

A Pilot Study Investigating Clinical Responses and Biological Pathways of Azelastine/Fluticasone in Nonallergic Vasomotor Rhinitis before and after Cold Dry Air Provocation

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Keywords

Antihistamines · Nonallergic vasomotor rhinitis · Environmental exposure chamber · Azelastine · Fluticasone · MicroRNA · Cold stimulation · Nasal lavage · Nasal mucosa · Substance P

Abstract

Background: Nonallergic vasomotor rhinitis (NAVMR) has been considered a diagnosis by exclusion due to unknown mechanisms or lack of diagnostic biomarkers. **Methods:** To determine clinical responses and biological pathways in NAVMR subjects challenged to cold dry air (CDA) in an environmental exposure chamber (EEC) pre- and posttreatment with azelastine/fluticasone (AzeFlu), 30 NAVMR subjects, prescreened for CDA-induced symptoms (approx. 14 °C, <15% relative humidity, ×1 h) were randomized to treatment with AzeFlu ($n = 20$) or placebo ($n = 10$) for 2 weeks. Total nasal symptoms scores, minimum cross-sectional area, cough, and conjunctival redness were recorded at visit 1 (pretreatment) and visit 2 (posttreatment) before, during, and after CDA challenge. At both visits, nasal lavage fluid (NLF) and nasal scrapings (NS) were collected pre- and post-CDA challenge. Substance P, neurokinin-A, and calcitonin gene-related peptide concentrations in NLF were analyzed

pre- and postchallenge at each visit. Their relationship with CDA-induced symptoms was determined by statistical analysis. MicroRNA sequencing from NS determined differentially expressed miRNA between the treatment groups post-CDA challenge at each visit. **Results:** The minimum cross-sectional area ($p < 0.05$), cough count ($p < 0.05$), and substance P ($p < 0.01$) improved posttreatment with AzeFlu versus placebo. Gene targets for differentially expressed miRNAs at visit 1 were enriched for biological pathways regulating epithelial ciliogenesis and cell integrity that were modified in the AzeFlu-treated group versus placebo posttreatment. **Conclusions:** This study demonstrated the feasibility of an EEC model to investigate CDA-induced clinical responses and pathobiology in NAVMR subjects pre- and posttreatment with AzeFlu. NAVMR disease mechanisms for other nonallergic triggers can be investigated similarly.

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Introduction

Nonallergic vasomotor rhinitis (NAVMR) is defined as a non-IgE mediated, chronic rhinitis condition with symptoms triggered by a spectrum of mechanical, osmotic, or irritant stimuli which can include extreme temper-

atures, changes in barometric pressure or temperature, perfumes, fragrances, smoke, cleaning agents, and other noxious odors or chemicals after other causes of non-IgE rhinitis have been excluded, such as gustatory factors, medications, exercise, and senile/atrophic rhinitis [1, 2]. Typical symptoms include rhinorrhea, congestion, post-nasal drip, headaches, facial pressure, and cough [3]. The disease mechanisms for NAVMR are poorly understood and currently there are no diagnostic biomarkers for this condition [4].

Disruption of the nasal epithelial barrier resulting in increased exposure of surface receptors on sensory nerve endings to irritants is a possible factor that contributes to the neuronal sensitivity to non-IgE triggers in NAVMR [5]. Neurogenic mechanisms involving imbalances between the parasympathetic and sympathetic nervous system have also been proposed in the pathogenesis of NAVMR [6]. Activation of trigeminal nerve endings between epithelial cells regulate blood flow in subepithelial blood vessels through antidromic reflexes [7]. Transient receptor potential (TRP) ion channels expressed on these nerve endings are activated by common nonallergic triggers [8, 9]. The subsequent antidromic depolarization of nociceptive nerve fibers releases neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), or neurokinin-A (NKA), resulting in the dilatation of submucosal blood vessels and hypersecretion of mucus from mucosal glands producing nasal congestion, anterior or posterior drainage, and cough, all of which are characteristic symptoms of NAVMR [10–13]. Studies have previously demonstrated that NAVMR symptoms are improved by desensitization of the calcium selective ion channel, TRPV1 (vanilloid 1 subtype), with continuous treatment with intranasal capsaicin [14]. This emphasizes the importance of TRP channel activation of nociceptive nerve fibers in NAVMR [6, 14–17]. Medications such as intranasal azelastine and fluticasone have both been demonstrated to improve NAVMR symptoms in clinical trials. In the case of azelastine, this effect may partly be due to the desensitization of TRP channels [18–20], providing further indirect evidence supporting TRP-mediated neurogenic mechanisms for NAVMR.

Ultrastructural nasal epithelial abnormalities in NAVMR patients include loss of ciliated epithelial cells, lack of tight junctions, and the widening of intercellular spaces [5]. Biological pathways associated with these histological features are largely unknown. Regulation of biological pathways by microRNAs (miRNAs), noncoding RNAs (<25 nucleotides long) that usually suppress coding gene expression at the posttranscriptional level [21],

is an accepted approach for investigating the molecular basis of disease mechanisms. Therefore, identification of differentially expressed miRNA can help further elucidate therapeutic targets, related pathomechanisms, and potential diagnostic biomarkers for NAVMR.

The environmental exposure chamber (EEC) is an advanced facility that can be used to provoke NAVMR subjects in response to common NAVMR triggers, such as simulated weather conditions, fragrance, ozone, and other irritants. The EEC has been developed to ensure that multiple subjects are uniformly exposed to these triggers under controlled temperature, humidity, and air flow while monitoring for changes in NAVMR symptoms and nasal resistance.

The primary objective of this pilot study was to determine whether NAVMR subjects challenged with cold dry air (CDA) in an EEC exhibit an attenuated clinical response posttreatment with azelastine/fluticasone (Aze-Flu) compared to placebo that correlates with changes in neuropeptide concentrations measured in nasal secretions. In addition, miRNA isolated from primary nasal epithelial cells before and after CDA challenge pre- and posttreatment with AzeFlu versus placebo were analyzed to identify potential relevant biological pathways associated with NAVMR.

