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Identification of nasal eosinophils using two-photon excited fluorescence

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INTRODUCTION

Allergic rhinitis is estimated to affect 30 to 60 million people in western countries and is associated with decreased quality of life, disorder in sleep hygiene, and impairment in work performance.¹ Eosinophils trigger rhinitis symptoms in numerous inflammatory diseases, including allergic rhinitis, non-allergic rhinitis eosinophilia syndrome, and chronic sinusitis.² The conventional method for diagnosing nasal eosinophilia is nasal cytology smear. While a nasal smear is easy to perform and well tolerated in general, some patients find this procedure unpleasant. A differential cell count from the sample provides information about relative cell populations. Nasal biopsy is a considerably more invasive procedure and requires technical expertise in tissue sampling and processing. However, it is the only sampling technique that provides direct information about viable cells. Furthermore, this approach is limited by sampling error, cytology cost, and bleeding risk. As a result, eosinophils are often missed. There is a need to improve diagnostic methods for rapid identification of nasal eosinophils, and novel *in vivo* imaging techniques with a miniature handheld instrument that can distinguish eosinophils from epithelial cells accurately may have great impact in the diagnosis and management of patients with rhinitis.

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Eosinophils normally comprise less than 2% of the peripheral blood population, but can be much greater in inflammatory conditions³. Human eosinophils contain granules that exhibit bright endogenous fluorescence, which allows them to be distinguished from other cells.^{4, 5, 6} Autofluorescence from these granules is associated flavin adenine dinucleotide (FAD)⁷. FAD is a coenzyme in the mitochondrial electron transport chain. The single photon absorption peak for FAD occurs at 445 nm, with peak fluorescence emission at 525 nm.⁸ Major basic protein, which makes up 50% of the eosinophil's total cellular content⁹ and is involved in eosinophil effector functions, has not been shown to account for fluorescence from eosinophils.^{7, 10}

Two-photon excitation is a powerful method for measuring the intrinsic fluorescence from cells and tissues, and microscopes based on this principle can achieve images with sub-cellular resolution, deep tissue penetration, and reduced photobleaching in comparison to single photon fluorescence. Excitation is provided by ultra-short pulses of light that localize energy in space and time to maximize the fluorescence signal. The two-photon effect occurs when two lower energy (longer wavelength) photons arrive at a biomolecule simultaneously to excite fluorescence.¹¹ Miniature imaging instruments are being developed to collect two-photon excited fluorescence *in vivo*.^{17–20} Flexible optical fibers have been developed to effectively transmit the excitation light and to efficiently collect fluorescence.²¹ In this study, we aim to demonstrate the use of TPEF to distinguish live unstained and unfixed eosinophils to support the design and development of a future miniature instrument that collects these images *in vivo*. This result provides proof-of-concept for eosinophils to be used as an *in vivo* biomarker to assess inflammatory diseases such as allergic rhinitis.

METHODS

Study Subjects

This study was approved by the Institutional Review Board at the University of Michigan. A total of 30 subjects were recruited. Inclusion criteria included: 1) age between 18 and 65 years, and 2) clinical symptoms consistent with rhinitis, including rhinorrhea, congestion, sneezing, and itching. Exclusion criteria included: 1) severe illness, including heart, lung, or kidney failure. Allergic status demonstrated by positive skin prick testing to our standard environmental panel (trees, grasses, weeds, mold, dust, cat, and dog) were recorded for each participant when available. A test was considered positive with a wheal greater than 3mm. The majority of subjects were tested during peak allergen season. All subjects had rhinitis at the time nasal cytology was obtained.

