

A Rapid Differential Display Analysis of Nasal Swab Fingerprints to distinguish Allergic from non-Allergic Rhinitis Subjects by Mesoporous Silica Particles and MALDI-TOF Mass Spectrometry

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List of abbreviations:

Abbreviations: **AR**, allergic rhinitis; **H**, healthy; **MPS**, mesoporous silica; **NF**, nasal fluid; **NLF**, nasal lavage fluid; **NAR**, non-allergic rhinitis; **NARES**, non-allergic rhinitis with eosinophils; **NARMA**, non-allergic rhinitis with mast cells; **NARNE**, non-allergic rhinitis with neutrophils; **SA**, sinapinic acid

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Abstract

Discriminating different rhinitis cases can sometimes be difficult as the diagnostic criteria used to identify the various subgroups are not always unambiguous. The nasal fluid (NF) highly reflects the pathophysiology of these inflammatory diseases. However, its collection, as nasal lavage fluid (NLF), may cause discomfort. Due to the non-invasiveness and rapidity of collection, nasal swab might represent an alternative to overcome these problems and also an ideal source of biomarkers. In this study we demonstrate that the combined use of mesoporous silica (MPS) with MALDI-TOF MS allows the rapid detection of differential nasal peptide profiles from nasal swabs of healthy (H), allergic rhinitis (AR) and non-allergic rhinitis (NAR) subjects. NF peptides from nasal swabs were captured by the mean of MPS then profiled by MALDI-TOF MS. As a proof-of-principle, we also explored the ability of our platform to discriminate between nasal swabs of patients with AR and NAR, and between these groups and H controls. Four peaks resulted differentially expressed between NAR and AR, two peaks discriminated AR from H while one peak segregated NAR from H group. Therefore,

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peptides selected and enriched by our platform could form part of a diagnostic “rhinomic” profile of the allergic and non-allergic patients.

Significance of the study

Peptidomics is increasingly being used as emerging platform in biomarker discovery.

The peptidomics-based platform here described might be an useful tool to capture naturally occurring circulating peptides acting as messengers in the control of various biological processes in particular, in the field of allergy. The protocol is based on the combined use of MPS and MALDI-TOF MS. For the first time, we demonstrate that complex peptide mixtures can be enriched from NF collected as nasal swab, depending on the pore diameter and on the chemical groups of the mesoporous surface. Different *rhinomic* fingerprints generated from subjects enrolled in this study in H, AR and NAR groups, were compared suggesting that they could be part of a possible profile for AR/NAR differential diagnosis. Preliminary findings seem to disclose a possible role of lysozyme and of naturally occurring statherin fragments in the pathobiology of AR and NAR. The differential display analysis of nasal swab fingerprints here described might also contribute to distinguish different vasomotor NAR sub-phenotypes. Finally, we auspicate that the *rhinomic* fingerprints extrapolated by our tool might in a future be able to reveal a distinctive pattern of molecular features correlated by specific relative expression in each analyzed subject and might possibly provide a more practical route to personalized treatment.

1 Introduction

Both AR and NAR are risk factors for the development of asthma [1]. NAR and AR are high prevalence chronic inflammatory diseases of the upper airways, characterized by sneezing, nasal congestion, nasal itching, and rhinorrhea, in any combination [2, 3]. AR, induced upon allergen exposure, is an IgE-mediated symptomatic inflammation of the nasal mucosa, with a prevalence of 10-20% in worldwide population [4]. NAR, with a prevalence of 5-10% worldwide [5] has a variable etiology with, among others, hormonal or medication-induced conditions and local AR [6,7]. The pathogenesis of NAR is not clearly elucidated. Specifically, NAR covers a heterogeneous pattern of conditions which vary dramatically in their pathogenesis and need different treatments. A number of pathologies, including nasal polyposis, anatomical/mechanical factors, autoimmune diseases, immunodeficiency, metabolic and genetic conditions, induce a clinical scenario similar to that caused by NAR or AR, which sometimes makes their clinical diagnosis quite difficult [8]. These conditions need to be accurately considered in the differential diagnosis, because therapies that are effective for AR (i.e., antihistamines and nasal corticosteroids) may be less effective for other types of rhinitis.

Apart from assessments of clinical signs and symptoms, the most common diagnostic tests for AR are skin prick test and dosage of serum allergen-specific immunoglobulin E (IgE) . So far, several potential nasal biomarkers, have been proposed for AR monitoring or treatment evaluation, including eosinophil derived markers [9], mast cell-derived markers such as tryptase [9], histamine [10], PGD2 [10], leukotrienes [11], chymase along with its inhibitor, cleaved secretory leucocyte protease inhibitor (cSLPI) [12], markers of nasal permeability (Albumin and α 2 macroglobulin), biomarkers of upper airway inflammation (cytokines and chemokines), and lastly nasal nitric oxide. However, none of the listed candidates has been validated because a substantial variability is introduced by both endogenous and exogenous factors [13].

On the other hand, NAR, usually characterized by pseudo-allergic symptoms, may be confused with AR and, as a consequence, its recognition still represents a serious problem from a clinical-diagnostic and therapeutic point of view. In particular, vasomotor NARs are classified on a cellular basis (also cited as cellular rhinitis) into different subtypes such as non-allergic rhinitis with eosinophils (NARES), non-allergic rhinitis with mast cells (NARMA), non-allergic rhinitis with neutrophils (NARNE), or non-allergic rhinitis with eosinophils and mast cells (NARESMA) [14]. Moreover, it is commonly observed that several patients with seasonal AR continue to have symptoms even when the exposure to allergens is expected to be low or absent [15]. In such cases an overlap of both AR and vasomotor NAR (mixed rhinitis or perennial NAR) is observed [16].

Given all these heterogeneous phenotypes, the various forms of NAR are more difficult to be unambiguously diagnosed compared to AR. To date the only strategy for vasomotor NAR diagnosis is based on the cytological analysis of nasal mucosa after exclusion of AR by routine diagnostic test (anamnesis, skin prick test, rhinoscopy, nasal cytology, rhinomanometry, etc.), which however maybe poorly informative in the case of perennial NAR.

Therefore, additional criteria for defining disease-specific differences based not only on the cellular features, but also on the molecular profiling underlying the onset and progression of rhinitis, are still an unmet medical need. Similarly, novel strategies allowing the phenotypic stratification of rhinitis, are barely required.

Human nasal epithelium, mucosa and its secretions are excellent sources of potential biomarkers for accurate molecular analysis of different forms of rhinitis [3]. Reliable biomarkers from nasal source, by providing a quick readout, may allow not only to investigate the still unclear

pathophysiology of such rhinitis types and subgroups, but also to speed up the early diagnosis in patients with AR and NAR.

Recently, the proteomics of nasal specimen has outlined a very powerful tool to shed light on AR and NAR [17-25]. However, little attention has been focused so far on NF peptidome and, in particular, no one has yet analyzed the NF peptidome using a MPS-based platform, which proved to be a very efficient tool to enrich the low molecular weight fraction of several bodily fluids [26-30]. Until some years ago, the peptidome was considered by the scientific community simply as the pool of the proteolytically derivatized peptide products that results from the action of active proteases, with an informational content equivalent to biological 'trash' or 'noise'. However, during the past years, a novel concept has been emerging, suggesting that the peptidome could instead be a goldmine for the discovery of novel biomarkers [31, 32]. Nowadays, it seems evident that many proteins precursors hide peptide substructures, which may exert relevant biological activities after their release [33]. Indeed, the low molecular weight fraction covered by peptidomics includes peptide hormones, cytokines and many other circulating peptides acting as messenger molecules involved in the control of various biological processes. In particular, in the field of allergy and asthma, cytokines and chemokines, peptide mediators released from effector cells and MHC classes I and II, play a pivotal role in disease pathobiology [34]. Therefore, peptidomics might be an excellent tool to discriminate at the molecular level AR from NAR cases.

On the basis of the above considerations, we designed our study with the aim of investigating the NF peptidome in patients with AR and different NAR subtypes. However, its collection, as NLF, may causes discomfort, above all, among younger patients. Due to the non-invasiveness and rapidity of the collection, nasal swab might represents an alternative mean to overcome these problems, as well as an ideal source of potential rhinitis biomarkers. Therefore, hereby we describe our efforts to

generate a protocol for efficient extraction of nasal peptidome from nasal swab specimen.

Moreover, we ascertain the usefulness of this methodology as potential diagnostic tool in identifying specific nasal fingerprints, characteristic of each rhinitis subtypes.

2 Materials and methods

2.1 Reagents. The MALDI matrix sinapinic acid (SA) was purchased from Fluka (St. Louis, MO, USA). Peptide and protein standards, for mass spectrometer external and internal calibration, were prepared from the Sequazime peptide mass standard kit (Applied Biosystems, Framingham, MA, USA). The water used was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). ACN (HPLC grade) and TFA (ACS grade) were purchased from Merck (Darmstadt, Germany). Protease inhibitor cocktail (PIC; P8340, Sigma, St. Louis, MI, USA) and Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) were used according to manufacturer instructions. Sodium chloride was purchased from Sigma-Aldrich (St. Louis, MI, USA) MCM-41 (Code 643645 hexagonal silica, mesostructured) and HMS (Code 541036 wormhole silica mesostructured) were purchased from Sigma-Aldrich (St. Louis, MI, USA).