Methods

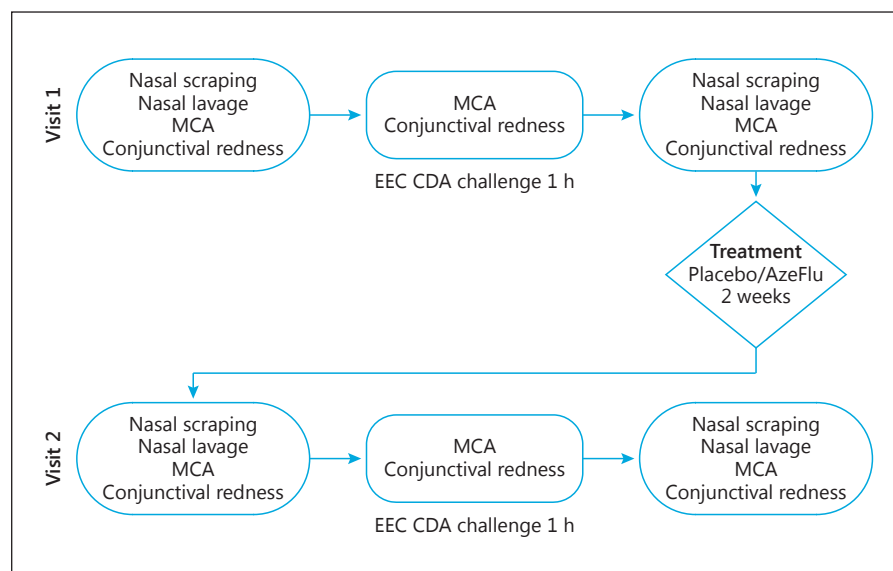
Study Design

A 2:1 randomized active treatment versus placebo-controlled, investigator and patient-blinded, 2-period, parallel study [22], approved by Health Canada, the Ontario Institutional Review Board, and University of Cincinnati IRB, was conducted in accordance with the guidelines for good clinical practice. The clinical EEC study and collection of biospecimens were conducted at Inflamax Research (Mississauga, ON, Canada) and the biospecimens were analyzed at the University of Cincinnati.

Subject Selection, Treatment Randomization, and CDA Challenge

Subjects were recruited and screened for NAVMR based on their symptoms and triggers. Those individuals with clinical features of NAVMR signed a written informed consent and were skin-prick tested to a panel of seasonal and perennial allergens to exclude atopy. Mechanical obstruction of the nasal cavity or infection was excluded by a physical exam. Those satisfying the above criteria and who reported nasal symptoms in response to a panel of nonallergic triggers, including CDA, in a NAVMR questionnaire were considered eligible to attend the pretreatment EEC visit. The randomization list was prepared by an unblinded member of the sample collection team. An unblinded pharmacist or designee under the supervision of a quality assurance manager completed the investigational product blinding.

Fig. 1. Study design listing the pre- and posttreatment sample collection before, during, and after challenge with cold dry air (CDA) in an environmental exposure chamber (EEC).



During pretreatment visit 1, all subjects underwent acoustic rhinometry measuring the minimum cross-sectional area (MCA) or nasal patency, collection of nasal lavage fluid (NLF), and a nasal scraping (NS) procedure prior to entering the EEC (Fig. 1). Bilateral ocular conjunctival redness was also assessed by trained staff before challenge based on a validated descriptive and photographic scale ranging from 0 to 4 in increments of 0.5 [23]. Subjects then entered the EEC and underwent CDA provocation ($14 \pm 5^\circ\text{C}$, $<15\%$ relative humidity, $\times 1\text{ h}$) as described by Bernstein et al. [24]. Four randomized NAVMR subjects were challenged simultaneously with CDA, which was directed into their face using air velocity tubes at a rate of $5 \pm 3\text{ ft/s}$, simulating a natural exposure similar to cold windy days. Before and after entering the EEC, subjects recorded their NAVMR total nasal symptoms scores (TNSS), which included anterior rhinorrhea, postnasal drip, and nasal congestion on a 4-point rating scale (0, none, to 3, severe) [20] using an electronic Patient Data Acquisition Tablet (ePDAT™) [25]. TNSS scoring was completed every 5 min during the first 30 min while in the EEC and every 10 min thereafter up to 1 h. During EEC exposure, subjects also recorded their cough frequency using a paper diary. Subjects were continuously monitored by staff to ensure they did not discuss their symptoms during the study. Nasal patency measurements (i.e., MCA) were taken using an acoustic rhinometer in the EEC during CDA exposure at the 30-min (± 5) time point. Following 60 min of CDA provocation, subjects exited the EEC and within 15 min trained staff collected their NLF, NS, and assessed the conjunctival redness. Based on criteria from our previous study and other meta-analyses [26, 27], in order to be eligible for treatment randomization and study continuation, subjects had to be deemed CDA responders by having at least a 10% decrease in nasal patency and a positive change from baseline of $\geq 20\%$ of the TNSS during the EEC CDA challenge.

Thirty subjects who passed the medical and EEC screening phase were randomized into AzeFlu ($n = 20$) or placebo ($n = 10$) treatment groups (Table 1) and were instructed to self-administer AzeFlu or placebo following the FDA-approved dosing regimen (1 spray in each nostril twice daily) at home for 2 weeks. AzeFlu was

supplied in a spray bottle containing a suspension of azelastine hydrochloride ($137\text{ }\mu\text{g}$) and fluticasone propionate ($50\text{ }\mu\text{g}$) per spray (0.137 mL). The inactive ingredients included preservatives (alcohols, benzalkonium) and a chelating agent (EDTA). Placebo contained the same excipients as the AzeFlu preparation without the active drug. During the study period, none of subjects used concomitant medications for NAVMR or medications that interact with AzeFlu.

After 2 weeks of dosing at home, the subjects returned to the clinic for the posttreatment EEC CDA provocation (visit 2). During this visit the nasal patency, NLF, NS, TNSS, cough counts, and conjunctival redness procedures were all repeated in the same manner as for the pretreatment visit.

NLF Collection

Nasal lavage was performed using a 10-mL syringe filled with 5 mL of warmed phosphate-buffered saline (PBS) solution with an adult nasal olive (20 mm) securely attached to the syringe. The syringe with the olive was inserted into the left nostril. Subjects were seated in a forward-flexed neck position (60° from the upright position) to prevent fluid from reaching the nasopharynx. PBS was flushed into the nasal cavity and withdrawn while ensuring an airtight seal around the olive. This procedure was repeated three times over a period of 1 min. The lavage sample was transferred from the syringe into a 15-mL centrifuge tube and preserved on ice. The total volume collected was recorded and centrifuged ($1,500\text{ rpm}$, 10 min , 4°C) to remove cellular fragments or mucus and stored at -80°C until further analysis.

NLF Processing and Neuropeptide Purification

The concentrations of 3 neuropeptides – SP, CGRP, and NKA – in NLF were measured. Because NLF is a heterogeneous mixture, the neuropeptides were purified using reverse phase chromatography columns (Waters Corp., Milford, MA, USA) followed by cryodesiccation and resuspension in a volume of PBS equal to the original NLF volume of each sample. Neuropeptide concentrations in resuspended samples were measured using

commercially available antibody-coated enzyme immunoassays kits (SP ELISA Kit, Cayman, Ann Arbor, MI, USA, LOD = 8.2 pg/mL; CGRP ELISA kit, SPI Bio, Bertin Pharma, Montigny le Bretonneux, France, LOD = 2 pg/mL; NKA ELISA Kit, Biomatik, Cambridge, ON, Canada, LOD = 4.28 pg/mL).

