

MUC4 impairs the anti-inflammatory effects of corticosteroids in patients with chronic rhinosinusitis with nasal polyps

Javier Milara, PhD, PharmD,^{a,b,c,d,*} Anselm Morell, PhD,^{c,d,*} Beatriz Ballester, PhD,^d Miguel Armengot, MD, PhD,^{e,f} Esteban Morcillo, MD, PhD,^{c,d,g} and Julio Cortijo, PhD^{c,d,h}
Valencia and Castellon, Spain

Background: Current evidence suggests that membrane-tethered mucins could mediate corticosteroid efficacy, interacting with glucocorticoid receptor (GR) in patients with chronic rhinosinusitis with nasal polyps (CRSwNP). Mucin 4 (MUC4)-tethered mucin is expressed in nasal polyp (NP) epithelial cells and upregulated under inflammatory conditions. Moreover, MUC4 β has the capacity to interact with other intracellular proteins. We hypothesized that MUC4 modulates corticosteroid efficacy of patients with CRSwNP.

Objective: We sought to analyze the role of MUC4 in corticosteroid effectiveness in different cohorts of patients with CRSwNP and elucidate the possible mechanisms involved.

Methods: Eighty-one patients with CRSwNP took oral corticosteroids for 15 days. Corticosteroid resistance was evaluated by using nasal endoscopy. Expression of MUC4 and MUC4 β was evaluated by means of real-time PCR, Western blotting, and immunohistochemistry. BEAS-2B knockdown with RNA interference for MUC4 (small interfering RNA [siRNA]-MUC4) was used to analyze the role of MUC4 in the anti-inflammatory effects of dexamethasone.

Results: Twenty-two patients had NPs resistant to oral corticosteroids. MUC4 expression was upregulated in these patients. In siRNA-MUC4 BEAS-2B airway epithelial cells dexamethasone produced higher anti-inflammatory effects, increased inhibition of phospho-extracellular signal-regulated kinase 1/2, increased mitogen-activated protein kinase phosphatase 1 expression, and increased glucocorticoid response element activation. Immunoprecipitation and immunofluorescence experiments revealed that MUC4 β forms a complex with GR α in the nuclei of NP epithelial cells from corticosteroid-resistant patients.

Conclusion: MUC4 β participates in the corticosteroid resistance process, inhibiting normal GR α nuclear function. The high expression of MUC4 in patients with CRSwNP might participate in corticosteroid resistance. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: MUC4, corticosteroid resistance, nasal polyp, chronic rhinosinusitis, glucocorticoid receptor

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory disease characterized by the presence of edematous masses of inflamed mucosa, leading to nasal obstruction, secretion, loss of smell, headache/facial pain, and reduced quality of life.¹ CRSwNP is frequently associated with asthma and aspirin intolerance, and T_H2 cytokine expression and eosinophilic infiltration are characteristics. Overall, the cause and pathogenesis of CRSwNP remain a matter of debate, suggesting that a role for either fungi or bacteria in patients with CRSwNP might play a key role.¹

Nasal topical corticosteroids are the first-line treatment for CRSwNP, reducing polyp size, nasal symptoms, and the number of recurrences after polypectomy.¹ In case of a lack of clinical response, a 2-week course of systemic corticosteroids generally improves polyp size and nasal symptoms.² However, there are subsets of patients who have resistance to oral corticosteroids and need sinus surgery to control the disease, which does not eliminate future recurrences. This scenario has increased the research on new therapies directed to those patients resistant to oral corticosteroids, particularly new biologic agents, such as omalizumab (anti-IgE), mepolizumab (anti-IL-5), or dupilumab (anti-IL-4/IL-13) among others.³ Therefore the greatest challenge for the future would be to identify biomarkers to appropriately select those corticosteroid-resistant patients who could benefit from new therapies.

Mucins are widely known key components of the airway epithelial mucus layer that contribute to the protection of the sinonasal mucosa from pathogens and irritants through their participation in the mucociliary clearance mechanism.⁴ However, recent evidence suggests that mucins are also involved in other less conventional roles, including host defense against airway infection and inflammation, morphogenesis and repair, and treatment response.⁵⁻⁷ In contrast to secreted mucins, membrane-tethered mucins are transmembrane proteins anchored to the apical surface of mucosal epithelial cells, with a highly glycosylated N-terminal domain in contact with the outside environment acting as a sensor receptor and a short cytoplasmic tail (CT) that enables its participation in intracellular signaling.⁴ Although mucin 1 (MUC1) and mucin 4 (MUC4) membrane-tethered mucins are overexpressed in nasal polyp (NP) epithelium in comparison with normal nasal mucosa,⁸ there is a lack of

From ^athe Department of Pharmacy, ^cthe Rhinology Unit, and ^hthe Research and Teaching Unit, University Hospital Consortium, Valencia; ^bthe Department of Pharmacology, Faculty of Medicine, Jaume I University, Castellon; the Departments of ^dPharmacology and ^eMedicine, Faculty of Medicine, University of Valencia; ^fCIBERES, Health Institute Carlos III, Valencia; and ^gInstituto de Investigación Sanitaria INCLIVA, Valencia.

*These authors contributed equally to this work.

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Corresponding author: Javier Milara, PhD, PharmD, Unidad de Investigación, Consorcio Hospital General Universitario, Avenida tres cruces s/n, E-46014 Valencia, Spain. E-mail: xmilara@hotmail.com.

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Abbreviations used

CRSwNP:	Chronic rhinosinusitis with nasal polyps
CT:	Cytoplasmic tail
Ct:	Cycle threshold
DMEM:	Dulbecco modified Eagle medium
EGF:	Epidermal growth factor
ERK:	Extracellular-signal-regulated kinase
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GR:	Glucocorticoid receptor
GRE:	Glucocorticoid response element
hsp:	Heat shock protein
LAR II:	Luciferase Assay Reagent II
MKP1:	Mitogen-activated protein kinase phosphatase 1
MUC4:	Mucin 4
NP:	Nasal polyp
NP-AIA:	Nasal polyps with aspirin-intolerant asthma
NP-ATA:	Nasal polyps with aspirin-tolerant asthma
NP-CR:	Nasal polyps resistant to oral corticosteroids
NPwA:	Nasal polyps without asthma or aspirin intolerance
PGN:	Peptidoglycan
siRNA:	Small interfering RNA
TLR:	Toll-like receptor

information about their role. In this regard we recently showed that MUC1 is significantly downregulated in patients with CRSwNP who are resistant to oral corticosteroids.⁶ Further analysis revealed that the CT fraction of MUC1 provides anti-inflammatory effects on NP epithelial cells through inhibition of Toll-like receptor (TLR) 2, TLR4, and TLR5. Moreover, MUC1-CT associates with the glucocorticoid receptor (GR) α protein complex, mediating GR α nuclear translocation in response to corticosteroids and allowing its anti-inflammatory effects.⁶ In contrast to MUC1, the role of MUC4 on corticosteroid efficacy in patients with CRSwNP is unknown.

The *MUC4* gene encodes a membrane-tethered mucin composed of 2 subunits: MUC4 α and MUC4 β .⁹ MUC4 α is the extracellular subunit featuring a typical hyperglycosylated region. MUC4 β is the transmembrane subunit containing 3 epidermal growth factor (EGF)-like domains (EGF3, EGF1, and EGF2 located from the C-terminus to the N-terminus) and a short CT fragment with potential protein-protein interactions, such as the well-described ErbB2-MUC4 β interaction in patients with different types of cancer.¹⁰

Recent evidence showed that MUC4 expression is upregulated in airways under inflammatory conditions¹¹ and that corticosteroids decrease MUC4 expression *in vitro*.¹¹⁻¹³ However, the association of oral corticosteroid efficacy with MUC4 expression, as well as the possible interactions among corticosteroids, GR, and MUC4, are currently unknown.

On the basis of this background, we hypothesized that MUC4 could modulate the systemic corticosteroid response in patients with CRSwNP. Thus we investigated the possible association of NP MUC4 expression with the response to oral corticosteroids in a cohort of patients with CRSwNP. In addition, we evaluated the possible mechanistic link between MUC4 β , GR α , and the anti-inflammatory efficacy of corticosteroids. Results observed in this work might help provide new biomarkers to explain corticosteroid response in patients with CRSwNP.