Specimen Preparation

After written informed consent, each patient had a bilateral nasal smear performed. The nasal anatomy was visualized with a nasal speculum (Sklar, West Chester, PA). The nasal specimen was obtained using an ASI Rhino-Probe® Curette (Arlington Scientific, Inc, Springville, UT) from the mid-inferior portion of the inferior nasal turbinate. The cells were placed in a 2 ml Eppendorf tube with normal saline and kept on ice during transport to the two-photon microscope, then spun down in a centrifuge for 5 min at 2000 rpm to remove debris. The supernatant was removed, leaving the cells in a pellet. The cells were then

resuspended in 100 μ l of normal saline and smeared over a standard #1.5 cover glass for imaging. Two circles with diameters of 1.5 and 0.6 cm were drawn in the center of the cover glass. The larger circle was drawn with a Super Pap Pen (Invitrogen, Carlsbad, CA) in order to keep the sample on the cover glass. The inner circle was drawn to help register the fluorescence and cytology images taken later.

Two-Photon Excited Fluorescence Imaging

The nasal cytology specimens were first imaged on a laboratory two-photon microscope (model# TCS SP5, Leica Microsystems, Bannockburn, IL) equipped with a tunable, ultrafast laser (Spectra-Physics, Mai Tai HP). We used a power of 162.5 mW on the cells at 700 nm excitation. This wavelength was chosen by maximizing the target-to-background ratio between the eosinophils and epithelial cells using a range of candidate excitation wavelengths between 700 and 900 nm in 50 nm increments. The fluorescence emission between 500 and 600 nm was collected to capture the peak emission wavelength of FAD in eosinophil granules at 525 nm. Fluorescence images were collected with a 40X objective with a numerical aperture of 1.25, resulting in a resolution of 0.6 μ m. Images were collected over a field of view (FOV) of $387.5 \times 387.5 \mu\text{m}^2$ with an image acquisition time of 5 seconds. The settings for the laser and the photomultiplier tube were kept constant for imaging of all specimens. After fluorescence imaging, the cells were allowed to dry overnight, and then processed with Hansel® stain (Lide Laboratories Inc., New Prague, MN). Using the one minute technique per manufacturer instructions, the slides were first immersed with Hansel® stain and allowed to stand for 30 seconds. Distilled water was then added to take up the stain and the cells were allowed to stand for 30 seconds. The stain was then poured off and the slide was rinsed with distilled water to remove the excess stain. Slides were then quickly rinsed with 95% methyl alcohol for fixation. The Hansel® stain contains methanol, eosin, methylene blue, and glycerin. Eosinophils stain red from eosin, neutrophils stain dark blue from methylene blue, and nasal epithelial cells stain light blue.

Cell Studies

Human cell lines including MoT neutrophils (ATCC, CRL-8055), Jurkat lymphocytes (ATCC, TIB-152), KB epithelial cells (ATCC, CLL-17), HL60 eosinophils (ATCC, CCL-240), and THP-1 monocytes (ATCC, TIB-202) were cultured. All cells were imaged with the two photon microscope using a range of candidate excitation wavelengths between 700 and 900 nm in 50 nm increments to obtain the optimal wavelength for excitation and obtain baseline data on fluorescent signature of other cell types commonly found in the nasal mucosa.

Image Analysis

The TPEF images were analyzed using the analyze and measure command in ImageJ® software (National Institutes of Health, Bethesda, Maryland). The mean and standard deviation of the fluorescence intensity and size of each cell were measured. Measurements were taken from 3 eosinophils and 3 epithelial cells per slide when available. Neutrophils, lymphocytes, and monocytes were only rarely seen, thus not included in this analysis. To avoid bias, a grid of squares with dimensions of 10 μ m was created and overlaid onto the fluorescence images. A random number generator was used to identify a square, and the

closest cell was chosen for analysis. The cytology (Hansel® stain) was viewed at 20X magnification, and the number of eosinophils per field were counted. The number of eosinophils on the TPEF image was counted and compared to that on the corresponding Hansel® stain.