2.2 Patient recruitment

All AR (n=8), NAR (n=7) patients and H subjects (n=7), with an average age of 39, 38 and 25 years respectively, recruited from the operative unit of Otorhinolaryngology of Magna Græcia University were enrolled in this study after the informed written consent was obtained from each participant

according to the Institutional Ethical Committee Board (Table 1). All subjects underwent anamnesis, otorhinolaryngological evaluation. The clinical evaluation included a complete traditional ENT examination of the upper airways and an endoscopic examination, with a flexible fiber rhinolaringoscope (Olympus Winter & Ibe GmbH, Berlin, Germany), of the nasal and nasopharyngeal cavities.

All the subjects were also tested for allergy by skin tests (prick method) with a full panel of common aeroallergens and were evaluated for respiratory function for asthma diagnosis by spirometry and bronchial challenge test (pulmonary function test with bronchodilation or methacholine challenge as appropriate).

The exclusion criteria for all subjects were the use of oral/nasal antihistamines and intranasal corticosteroids within 10 and 20 days respectively prior to the NF collection and during the study, detection of active rhinitis at the time of collection, smoking habits, pregnancy, lactation, diagnosis of systemic, oncologic and autoimmune diseases. Patients were classified as H, AR or NAR based on skin prick test (Lofarma, Milan, Italy) and specific IgE (Immulite 2000 XPI Siemens Healthcare, Milan, Italy) measurement. In particular, allergic sensitization was assessed by skin prick test using standardized kit of allergens (100 DBU/mL), including *Parietaria officinalis*, *Olea europea*, *Graminaceae* mix, *Dermatophagoides* mix, *Compositae* mix, *Cynodon dactylon*, derived epithelial cat and dog, *Cupressus arizonica*, *Alternaria alternata*.

Subjects in the H control group had negative skin test results to the panel of allergen extracts, did not have allergy histories and no history of rhinitis symptoms (congestion, sneezing, itching, rhinorrhea), without nasal polyposis and infectious rhinopathy.

Nasal cytology was performed by nasal scraping using a Rhino-Probe® 17 18 according to the method by Gelardi *et al* [35]. Briefly, samples were collected from the middle portion of the inferior turbinate and were placed on a glass slide. Then, the samples were fixed by air drying and stained by May-Grunwald Giemsa method (Carlo Erba®, Milan, Italy). The slides were examined under a light microscope (Leica DFC 500 Wetzlar, Germany) equipped with a digital camera. Cell count was performed on 50 microscopic fields at 1,000 x. The cellular forms of rhinitis were differentiated on the basis of their cellular pattern into NARES (eosinophils > 20% of total cells) NARMA (mast cells > 10% of total cells), NARNE (neutrophils > 50% of the total cells, without spores and bacteria).

2.3 NF collection by nasal swab

NF was obtained by non-invasive procedure from inferior turbinates of AR and NAR patients and H controls (Fig. 1A and Fig. 1B) out of the pollen season. No decongestant or topical anesthesia was instilled before collection. A cotton swab with wood rod (Biomérieux, Marcy-l'Etoile, France) was gently introduced in the nasal fossae covering the middle portion of the inferior turbinate on both sites. Then the swab was gently rotated against inside of nostril for 5 seconds. A light pressure was applied on the outside of the nose while rotating swab (Fig. 1B). Care was taken to avoid touching the nasal mucosa to avoid contamination due to bleeding. The swab containing the NF was immediately placed in a Falcon tube dipped on ice and immediately carried to laboratory for analysis.

2.4 NF elution procedure from nasal swabs

The recovery of NF from the swabs was performed by a three steps elution procedure with hypertonic saline solution of sodium chloride (Fig.1A). Briefly, before sample collection, one sterile cotton swab was placed in a 50 mL Falcon tube and weighted on a Gibertini semimicro analytical balance with a readability of ± 0.01 mg and maximum capacity of 90 g (Mod. E50S/2 DUAL RANGE, Gibertini Elettronica, Novate (MI), Italy). The total amount of NF adsorbed on the cotton swab was determined by differential weighing according to the equation:

$$M_{NF} = M_2 - M_1$$

where M_2 and M_1 represent the weight of the Falcon tube containing the NF, after and before the NF collection, respectively. NF was extracted from cotton swab with 0.9% or 30 % sodium chloride solution by a three steps centrifugal elution procedure at 2000 *g*. Forensic Micro-Spin Filter (MIDSCI 280 Vance Road, St. Louis, MO 63088, code 8500-01) were used for the extraction of soluble fraction of the NF collected on the cotton swab.

In particular, the tip of the cotton swab containing the collected NF was cut and placed inside the filter unit of the microspin tube (Fig. 1C). The Forensic Micro-Spin Filter devices used have an 800 μ L capacity filter unit, and a 2 mL graduated micro centrifuge tube with an attached cap. The filter unit has an integral perforated bottom that acts like a strainer and allows fluids to pass but retains small cloth samples in the filter unit (Fig. 1D). Appropriate volume of sodium chloride solution was added to the tip of the swab in the filter unit in order to allow the extraction of the soluble fraction which was recovered in the underlining 2 mL micro centrifuge receiver tube. Following the first centrifugation step (5 min), the filter unit was removed from the receiver tube, and first eluted solution was separated from the cellular debris in the bottom of the tube and it was placed inside a new Eppendorf tube. This operation was repeated for a total of three steps (15 min overall). The

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volume of the elution solution was determined as a function of the total amount of NF on the cotton swab (3 μ L or 30 μ L *per* mg of weighted sample). The procedure was repeated three times, the eluted solutions were pooled, protein concentration was determined by Bio-Rad Protein Assay Kit and the resulting pool was divided into aliquots and a part was supplemented with PIC in a 1:100 v/v ratio. All the aliquots with and without PIC were stored at -80°C , except for the samples immediately analyzed by MALDI-TOF MS.

2.5 Samples normalization

The concentrations of eluates from nasal swabs from each donor patient, ranged from 0.6 to 2.8 $\mu\text{g}/\mu\text{L}$. A total amount of 75 μg of unfractionated protein from each clinical sample was either concentrated (by vacuum centrifugation) or diluted (by the addition of deionized water) to 30 μL , in order to obtain a final concentration of 2.5 $\mu\text{g}/\mu\text{L}$ for further processing described below.

2.6 NF peptidome enrichment by MPS.

NF eluate from swabs was incubated with MPS for 1 h at room temperature. The beads were subsequently separated from the supernatant by centrifugation, and the washing step was performed according to the protocol optimized for each bead functionality (see Fig. 1E and the next subsections).

MCM-41. For MCM-41, hexagonal silica mesostructured, the following protocol was performed.

Aliquots (5 mg) of beads were mixed with 30 μL of NF eluate and shaken at room temperature for 1 hour. The suspension was centrifuged at $2000 \times g$ for 2 min, and then MPS were separated from the

supernatant and washed with 30 μ L of 0.1% TFA. After the wash, bound species were extracted in 30 μ L of a 1:1 (v/v) ACN and 0.1% TFA solution.

HMS. For HMS, wormhole silica mesostructured, we used the following protocol. Aliquots (2 mg) of HMS were mixed with 30 μ L of NF swab eluate and shaken at room temperature for 1 hour. The suspension was centrifuged at 2000 g for 2 min, and then HMS particles were separated from the supernatant and washed twice with 0.1% TFA (20 μ L). After the last wash, species retained on silica surface were extracted in 30 μ L of a 1:1(v/v) mixture of ACN and 0.1% TFA.

MPS-A. For MPS-Aliquots of MPS-A (2 mg) were mixed with 30 μ L of NF eluate sample and shaken at room temperature for 1 h. The suspension was centrifuged at 2000 $\times g$ for 2 min, and then the MPS were separated from the supernatant and washed with deionized water ($2 \times 30 \mu$ L). After the last wash, adsorbed peptides were extracted into 15 μ L of a 1:1 (v/v) ACN and 0.1% TFA solution.

MPS-C. For aminopropyl functionalized MPS (MPS-C), aliquots (2 mg) of particles were preconditioned with 50 μ L of 25 mM Tris HCl at pH 8.8 for 5 min. Mesoporous silica were then mixed with 30 μ L of NF and shaken at room temperature for 1 h. The suspension was centrifuged at 2000 $\times g$ for 2 min, and then the particles separated from the supernatant were washed with 25 mM Tris HCl at pH 8.8 ($2 \times 30 \mu$ L). After the last wash, the bound species were extracted into 20 μ L of a 1:1(v/v) mixture of ACN and ammonium formate (100 mM, pH=3).

2.7 MALDI-TOF MS Analysis.

We performed exploratory experiments on matrix-sample preparation, in order to increase the analytical performances and the robustness of the results. 1 μ L of the solution containing the bound

peptides was mixed with 4 μ L of 4 mg/mL SA solution (50/50 ACN/0.1% TFA) and 1 μ L of the obtained mixture spotted on the MALDI target plate and analyzed by MALDI-TOF MS.

