Program of Specific Action for the Prevention and Control of Respiratory Diseases and Influenza (PAE-ERI), of the National Center for Preventive Programs and Disease Control (CENAPRECE), for the facilities provided for Certification in spirometry taking by The National Institute for Occupational Health and Safety (NIOSH).

David Vázquez Rivera, technician respiratory therapy, for his participation in the performance of spirometry sampling.

Anton Terekhov. Managing Director of Analytical Services, for the revision of the grammar English manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pdpdt.2019.05.009.

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