Neuropeptide Concentration Measurements

The neuropeptide concentration differences (i.e., the measured concentration of a neuropeptide in NLF post-CDA challenge compared to its prechallenge concentration reference level) was a primary endpoint for determining the CDA-induced pathogenic effects on NAVMR subjects. Neuropeptide concentration differences for SP ($SP_{\text{post-pre}}$), CGRP ($CGRP_{\text{post-pre}}$), and NKA ($NKA_{\text{post-pre}}$) were measured for each subject at each visit. For example, the visit 2 $SP_{\text{post-pre}}$ was calculated as: (SP concentration post-CDA challenge) – (SP concentration pre-CDA challenge) for each subject at visit 2.

NS for Nasal Epithelial Cells

Excess mucus from the subject's nose was wiped with a facial tissue. With the subject's neck extended approximately 30° from the horizontal a single-use cytology brush (BC-202D-3010, working channel diameter 2.0 mm, working length 1,150 mm, brush diameter 3.0 mm, Olympus), was advanced into 1 nostril. The inferior surface of the middle turbinate of the nostril was brushed twice with the cytology brush using rotatory and linear movements. Both nostrils were brushed once – the left nostril was brushed pre-EEC entry and the right nostril was brushed post-EEC exit to avoid confounding the results caused by inflammatory changes in nasal epithelial cells provoked by scraping. The brush was taken out and put immediately in cryopreservation medium (CryoSofree™) in sterile polypropylene cryogenic vials. The brush was shaken vigorously within the tube multiple (approx. 40) times, so that the cells became detached from the brush. Each container was labeled and flash frozen using ethanol and dry ice, and preserved at –80°C until analysis.

miRNA Expression Profiling

RNA Extraction

The NS were processed to extract total RNA and stored at –70°C until further analysis. Nasal cells in the cryopreservation medium were centrifuged to remove mucus and any foreign particles. The presence of nasal cells was confirmed under optical microscopy. Although differential staining and histology was not performed, the epithelial sample was expected to be nonhomogeneous.

Library Preparation and miRNA Sequencing [28, 29]

miRNA sequencing was performed by the Genomics, Epigenomics and Sequencing Core at the University of Cincinnati. A NEBNext small RNA sample library preparation kit (NEB, Ipswich, MA, USA) was used in the library preparation. The protocol for this kit takes advantage of the natural structure common to most known miRNA molecules. The adaptors compatible to Illumina next-generation sequencing in this kit are directly and more specifically ligated to miRNAs. First, RNA 3' adaptor was specifically modified to target miRNAs and other small RNAs that have a 3' hydroxyl group resulting from enzymatic cleavage by Dicer or other RNA processing enzymes. After ligation, the excess 3' adaptor was removed by hybridization to prevent adaptor-dimer formation.

The 5' ends of miRNAs that have a 5' phosphate were then ligated to the 5' adaptor. Following 3' and 5' ligation, reverse transcription was performed to convert the ligated small RNAs into cDNA, and then uniquely indexed by PCR to generate the sequencing library. Next, the concentration of the individual miRNA library, ranging from 135 to 146 bp, was measured with a bioanalyzer. Based on this measurement, the libraries with different barcodes were equal-molar pooled and mixed with the newly designed DNA ladder that contains 135–146 bp DNA for better positioning and size selection. After running 3% agarose gel electrophoresis at 7 V/cm for approximately 1.5 h, the libraries ranging from 135 to 146 bp, including the DNA marker, were cut, gel purified, and eluted in 20 µL. After gel purification, 2 µL of the libraries were 1:104 diluted in dilution buffer (10 mM Tris-HCl, pH 8.0, with 0.05% Tween 20), and qPCR analyzed with a Kapa Library Quantification kit (Kapabiosystem) using ABI's 9700HT Fast Real-Time PCR system.

Cluster Generation and HiSeq Sequencing

The quantified libraries were clustered onto a flow cell at the concentration of 10 pM using Illumina's TruSeq SR Cluster Kit v3, and sequenced for 50 cycles using a TruSeq SBS kit on an Illumina HiSeq system. Depending on the quality of the input RNA, the different samples generated between 5 and 30 million reads that were sufficient for downstream bioinformatic analysis.

Bioinformatic Analysis

Sequence reads were aligned to the miRNA database and analyzed. Analysis included miRNA-seq data quality control and sample clustering analysis, total miRNA expression level in RNA samples, and the FDR-corrected differentially expressed miRNAs between treatment groups pre- and postexposure.

Statistical Methods

Comparison of TNSS

The mean total changes of TNSS during CDA challenge at post-treatment visit 2 compared to pretreatment visit 1 in both the Aze-Flu and placebo groups were analyzed by ANOVA.

Comparison of Nasal Patency (as Measured by MCA) between Groups Pre- and Posttreatment

Differences between MCA measured at 30 min after continuous CDA challenge inside the EEC and baseline MCA measurements before EEC entry (i.e., $\Delta MCA_{30 \text{ min-baseline}}$) were calculated. This variable was compared between groups at visits 1 and 2. Nonparametric tests for location and scale differences across treatments based on the Wilcoxon scores of $\Delta MCA_{30 \text{ min-baseline}}$ were used in such comparisons. The nonparametric (Spearman) correlation between $\Delta MCA_{30 \text{ min-baseline}}$ and $SP_{\text{post-pre}}$ (difference in NLF SP concentration post- and pre-CDA challenge) as well as the expression levels of differentially expressed miRNAs between the groups at visit 2 was also determined.

Comparison of Conjunctival Redness during Challenge between Groups at Visits 1 and 2

The differences in values of conjunctival redness scale post/prechallenge at visits 1 and 2 were calculated. The distribution of these differences did not meet assumptions of normality and, therefore, these values between groups at each visit were analyzed by 1-way nonparametric analysis (proc npar1way, SAS 9.4).