METHODS**Patients**

This study analyzed data from patients with CRSwNP ($n = 81$) enrolled from the Rhinology Unit of Valencia University General Hospital during a 4-year period (2010-2014). This work was approved by the local ethics committee, and informed consent was obtained from each subject. Patients with CRSwNP with no clinical and endoscopic response to intranasal corticosteroids for 3 months were cross-sectionally recruited. At the moment of recruitment, all patients had extensive polyps (score = 2-4 on both sides, see [Table E1](#) in this article's Online Repository at www.jacionline.org). Patient phenotypes were classified as nasal polyps without asthma or aspirin intolerance (NPwA), nasal polyps with aspirin-tolerant asthma (NP-ATA), and nasal polyps with aspirin-intolerant asthma (NP-AIA). Patients started oral corticosteroids (deflazacort) at 1 mg/kg/d for 8 days, followed by 0.5 mg/kg/d for another 7 days, according to routine clinical practice. Corticosteroid resistance was evaluated at day 15 after corticosteroid therapy. Patients unable to reduce more than 1 NP endoscopic score after the oral corticosteroid course were considered resistant to corticosteroids and classified as the nasal polyps resistant to oral corticosteroids (NP-CR) group. The clinical characteristics of all patients before starting oral corticosteroids and at the end of treatment are presented in [Table E2](#) in this article's Online Repository at www.jacionline.org. NP tissues were sampled before and after oral corticosteroid therapy to isolate nasal epithelial cells and analyze tissue mRNA and protein expression, as well as protein epithelial location, by means of immunohistochemistry. Further information on the NP epithelial cell culture, immunohistochemistry/immunofluorescence, real-time RT-PCR and small interfering RNA (siRNA) experiments, Western blot analysis, immunoprecipitation, ELISAs for IL-8 and GM-CSF, experimental protocols, inducible MUC4-plasmid construct, and statistical analysis is provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS**Expression and distribution of MUC4 is altered in patients with CRSwNP resistant to oral corticosteroids**

Eighty-one patients with CRSwNP and no response to intranasal corticosteroids were recruited to initiate a 15-day course of oral corticosteroids. Patients started oral corticosteroids (deflazacort) at 1 mg/kg/d for 8 days, followed by 0.5 mg/kg/d for another 7 days, according to routine clinical practice. Therapy compliance was evaluated by a clinical research pharmacy by means of direct recount of drug containers and personal interview with the patients after therapy ended. At the end of oral therapy, 22 patients were classified as having NP-CR because their NP endoscopic scores were not reduced by more than 1 unit (see the polyp scoring system in [Table E1](#)). Responder patients were classified as patients with NPwA ($n = 14$), NP-ATA ($n = 12$), and NP-AIA ($n = 33$). Among the 22 patients with NP-CR, 6 of them had NPwA (30% of total NPwA), 2 had NP-ATA (14.3% of total NP-ATA), and 14 had NP-AIA (29.8% of total NP-AIA). The clinical characteristics of all patients before and after oral corticosteroid therapy are defined in [Table E2](#), and the characteristics of the patients with NP-CR are shown in [Table E3](#) in this article's Online Repository at www.jacionline.org.

NP biopsy specimens were taken before and after a 2-week course of oral corticosteroids to analyze the possible association of *MUC4* expression with corticosteroid response. *MUC4* mRNA expression was significantly upregulated before and after oral corticosteroid therapy in the NP tissue of patients with NP-CR ([Fig 1, A](#), and [Table E2](#)) compared with the other groups, whereas no differences were observed among responders in the NPwA, NP-ATA, and NP-AIA groups ([Fig 1, A](#), and [Table E2](#)). The

Nasal polyp tissue

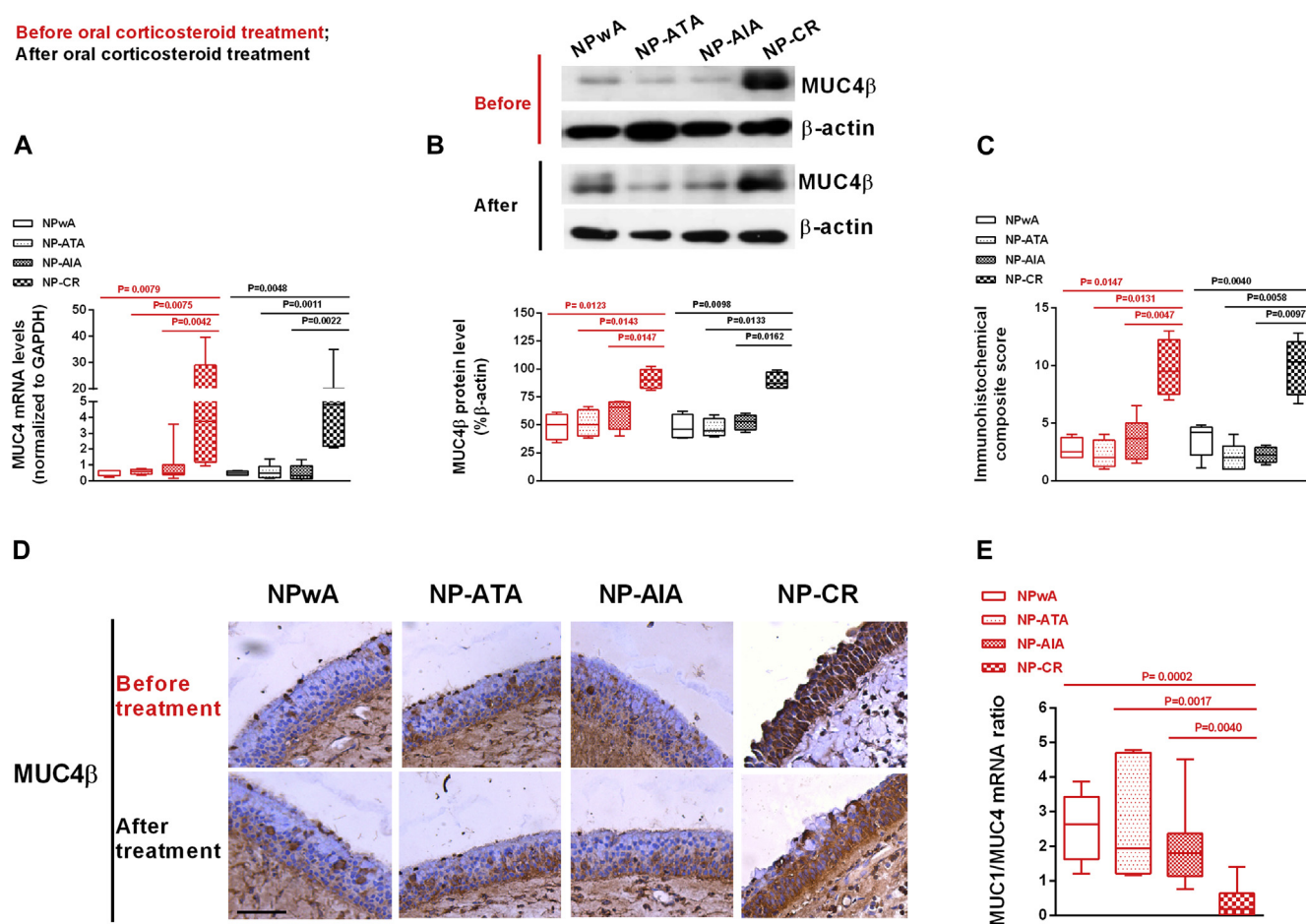
Before oral corticosteroid treatment;
After oral corticosteroid treatment

FIG 1. MUC4 expression is upregulated in patients with NP-CR. **A**, mRNA expression of MUC4 in NP tissue of patients with NPwA (n = 14), NP-ATA (n = 12), NP-AIA (n = 33), and NP-CR (n = 22) before (red) and after (black) oral corticosteroid treatment. Data are expressed as $2^{-\Delta\Delta C_t}$ MUC4 mRNA levels normalized to GAPDH mRNA levels. **B**, Western blot analysis of MUC4β protein expression in patients with NPwA, NP-ATA, NP-AIA, and NP-CR. A representative Western blot is shown. Data are expressed as MUC4β protein levels, as determined by means of densitometry relative to β-actin levels. **C**, Box plot representing the composite score of the MUC4β marker across NPs. **D**, NPs were immunostained for MUC4β (brown) and counterstained with hematoxylin. Representative immunohistochemistry images are shown. Scale bar = 50 μm. **E**, MUC1/MUC4 gene expression ratio of NP tissue. Data are presented as a box and whisker plot of medians, interquartile ranges, and minimum and maximum values. P values were obtained by using the Kruskal-Wallis test, followed by the Dunn *post hoc* test.

same pattern of MUC4 expression was observed between patients with NP-CR showing NPwA, NP-ATA, and NP-AIA (see Table E3). In a similar manner MUC4β protein expression was significantly overexpressed in patients with NP-CR, mainly in the NP epithelium, regardless of the stage of oral therapy (Fig 1, B-D). Furthermore, patients with NP-CR had a decreased MUC1/MUC4 gene expression ratio compared with responder patients, indicating a differential role of MUC1 and MUC4 in corticosteroid responsiveness (Fig 1, E).