Statistical Analysis

The performance for using the mean two-photon excited fluorescence intensity to distinguish between eosinophils and the epithelial cells was evaluated by varying a threshold over a range from 0 to 30 arb units (au). Each cell was designated as positive if the mean fluorescence intensity was greater than the threshold and as negative if below. Diagnostic performance was defined by sensitivity, specificity, positive predictive value, and total accuracy, given the number of true positives, true negatives, false positives, and false negatives.²¹ Statistical significance (p-value) was calculated using a two-sided Student's t-test with unequal variance. All results are shown as mean $\pm$ standard deviation. Culture cells were also analyzed for mean fluorescence intensity. Statistical significance was calculated using single factor ANOVA, with cell to cell comparison using Student's t-test with a Bonferroni correction due to multiple comparisons. Significance was assessed at an alpha level of 0.0125. Allergic status for each study participant was recorded to our standard environment panel. Allergic status was correlated to number of eosinophils on both TPEF images and Hansel® Stain images through the Wilcoxon rank-sum test.

RESULTS

Study Subjects

A total of 30 subjects were recruited into this study, ages 24 to 59, with an average age of 41 ± 11 . A total of 14 males and 16 females participated. Rhinitis was the primary complaint of each patient. In addition, some subjects complained of congestion and headache. 23 subjects were skin prick test positive for inhalant allergens. This data is shown in Table 1 along with eosinophil cell count from Hansel® stain and TPEF images. Using the Wilcoxon rank-sum test, eosinophil cell count using TPEF correlates to allergic status, $p=0.027$, as does the current standard Hansel® stain, $p=0.008$.

Two-Photon Excited Fluorescence Imaging

Cell studies revealed that the HL 60 cells have significantly greater autofluorescence (43.9 ± 9.29 au) at 700 nm than other cells including MoT (8.03 ± 3.47 au), Jurkat (9.43 ± 2.10 au), KB (8.14 ± 1.46 au), and THP-1 (2.49 ± 0.26 au) cell lines, all $p < 0.001$, as seen in Fig. 1, with a target to background ratio of 5.5, 4.7, 5.4, and 17.6, respectively.

We also observed a significantly greater mean TPEF intensity from eosinophils compared to epithelial cells in nasal cytology specimens, 13.8 ± 4.3 (range 7.8 to 25.2) versus 3.7 ± 1.8 (range 1.3 to 10.5) au, $p < 0.01$, resulting in a target-to-background ratio of 3.7. The TPEF image and Hansel® stain from a patient with eosinophilia and positive skin prick testing are shown in Fig. 2A and 2B, respectively. The fluorescence image shows many brightly fluorescent eosinophils, and the corresponding Hansel® stain shows that the eosinophils stain red, are circular in shape, and have dimensions $< 10 \mu\text{m}$. In comparison, the epithelial

cells stain light blue, are elliptical in shape, and have dimensions $>10\ \mu\text{m}$. In comparison, a TPEF image and Hansel® stain from a patient with no eosinophils is shown in Fig. 2C and 2D, respectively. Several epithelial cells can be barely visualized on the fluorescence image, and are clearly evident on the corresponding Hansel® stain. Incomplete registration of the cells in these images results from small shifts in the position of cells during cytological processing.

Moreover, numerous regions of high fluorescence intensity can be seen in the cytoplasm of the eosinophils (arrows) which likely corresponds to the FAD-containing granules, as shown in the high magnification image in Fig. 2E. In addition, an epithelial cell (arrowhead) can be seen that demonstrates much less fluorescence intensity. In addition, we measured a significantly greater size for epithelial cells in comparison to that for eosinophils from the fluorescence images, 392.0 ± 214.6 versus $27.0 \pm 10.2\ \mu\text{m}^2$, $p < 0.01$. The differences in the fluorescence intensity and size between the eosinophils and epithelial cells were both statistically significant ($p < 0.01$).