Table 1. Subject demographics: description of age, gender, and race between the comparison groups (AzeFlu vs. placebo)

Treatment	<i>n</i>	Mean age, years
<i>AzeFlu treatment</i>	20	
Female	12	
African American	2	47.5
White	10	41.3
Male	8	
African American	4	33.25
White	4	37.25
<i>Placebo treatment</i>	10	
Female	6	
African American	2	54.5
White	4	47.5
Male	4	
White	4	50.25

Comparison of Cough Frequencies during Challenge between Groups at Visits 1 and 2

CDA-induced cough frequencies inside the EEC were measured as “cough counts.” The difference in cough count during the CDA challenge at the posttreatment visit from pretreatment visit (i.e., cough count_{post-pretreatment}) between treated and placebo groups were compared using the Wilcoxon rank sum test (due to a skewed data distribution).

Comparison of CDA-Induced Neuropeptide Concentration Differences between Groups at Pre- and Posttreatment Visits

The distribution of SP_{post-pre} values did not meet assumptions of normality. CGRP_{post-pre} and NKA_{post-pre} were distributed normally. Therefore, the comparison of median SP_{post-pre} between groups at each visit was performed using the Wilcoxon rank-sum test. CGRP_{post-pre} and NKA_{post-pre} between groups at each visit were compared by unpaired *t* test.

Multivariate Analysis of Associations between Posttreatment (Visit 2) Differentially Expressed miRNAs and Cough Frequencies Induced by CDA Challenge

Possible associations of intergroup differences in posttreatment cough frequency with posttreatment differential miRNA expression pre- and post-CDA challenge were determined by negative binomial regression analysis. The goodness of fit was assessed by the Pearson χ^2 test and a nonsignificant *p* value (0.49) for this test indicated that the negative binomial model is a good fit for the cough count data.

miRNA Target Prediction and Pathway Analysis

Differentially expressed miRNAs after CDA challenge pre- and posttreatment between the groups were determined based on cut-off false discovery rates ≤ 0.05 and a log₂-fold change $< \text{or } > 1.5$. Target genes of the significant differentially expressed miRNAs were further analyzed for gene ontology and KEGG pathway enrichment by DIANA mirPath [30]. This web-based application uses microT-CDS, and miRTarBase algorithms to identify miRNA target genes. Targets can either be predicted or experimentally val-

idated through base-pair matching. Multiple miRNAs can be analyzed simultaneously to determine the combinatorial effect of co-expressed miRNAs in the regulation of a specific pathway [31]. The statistical significance value of identified biological pathways is calculated by mirPath ($p < 0.05$) [30].

Results

Demographics

There were no significant differences between randomized groups with respect to average age or gender distribution (Table 1). Thirty percent ($n = 9$) of subjects reported a history of cough and 80% ($n = 24$) a history of nasal congestion; 20% ($n = 6$) reported previous symptomatic relief with nasal antihistamines and 13% ($n = 4$ patients) with nasal corticosteroids.

Total Nasal Symptoms Scores

The mean change in TNSS (TNSS_{visit 2} – TNSS_{visit 1}) was reduced for the AzeFlu cohort (vs. placebo cohort), but did not reach statistical significance. A similar numerical trend was observed, albeit to a lesser extent, for individual symptom scores of runny nose, post nasal drip, and nasal congestion (online suppl. Fig. 1, see www.karger.com/doi/10.1159/000478698).

Minimal Cross-Sectional Area

At visit 1, the average values of MCA (cm²) before CDA challenge in the 2 groups were 0.42 (AzeFlu) and 0.44 (placebo), and at 30 min after challenge were 0.33 (AzeFlu) and 0.34 (placebo). At visit 2, the corresponding values before CDA challenge were 0.40 (AzeFlu) and 0.48 (placebo), and at 30 min after challenge were 0.38 (AzeFlu) and 0.33 (placebo). Therefore, during pretreatment visit 1, both the AzeFlu and placebo groups exhibited a comparable decrease in $\Delta\text{MCA}_{30 \text{ min-baseline}}$ (the median decreases in MCA for the AzeFlu and placebo groups were -0.10 and -0.11 , $p = 0.692$; Fig. 2). In contrast, at posttreatment visit 2, the decrease in $\Delta\text{MCA}_{30 \text{ min-baseline}}$ in the placebo group (-0.16) was significantly less than that in the AzeFlu group (-0.04 , $p = 0.02$). In previous studies, an absolute difference of 0.05 cm^2 or a percent change between 16 and 29% in MCA was shown to be clinically significant [32, 33]. In our study, the values of percent change in $\Delta\text{MCA}_{30 \text{ min-baseline}}$ for the AzeFlu-treated and placebo-treated subjects were 2.8% (± 8) and -28.6% (± 11), respectively ($p = 0.037$) (experimental data available in online suppl. Fig. 1). This result suggests that AzeFlu was clinically effective at preventing decreased nasal patency during CDA challenge.

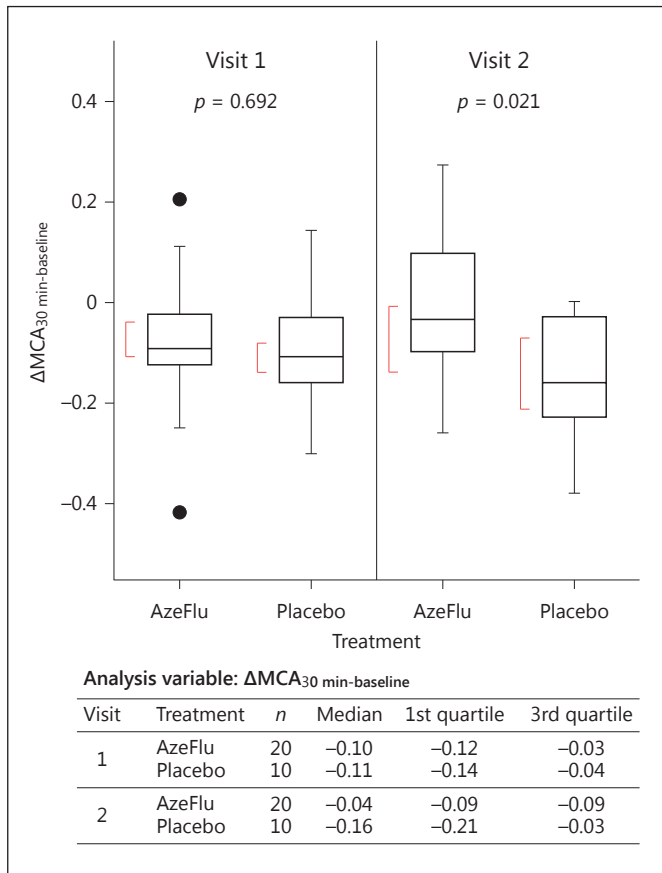


Fig. 2. The effect of AzeFlu (vs. placebo) on preventing CDA-induced nasal congestion was measured by determining the MCA inside the nasal cavity at baseline and after 30 min during CDA challenge ($\Delta MCA_{30 \text{ min-baseline}}$). Before the treatment, both the AzeFlu- and placebo-treated groups had a similar reduction in MCA, whereas after treatment reduction in the placebo group was significantly greater than in the AzeFlu group.