MALDI-TOF mass spectra were acquired using a AB SCIEX MALDI-TOF/TOF 5800 System (ABSciex, Framingham, MA, USA) equipped with a diode-pumped, Nd:YLF laser with $\lambda = 345$ nm wavelength.

Mass spectra were acquired both linear and reflector mode. For MALDI MS measurements in SA the following settings were applied: bin size was set at 4 ns, final detector voltage was 2.070 kV with multiplier value at 0.75; 2000 laser shots were accumulated for each spectrum. MS data were calibrated via external calibration using the 5800 Mass Standards kit (AB SCIEX, Framingham, MA, USA) containing insulin bovine (MH^+ 5734.59), thioredoxin (MH^+ 11674.48), and horse apomyoglobin (MH^+ 16952.56).

In order to enhance the reproducibility of the peaks in the profiles generated, all of our samples were processed and analyzed on the same day, in the same experimental session to minimize inter-day variation of the peak intensities.

For data acquisition and processing, Data Explorer version 4.11 software (AB SCIEX, Framingham, MA, USA) was used. Each spectrum was baseline corrected, a smoothing was applied and manual calibration was performed. The peak detection criteria used were a minimum S/N of 10 with a centroid percentage of 50%. Normalization of all spectra to their own total ion count was performed.

Spectra peak lists from Data Explorer, containing centroid masses, heights, relative intensities, areas, S/N, for the m/z ranging from 2 to 20 kDa, were exported as pkt files. The alignment was performed by an in-house software conceptually developed for finding, for each observed spectrum, a warping

function in order to synchronize all spectra before applying statistics. In particular our algorithm used high-abundance proteins (usually present in all samples) to identify the warping function, then use the warping function to compute the shifts associated with low-abundance proteins. Another advantage of high-abundance proteins is their high S/N which makes the alignment result much more reliable compared to that of small peaks. Therefore, these large peaks can be used as the calibration peaks to estimate the warping function accurately as also described by He *et al.* [36]. In order to confirm the consistency of the spectra alignment, manual alignment in Excel (Microsoft Excel 2003) data sheets was also carried out.

2.8 Statistical analysis across groups

Data from H, AR and NAR groups, once pre-processed by the already described in-house software were subsequently analyzed for differential peptide patterns. In particular, for statistical analysis, peaks with a similar m/z value across all spectra (within 0.1%) were considered the same. The m/z values representing the peaks were filtered by first applying an Anderson-Darling test to assess the distribution of peak areas. Significance in the change in average peak area was then evaluated using the two-tailed Student's t-test (Anderson-Darling $P > 0.05$ = normal distribution). Significance was set at a p value of less than 0.05 [37].

3 Results and discussion

High-throughput MS-based profiling strategies for clinical samples such as tissue and bodily fluids are widely recognized as a valuable research tool for biomarker discovery. MALDI-TOF and SELDI-TOF have recently captured significant attention for the ability of screening with high-

throughput small amounts of clinical samples. Like the proteome, the so-called low-molecular-weight proteome, or peptidome, may also have the potential to contain disease-specific information [38]. Both MALDI-TOF and SELDI-TOF are very versatile for the detection of endogenous and naturally occurring fragments of proteins. In particular by MALDI-TOF/TOF platform adopted in our study it is also possible to achieve the structural characterization of peptides of interest by MS/MS fragmentation.

3.1 Nasal swabs procedure for peptidomics: the rationale

The study workflow shown in Fig. 1A outlines the main steps of our procedure for the rapid displaying of nasal peptidome profiles of normal, allergic and non-allergic-matched rhinitis cases. Our strategy was to interrogate by MALDI MS a specimen which specifically reflects the local pathophysiology of nasal mucosa, in our case the fluid covering the surface of the inferior turbinate based on the rationale that in general the proximal fluids, being much closer to the inflamed sites, may be enriched with cell secreted and/or shed proteins relevant for the disease. To the best of our knowledge only few investigations are present in literature about the NF protein composition in the turbinates [39]. The greatest of specific literature is focused on NLF [20, 21, 40-43] or nasal secretions [23, 25, 44-46] and nasal epithelium or nasal mucosa [17, 24, 47, 48]. The inferior turbinate plays a pivotal role in the air conditioning activity of the nasal cavity (http://care.american-rhinologic.org/nasal_anatomy; last update: 01/20/2015).

As showed in Fig. 1B, we collected the fluid by the mean of a nasal swab which is a practical device easily to manage and well tolerated also from younger patients in comparison to the NLF collection which resulted a little more invasive. Additionally, nasal swab collection is common standard

practice in microbiology and toxicology for the analysis of inflammatory biomarkers of NF such as cytokines [49]. As shown in Fig. 1B, the swab is inserted approximately in the middle portion of the inferior turbinate. The use of alternative devices such sinus pack, was excluded based on the difficulties which may occur in cases of anterior septal deviation or narrow *vestibulum nasi* [50].

Although the nasal swab collection from the inferior turbinate might appear less representative of the respiratory tract for the study of lung pathologies, in comparison to the NLF this procedure overcomes the major drawbacks of NLF collection, which suffers from high amounts of blood serum proteins [21, 51]. Additionally, the use of hypertonic saline in NLF as washing solution if on one hand yields enhanced protein concentrations in NLF samples on the other leads to intense irritation of nasal mucosa and therefore to falsified results [52, 53]. We believe that soft swabbing, as adopted in our study, constitutes a simple tool for representative NF which can prevent vascular trauma and consequent contamination.

3.2 A MPS-based profiling strategy: selectivity and reproducibility

Recently, we have described the combined use of MPS and MALDI-TOF MS for the selective binding and enrichment of plasma endogenous peptides [26], procedure that has also been applied to human urine [27], human induced sputum [29] and human synovial fluid [30]. MPS are able to “capture” peptides present in human body fluids as a result of a variety of interactions and a cut-off mechanism by which only small-sized molecules, such as peptides that are able to diffuse into the mesoporous channels (nanometric in size), are harvested. The same mechanism also excludes from the adsorptive process larger-sized proteins that, if abundant, compete with desorption/ionization process and could mask and/or suppress peptidome signals. In the present investigation we test the

efficiency of this platform to enrich the precious and still underrepresented NF peptidome. We selected four different MPS (MCM-41, HMS, MPS-A and MPS-C) characterized by different pore size and surface area (see Supporting Information Table S1). However, the use of cotton swab devices requires an efficient extraction methodology to maximize sample recovery prior to MPS enrichment and MALDI-TOF MS analysis. In this case, highest protein recovery was obtained when the elution from the cotton swab device was performed in hypertonic saline solution by a three-step centrifugal elution procedure fixing the time to 5 min for each step to achieve a good compromise in terms of protein recovery (three steps guarantee a better protein recovery than one step only, data not shown.) and rapidity of protocol.

In order to ensure to our protocol a better yield in protein recovery, different volumes ratios (3 or 30 μ L of elution volume *per* 1 mg of swab content) and different saline compositions (30% hypertonic saline and 0.9% physiological solution) were tested. The use of 30 μ L of hypertonic saline 30% *per* 1 mg of swab content gave 55% higher yield of protein recovery as compared to the use of 30 μ L of 0.9 % sodium chloride solution (see Supporting Information section S2 and Fig. S1).

The nasal swab eluate was processed using the four different MPS according to the steps illustrated in Fig. 1E: adsorption, separation, washing, and extraction.

In the first step, MPS were incubated with equal volumes of NF eluate and with the same total protein content. During this step, the MPS act as sponges: only peptides and proteins with sizes smaller than or comparable to the pore size are adsorbed into the mesoporous channels. Moreover, given the specific charge and chemical group present on the mesoporous surface, the adsorption process will also be driven by the electrostatic interactions [27]. Typically, the adsorption of body fluid peptides into MPS can often change with pH. Thus, in a neutral solution, MPS-C containing

primary amine groups (pKa around 10) retain a positively charged surface, which can adsorb negatively charged peptides. Different adsorption pH values were also tested in order to ensure that the maximum number of peptides were selectively captured from NF. We found that, with the exclusion of MPS-C, better working conditions (in terms of MALDI-TOF peak number detection) for all MPS were neutral. For MPS-C containing primary amine groups, optimal adsorption conditions were obtained at pH=9, rather than under neutral conditions (data not shown). In our previous experience with MPS bodily fluid protocols, we found that the separation and washing steps were critical in order to ensure good quality MALDI spectra. Separation of the beads from the supernatant requires gentle centrifugation in order to avoid diffusion of adsorbed peptides. At the same time, washing needs to be as fast as possible. Since we use an hypertonic saline as working solution, the washing step of the MPS after the separation from the working solution is crucial in order to eliminate salts, which can interfere with MALDI-TOF MS analysis. At the same time, too many washes should be avoided due to the risk of sample loss. As described in Materials and methods section, the best compromise was found when the number of washes did not exceed three.