Anti-inflammatory effects of dexamethasone on NP epithelial cells from corticosteroid responders and nonresponders

NP epithelial cells were isolated from NP biopsy specimens of responders and nonresponders to oral corticosteroids to explore

the anti-inflammatory effects of dexamethasone. Nasal sinus microorganism colonization is present in patients with CRSwNP and contributes to the progression and severity of the disease. Both gram-positive and gram-negative bacteria are common in NP tissue, promoting inflammation through TLR activation. In particular, TLR2 and TLR4 are activated by gram-positive peptidoglycan (PGN) and gram-negative LPS, respectively. In NP epithelial cells isolated from responder patients, both 10 μg/mL PGN or 1 μg/mL LPS significantly induced overexpression of MUC4, which was inhibited by dexamethasone, even to less than control levels in the absence of stimulus, after 24 hours of exposure (Fig 2, A). Mitogen-activated protein kinase phosphatase 1 (MKP-1) was upregulated by dexamethasone in the presence or absence of the bacterial stimulus (Fig 2, B). Furthermore, dexamethasone inhibited the release of IL-8 induced by PGN and LPS by $49.9\% \pm 16.6\%$ and

Nasal polyp primary epithelial cells

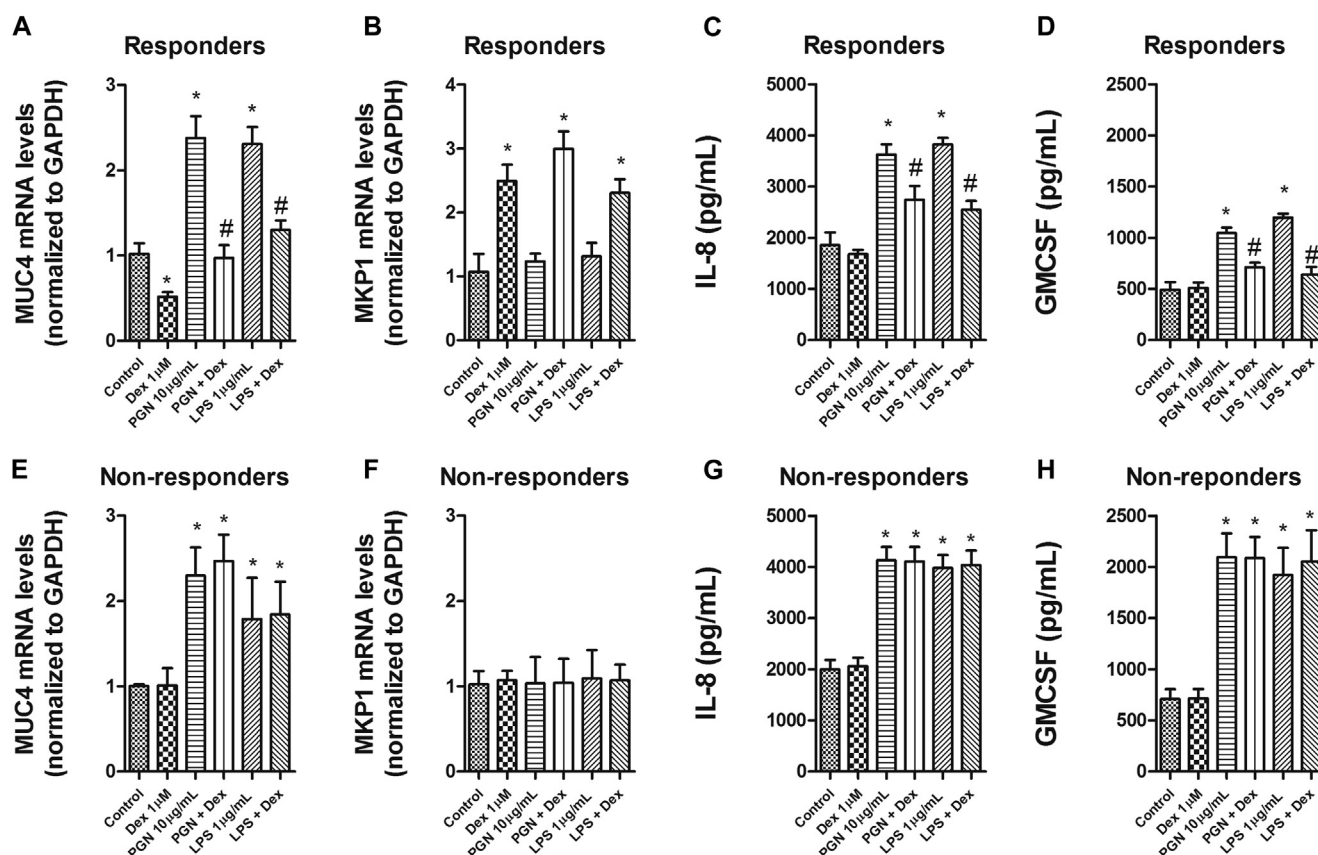


FIG 2. NP epithelial cells from corticosteroid-resistant patients are insensitive to dexamethasone. Primary NP epithelial cells from responder patients ($n = 7$; **A-D**) and nonresponder patients with NP-CR ($n = 8$; **E-H**) were incubated with or without $1 \mu\text{mol/L}$ dexamethasone for 2 hours before LPS ($1 \mu\text{g/mL}$) or PGN ($10 \mu\text{g/mL}$) stimulation for 24 hours. Fig 2, **A** and **E**, MUC4 gene expression in NP epithelial cells. Fig 2, **B** and **F**, MKP1 gene expression in NP epithelial cells. Fig 2, **C**, **D**, **G**, and **H**, IL-8 and GM-CSF concentrations were measured by means of ELISA. Results are expressed as means (SEM) of 3 independent experiments in each population. Two-way ANOVA was followed by the *post hoc* Bonferroni test. * $P < .05$ related to control release in the same subject group. # $P < .05$ related to the stimulus LPS or PGN.

$64.7\% \pm 15.2\%$, respectively, and the release of GM-CSF induced by PGN and LPS by $60.5\% \pm 14.3\%$ and $79.4\% \pm 19.3\%$, respectively (Fig 2, **C** and **D**). Unlike responder patients, NP epithelial cells isolated from patients resistant to oral corticosteroids were insensitive to dexamethasone. In NP epithelial cells from patients with NP-CR, dexamethasone did not show any effect on MUC4 overexpression induced by PGN or LPS (Fig 2, **E**). Furthermore, dexamethasone was unable to induce MKP1 expression and did not inhibit IL-8 and GM-CSF secretion (Fig 2, **F-H**). In addition, NP epithelial cells from nonresponder patients released higher IL-8 and GM-CSF amounts than those cells from responder patients in response or absence to PGN or LPS (Fig 2, **C**, **D**, **G**, and **H**).

Effects of MUC4 on dexamethasone anti-inflammatory pathways

Next, experiments were conducted to evaluate the contribution of MUC4 to the anti-inflammatory effects of dexamethasone. Neither MUC4 knockdown nor MUC1 knockdown modified MUC1 or MUC4 expression, respectively, which ruled out that

Muc1 and Muc4 regulate each other's expression (see Fig E1 in this article's Online Repository at www.jacionline.org). MUC4 knockdown by RNA interference (siRNA-MUC4) was assayed in BEAS-2B cells after stimulation with LPS or PGN in the presence or absence of dexamethasone. siRNA-MUC4 cells showed significantly lower IL-8 and GM-CSF release after LPS or PGN stimulation than did siRNA(-) stimulated cells ($P < .05$; see Fig E2, **A**, and E3, **B**, in this article's Online Repository at www.jacionline.org). Dexamethasone inhibited IL-8 secretion by $57.7\% \pm 6.6\%$ and $37.3\% \pm 16.1\%$ after LPS and PGN stimulation in siRNA(-) cells, respectively. The percentage inhibition of dexamethasone in siRNA-MUC4 cells was significantly higher, reaching $92.3\% \pm 14.4\%$ and $99.5\% \pm 20.9\%$ after LPS and PGN stimulation, respectively ($P < .05$; see Fig E2, **A**, and E3, **B**). Similar results were found for GM-CSF release. Dexamethasone inhibited GM-CSF secretion by $62.3\% \pm 9.3\%$ and $65.3\% \pm 18\%$ in siRNA(-) cells and by $88.2\% \pm 1\%$ and $80.3\% \pm 5\%$ in siRNA-MUC4 cells after LPS and PGN stimulation, respectively ($P < .05$; see Fig E2, **C**, and E3, **D**). Further experiments showed that the increase in MKP1 expression induced by dexamethasone was higher in

siRNA-MUC4 cells when compared with siRNA(–) control cells, suggesting that *MUC4* gene expression impairs anti-inflammatory effects of dexamethasone (see Fig E2, E and F).