Conjunctival Redness

During visit 1, both the AzeFlu and placebo groups exhibited comparable changes in post-prechallenge conjunctival redness. Median (first, third quartile) values for differences in the AzeFlu and placebo groups were 0 (0, 0.5) and 0 (-0.5, 0), $p = 0.14$. The differences between groups at visit 2 were also not significant ($p = 0.18$).

NLF Neuropeptide Concentrations

Among the neuropeptides analyzed, only $SP_{\text{post-pre}}$ was found to be statistically significant. The median value of $SP_{\text{post-pre}}$ in the AzeFlu group (2.01) was not significantly

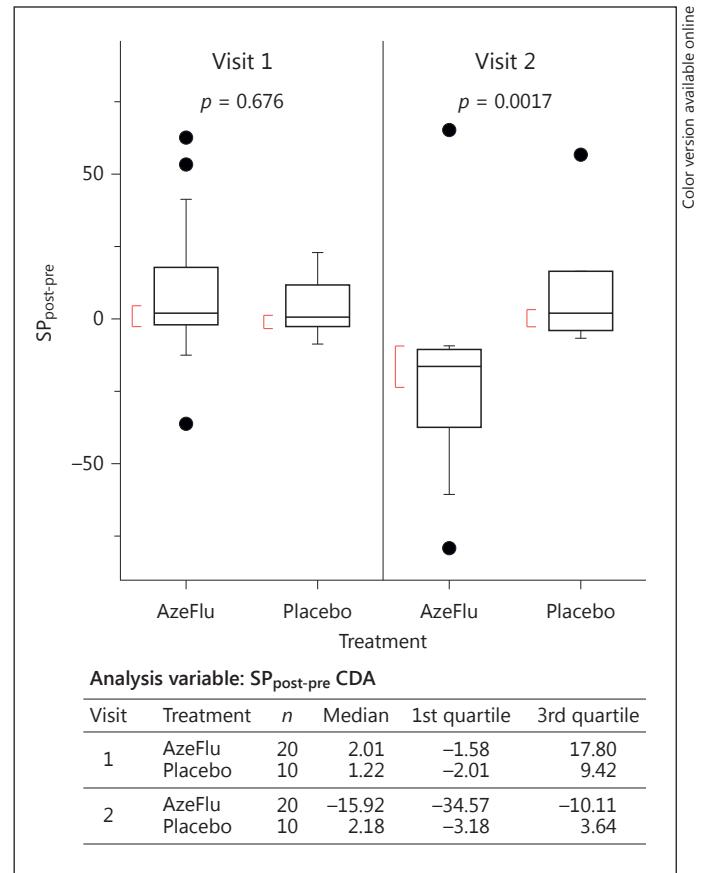


Fig. 3. The effect of AzeFlu (vs. placebo) on preventing CDA-induced SP release in nasal secretion was measured by determining the difference in SP concentration in nasal lavage before and after CDA challenge ($\times 1 \text{ h}$). The y axis represents changes in SP concentration in nasal lavage after challenge compared to the baseline (prechallenge) concentration ($SP_{\text{post-pre}}$). Before treatment (visit 1), both the AzeFlu- and placebo-treated groups had a similar increase in CDA-induced SP concentration. After treatment (visit 2), $SP_{\text{post-pre}}$ was significantly greater in the placebo compared to the AzeFlu group.

different ($p = 0.67$) from that in the placebo group (1.22) at visit 1, but was significantly decreased at visit 2 (AzeFlu = -15.92, placebo = 2.18, $p = 0.002$; Fig. 3). $CGRP_{\text{post-pre}}$ and $NKA_{\text{post-pre}}$ between groups at either visit were not statistically significant (results not shown).

Cough Count

$\Delta \text{Cough count}_{\text{post-pretreatment}}$ was significantly ($p = 0.0425$) reduced in the AzeFlu-treated group (mean = -28.8 ± 30.6 , median = -17.5) compared to that in the placebo-treated group (mean = -9.1 ± 12.1 , median = -5) as shown in Figure 4.

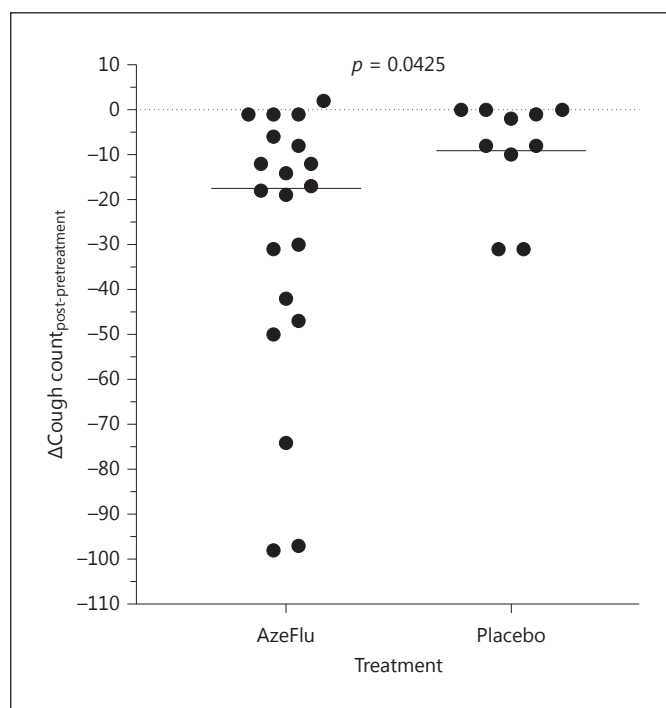


Fig. 4. The effect of AzeFlu on reducing CDA-induced cough was measured by comparison of cough count during a CDA challenge ($\times 1$ h) before and after treatment ($\Delta\text{cough count}_{\text{post-pretreatment}}$) with AzeFlu or placebo ($\times 2$ weeks). AzeFlu-treated subjects showed a significant reduction in cough count during CDA challenge when compared to the placebo group.