Representative examples of the NF profiles obtained after processing with different MPS, are shown in Fig. 2. The use of MPS appears to provide sufficiently rich fingerprints of NF, as revealed by comparing the mass spectrum of NF eluate from cotton swab (Fig. 2A) to mass spectra generated after MPS processing (Fig. 2B-E and Table 1 and Supporting Information Table S2). All MALDI spectra were acquired in the m/z range from 800 to 20000 and the number of the peaks detected with $S/N > 10$ in this range for NF from one H subject processed with the various silica, are reported in Table 2. These data for a subject suffering of NARNE are shown in Table S2 (Supporting Information). For all kind of MPS, most of the signals were detected in the range from 2000-6000 exhibiting also a good coverage in the lower m/z range from 800 to 2000, while, with the only exception of MPS-C, no

peaks or some sporadic peaks were detected over 6000 Da. Horizontal arrows in the highlighted area of Fig. 2 show the progressive molecular mass increment of enriched peptidome from MCM-41 (Fig. 2B) up to MPS-C (Fig. 2E). In order to demonstrate the efficacy of our platform in performing exclusion by molecular cutoff, we purposely show in Fig. 2 A one of the very few sample characterized by a slight blood contamination. Vertical arrows in panel (B-E) highlight the exclusion by molecular cutoff of Hemoglobin α and β chains. No peaks at m/z 15000 and at higher m/z were detected after processing with MPS, indicating, as expected, the selectivity of MPS toward low molecular weight peptides. Among all bead types, aminopropyl-derivatized MPS-C provided the best performance with 89 total peaks detected. However, due to the biological variability, the number of detected peaks were different among subjects analyzed (see Table S2). We speculated that the higher capacity of MPS-C over the other silica particles was the results of a good combination between the pore size (roughly 4 nm) and the surface area (270 m²/g). The comparison of the peaks yield between the unprocessed and the MPS extracted samples, for all the subjects analyzed, indicated that our platform was able to amplify the NF peptidome, increasing by more than two-fold the number of molecular species in the m/z range from 800 to 2000 and by more than four- up to eight-fold the number of peptides in the middle range from 2000 to 6000 compared to the control sample. As an example, data referring to single subjects are shown in Table 2 for an H subject and in Table S2 (see Supporting Information) for a patient suffering from NARNE. MCM-41 showed the most intense peaks in the lower m/z range from 800 to 2000 (Fig. 1), thus demonstrating its high selectivity for the lower molecular weight peptidome of the NF. Satisfactory peak distribution of NF peptidome was obtained in the medium m/z range (from 2000 to 6000) when the extraction was performed by the mean of HMS showing for this range higher S/N peaks in comparison to the other MPS (see Fig. 3). Interestingly, MPS-A and MPS-C showed in this range almost the same selectivity

with about 90% of common shared peaks (Fig. 3); however, these two silica, which differ for pore size distribution and surface area and functionalization (see Table S1), differentiated their selectivity in the upper m/z range. In fact, an increment of over seven fold in peak number for MPS-C in comparison to MPS-A was detected in the range from 6000 to 20000; while no significant variation was observed in the overall analyzed range (Table 2 and Table S2). Several adjustments were performed in order to ensure optimal mass signals reproducibility, which in protein profiling comparative studies by MALDI-TOF or SELDI-TOF, are of extreme importance [54, 55]. Therefore, analytical variables related to instrument settings and sample preparation (MALDI matrix composition, sample dilution in MALDI matrix solution, sample spotting on target plate) for MALDI-TOF MS analysis were optimized. The reproducibility of the mass spectra was evaluated with respect to the peak intensities, S/N and area variability measured by CV. Tables 3 lists CVs of several peaks randomly selected over the whole mass range. We observed similar reproducibility among the different MPS particles with CVs ranging from 2 to 25% for the peak height and from 1 to 28% for the peak area. A low variability comparable to those of peak height and peak area was also observed for the S/N ratio (2 to 29%). Overall, these data reveal a satisfactory analytical reproducibility, suggesting that these peptide peaks could be part of a diagnostic profile for correctly evaluating biologically significant differences between disease-specific groups.

3.3 Differential display analysis of AR, NAR and H: a proof of concept

In order to demonstrate the feasibility of our MPS-MALDI-TOF differential display analysis to discriminate between AR, NAR and H, we performed an initial study on a small cohort of normal

individuals (n=7), AR subjects (n=8) and from subjects suffering from vasomotor NAR with different cytological profile (n=7).

The clinical characteristics of these subjects are shown in Table 1. Before performing MPS enrichment, we normalized the total protein content as reported in material and method section. For this comparative study, we selected HMS and MPS-C because these MPS particles showed the best performance in the lower-medium (800-6000) and in the whole mass range (800-20000) respectively (Table 2, Table S2 Fig. 2 and Fig. 3). Typical MALDI-TOF mass spectra of the NF extracted by MPS-C and HMS from a H individual and subjects suffering from AR and NAR subtypes groups are illustrated in Fig. 4 and 5 respectively. In particular, in Fig. 4 are shown together with the “rhinomic” fingerprints in the m/z range from 3000 to 16000, the rhino-cytograms obtained from the same healthy (Fig. 4F), AR (Fig. 4G), NARES (Fig. 4H), NARMA (Fig. 4I) and NARNE (Fig. 4J) subjects.

Different group comparisons were assessed in order to perform a profiling analysis of the NF peptidome and to obtain statistical patterns of putative discriminant biomarkers. Considering that we always compare NF from healthy subjects against NF of diseased subjects enriched with the same MPS, the fact that different volumes and solutions are used for different MPS does not affect the results we obtained. From this preliminary analysis a pattern of four m/z peaks was found to differentiate NAR versus AR group while two peaks discriminated the AR from the H group. Finally, one peak was significantly different between the NAR and the H group. Fig. 6 shows the normalized peak area for each group together with the comparison between groups and the statistical significance of the difference.

Interestingly, all the m/z peaks discriminating NAR from AR (m/z =3298, 3460, 4633 and 4712), were identified as statherin fragments (Supporting Information Table S3 and Fig. S2) and were found

increased in the NAR group compared to AR group. Additionally, the peak at $m/z=4712$ also resulted to be decreased in the AR group with respect to the control group (H), regardless of the kind of silica used for the selection (Fig. 6D and Fig. 6E). We found only one peak ($m/z=14692$) significantly increased in the AR group in comparison to the H group, which was identified as Lysozyme C (Fig. 6F and Supporting Information Table S3, Table S4, Fig. S3 and Fig. S4). Finally, the peak at $m/z= 3298$ was significantly increased in the NAR vs the H group. Of note, four peaks were selected by HMS ($m/z= 3298$, $m/z= 3460$, $m/z= 4633$ and $m/z= 4712$) and two peaks ($m/z =4712$ and $m/z =14692$) were selected by MPS-C. We previously observed that HMS was able to select specifically lower peptidome range in comparison to MPS-C (see Table 2). In line with the previous general observation, of the five significantly different peaks, three with a lower MW were selected by HMS, one with the highest MW was selected by MPS-C and one with an intermediate MW ($m/z=4712$) was selected by both materials.

Interestingly, although four fragments derived from statherin were found to be significantly increased in the NAR over the AR group, we detected also the intact peptide ($m/z=5380$) which, instead, did not evidence any significant change between any of the analyzed groups.

Originally identified in parotid and submandibular- sublingual secretions, statherin is a molecule expressed in submucosal glands. It plays an important role for oral health by protecting tooth enamel through lubrication and in the maintenance of mineral homeostasis and, in the adsorbed state, by contributing to the early phases of microbial colonization [56]. Statherin is also expressed in nasal secretions and together with other proteins such as secretory phospholipase A2 and defensins likely contribute to their total antimicrobial properties [57]. So far, very few investigations appeared in the literature describing how statherin changes at the protein level as a function of a respiratory tract pathology as compared to H controls [21, 22, 58].

In particular, in a very recent study, in which nasal mucus was collected in and out of pollen season from AR patients (n=10) and from H controls (n=12) and was analyzed by LC-MS/MS, a trend towards higher statherin concentration in AR with respect to H groups was found out of pollen season [44]. This finding appears in disagreement with our investigation. However, the following considerations should be taken into account: first, the increase observed by these authors [44] appears not to be statistically significant; second, as stated above, in our study we found no significant changes between AR patients and H controls not only for the full length peptide ($m/z=5380$), but also for the fragments with $m/z=3298$, $m/z=3460$ and $m/z=4633$, (Fig. 6); third, only three tryptic fragment of statherin were found to contribute to the mean percentage AUC used for comparative analysis between the two groups, leaving not determined whether the difference measured reflects the different amount of the full length species or the differential expression of endogenous fragments generated *in vivo* prior to tryptic digestion [44]. In fact, a bottom-up LC-MS/MS approach fails to address the levels of the statherin endogenous fragments which, by using a top down MALDI-TOF platform, we found differentially expressed as compared to the full-length peptide.

Statherin changes as a function of various pathologies were studied also at gene expression level [59-61]. Both protein and gene expression level data reported so far were contradictory, probably due to differences in the enrollment criteria and in patients classification and to the different techniques used. Moreover, to the best of our knowledge, no study of the upper airways appeared so far was able to address the levels of the various statherin fragments which we found differentially expressed as compared to the full-length peptide. It is worthwhile to underline that in previous studies different fragments of statherin were detected in saliva with a probably (not confirmed) presecretory origin, because directly collected from parotid or submandibular/sublingual gland ducts [62].