The performance for use of TPEF intensity to distinguish between eosinophils and epithelial cells was determined as a function of an intensity threshold that ranges from 0 to 30. The fluorescence intensity from a total of 159 cells (72 eosinophils and 87 epithelial cells) were used from the 30 subjects. The data was analyzed at thresholds that ranged from 0 through 30 au, which spanned the full range of the fluorescence intensities for both cells. At each threshold, the sensitivity, specificity, positive predictive value, negative predictive value, and total accuracy were evaluated, as shown in Fig. 3. The sensitivity (blue) and NPV (green) achieve maximum values at low threshold values, and decrease to minimum levels at high thresholds. On the other hand, the specificity (red) and PPV (yellow) are at a minimum at low threshold values, and increase to 100% at high thresholds. This behavior represents a tradeoff between sensitivity and specificity as a function of the threshold, and provides the clinician a variable to optimize the results, depending on the parameter that is most relevant to the clinical application. A maximum total accuracy of 97% was achieved at a threshold of 7.5 au. This threshold was also found to produce the optimal tradeoff between sensitivity and specificity of 100% and 94%, respectively, as shown on the ROC curve in Fig. 4. The resulting area under the curve is 98%.

Even better discrimination between eosinophils and epithelial cells was achieved by including the cell size in addition to fluorescence intensity, as shown by the scatter plot in Fig. 5. We found a significantly greater size for epithelial cells compared to that for eosinophils in the nasal cytology specimens, 392 ± 214 (range 119 to 1125) versus 27 ± 10 (range 13 to 68) μm^2 , $p < 0.01$, on TPEF images. The eosinophils can be distinguished from the epithelial cells using intensity and size determined by TPEF images with a sensitivity, specificity, PPV, NPV, and total accuracy of 100% each using the dashed line in Fig. 5.

The relationship between the number of eosinophils found on the TPEF images and that seen on the Hansel® stain images appears linear, as shown in Fig. 6, and the linear regression model revealed a correlation coefficient of $R^2 = 0.91$. The line drawn represents a one-to-one correspondence. Correlation between allergic status and eosinophil cell count on

both TPEF images and Hansel® stain images was found to be statistically significant for both imaging techniques, $p < 0.05$.

DISCUSSION

To the best of our knowledge, this is the first study to demonstrate the use of TPEF to distinguish eosinophils on human nasal cytology. We were able to detect auto fluorescence from FAD contained within cytoplasmic granules of the cells and to use this signal to measure parameters of intensity and size to distinguish eosinophils from epithelial cells with 100% accuracy. These results were used to establish a cell count on fluorescence that is highly correlated with cytology. Moreover, an intensity threshold can be provided to vary the tradeoff between the sensitivity and specificity of detection.

For many patients, the diagnosis of rhinitis can be achieved from a careful history, physical exam, and allergy skin testing. Clinical symptoms can reveal rhinitis, and history can help elicit signs that may suggest the diagnosis. Nasal smears can be useful as an adjunct when the diagnosis of rhinitis is in question.¹⁴ The ability to detect and quantify nasal eosinophils with a future miniature two-photon imaging instrument *in-vivo* offers a promising diagnostic tool to further assist our clinical decision making in patients who present with persistent rhinitis unresponsive to treatment or when the diagnosis is unclear. Two-photon imaging offers real time data without the need for additional time for fixation and staining. As previous studies have established the use of quantification of nasal eosinophils in the diagnosis of different forms of rhinitis,^{1,14} our study also found nasal eosinophil count to correlate significantly with allergic status. Other disease states including eosinophilic esophagitis can also benefit from this new diagnostic method when future endoscope compatible *in vivo* studies are undertaken. We found 700 nm to be ideal for imaging of eosinophils based on our preliminary studies, and this result is consistent with the blue shift that has been observed by others as well.¹³ Our cell culture studies confirmed increased autofluorescence from eosinophils as compared to neutrophils, lymphocytes, monocytes, and epithelial cells.

The TPEF and Hansel® stain images were obtained by two different microscopes at separate times; therefore, the FOV was not identical. This margin of error was minimized by looking at the same general area on the slide, within 3–5 FOV, as described in the methods. Nasal cytology samples were first imaged with the two-photon microscope while the samples were still moist. The samples were then allowed to dry before Hansel® stain images were obtained. During the drying process, cells may have incurred a slight shift. This effect was mitigated by using a large FOV that could capture up to several hundred cells, and had little influence on the conclusions of our study, as the eosinophil count on fluorescence was still found to be highly correlated to that on cytology.