Corticosteroids mediate part of through anti-inflammatory effects through transactivation of genes that encode proteins with anti-inflammatory properties through direct interaction of the agonist-bound GR to glucocorticoid response elements (GREs). In this work cells were transfected with GRE reporter construct in siRNA-MUC4 cells and siRNA(–) cells and incubated with 1 $\mu\text{mol/L}$ dexamethasone for 6 hours. Treatment of 2xGRE BEAS-2B reporter cells with dexamethasone (0.1 nmol/L to 1 $\mu\text{mol/L}$) for 6 hours induced GRE-dependent transcription in a concentration-dependent manner that was significantly higher in siRNA-MUC4 than in siRNA(–) control cells (see Fig E2, G).

In siRNA(–) cells both PGN and LPS increase the levels of phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 over that seen in siRNA-MUC4 cells (see Fig E3, A). Dexamethasone partially inhibited PGN- and LPS-induced phosphorylation of ERK1/2 in siRNA(–) cells, whereas in siRNA-MUC4 cells the ERK1/2 phosphorylation was completely suppressed (see Fig E3, A). Contrary effects were observed for MKP1 expression. In siRNA(–) control cells the upregulation of MKP1 protein induced by dexamethasone was higher than that observed in siRNA-MUC4 cells (see Fig E3, B).

MUC4 β interacts differently with the GR α complex in NP epithelial cells from patients with CRSwNP, both responders and nonresponders to oral corticosteroids

NP primary epithelial cells were treated with or without 1 $\mu\text{mol/L}$ dexamethasone for 4 hours to promote GR α nuclear translocation. Nuclear, cytoplasmic, or total protein was extracted and immunoprecipitated with MUC4 β or GR α , followed by an immunoblot with the GR α antibody or MUC4 β immunoblot. In NP epithelial cells from responders with CRSwNP, coimmunoprecipitation of MUC4 β -GR α was absent in the nucleus independent of dexamethasone treatment. In contrast, cytoplasm expressed the MUC4 β -GR α complex, expression of which decreased after dexamethasone exposure (Fig 3, A). Confocal immunofluorescence for GR α and MUC4 β revealed that both proteins are coexpressed in the cytoplasm of NP epithelial cells around the nucleus. After addition of dexamethasone, the GR α -MUC4 β complex was dissociated, and GR α translated to the nucleus, whereas MUC4 β remained in the cytoplasm (Fig 3, A). Different results were observed in NP epithelial cells from corticosteroid-resistant patients. NP epithelial cells showed a broad MUC4 β distribution in cytoplasm and nucleus. MUC4 β -GR α complex was present in nucleus and cytoplasm. Confocal images corroborated immunoprecipitation experiments, suggesting that in NP epithelial cells from corticosteroid-resistant patients, dexamethasone is unable to induce GR α -MUC4 β complex dissociation.

Effect of MUC4 overexpression on anti-inflammatory effects of dexamethasone in the HEK293/Tet3G/TRE3G-MUC4 stable cell line

The HEK293/Tet3G/TRE3G-MUC4 stable cell line was created with the doxycycline-inducible MUC4 expression system

and the full-length MUC4-inducible plasmid CS-H2247-pTREG (see Fig E4 in this article's Online Repository at www.jacionline.org). Doxycycline at 1 nmol/L, 10 nmol/L, 100 nmol/L, and 1 $\mu\text{mol/L}$ induced 2-, 19-, 42-, and 100-fold MUC4 increased expression, respectively, after 24 hours of incubation (see Fig E5, A, in this article's Online Repository at www.jacionline.org). The HEK293/Tet3G/TRE3G-MUC4 stable cell line was incubated with or without doxycycline (100 nmol/L and 1 $\mu\text{mol/L}$) for 24 hours, followed by the addition of dexamethasone (0.1 nmol/L–1 $\mu\text{mol/L}$) for 2 hours before LPS stimulation for 24 hours.

The IL-8 percentage inhibition of dexamethasone in control cells was 100%, which is significantly higher than in cells pretreated with 100 nmol/L and 1 $\mu\text{mol/L}$ doxycycline, reaching $73.6\% \pm 4\%$ and $48.7\% \pm 0.8\%$ inhibition, respectively (see Fig E5, B). In other experiments HEK293/Tet3G/TRE3G-MUC4 cells were transfected with GRE reporter construct and incubated with doxycycline (100 nmol/L or 1 $\mu\text{mol/L}$) for 24 hours, followed by the addition of dexamethasone (0.1 nmol/L to 1 $\mu\text{mol/L}$) for 6 hours. Treatment of 2xGRE HEK293/Tet3G/TRE3G-MUC4 cells with dexamethasone (0.1 nmol/L to 1 $\mu\text{mol/L}$) for 6 hours induced GRE-dependent transcription in a concentration-dependent manner that was significantly higher in the absence of doxycycline (see Fig E5, C). Finally, expression of the corticosteroid-inducible gene MKP1 was concentration-dependently induced by dexamethasone (0.1 nmol/L to 1 $\mu\text{mol/L}$) in HEK293/Tet3G/TRE3G-MUC4 cells and inhibited in the presence of doxycycline (see Fig E5, D). Coimmunofluorescence analysis showed that under basal control conditions, MUC4 β colocalized with GR α in the cytoplasm in the absence of dexamethasone. After stimulation with 1 $\mu\text{mol/L}$ dexamethasone, GR α dissociated from MUC4 β and translocates to the nucleus. In HEK293/Tet3G/TRE3G-MUC4 preincubated with 1 $\mu\text{mol/L}$ doxycycline, MUC4 β colocalized with GR α throughout the cell and remained colocalized with MUC4 β in the cytoplasm and nucleus after dexamethasone stimulation, suggesting a similar pattern of distribution as seen in NP epithelial cells from nonresponder patients (see Fig E5, E).

DISCUSSION

Findings showed in this work provide first evidence on the participation of MUC4 in the corticosteroid resistance seen in patients with CRSwNP. MUC4 was significantly overexpressed in NP epithelial cells of corticosteroid-resistant patients when compared with those of CRSwNP responder patients. Mechanistic experiments revealed that TLR2 and TLR4 bacterial agonists induce MUC4 overexpression that is reversed by dexamethasone in responder patients but not in corticosteroid-resistant patients. MUC4 knockdown attenuated the inflammatory response elicited by TLR2 and TLR4 agonists and improved the anti-inflammatory effects of dexamethasone. In NP epithelial cells from responder patients, MUC4 β formed a complex with GR α in the cytoplasm that was dissociated in response to dexamethasone to promote translocation of GR α to the nucleus. However, in NP epithelial cells from corticosteroid-resistant patients, MUC4 β was overexpressed, and MUC4 β -GR α complex was present in cytoplasm and nucleus remaining as a complex, regardless of the addition of dexamethasone, thus impairing the correct binding of GR α to GRE in promoter regions of glucocorticoid-inducible genes, such as MKP1, which might