Although we detected intact statherin ($m/z=5380$), in this study only four fragments derived from this protein were found to be significantly increased in the NAR over the AR group. Therefore, we hypothesize a possible role of statherin proteolytic fragments in the rhinitis pathophysiology. This speculation, which warrants further investigations, appears not totally isolated. In fact, a synthetic fragment of statherin (Stath fr. 6–21) was found to elicit broad-spectrum antimicrobial activity against oral pathogens, whereas the antimicrobial activity of the intact statherin was minimal (Raj, P. A., Antonyraj, K. J., 79th Gen. Sess. IADR, June 27–30 2001, <http://iadr.confex.com>).

Another consistent finding of this study was that our platform is able to detect significant overexpression of lysozyme in AR vs H controls (Fig. 6F)

Lysozyme is the most abundant antimicrobial polypeptide of airway fluid. It is stored in and secreted by serous cells in submucosal glands and it is also secreted by eosinophils and neutrophils present in inflamed secretions [63]. In particular, electron microscopy investigations of inferior turbinate tissue identified lysozyme in serous cells from glands of the nasal mucosa [64] and also in the goblet cells of the nasal epithelium. Lysozyme hydrolyzes glycosidic bonds in the peptidoglycan cell wall of bacteria, thereby determining enzymatic lysis, and it is highly effective against many common upper airway gram-positive bacteria, such as streptococci [65]. It acts as antibacterial agent non only via its enzymatic activity but also as a cationic protein [66]. Moreover, lysozyme is normally associated with the monocyte-macrophage system and enhances the activity of immunoagents [67].

Lysozyme overexpression, which we observed in AR group versus H group, seems to be consistent with the results of Yuta *et al.* [68]. In particular, these authors found a trend towards higher lysozyme concentration in NLF of AR with respect to H groups. In another study by Kalfa *et al.*, patients with perennial AR showed a reduced concentration of lysozyme compared to controls,

however the difference was not significant ($P>0.05$) [69]. This apparent discrepancy with our results can be easily reconciled as the perennial AR patients enrolled by Kalfa *et al.* were using topical corticosteroids. On the contrary, all the subjects enrolled in our study were asked not to use oral/nasal antihistamines and intranasal corticosteroids within 10 and 20 days prior to nasal swab collection, respectively. Indeed, there is evidence that topical corticosteroids suppress cellular infiltrates in the edematous nasal tissues [70] and the release of inflammatory mediators [71].

For this reason, we decided to adopt, among the exclusion criteria, the use of topical corticosteroids prior NF swab collection in order to rule out a potential bias in the identification of pathology specific biomarkers caused by the response to the pharmacological treatment.

Interestingly, Tomazic *et al.*, in an above cited investigation on nasal mucus, found a not significant increase in the expression of lysozyme in the AR group towards the H group out of the pollen season [44]. Despite the previously described limitations of the bottom-up LC-MS/MS platform, such finding can be considered reliable because of the high number of detected tryptic lysozyme fragments and of the absence of endogenously occurring lysozyme fragments, contrary to what has been observed by us and by others for statherin [62].

The observation that we found lysozyme levels significantly higher in AR patients compared to controls is particularly relevant considering that NF was collected in the absence of active rhinitis as described in the materials and methods (2.2 section). Therefore, it is highly unlikely that the increased lysozyme levels may be due to an active immune response against bacterial infections. It is well known that lysozyme is an index of serous glandular secretions [72, 73]. It can thus be speculated that allergic subjects may display hypertrophy or hyper-activation of the nasal

submucosal glands. This hypothesis and its eventual contribution to the increased response to the antigens observed in allergic subjects warrants further investigations.

Interestingly, we observed a not significantly increased lysozyme expression in the NAR group towards the H controls. The absence of statistical significance can be attributed to the evident differences in the quali-quantitative expression pattern which were observed in the NAR group despite a significant overlap of common peaks (like peaks with $m/z=4712$, 4965, 5232 and 5380 see Fig. 4C-E). In fact, the lysozyme C (see peak at $m/z=14692$ Fig. 4) was shared by all subjects (Fig. 4A-E) but this peptide was dramatically overexpressed in the only subject suffering from NARMA (Fig. 4D and Table 1) and almost absent in our patients with NARES and NARNE (Fig. 4 C).

Even more evident, peak at $m/z=10761$, identified in other investigations as β -seminomicroprotein [57] is totally absent in 2 out of 3 spectra derived from patients diagnosed with NARNE (one of which is shown in Fig. 4, panel E), while peak at $m/z=11711$, identified in other investigations as antileukoproteinase [57] is detected at high levels only in spectra derived from the patient diagnosed with NARMA (Fig. 4, panel D). Owing to these individual differences, maybe correlated with the specific subtype of NAR, these peaks were not found significantly changed in the NAR group as a whole when compared against the H or the AR groups. However, by increasing the sample size, there is the possibility that they may emerge in the future as biomarkers able to discriminate a NAR phenotype from the other.

Indeed, our group is working in this direction by enrolling a higher number of NAR patients in order to overcome this limitation. Although only exploratory results, these preliminary findings demonstrate the potentials of an high-throughput screening MS-based platform in discovering key

peptidic-biomarkers able to distinguish different vasomotor NAR sub-phenotypes by using nasal swab collection which in near future may be adopted in standard clinical settings.

We are aware that the relatively small sample size is a limitation of this study, whose main focus is to demonstrate that the combined use of MPS and MALDI-TOF MS allows the rapid detection of nasal peptide profiles. However, as we continue the current study to include more patients, we expect that the specific peptide fingerprints able to differentiate the different NAR subtypes can be appropriately adjusted, optimized, and validated. On the other hand, the rhino-secretions originating in proximity of the turbinate makes our nasal swab collection extremely specific as an optimal source of biomarkers for both NAR and AR.

Therefore, all the listed drawbacks do not decrease the importance of collecting molecular panels or *rhinomic* suits of peptides into clinical databanks for the diagnosis and treatment of the various NAR subtypes.

4 Conclusions

Change in protein expression may be associated to the pathobiology of a disease. The possibility to capture variations in the molecular profiling of fluids proximal to the specific target tissue both in normal and in pathologic conditions may be useful to delineate the pathobiology of the disease.

We have described a rapid procedure for displaying differential patterns of endogenous peptides in the NF of H, AR and NAR subjects. In the present study, we propose a biomarker discovery strategy based on NF collected from the inferior turbinates by the mean of nasal swab devices. The nasal swab collection has the advantage of being a non-invasive diagnostic procedure compared to NLF

collection. The protocol is based on the combined use of MPS and MALDI-TOF MS. Depending on the pore diameter and on the chemical groups of the mesoporous surface, complex peptide mixtures were extracted from NF collected as nasal swab and converted into reproducible MALDI-TOF fingerprints. As a demonstration of feasibility of our platform, we compared the *rhinomic* fingerprints generated from each subject of H, AR and NAR groups. Different panels of nasal peptide repertoires have been obtained, suggesting that they could be part of a possible profile for AR/NAR differential diagnosis.

These preliminary findings may also contribute to defining a high-throughput screening MS-based platform for monitoring key peptidic-biomarkers able to distinguish different vasomotor NAR subtypes in NF samples.

Finally, we also auspicate that the *rhinomic* fingerprints extrapolated by our tool might in a future be able to reveal a distinctive pattern of molecular features correlated by specific relative expression in each analyzed subject and might possibly provide a more practical route to personalized treatment.

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Figure Captions

Figure 1. (A) Experimental workflow of the study. Peptidome profiling steps are outlined from collection of nasal fluid samples to enrichment by MPS before MALDI-TOF analysis. (B) A rhinoscopic image of the left nasal nostril showing the collection of NF by nasal swab. The swab is inserted approximately in the middle portion of inferior turbinate. Then the swab is gently rotate against inside of nostril for 5 seconds. A light pressure is applied on the outside of the nose while rotating swab. (C) The tip of the cotton swab containing the collected NF is cut and placed into the filter unit of the microspin tube for further processing. (D) An image showing the elution step for the recovery of the soluble fraction of NF collected on the cotton swab. The zoom displays the filter unit in the upper side of the device used for the centrifugal elution. The filter unit has an integral perforated bottom that acts like a strainer and allows fluids to pass but retains small cloth samples in the filter unit. (E) Illustration of the four steps MPS procedure for NF peptidome enrichment.