Interestingly, as noted in Fig. 5, there was a wide range of fluorescence intensities for both eosinophils and nasal epithelial cells. We found some samples to have greater fluorescence intensity from both cell populations than others. This observation may occur as a result of genetic variations among patients.

This study demonstrates the use of TPEF to accurately identify eosinophils on nasal cytology specimens collected from patients with rhinitis. This *ex vivo* study provides proof of concept for this technique. In the future, we aim to develop a rhinoscope compatible two-photon imaging instrument for *in vivo* detection and quantification of eosinophils in the nasal mucosa. This project has the potential for broad impact in clinical medicine by resulting in the development of an innovative instrument that can perform rapid, accurate diagnosis and monitoring of inflammatory diseases.

Several research groups are developing a flexible fiber two-photon excited fluorescence instrument.^{15,16,17} Future development of such a rhinoscope-compatible microscope could aid the clinician in the diagnosis of rhinitis subtypes and other eosinophilic disease states such as eosinophilic esophagitis (EE). These instruments would offer an ideal non-invasive imaging tool that could be utilized in association with nasal rhinoscopy, a tool increasingly used in many Allergy and Immunology practices.

CONCLUSION

TPEF imaging offers a promising, new technique to identify nasal eosinophils from increased endogenous fluorescence from FAD present in eosinophil granules. This new method was found to be equivalent to the current gold standard staining technique, and offers the potential to perform real time imaging without fixation or staining. This pilot *ex vivo* study demonstrates feasibility for a future *in vivo* diagnostic strategy to aid the physician in nasal eosinophilia diagnosis.

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Abbreviations used

Arb units	arbitrary units
Eos	Eosinophil
EE	Eosinophilic Esophagitis
Epi	Epithelial
FN	false negatives
FP	false positives
FAD	flavin adenine dinucleotide
FOV	field-of-view
MBP	Major basic protein
ROC	Receiver Operating Characteristic
TN	true negatives