Differential miRNA Profiles between Groups Pre- and Posttreatment

Distinct miRNAs were concurrently over- or under-expressed after CDA challenge in the NAVMR subjects that can be associated with distinct histopathological abnormalities (Fig. 5a). At visit 2, there was significant ($\text{FDR} \leq 0.05$) overexpression ($\log_2\text{-fold} > 1.0$) of miR-543, miR-129-5p, and miR-126-3p, and under-expression ($\log_2\text{-fold} < [-1.0]$) of miR-2110, miR-449c-5p, miR-449b-5p, miR-190b, and miR-92b-5p. Gene targets for the overexpressed miRNAs are enriched in the mucin type O-glycan biosynthesis pathway and mTOR (mammalian target of rapamycin) pathway. Other interrelated signaling pathways, e.g., mTOR, ErbB, adherens junctions, and focal adhesion between cells, are also regulated by the overexpressed miRNAs. Target genes for under-expressed miRNAs are enriched in the ErbB, Fcγ-R mediated phagocytosis, adherens junctions, p53, and Notch signaling pathways. Altogether, these miRNAs regulate pathways involved in ciliogenesis (PDGFα, hedgehog, mTOR, and

Notch signaling), a process relevant to NAVMR pathogenesis based on histopathological changes described in a previous study [5].

Comparison of the differentially expressed miRNAs at posttreatment between the groups can elucidate potential specific NAVMR mechanisms affected by AzeFlu treatment (Fig. 5a, right). Differentially expressed miRNAs for the AzeFlu-treated subjects may be representative signatures for the pharmacotherapeutic response to AzeFlu in NAVMR. miR-299-5p was under-expressed in the placebo compared to the AzeFlu group. This miRNA targets the transient receptor potential ankyrin 1 (TRPA1) gene. Because TRPA1 channels perceive noxious cold temperatures ($< 14^\circ\text{C}$) [32] and their activation increases mucin secretion [33], this finding highlights the possible role of AzeFlu in diminishing the TRPA1-mediated neurogenic effects in NAVMR.

Correlation of ΔMCA with $\text{SP}_{\text{post-pre}}$ and miRNA Expression

The decrease in MCA during posttreatment challenge correlated with higher $\text{SP}_{\text{post-pre}}$ values (Spearman $\rho = -0.54$, $p = 0.003$) and with high expression levels of specific miRNAs that were overexpressed in the placebo group compared to the AzeFlu group posttreatment, such as miR-590-3p ($\rho = -0.54$, $p = 0.004$) and miR-32-5p ($\rho = -0.42$, $p = 0.031$; Fig. 5c).

Associations and Correlation between Cough Counts and miRNA Expression

Differentially expressed miRNAs are predicted to regulate biological pathways that have relevance in the mechanism of NAVMR and manifestation of clinical symptoms. Here we have explained the significant clinical symptoms, such as cough, by the expression levels of differentially expressed miRNAs between groups. At baseline (i.e., before CDA challenge) and posttreatment, the overexpressed miRNA in the placebo group included miR-3591-3p and miR-129-1-3p (Fig. 5b). Higher expression levels of both these miRNAs were predictive of higher cough frequencies during the subsequent CDA challenge (Table 2). The expression levels of miR-3591-3p also correlated fairly well with $\text{SP}_{\text{post-pre}}$ at this visit (pairwise correlation $\rho = 0.53$, $p = 0.004$; Spearman $\rho = 0.28$, $p = 0.164$). Overexpression of this miRNA in the placebo group may be related to higher $\text{SP}_{\text{post-pre}}$ in this group compared to the AzeFlu group at visit 2.

CDA challenge at visit 2 resulted in the relative overexpression of miR-3613 and miR-2355 in the placebo

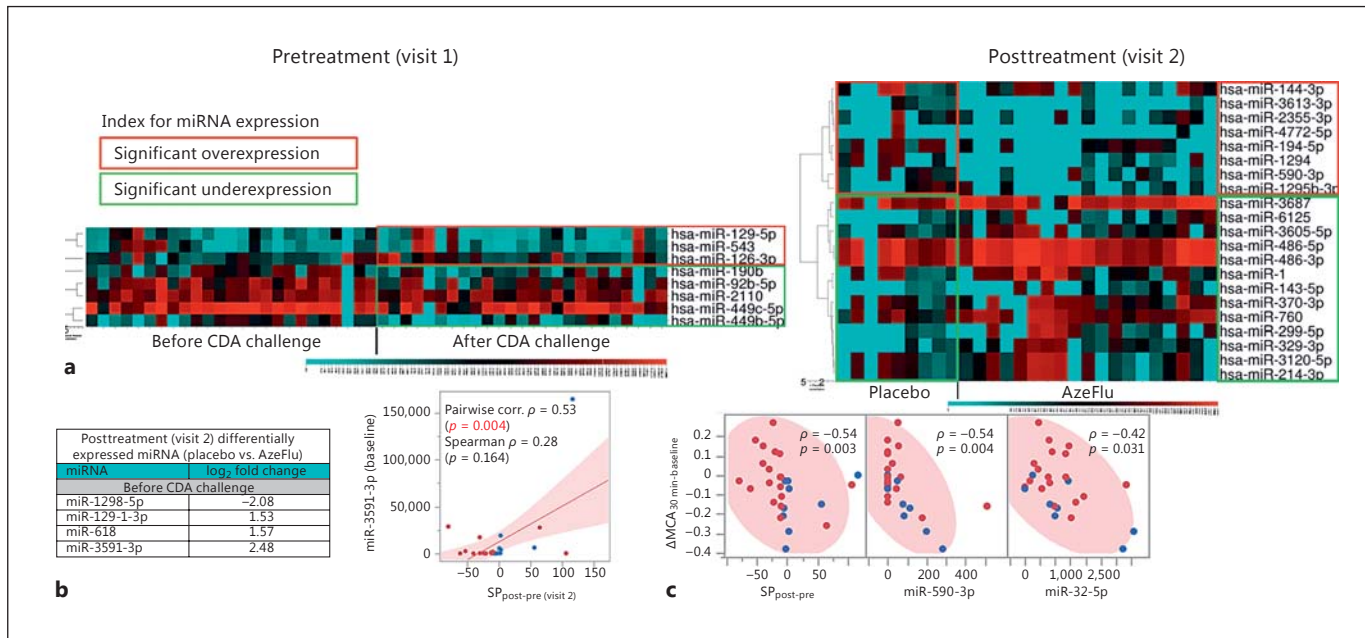


Fig. 5. **a** Differentially expressed miRNA (log₂-fold >1 or <-1, FDR ≤0.05) in the nasal epithelium of NAVMR subjects (*n* = 30) challenged with CDA, at visit 1 (before treatment) (left panel) and at visit 2 (after treatment for 2 weeks with either AzeFlu, *n* = 20, or placebo, *n* = 10) (right panel). **b** Differentially expressed miRNA (log₂-fold change) in the placebo versus AzeFlu group at posttreatment (visit 2) before CDA challenge. The correlation between

miR-3591-3p and the SP ratio is presented ($\rho = 0.66$, $p = 0.05$). **c** Correlation graphs between ΔMCA_{30 min-baseline} and SP_{post-pre} and the overexpressed miRNAs in the placebo group compared to the AzeFlu group at posttreatment (visit 2). Red dots represent AzeFlu, blue dots indicate placebo.