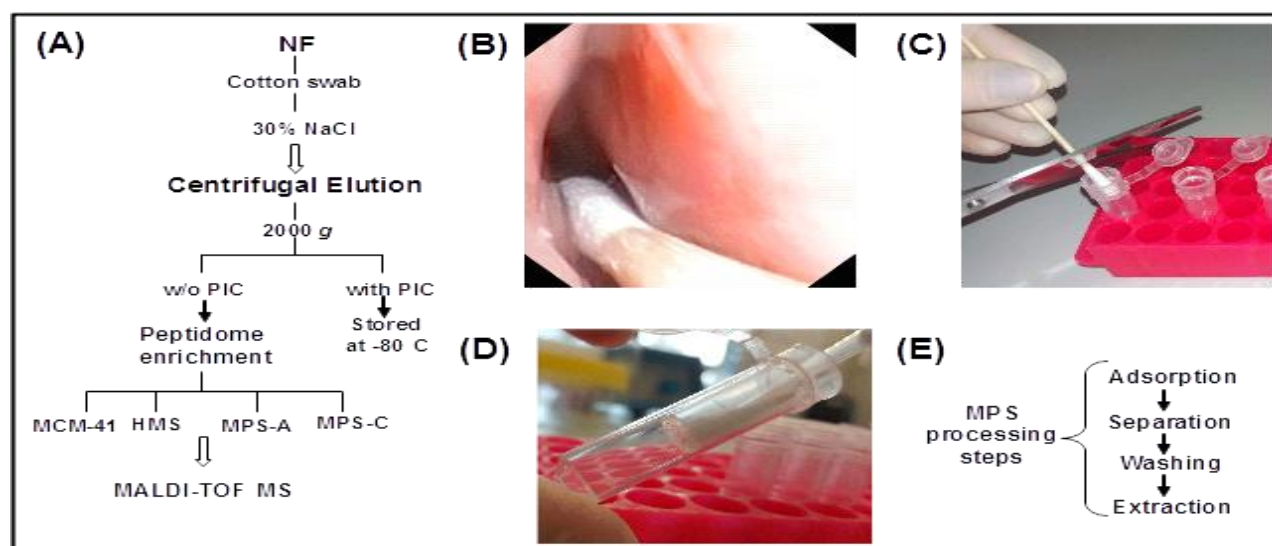


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Figure 2. MALDI-TOF mass spectra of NF before (A) and after processing by MPS MCM-41 (B), HMS (C), MPS-A (D) and MPS-C (E). NF from one H participant to the study, was collected with nasal swab and 30% of sodium chloride was used as eluent (A). The eluate was then incubated with the different MPS particles for the selective enrichment of nasal peptidome. Horizontal arrows in the highlighted area show the progressive molecular mass increment of enriched rhino-peptidome from MCM-41 (B) up to MPS-C (E). Vertical arrows in panels (B-E) underline the exclusion by molecular cutoff of hemoglobin α and β chains detected in the control sample. MALDI measurements were performed in SA and the spectra were recorded in linear mode in the m/z range 2000-20000.

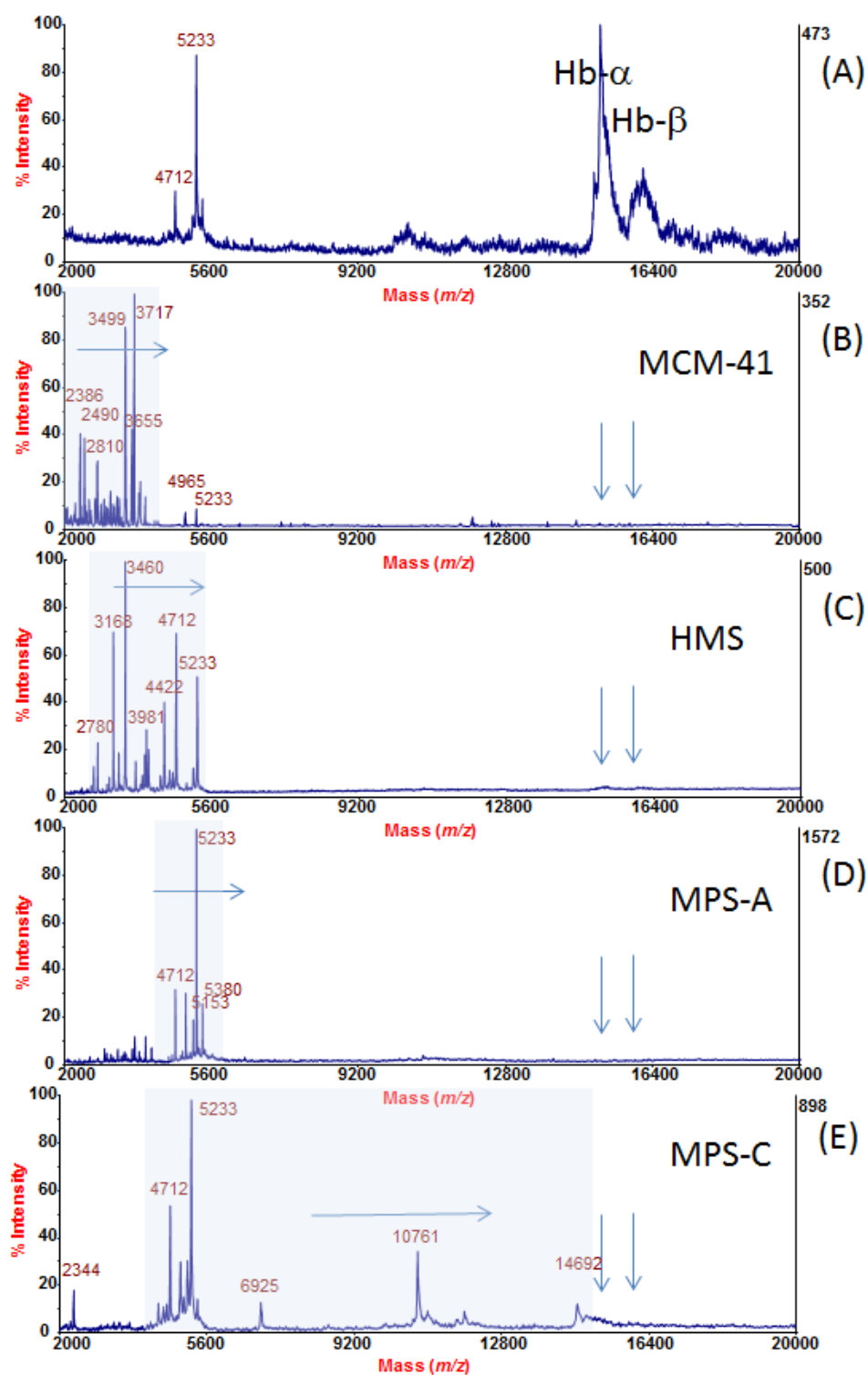


Figure 3. MALDI-TOF MS spectra of MPS-enriched nasal swab peptidome. The zoom in the m/z range from 2000 to 6000 highlights the different selectivity of the MPS particles towards the same sample of NF. MALDI measurements were performed in SA and the spectra were recorded in linear mode in the m/z range 2000-20000.