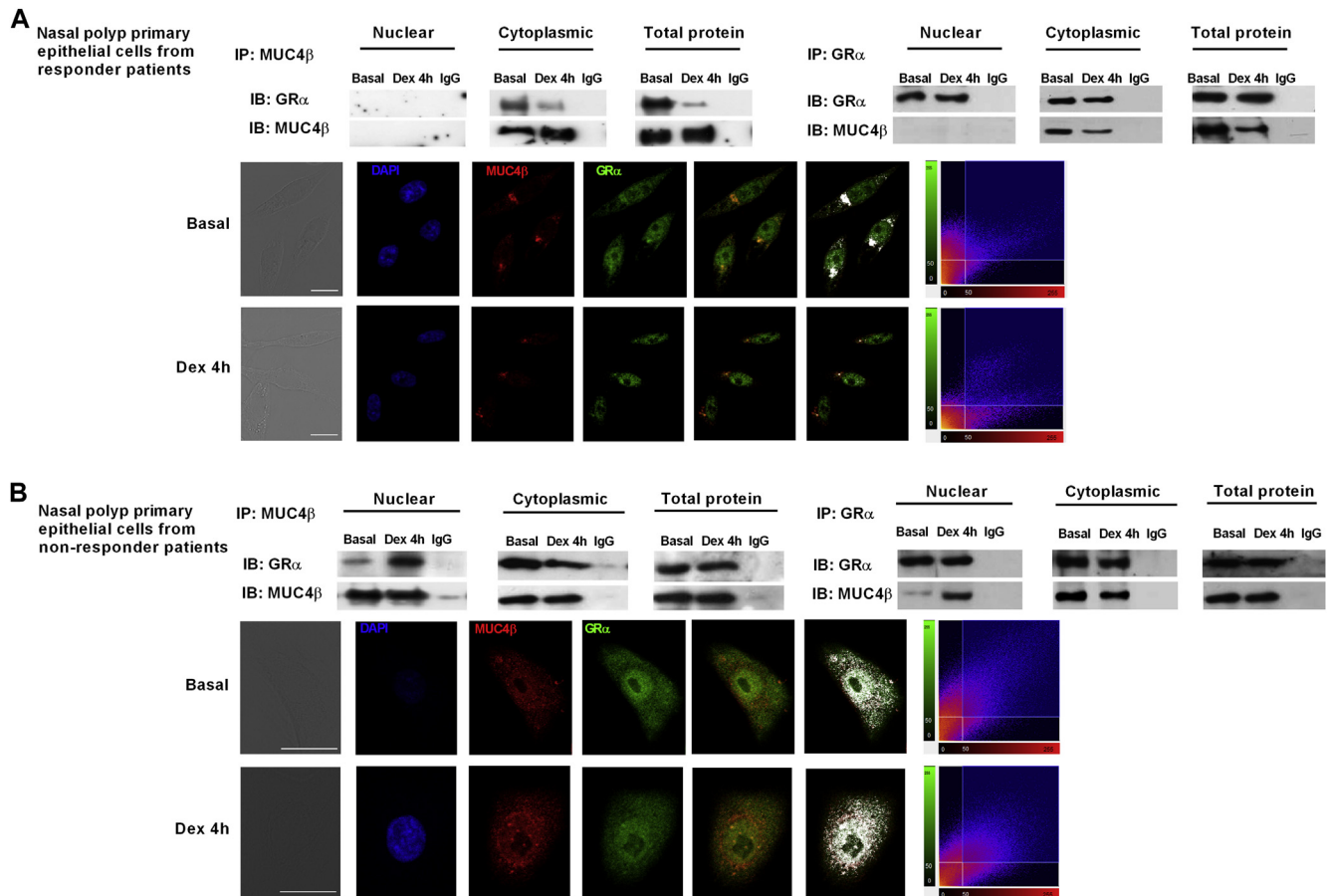


FIG 3. MUC4 β is coexpressed with GR α in NP epithelial cells. The nuclear, cytoplasmic, or total protein immunoprecipitates (IP) of MUC4 β or GR α were collected from NP primary epithelial cells from responder (n = 6; **A**) and nonresponder (n = 8; **B**) patients and incubated in the presence or absence (basal) of dexamethasone (Dex; 1 μ mol/L) for 4 hours. Immunoprecipitates were immunoblotted (IB) with the opposite antibody GR α or with the same antibodies used for immunoprecipitation as controls. The IgG isotype was used as a negative control for immunoprecipitation. NP epithelial cells were fixed, permeabilized, and used for MUC4 β (red) and GR α (green) immunostaining. Under basal conditions, MUC4 β colocalized with GR α in the cytoplasm of responder patients and throughout the cell in nonresponder patients. After dexamethasone stimulation, GR α dissociated from MUC4 β and translocated to the nucleus in cells from responder patients but remained colocalized with MUC4 β in cytoplasm and nucleus in nonresponder patients. Colocalization was analyzed with a confocal spectral microscope (Leica TCS SP2; Leica, Wetzlar, Germany) that generated a bidimensional cytofluorogram that selected common localized points of both antibodies (white color). 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI), blue nuclei. Scale bars = 10 μ m.

explain, in part, the effect of MUC4 on corticosteroid resistance of patients with CRSwNP.

This work is an extension study of previously reported clinical translational research⁶ in which the membrane-tethered MUC1-CT was identified as a molecular tool that forms a complex with GR α , enabling GR α nuclear translocation in response to dexamethasone and the subsequent corticosteroid anti-inflammatory effects.⁶ In this regard patients with NP-CR had lower MUC1-CT-GR α complexes in NP tissue along with lower expression of MKP1 and GR α than that observed in responder patients. Furthermore, dexamethasone increased MUC1-CT expression as part of its anti-inflammatory effects because MUC1-CT also inhibited activation of TLR proinflammatory signals.¹⁴ The hypothesis derived from this previous study was that the membrane-tethered MUC4 mucin could play some role in corticosteroid efficacy in patients with CRSwNP.

In contrast to MUC1, the role of MUC4 has been described only in the context of different types of cancers.¹⁵ Experimental evidence indicates that MUC4 β binds the receptor ErbB2 in plasma membrane and modulates its localization,¹⁶ phosphorylation, and downstream signaling,¹⁷ thus promoting growth, motility, or differentiation of cancer cells.¹⁸ However, MUC4 β can play different roles independently of ErbB2, promoting, for example, the activation of c-Jun N-terminal kinase pathway and cell cancer migration independently of the ErbB2 receptor interaction.¹⁹ As occurs with MUC1 mucin, MUC4 is expressed in nasal and bronchial epithelial cells of healthy subjects.²⁰ Previous reports have shown that MUC4 is overexpressed in NPs when compared with nasal mucosa,^{8,11} and its expression is upregulated under inflammatory conditions^{13,21} and after fungal, bacterial, or rhinovirus injury.^{11,21,22}

Recent data also indicate that MUC4 is overexpressed in bronchial epithelium of patients with severe uncontrolled asthma when compared with that of healthy subjects or patients with mild-to-moderate asthma.²³ In this work we analyzed the role of MUC4 on corticosteroid efficacy. The overexpression of MUC4 β was significantly associated with the lack of response to oral corticosteroids in patients with CRSwNP. Opposite results were observed for MUC1 expression. In fact, the lower ratio between MUC1/MUC4 expression in NPs clearly differentiated responder and nonresponder patients. Although the effect of MUC4 on corticosteroid efficacy is not known, previous observations could indicate some contribution. Thus, for example, airway bacterial (mainly *Staphylococcus aureus*) and rhinovirus airway colonization increase corticosteroid insensitivity^{24,25} and MUC4 expression.^{11,22} In addition, MUC4 is overexpressed in patients with severe uncontrolled asthma²³ that is particularly resistant to corticosteroids.²⁶ In this work the incidence of corticosteroid resistance was similar among patients with NPs without asthma and patients with asthma, perhaps because asthmatic patients participating in this study had mild-to-moderate well-controlled asthma.²³

However, the association of MUC4 overexpression with corticosteroid resistance could be only an observation secondary to NP inflammation without any direct relation to corticosteroid mechanistic pathways. In this regard several mechanisms have been proposed to contribute to the loss of corticosteroid efficacy in patients with CRSwNP, such as the loss of MUC1, GR α , and MKP1 expression in NP tissue⁶ or the increase of nuclear factor κ B, GR β , phospho-ERK1/2, c-Jun N-terminal kinase, and GR-Ser226 hyperphosphorylation.^{6,27-29} This fact indicates that corticosteroid resistance is an acquired and progressive condition that can depend on several multiple molecular pathways. In this work we focused on the effect of MUC4 on dexamethasone's anti-inflammatory effects. In NP epithelial cells from responder patients, we observed that TLR2 and TLR4 agonists increased the expression of MUC4 and the release of IL-8 and GM-CSF, which were significantly inhibited by dexamethasone. Similar results have been observed previously in human NP epithelial cells, showing an inhibitory effect of dexamethasone on IL-1 β or LPS-induced MUC4 overexpression.^{11,13} Unlike in responder patients dexamethasone did not reduce the expression of MUC4 and release of IL-8 and GM-CSF in NP epithelial cells from corticosteroid-resistant patients. To identify the contribution of MUC4 to the anti-inflammatory effects of corticosteroids, we conducted mechanistic experiments by using MUC4 knockdown by RNA interference. Thus in siRNA-MUC4 cells TLR2 and TLR4 agonists significantly increased the release of IL-8 and GM-CSF when compared with siRNA(–) control cells, suggesting a role for MUC4 in the inflammatory process. Furthermore, dexamethasone showed an improved anti-inflammatory effect in siRNA-MUC4 cells. These results were mechanistically corroborated in siRNA-MUC4 cells, in which the activation of TLR2/TLR4 induced lower ERK1/2 phosphorylation that was nearly suppressed by dexamethasone. These results suggested that MUC4 β could play some role collaborating in TLR activation or inhibiting corticosteroid efficacy. The last hypothesis was tested measuring levels of the glucocorticoid-enhanced MKP1 protein, which is induced as a consequence of the interaction of GR α with GRE in promoter regions of corticosteroid-inducible genes to inhibit ERK1/2 phosphorylation and the consequent inflammation.³⁰ In this