Table 2. Significant associations between CDA-induced cough count and differentially expressed miRNA at visit 2: analysis of maximum likelihood parameter estimates

Parameter	Estimate	Pr > χ^2
Pre-CDA challenge		
Intercept	1.339	<0.0001
miR-3591-3p	0.000	0.0164
miR-129-1-3p	0.004	0.0018
Post-CDA challenge		
Intercept	3.042	<0.0001
miR-3120-5p	0.003	0.0009
miR-329-3p	-0.007	0.0005
miR-760	-0.002	0.0022
miR-3687	0.000	0.0094
miR-1295b-3p	-0.022	0.0006
miR-144-3p	-0.001	<0.0001
miR-3613-3p	0.050	<0.0001

group (Fig. 5a, right). In our analysis, the mean difference in cough count between visits 1 and 2 (Δ cough count_{post-pretreatment}) in the 2 groups were significantly associated with the expression levels of miR-3613 ($p = 0.04$) and miR-2355 ($p = 0.003$). These miRNAs regulate ion binding (GO:0043167) and synaptic vesicle cycling (hsa04721, KEGG pathway). These results imply dysregulated SP release and exaggerate cough reflex in NAVMR [34, 35].

Discussion

This is the first study to perform controlled provocation using a nonallergic trigger in an EEC to investigate biomarkers and differentially expressed miRNAs in NAVMR subjects. The results of this study support previous work suggesting that nasal symptoms elicited by non-allergic triggers in NAVMR result from activation of neurogenic and nonneurogenic pathways [36, 37]. We demonstrated that the neuropeptide SP, and to a lesser extent

NKA, are released in response to CDA. Furthermore, treatment with the AzeFlu compound, the individual components of which have both been demonstrated in clinical trials to be effective for the treatment of NAVMR, resulted in a decreased SP release and cough frequency during CDA challenge. The objective worsening of nasal resistance during the CDA challenge, as measured by Δ MCA, was also substantially diminished after AzeFlu treatment compared to placebo. Among the biochemical markers, the SP ratio, i.e., the measure of change in SP concentration during challenge, was decreased significantly with AzeFlu (posttreatment vs. pretreatment) but not with placebo.

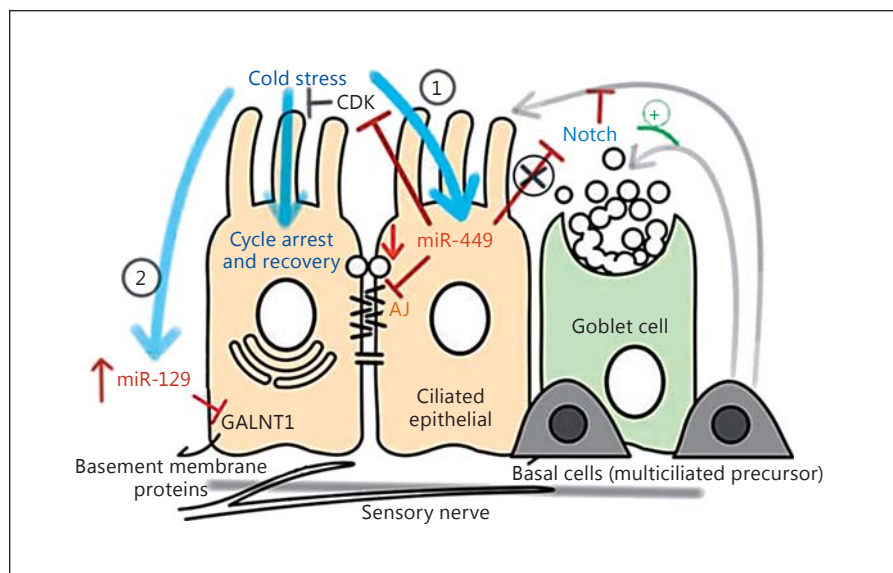
Despite the relatively greater SP localization in nasal epithelial cells in NAVMR compared to allergic rhinitis (AR) [11], it is evident that increased neuropeptides in nasal secretions are not specific biomarkers for NAVMR. Baraniuk et al. [38] previously demonstrated that hypertonic saline increased SP in nasal secretions in normal human subjects, and Mosimann et al. [39] demonstrated increased SP in the nasal secretions of AR patients following histamine or graded allergen provocation. The non-specific SP release reported for AR and NAVMR is not surprising as it is well known that a spectrum of nonspecific triggers can potentially activate TRP channels (e.g., TRPA1) on sensory nerve endings resulting in the depolarization of nociceptive nerve fibers and local neuropeptide release [40–43]. This later observation, in addition to the overexpression of miR-7 for the NAVMR subjects in this study, which was reported for AR subjects in other studies, implicating dysregulation of the TRPA1 gene expression in the nasal epithelium [44–47], could explain the coexistence of concomitant IgE-mediated and neurogenic [11] clinical manifestations in mixed rhinitis patients. However, the differentially expressed miRNAs observed post-CDA challenge in the NAVMR subjects, not previously reported for AR, which were subsequently restored following AzeFlu treatment, signal unique biological pathways involved in NAVMR that require further investigation. Predicted biological pathways for these miRs include the formation of intercellular (adherens, gap) junctions and glycosphingolipid biosynthesis that is known to be associated with tissue damage with edema and congestion [34, 35]. It should be noted that the miRNA sequencing results in this study reflect the changes in nasal cells from nasal epithelium in NAVMR subjects. The nasal epithelium is nonhomogeneous and NAVMR inflammation likely involves tissues other than the epithelium. In these tissues, TRPV1 is coexpressed with TRPA1 as well as SP and CGRP, but is also highly

expressed in the trigeminal sensory nerve. TRPA1 is a major oxidative stress sensor and chemosensor (endogenous or exogenous) on sensory nerves. It has also been demonstrated to be present in nonneuronal cells, such as pulmonary fibroblasts, airway smooth muscle cells, and pulmonary alveolar epithelial cells. TRPA1 activation has been proposed to contribute to neuronal as well as non-neuronal airway inflammation [48]. Therefore, the pathological changes in NAVMR should be interpreted in this broader context. In addition, damaged nasal epithelium releases acetylcholine [49] that can reduce the activation thresholds for TRPA1 and TRPV1 [50], which may inhibit reepithelialization by acting on the M3 subset of acetylcholine receptors [51, 52].