TP	true positives
TPEF	two photon excited fluorescence

References

1. Dykewicz MS, Hamilos DL. Rhinitis and sinusitis. *J Allergy Clin Immunol*. 2010 Feb; 125(2 Suppl 2):S103–15. [PubMed: 20176255]
2. Howarth PH, Persson CG, Meltzer EO, Jacobson MR, Durham SR, Silkoff PE. Objective Monitoring of Nasal Airway Inflammation in Rhinitis. *J Allergy Clin Immunol*. 2005; 115:S414–41. [PubMed: 15746881]
3. Moqbel, R.; Lacy, P.; Adamko, DJ.; Odemuyiwa, SO. Chapter 18: Biology of Eosinophils. In: Adkinson, NF., Jr, et al., editors. Middleton's Allergy Principles and Practices. Mosby, An Imprint of Elsevier; 2008.
4. Weil GJ, Chused TM. Eosinophil autofluorescence and its use in isolation and analysis of human eosinophils using flow microfluorometry. *Blood*. 1981; 57:1099–104. [PubMed: 7225570]
5. Samoszuk MK, Espinoza FP. Deposition of autofluorescent eosinophil granules in pathologic bone marrow biopsies. *Blood*. 1987; 70:597–9. [PubMed: 3607291]
6. Barnes D, Aggarwal S, Thomsen S, et al. A characterization of the fluorescent properties of circulating human eosinophils. *Photochem Photobiol*. 1993; 58:297–303. [PubMed: 8415921]
7. Mayeno AN, Hamann KJ, Gleich GJ. Granule-associated flavin adenine dinucleotide (FAD) is responsible for eosinophil autofluorescence. *J Leukoc Biol*. 1992; 51:172–5. [PubMed: 1431554]
8. Rhee HW, Choi HY, Han K, Hong JI. Selective fluorescent detection of flavin adenine dinucleotide in human eosinophils by using bis(Zn²⁺-dipicolylamine) complex. *J Am Chem Soc*. 2007; 129:4524–5. [PubMed: 17381094]
9. Gleich GJ, Loegering DA, Maldonado JE. Identification of a major basic protein in guinea pig eosinophil granules. *J Exp Med*. 1973; 137:1459–1471. [PubMed: 4350835]
10. Fuerst DE, Jannach JR. Autofluorescence of eosinophils: a bone marrow study. *Nature*. 1965; 2–5:1333.
11. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. *Science*. 1990; 248:73–6. [PubMed: 2321027]
12. Helmchen F, Denk W. Deep tissue two-photon microscopy. *Nat Methods*. 2005; 2:932–40. [PubMed: 16299478]
13. Bestvater F, Spiess E, Stobrawa G, Hacker M, Feurer T, Porwol T, et al. Two-photon fluorescence absorption and emission spectra of dyes relevant for cell imaging. *Journal of Microscopy*. Nov; 208(Pt 2):108–115. [PubMed: 12423261]
14. Diagnosis and Management of Rhinitis: An Updated Practice Parameter. *J Allergy Clin Immunol*. 2008; 122:S1–S84. [PubMed: 18662584]
15. Ye JY, Myaing MT, Norris TB, Thomas TP, Baker JR. Biosensing based on two-photon fluorescence measurements through optical fibers. *Optics Letters*. 2002; 27:1412–1414. [PubMed: 18026463]
16. Myaing MT, Ye JY, Norris TB, Thomas TP, Baker JR, Wadsworth WJ, Bouwmans G, Knight JC, Russell PS. Enhanced two-photon biosensing with double-clad photonic crystal fibers. *Optics Letters*. 2003; 28:1224–1226. [PubMed: 12885028]
17. Engelbrecht CJ, Johnston RS, Seibel EJ, Helmchen F. Ultra-compact fiber-optic two-photon microscope for functional fluorescence imaging in vivo. *Optics Express*. 2008; 16(8):5556–5564. [PubMed: 18542658]
18. Chang F, Anderson S. Clinical and pathological features of eosinophilic esophagitis: a review. *Pathology*. 2008; 40:3–8. [PubMed: 18038308]
19. Bao H, Allen J, Pattie R, Vance R, Gu M. Fast handheld two-photon fluorescence microendoscope with a 475 micron x 475 micron field of view for in vivo imaging. *Opt Lett*. 2008; 33:1333–5. [PubMed: 18552949]

20. Fu L, Jain A, Cranfield C, Xie H, Gu M. Three-dimensional nonlinear optical endoscopy. J Biomed Opt. 2007; 12:040501. [PubMed: 17867789]
21. Rosner, B. Fundamentals of Biostatistics. 6. Belmont, CA: Duxbury Press; 2005.

Fig. 1.

Two-photon excited fluorescence images from cells in culture over excitation wavelengths ranging from 700 to 900 nm show that eosinophils (HL 60) have greater autofluorescence intensity than other cells, including neutrophils (MoT), lymphocytes (Jurkat), epithelial cells (KB), and monocytes (THP-1). At 700nm, average intensities for each cell line were as follows: HL 60 43.9 ± 9.29 au, MoT 8.03 ± 3.47 au, Jurkat 9.43 ± 2.10 au, KB 8.14 ± 1.46 au, and THP-1 2.49 ± 0.26 au, $p < 0.001$.

Fig. 2.

A) Two-photon excited fluorescence image of a nasal smear obtained from a patient with allergic rhinitis shows intense autofluorescence from eosinophils. B) Eosinophils on the corresponding Hansel® stain appear red in color and are circular in shape. C) Two-photon excited fluorescence image from a negative patient shows no eosinophils. D) Corresponding Hansel® stain shows no eosinophils but many epithelial cells can be seen that appear light blue in color and elliptical in shape. E) Magnified view of the two-photon excited fluorescence image shows numerous high intensity cytoplasmic granules seen in two eosinophils (arrows) as compared to lower intensity regions from a nearby nasal epithelial cell (arrowhead), scale bar 10 μ m.