regard dexamethasone significantly increased MKP1 expression in NP epithelial cells from responder patients and in siRNA-MUC4 cells when compared with cells from nonresponders or siRNA(–) cells, respectively. Furthermore, dexamethasone significantly increased GRE reporter signal in siRNA-MUC4 cells when compared with that in siRNA(–) cells, confirming the hypothesis by which MUC4 can inhibit transactivation of GR α . Next, we analyzed the possible interaction of MUC4 β with the GR α complex, as well as its possible effect on nuclear translocation. As occurs with MUC1-CT, the mucin MUC4 β also contains a cytoplasm tail that can interact with other intracellular proteins to modulate different signal pathways.¹⁵ However, in contrast to MUC1-CT, there is no current evidence of MUC4 β intracellular interactions. In this work we immunoprecipitated MUC4 β in NP epithelial cells from responders and from corticosteroid-resistant patients after 4 hours of dexamethasone exposure. In NP epithelial cells from responder patients, the MUC4 β -GR α complex was absent in the nucleus and restricted to cytoplasm, mainly around the nucleus. After dexamethasone exposure, the MUC4 β -GR α complex was dissociated, and GR α was released to the nucleus. However, in cells from nonresponder patients, the MUC4 β -GR α complex was present in the cytoplasm and nucleus and was not dissociated by dexamethasone. These results indicate that MUC4 β could work by blocking the correct interaction of GR α with GRE DNA regions. GR α forms different dynamic complexes with multiple proteins that modulate its correct function. In the absence of corticosteroids, GR α resides in the cytoplasm in a multimeric chaperone complex consisting of heat shock protein (hsp) 90, hsp70, hsp90-binding protein p23, immunophilins, MUC1-CT, and other factors to prevent its degradation and assist in its maturation.^{6,31} The GR α complex translocates to the nucleus in response to corticosteroids and with the participation of nuclear pore complex, including importins and nucleoporins. GR α also builds a complex with the chaperone calreticulin, forming an inactive complex in the nucleus to export GR α to the cytoplasm.^{32,33} Interestingly, MUC4 β can also form a complex with calreticulin, which might explain, in part, the inactivation of GR α by a multimeric MUC4 β -calreticulin-GR α complex.³⁴ However, the last proposed mechanism is only a speculation and reveals the necessity of further investigations to appropriately dissect the exact mechanism mediating the MUC4 β -GR α relation.

To better understand the direct effect of MUC4 overexpression on corticosteroid resistance, we created a HEK293/Tet3G/TRE3G-MUC4 stable cell line based in tetracycline transactivator-inducible gene expression system. Cells incubated with doxycycline increased the expression of MUC4 to up of 100-fold. In doxycycline-treated HEK293/Tet3G/TRE3G-MUC4 cells dexamethasone showed less inhibitory effect on LPS-induced IL-8 release and less GRE induction and MKP1 expression. Furthermore, the MUC4-GR α complex was not dissociated in doxycycline-treated HEK293/Tet3G/TRE3G-MUC4 cells overexpressing MUC4, which mimicked the observations in NP epithelial cells from nonresponder patients and corroborated the effect of MUC4 overexpression on corticosteroid insensitivity. However, there is a lack of evidence whether MUC4 might play a role in the corticosteroid resistance seen in other tissues or inflammatory cells, as well as in patient with other airway diseases in which it is possible to observe a loss of corticosteroid efficacy, such as severe asthma or chronic obstructive pulmonary disease.²⁶ It should be emphasized that

mechanistic experiments were done in the BEAS-2B bronchial epithelial cell line and the MUC4-overexpressing HEK293 cell line. Both are biologically different from NP primary epithelial cells, and this represents a potential limitation of our study.

In summary, we demonstrated a new role for MUC4 in modulating the loss of the anti-inflammatory effects of corticosteroids in patients with CRSwNP, which might help explain the interindividual differences in response to corticosteroids.

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Key messages

- MUC4 overexpression on NP epithelium of patients with CRSwNP is a biomarker of oral corticosteroid resistance.
- MUC4 overexpression impairs the correct anti-inflammatory effects of corticosteroids by forming a MUC4-GR α inhibitory complex.

REFERENCES

- Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. European position paper on rhinosinusitis and nasal polyps 2012. *Rhinol Suppl* 2012;3 p preceding table of contents:1-298.
- Hissaria P, Smith W, Wormald PJ, Taylor J, Vadas M, Gillis D, et al. Short course of systemic corticosteroids in sinonasal polyposis: a double-blind, randomized, placebo-controlled trial with evaluation of outcome measures. *J Allergy Clin Immunol* 2006;118:128-33.
- Pauwels B, Jonstam K, Bachert C. Emerging biologics for the treatment of chronic rhinosinusitis. *Expert Rev Clin Immunol* 2015;11:349-61.
- Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 2006;86:245-78.
- Kim KC. Role of epithelial mucins during airway infection. *Pulm Pharmacol Ther* 2012;25:415-9.
- Milara J, Peiro T, Armengot M, Frias S, Morell A, Serrano A, et al. Mucin 1 down-regulation associates with corticosteroid resistance in chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2015;135:470-6.
- Senapati S, Das S, Batra SK. Mucin-interacting proteins: from function to therapeutics. *Trends Biochem Sci* 2010;35:236-45.
- Martinez-Anton A, Debolos C, Garrido M, Roca-Ferrer J, Barranco C, Alobid I, et al. Mucin genes have different expression patterns in healthy and diseased upper airway mucosa. *Clin Exp Allergy* 2006;36:448-57.
- Jonckheere N, Van Seuningen I. The membrane-bound mucins: how large O-glycoproteins play key roles in epithelial cancers and hold promise as biological tools for gene-based and immunotherapies. *Crit Rev Oncog* 2008;14:177-96.
- Carraway KL 3rd, Funes M, Workman HC, Sweeney C. Contribution of membrane mucins to tumor progression through modulation of cellular growth signaling pathways. *Curr Top Dev Biol* 2007;78:1-22.
- Bai CH, Song SY, Kim YD. Effect of glucocorticoid on the MUC4 gene in nasal polyps. *Laryngoscope* 2007;117:2169-73.
- Seo KY, Chung SH, Lee JH, Park MY, Kim EK. Regulation of membrane-associated mucins in the human corneal epithelial cells by dexamethasone. *Cornea* 2007;26:709-14.
- Woo HJ, Bae CH, Song SY, Lee HM, Kim YD. Expression of membrane-bound mucins in human nasal mucosa: different patterns for MUC4 and MUC16. *Arch Otolaryngol Head Neck Surg* 2010;136:603-9.
- Ueno K, Koga T, Kato K, Golenbock DT, Gendler SJ, Kai H, et al. MUC1 mucin is a negative regulator of toll-like receptor signaling. *Am J Respir Cell Mol Biol* 2008;38:263-8.
- Bafna S, Kaur S, Batra SK. Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. *Oncogene* 2010;29:2893-904.
- Ramsauer VP, Carraway CA, Salas PJ, Carraway KL. Muc4/sialomucin complex, the intramembrane ErbB2 ligand, translocates ErbB2 to the apical surface in polarized epithelial cells. *J Biol Chem* 2003;278:30142-7.
- Ramsauer VP, Pino V, Farooq A, Carothers Carraway CA, Salas PJ, Carraway KL. Muc4-ErbB2 complex formation and signaling in polarized CACO-2 epithelial cells indicate that Muc4 acts as an unorthodox ligand for ErbB2. *Mol Biol Cell* 2006;17:2931-41.
- Carraway KL, Theodoropoulos G, Kozloski GA, Carothers Carraway CA. Muc4/MUC4 functions and regulation in cancer. *Future Oncol* 2009;5:1631-40.
- Jonckheere N, Skrypek N, Merlin J, Dessein AF, Dumont P, Leteurtre E, et al. The mucin MUC4 and its membrane partner ErbB2 regulate biological properties of human CAPAN-2 pancreatic cancer cells via different signalling pathways. *PLoS One* 2012;7:e32232.
- Jonckheere N, Van Seuningen I. The membrane-bound mucins: from cell signalling to transcriptional regulation and expression in epithelial cancers. *Biochimie* 2010;92:1-11.
- Kim ST, Ye MK, Shin SH. Effects of Asian sand dust on mucin gene expression and activation of nasal polyp epithelial cells. *Am J Rhinol Allergy* 2011;25:303-6.
- Shin SH, Ye MK, Kim JK. Effects of fungi and eosinophils on mucin gene expression in rhinovirus-infected nasal epithelial cells. *Allergy Asthma Immunol Res* 2014;6:149-55.
- Hattori T, Zhou X, Trudeau JB, Wenzel SE. MUC4 protein is increased in severe asthmatic bronchial epithelial cells. *Am J Respir Crit Care Med* 2013;187:A2404.
- Li LB, Goleva E, Hall CF, Ou LS, Leung DY. Superantigen-induced corticosteroid resistance of human T cells occurs through activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK-ERK) pathway. *J Allergy Clin Immunol* 2004;114:1059-69.
- Papi A, Contoli M, Adcock IM, Bellettato C, Padovani A, Casolari P, et al. Rhinovirus infection causes steroid resistance in airway epithelium through nuclear factor kappaB and c-Jun N-terminal kinase activation. *J Allergy Clin Immunol* 2013;132:1075-85.e6.
- Barnes PJ. Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 2013;131:636-45.
- Choi BR, Kwon JH, Gong SJ, Kwon MS, Cho JH, Kim JH, et al. Expression of glucocorticoid receptor mRNAs in glucocorticoid-resistant nasal polyps. *Exp Mol Med* 2006;38:466-73.
- Hamilos DL, Leung DY, Muro S, Kahn AM, Hamilos SS, Thawley SE, et al. GRbeta expression in nasal polyp inflammatory cells and its relationship to the anti-inflammatory effects of intranasal fluticasone. *J Allergy Clin Immunol* 2001;108:59-68.
- Valera FC, Queiroz R, Scrideli C, Tone LG, Anselmo-Lima WT. Evaluating budesonide efficacy in nasal polyposis and predicting the resistance to treatment. *Clin Exp Allergy* 2009;39:81-8.
- Vandevyver S, Dejager L, Libert C. On the trail of the glucocorticoid receptor: into the nucleus and back. *Traffic* 2012;13:364-74.
- Grad I, Picard D. The glucocorticoid responses are shaped by molecular chaperones. *Mol Cell Endocrinol* 2007;275:2-12.
- Burns K, Duggan B, Atkinson EA, Famulski KS, Nemer M, Bleackley RC, et al. Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* 1994;367:476-80.
- Roderick HL, Campbell AK, Llewellyn DH. Nuclear localisation of calreticulin in vivo is enhanced by its interaction with glucocorticoid receptors. *FEBS Lett* 1997;405:181-5.
- Lomako J, Lomako WM, Carothers Carraway CA, Carraway KL. Regulation of the membrane mucin Muc4 in corneal epithelial cells by proteosomal degradation and TGF-beta. *J Cell Physiol* 2010;223:209-14.