Previous findings in which azelastine was demonstrated to attenuate airway hyperreactivity to a noxious cold stimulus mediated through phospholipase A2 and phospholipase C pathways further support our observation of AzeFlu improving CDA-induced cough [53]. This effect can be attributed to decreased TRPA1 trafficking into neuronal membranes [54] or to downregulation in the synthesis and/or release of SP from the nerve endings [55] with a subsequent effect on smooth muscle induction in the upper respiratory tract [56]. Interestingly, azelastine has also been demonstrated previously to desensitize TRPV1 channels in vitro [19] and fluticasone has been shown to increase the degradation of neuropeptides [57, 58]. Fluticasone can also reduce leukocyte-derived nerve growth factor, the major neurokinin that regulates expression of TRPA1 and TRPV1 [59–62], and can reduce the epithelial acetylcholine release from damaged nasal epithelium [63]. Thus, the combined effects of AzeFlu demonstrated here on reducing the NLF SP concentration post-CDA challenge is consistent with previous mechanistic activities reported for each monotherapy.

Basic mechanisms of NAVMR are emphasized by a gene set enrichment analysis of target genes for the differentially expressed miRNA during pretreatment CDA challenge. The gene sets are enriched in pathways maintaining cell integrity, physiological response to stress and epithelial ciliogenesis [57, 64], and the adaptive responses in nasal epithelium (e.g., inhibition of mTOR signaling) to support cell recovery after CDA-induced genotoxic stress [57, 64–66] (Fig. 6). These mechanisms include the dysregulation of signaling through ErbB receptors that are constitutively expressed on nasal epithelium [67–69], leading to the degradation of tight junction proteins and widening of intercellular spaces, consistent with the pathological features of NAVMR. Additionally, the dysregulation of ciliary protein expression and mucin oversecre-

Fig. 6. Roles of differentially expressed nasal epithelial miR-449 and miR-129 in NAVMR pathogenesis. CDA-induced miR-449 underexpression may: (1) facilitate Notch signaling, hindering ciliogenesis, and promote goblet cell mucin secretion; (2) disintegrate adherens junctions (AJ) and widen cell junctions; (3) inhibit cyclin-dependent kinase (CDK), impeding cell recovery during stress. miR-129 ion overexpression inhibits GALNT1 gene expression, which weakens cell adherence.



tion in NAVMR is implied by CDA-induced under-expression of miR-449 with a consequent lack of Notch signaling control [5, 70, 71] (Fig. 6). Future studies can validate these biological pathways by PCR arrays designed for pathway-focused gene expression profiling [72] and subsequently the pathways specific to NAVMR can be distinguished by comparing gene expression profiles specific to AR.

The strengths of this study include that it was patient and investigator blinded, placebo controlled, and enrolled very well-characterized NAVMR subjects based on questionnaire responses and negative skin-prick test results. Other noteworthy strengths include the use of a precisely engineered and validated EEC that ensured all subjects, regardless of their EEC seating position, were exposed to identical environmental conditions, and that NLF and NS were collected uniformly using well-established methodologies by trained staff at similar time points. Furthermore, the study design allowed the comparison of clinical and mechanistic responses pre- and posttreatment with a combination medication, the monotherapies of which have previously been demonstrated to be effective for the treatment of NAVMR [20, 73].

Nonetheless, there are several limitations of this study, mostly due to financial constraints. These include the small study population, which resulted in a low power for many of the clinical responses, lack of an alternative control, such as AR without nasal symptoms

following CDA, and azelastine and fluticasone monotherapy treatment arms. The omission of these control treatment arms was somewhat mitigated by collecting samples before CDA challenge, which provided baseline values for comparing significant effects post-CDA challenge, and the use of a placebo treatment group to compare the effect of AzeFlu on NAVMR pre- and post-CDA. Finally, although the differentially expressed miRNAs provide an outline of the gene regulation in NAVMR, the potential for significant cellular heterogeneity between individual biopsy samples despite using a homogeneous collection method for all subjects must also be considered.

In conclusion, the EEC is a consistent and reliable model with which to assess clinical and mechanistic responses pre- and postexposure to nonallergic triggers like CDA and related changes after treatment with targeted therapies like AzeFlu. The findings from our study data, though preliminary, are hypothesis generating and provocative. Larger studies are needed to confirm these clinical findings and to further investigate the relevant biological pathways that were identified to be dysregulated in NAVMR subjects in response to CDA. Ultimately, a better understanding of the pathomechanistic pathways of NAVMR in response to CDA and other nonallergic triggers will lead to more effective targeted therapies for this commonly encountered clinical condition.

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Disclosure Statement

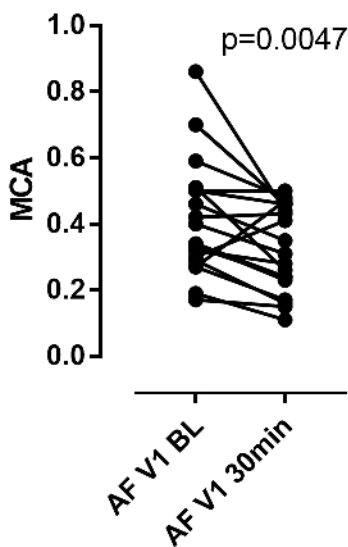
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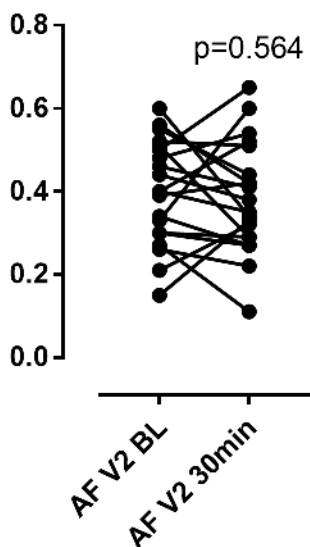
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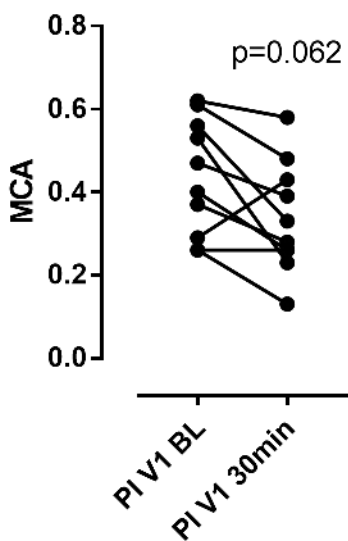
**paired t-test
AzeFlu Visit 1**



**paired t-test
AzeFlu Visit 2**



**paired t-test
Placebo Visit 1**



**paired t-test
Placebo Visit 2**