Fig. 3.

The performance for detection of eosinophils from two-photon excited fluorescence as a function of an intensity threshold is shown. Tradeoffs among sensitivity, specificity, positive predictive value, and negative predictive value can be appreciated. A maximum total accuracy of 97% was achieved at a threshold of 7.5 au.

Fig. 4. ROC curve. An optimal tradeoff between sensitivity and specificity of 100% and 94%, respectively, can be achieved at a threshold of 7.5 au. The area under the curve (AUC) is 98%.

Fig. 5.

Scatter plot. The distribution in intensity and size of $n = 72$ eosinophils and $n = 87$ nasal epithelial cells from $n = 30$ human subjects is shown. A sensitivity, specificity, positive predictive value, negative predictive value and total accuracy of 100% each for distinguishing between eosinophils and epithelial cells can be achieved using the dashed line.

Fig. 6. Cell Counts. The correlation between the number of eosinophils found on two-photon excited fluorescence and Hansel® stain using linear regression is shown and reveals a $R^2 = 0.91$. The line drawn represents a one-to-one correspondence.

Table 1

Demographic information for study subjects in addition to their clinical symptoms, allergic status to our standard environmental panel when available, eosinophil cell count on TPEF and Hansel® stain images.

No	Sex	Age	Clinical Symptoms	Hansel® stain	Eos cell count per field	2P Eos cell count per field	Allergic Status	Immunotherapy
1	M	43	Rhinitis	22		33	(+) SPT	Yes
2	F	54	Rhinitis	0		2	(+) SPT	Yes
3	F	53	Rhinitis	400		117	(+) SPT	Yes
4	M	47	Rhinitis	832		992	(+) SPT	Yes
5	F	46	Rhinitis	544		288	(+) SPT	No
6	F	32	Rhinitis	272		608	(+) SPT	No
7	M	32	Rhinitis	164		244	(+) SPT	Yes
8	M	37	Rhinitis	120		256	(+) SPT	Yes
9	M	44	Rhinitis	144		224	(+) SPT	Yes
10	F	26	Rhinitis, C	0		1	(-) SPT, irritant	No
11	F	25	Rhinitis	81		67	(+) SPT	Yes
12	F	59	Rhinitis	272		560	(+) SPT	Yes
13	F	50	Rhinitis	400		528	(+) SPT	Yes
14	M	40	Rhinitis	52		100	(+) SPT	Yes
15	F	44	Rhinitis	8		11	(+) SPT	Yes
16	M	54	Rhinitis, C	98		52	(+) SPT	Yes
17	M	31	Rhinitis	160		320	(+) SPT	Yes
18	F	56	Rhinitis	132		96	(+) SPT	Yes
19	F	24	Rhinitis, HA	92		47	(+) SPT	Yes
20	F	58	Rhinitis	2		0	(+) SPT	Yes
21	M	51	Rhinitis	52		19	(-) SPT	No
22	F	54	Rhinitis	248		224	Unknown	No
23	M	32	Rhinitis	9		14	(+) SPT	Yes
24	M	30	Rhinitis, C	160		336	(+) SPT	No
25	M	28	Rhinitis	1		0	(+) SPT	Yes
26	F	52	Rhinitis	0		0	Unknown	No

No	Sex	Age	Clinical Symptoms	Hansel® stain	Eos cell count per field	2P Eos cell count per field	Allergic Status	Immunotherapy
27	M	31	Rhinitis	37		48	Unknown	No
28	F	30	Rhinitis	42		76	(+) SPT	No
29	F	40	Rhinitis	1		0	Unknown	No
30	M	26	Rhinitis	2		0	Unknown	No

No: patient number, C: congestion, HA: headache, SPT: Skin prick test