METHODS

Patients

This study analyzed data from patients with CRSwNP ($n = 81$) enrolled from the Rhinology Unit of Valencia University General Hospital during a 4-year period (2010–2014). This work was approved by the local ethics committee, and informed consent was obtained from each subject. This study incorporated 22 patients from a previously published work on MUC1's role in corticosteroid efficacy, which shares the same clinical design as the present study.^{E1} Patients with CRSwNP with no clinical and endoscopic response to intranasal corticosteroids for 3 months were cross-sectionally recruited. In this study NPs were defined based on endoscopic findings of bilateral polyps originating from the middle meatus and/or confirmation of NPs by using computed tomography, according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines.^{E2} All patients had extensive polyps (score, 2–4 on both sides; Table E1). Atopic status was evaluated by using a skin prick test or assays for specific IgEs against common inhaled allergens. The diagnosis of aspirin intolerance was confirmed by means of lysine-aspirin nasal challenge, as previously described.^{E3} The diagnosis of asthma was determined by a pneumologist. All subjects were free of upper respiratory tract clinical infections for the 4 weeks preceding the oral corticosteroid therapy. None of the subjects used other medications (eg, antibiotics or antileukotrienes) for 4 weeks before starting oral corticosteroids.

At the moment of recruitment, patient phenotypes were classified as NPwA, NP-ATA, and NP-AIA. Patients started oral corticosteroids (deflazacort) at 1 mg/kg/d for 8 days, followed by 0.5 mg/kg/d for another 7 days, according to routine clinical practice. Therapy compliance was evaluated by a clinical research pharmacy using direct recount of drug containers and personal interviews with the patients after therapy ended. Corticosteroid resistance was evaluated at day 15 after corticosteroid therapy. Patients who were unable to reduce more than 1 NP endoscopic score after oral corticosteroid course were considered resistant to corticosteroids and classified as the NP-CR group. The clinical characteristics of all patients before starting oral corticosteroids and at the end of treatment are presented in Table E2. Patients of the NP-CR group had the NPwA ($n = 6$), NP-ATA ($n = 2$), and NP-AIA ($n = 14$) phenotypes, and their characteristics are defined in Table E3.

NP tissues were sampled before and after oral corticosteroid therapy to isolate nasal epithelial cells and to analyze tissue mRNA and protein expression, as well as protein epithelial location, by means of immunohistochemistry.

NP epithelial cell isolation and cell culture

NP samples (approximately 5×5 mm) were washed with sterile physiologic serum and plated on collagen-coated culture dishes (10 $\mu\text{g}/\text{cm}^2$ rat type I collagen; Sigma, St Louis, Mo) in bronchial epithelial growth medium (comprising bronchial epithelial basal medium supplemented with bovine pituitary extract at 52 $\mu\text{g}/\text{mL}$, hydrocortisone at 0.5 $\mu\text{g}/\text{mL}$, human recombinant EGF at 25 ng/mL, epinephrine at 0.5 $\mu\text{g}/\text{mL}$, transferrin at 10 $\mu\text{g}/\text{mL}$, insulin at 5 $\mu\text{g}/\text{mL}$, retinoic acid at 50 nmol/L, triiodo-L-thyronine at 6.5 ng/mL, gentamicin at 40 $\mu\text{g}/\text{mL}$, amphotericin B at 50 ng/mL, BSA at 1.5 $\mu\text{g}/\text{mL}$, and retinoic acid at 0.05 $\mu\text{mol}/\text{L}$).

NPs were oriented with the epithelial layer to be in contact with the culture plate. Cultures were kept in a 5% CO_2 humidified incubator at 37°C. After a period of approximately 1 to 2 weeks, NP epithelial cells were observed around the polyps and used for *in vitro* mechanistic experiments. The identity of the monolayer as NP epithelial cells was affirmed by using morphologic criteria and immunofluorescence for cytokeratin 18 (KRT18), as well as subsequent *in vitro* differentiation in an air-liquid interface as pseudostratified NP epithelium with basal cells, ciliated cells, columnar cells, and goblet cells, as previously outlined.^{E4} Cell viability was assessed by means of vital trypan blue exclusion analysis with the Countess automated cell counter (Life Technologies, Madrid, Spain). Cell viability was greater than 98% in all cell cultures tested in this work. Experiments with primary nasal epithelial cells were performed at passage 1.

The bronchial epithelial BEAS-2B cell line was obtained from the American Type Culture Collection and cultured with the same culture medium and conditions as the primary NP epithelial cells described above at 37°C in a 5% CO_2 atmosphere in humidified air. The culture medium was replaced every 48 hours.

Immunohistochemistry and immunofluorescence analysis

For immunohistochemical analysis of human NPs, the tissue was fixed, embedded in paraffin, cut into sections (4–6 μm), and stained with hematoxylin, as reported previously.^{E5} The sections were incubated with rabbit anti-human polyclonal MUC4 β antibody (NBP1-52193; Novus Biologicals, Littleton, Colo) for 24 hours at 4°C. A secondary anti-rabbit antibody (1:100; Vector Laboratories, Burlingame, Calif) with avidin-biotin complex/horseradish peroxidase was used for immunohistochemistry. The nonimmune IgG isotype was used as a negative control and yielded no signal in all cases (data not shown). Stained slices were scored by a blinded pathologist under a Nikon Eclipse TE200 light microscope (Nikon, Tokyo, Japan), and representative photographs were taken (10 slices per patient). Staining intensity was analyzed in NP epithelium. Staining intensity for MUC4 β antibody was scored on a scale of 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong immunoreactivity). The percentage of cells positive for MUC4 β within NP epithelium was scored on a scale of 1 to 4 as follows: 1, 0% to 25% positive; 2, 26% to 50% positive; 3, 51% to 75% positive; and 4, 76% to 100% positive. The score of the staining intensity and percentage of immunoreactive cells were then multiplied to obtain a composite score ranging from 0 to 12.