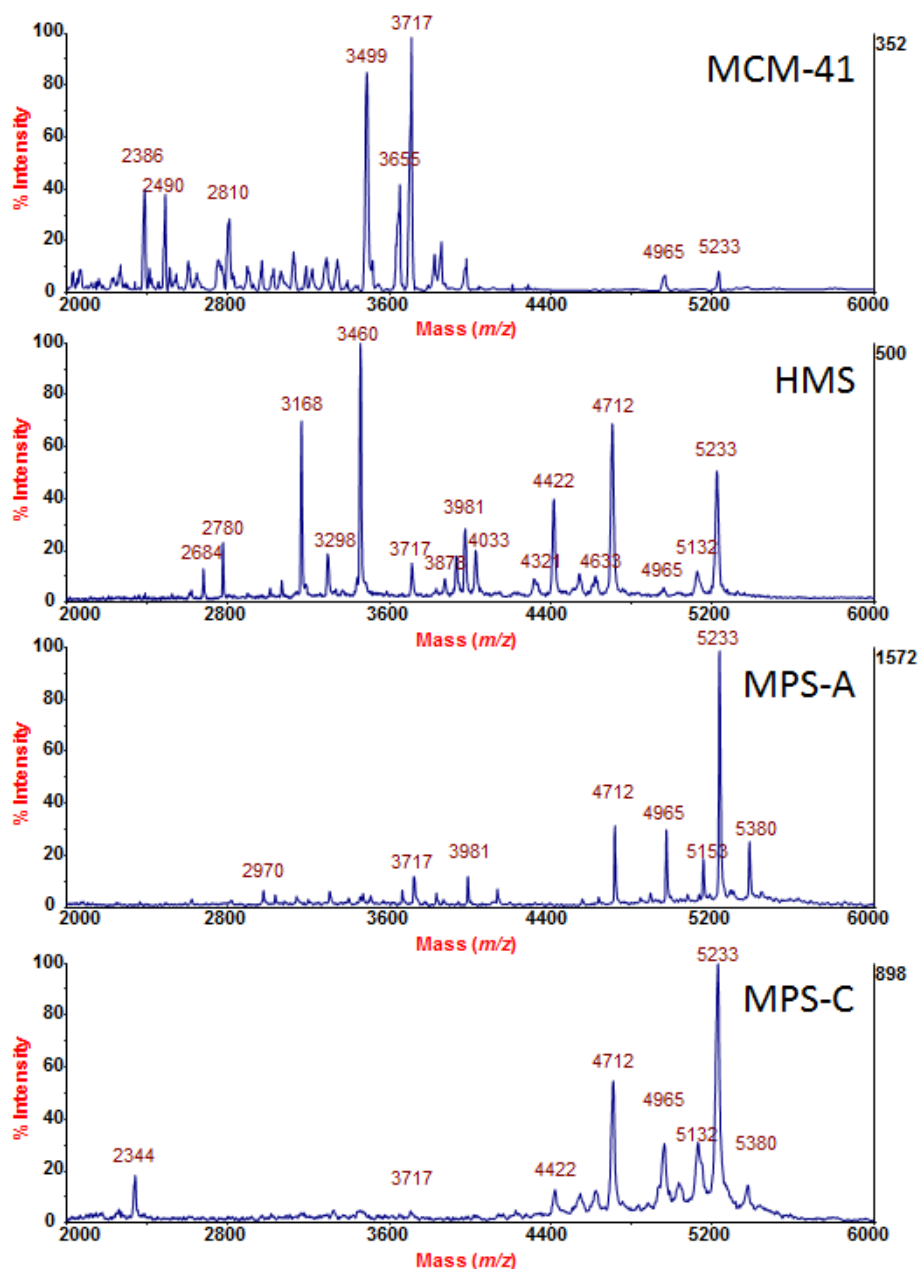


Figure 4. MALDI-TOF (left side) and cytological (right side) analysis from one H and four different rhinitis subjects. Rhino-peptidomic fingerprints selectively extracted by MPS-C from the H individual (A) and from subjects suffering from AR (B), NARES (C), NARMA (D), NARNE (E). The spectra, recorded in SA, are shown in the m/z range from 3000 to 16000. Rhino-cytograms from the same H (F), AR (G), NARES (H), NARMA (I) and NARNE (J) subjects.

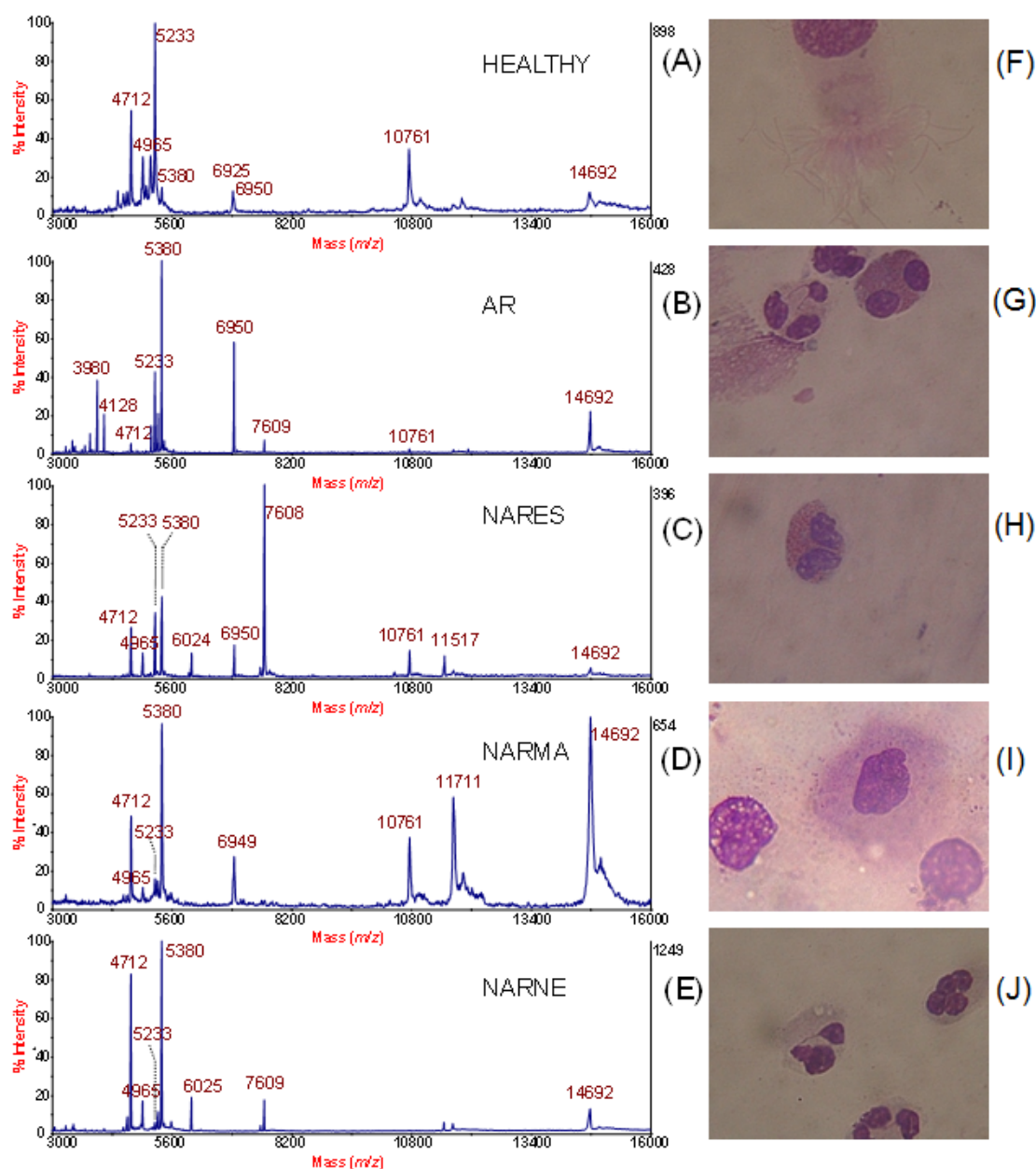
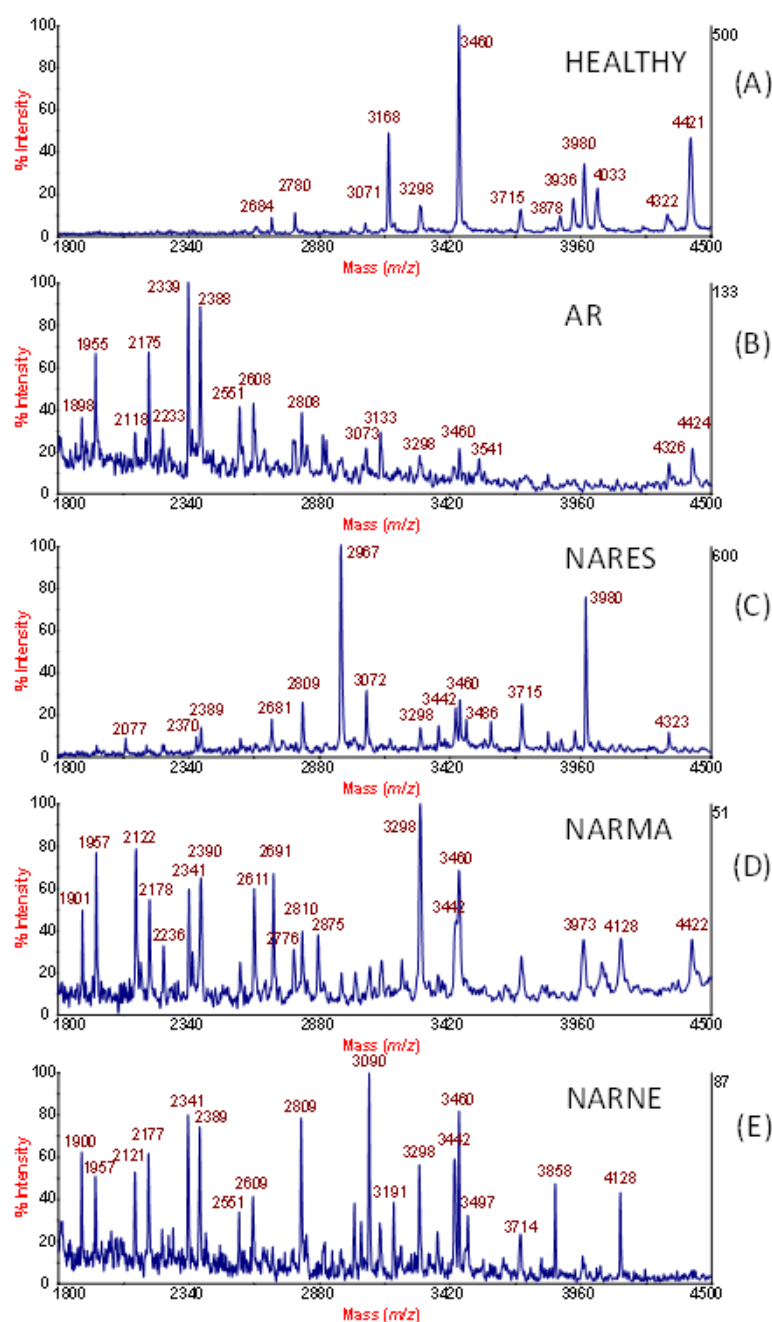


Figure 5. Rhino-peptidomic fingerprints selectively extracted by HMS from one H individual (A) and from subjects suffering from AR (B) NARES (C), NARMA (D), NARNE (E). The spectra were recorded in SA and were acquired in a m/z range from 800 to 20000. The zoom from 2000 to 6000 better highlights the selectivity performances of HMS in this m/z range.



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Figure 6. Normalized peak area for each group, comparison between H, AR and NAR groups and level of significance between groups. The *p* values were calculated with paired *t*-test on normalized peak area; **p* values < 0.05, ***p* values < 0.01.

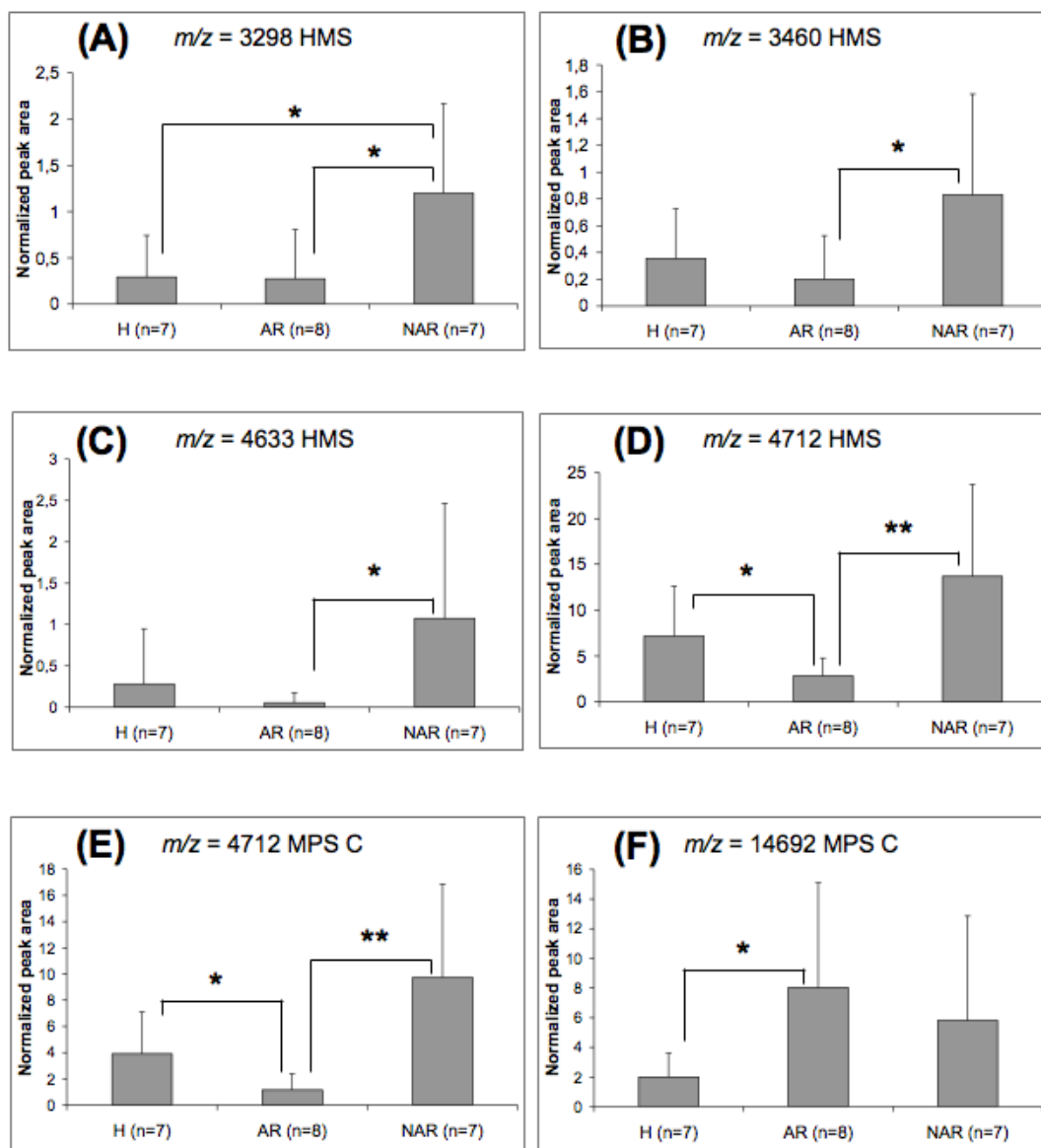


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Table 1. Clinical characteristics of the subjects enrolled in the study.

Subject code ^{a)}	Gender	Age	Skin Prick test	Nasal Cytology ^{b)}	Diagnosis ^{c)}	NP ^{d)}	Asthma
H1	M	38	Neg.	Neg.	Neg.	No	No
H2	F	30	Neg.	Neg.	Neg.	No	No
H3	F	17	Neg.	Neg.	Neg.	No	No
H4	F	28	Neg.	Neg.	Neg.	No	No
H5	F	23	Neg.	Neg.	Neg.	No	No
H6	F	19	Neg.	Neg.	Neg.	No	No
H7	F	19	Neg.	Neg.	Neg.	No	No
AR1	M	47	<i>Olea Europaea, Parietaria, Graminaceae mix</i>	E++ N+++ M+	AR	No	No
AR2	F	38	<i>Epithelial dog, Dermatophagoides mix.</i>	E+ N++ M++	AR	No	No
AR3	F	33	<i>Epithelial dog, Graminaceae mix</i>	E++ N++ M++	AR	No	No

AR4	M	32	<i>Olea Europaea,</i> <i>Graminee mix,</i> <i>Alternaria Alternata,</i> <i>Cupressus Arizonica,</i> <i>Dermatophagoides</i> <i>mix, Epithelial cat</i>	E+++ N+++ M++	AR	No	No
AR5	M	51	<i>Graminacee mix,</i>	E++++ N+++ M++	AR	No	No
AR6	M	39	<i>Graminacee mix,</i> <i>Parietaria,</i> <i>Epithelial cat</i>	E++ N++ M+	AR	No	No
AR7	F	51	<i>Dermatophagoides</i> <i>mix</i>	M+ E+	AR	No	No
AR8	M	22	<i>Parietaria,</i> <i>Olea Europaea,</i> <i>Graminacee mix,</i> <i>Alternaria Alternata,</i> <i>Compositae mix,</i> <i>Cynodon Dactylon,</i> <i>Cupressus Arizonica,</i> <i>Dermatophagoides</i> <i>mix, Epithelial cat</i>	M+	AR	No	No
NAR1	M	51	Neg.	E+++	NARES	Yes	Yes
NAR2	M	26	Neg.	E++	NARES	No	No
NAR3	F	30	Neg.	E++	NARES	No	No
NAR4	M	60	Neg.	M+++	NARMA	Yes	No
NAR5	M	16	Neg.	N+++	NARNE	No	No
NAR6	F	61	Neg	N++	NARNE	Yes	No

NAR7	M	23	Neg.	N++++	NARNE	No	No
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- a) H, Healthy; AR, Allergic Rhinitis; NAR, Non Allergic Rhinitis.
- b) E, eosinophils infiltration; N, neutrophils infiltration; M, mast cells infiltration.
- c) NARES, Non Allergic Rhinitis with Eosinophils, NARMA, Non Allergic Rhinitis with Mast cells; NARNE, Non Allergic Rhinitis with Neutrophils.
- d) Nasal polyposis

Table 2. Number of peaks detected for different MPS particles from NF of an healthy subject.

MPS	Number of peaks 0.8-2 kDa	Number of peaks 2-6 kDa	Number of peaks 6-20 kDa	Total peaks
NOT APPLIED	10	5	10	25
MCM-41	24 ± 3	36 ± 2	0	60
HMS	16 ± 4	22 ± 1	0	38
MPS-A	21 ± 5	41 ± 5	4 ± 1	66
MPS-C	25 ± 1	35 ± 10	29 ± 5	89

Mean number of peaks detected with $S/N \geq 10$ for three independent experiments on nasal fluid from the same subject. Mass spectra were acquired in sinapinic acid in a m/z range from 800 to 20000.

Table 3. Reproducibility assessment for signal intensity, area and S/N in acquired MALDI-TOF spectra from replicate analyses (n=3) of NF specimens processed by MPS.

MCM41				HMS			
<i>m/z</i>	CV% ^a Height	CV% Area	CV% S/N	<i>m/z</i>	CV% Height	CV% Area	CV% S/N
2386	9	17	8	3168	14	12	14
2490	4	8	10	3460	14	11	8
2810	4	15	9	3717	17	28	10
3499	5	13	8	3936	8	14	6
3655	8	19	9	3981	21	14	6
3717	7	4	7	4033	14	13	3
4712	18	3	2	4321	25	9	19
3981	24	21	23	4422	6	6	8
4965	17	25	11	4553	18	2	11
5233	3	17	3	4712	15	21	4
5380	15	28	26	5132	7	5	6
10761	11	11	17	5233	10	10	15
MPS-A				MPS-C			
<i>m/z</i>	CV % Height	CV % Area	CV % S/N	<i>m/z</i>	CV % Height	CV % Area	CV % S/N

2970	2	7	17	2344	7	2	15
3460	9	7	4	3717	16	2	17
3717	10	9	13	4712	10	3	16
3981	14	4	16	4965	16	9	18
4127	7	14	13	5140	7	1	9
4712	9	11	12	5233	11	16	14
4965	11	15	15	5380	6	12	13
5132	12	15	24	6925	23	20	29
5153	11	12	15	8565	20	17	13
5233	9	14	12	10761	15	17	12
5380	13	15	12	11914	6	8	2
10761	24	6	28	14692	15	11	3

a. Coefficient of variation