The *in vitro* cell culture of NP epithelial cells was fixed (4% paraformaldehyde for 30 minutes at room temperature) after appropriate treatments. Cells were permeabilized (20 mmol/L HEPES at pH 7.6, 300 mmol/L sucrose, 50 mmol/L NaCl, 3 mmol/L MgCl_2 , and 0.5% Triton X-100), blocked (10% goat serum in PBS), and incubated with the primary antibodies rabbit anti-human polyclonal MUC4- β antibody (NBP1-52193, Novus Biologicals) and mouse anti-human monoclonal GR α antibody (catalog no. 611227; BD Biosciences, San Jose, Calif) overnight at 4°C, followed by secondary antibody anti-rabbit/mouse rhodamine/fluorescein isothiocyanate (1:100; Molecular Probes, Eugene, Ore).

In Fig 3, colocalization of MUC4- β and GR α was performed with a confocal spectral Leica TCS SP2 microscope at $\times 1000$ magnification and $\times 3$ zoom. Red (HeNe 543 nm), green (HeNe 488 nm), and blue (Ar 351 nm and 364 nm) lasers were used. Colocalization studies were performed with Leica confocal software v2.61. A bidimensional cytofluorogram was created between different green and red intensity points of the cell image. The cell images with colocalized points of the 2 laser canals were changed to a white color in the cell image.

Real-time RT-PCR and siRNA experiments

Total RNA was isolated from NP tissue by using TriPure Isolation Reagent (Roche, Indianapolis, Ind). The integrity of the extracted RNA was confirmed with the Bioanalyzer (Agilent, Palo Alto, Calif). Reverse transcription was performed in 300 ng of total RNA with a TaqMan reverse transcription reagent kit (Applied Biosystems, Perkin-Elmer, Foster City, Calif). cDNA was amplified with specific primers and probes predesigned by Applied Biosystems for MUC1 (Hs00159357_m1), MUC4 (Hs00366414_m1), and MKP1 (Hs00610256_g1) in a 7900HT Fast Real-Time PCR System (Applied Biosystems) by using Universal Master Mix (Applied Biosystems).

Expression of the target gene was expressed as the fold increase or decrease relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an endogenous control (4310884E, Applied Biosystems). The mean value of the replicates for each sample was calculated and expressed as the cycle threshold (Ct). The level of gene expression was then calculated as the difference (ΔCt) between the Ct value of the target gene and the Ct value of *GAPDH*. The fold changes in the target gene mRNA levels were designated $2^{-\Delta\text{Ct}}$.

siRNA, including the scrambled siRNA control, was purchased from Ambion (Cambridge, United Kingdom). *MUC4* gene-targeted siRNA (identification no. s9073) was designed by Ambion. BEAS-2B cells were transfected with siRNA (50 nmol/L) in serum and antibiotic-free medium. After 6 hours, the medium was aspirated and replaced with medium containing serum for a further 42 hours before cell stimulation. The transfection reagent used was lipofectamine-2000 (Invitrogen, Paisley, United Kingdom) at a final concentration of 2 µg/mL. *MUC4* knockdown was evaluated in BEAS-2B cells by means of Western blotting and RT-PCR by using *MUC4*β antibody and *MUC4* primers and probe, as described above. *MUC4* expression in *MUC4* knockdown experiments was always less than 10% of *MUC4* expression in siRNA(−) control cells (data not shown).

Western blot analysis

Western blot analysis was used to detect changes in NP tissue and BEAS-2B protein expression of *MUC4*β, mouse anti-human monoclonal phospho-ERK1/2 (catalog no. M9692, Sigma-Aldrich), rabbit anti-human polyclonal MKP1 (B1099; Assay Biotech, Sunnyvale, Calif), and GRα antibody (GRα; catalog no. 611227, BD Biosciences). NP tissue or BEAS-2B cells were homogenized or scraped from a confluent 25-cm² flask and lysed on ice with a lysis buffer comprising complete inhibitor cocktail plus 1 mmol/L EDTA (Roche Diagnostics, West Sussex, United Kingdom) with 20 mmol/L TRIS base, 0.9% NaCl, 0.1% Triton X-100, 1 mmol/L dithiothreitol, and 1 mg/mL pepstatin A. Nuclear and cytoplasm protein extraction was performed with a nuclear active motif extraction kit (Active Motif Europe, Rixensart, Belgium), according to the manufacturer's protocol. The Bio-Rad assay (Bio-Rad Laboratories, Herts, United Kingdom) was used according to the manufacturer's instructions to quantify the level of protein in each sample to ensure equal protein loading. SDS-PAGE was used to separate the proteins according to their molecular weight. Briefly, 15 µg of proteins (denatured) along with a molecular weight protein marker (Bio-Rad Kaleidoscope marker, Bio-Rad Laboratories) was loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel stacked on top of a 10% acrylamide resolving gel and run through the gel by means of application of 100 V for 1 hour. Proteins were transferred from the gel to a polyvinylidene difluoride membrane by using a wet-blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween 20, probed with the antibodies described above, and normalized to total mouse anti-human β-actin (1:1000) antibody (mAb, catalog no. A1978; Sigma) or total rabbit anti-human ERK1/2 (1:1000) antibody (mAb; catalog no. 4695; Cell Signaling, Boston, Mass) as appropriate. The enhanced chemiluminescence method of protein detection with enhanced chemiluminescence reagents (ECL Plus; Amersham GE Healthcare, Buckinghamshire, United Kingdom) was used to detect labeled proteins. Densitometry of films was performed with Image J 1.42q software (available at <http://rsb.info.nih.gov/ij/>; National Institutes of Health, Bethesda, Md). Results of target protein expression are expressed as the percentage of the densitometry of the endogenous controls β-actin or total ERK1/2, as appropriate.

Immunoprecipitation

Equal amounts of protein (200 µg) from total, cytoplasmic, or nuclear extracts were incubated with 2 µg of anti-GRα or anti-*MUC4*β antibodies and the IgG isotype control. Immune complexes were precipitated with protein G on Sepharose 4B fast flow beads (P-3296; Sigma) overnight at 4°C. After washing 3 times with NET buffer containing 50 mmol/L Tris-HCl at pH 8.0, 150 mmol/L NaCl, and 0.1% Nonidet P-40, the bound materials were eluted from the immunoprecipitates in reducing SDS-PAGE loading buffer containing 10% SDS, 1 mol/L Tris-HCl at pH 6.8, 50% glycerol, 10% 2-mercaptoethanol, and 2% bromophenol blue at 100°C for 10 minutes. Immunoprecipitated protein complexes were assayed by means of Western blotting, as described above, and probed with anti-GRα or anti-*MUC4*β antibodies, as appropriate.

GRE transfection assay

BEAS-2B epithelial cells were seeded (40,000 cells/well) and cultured for 24 hours in a 5% CO₂/air atmosphere at 37°C in 96-well plates containing Dulbecco modified Eagle medium (DMEM). The Cignal GRE Reporter Assay Kit (catalog no. 336841; Qiagen, Hilden, Germany) was used to monitor the activity of GR-induced signal transductions pathways in cultured cells, according to the manufacturer's indications. First, cells were transfected with *MUC4* gene-targeted siRNA or scrambled siRNA control, as described above. Twenty-four hours later, cells were transfected with Cignal Reporter (100 ng), Cignal negative control (100 ng) and Cignal positive control (100 ng) in Opti-MEM serum-free culture medium using Lipofectamine 2000 Reagent (Invitrogen) as a transfection reagent. Subsequently, cells were incubated with the transfection reagents at 37°C in a 5% CO₂ incubator for 16 hours. After this incubation period, cells were preincubated for 6 hours with different dexamethasone concentrations (0.1 nmol/L–1 µmol/L) in DMEM.

After this 6-hour period, the luciferase assay was developed by using the Dual-Luciferase Reporter Assay System (catalog no. 1910; Promega, Madison, Wis), according to the manufacturer's protocol for developing the assay. In brief, growth medium was removed from the cultured cells, and surfaces of the cultured cells were washed gently with PBS. After removing completely the rinse solution, Passive Lysis Buffer 1× was added. The culture plate was then placed on an orbital shaker, with gentle shaking at room temperature for 15 minutes. In the meantime, Luciferase Assay Reagent II (LAR II) was prepared by resuspending the provided lyophilized Luciferase Assay Substrate in 10 mL of the supplied Luciferase Assay Buffer II. A volume of 100 µL of LAR II was dispensed in the appropriate number of wells of a white 96-well plate. Then a volume of 20 µL of cell lysate was added to the wells containing LAR II and mixed by means of pipetting 2 or 3 times. The white plate was placed in the luminometer (Victor, PerkinElmer Inc, Zaventem, Belgium), reading was initiated, and firefly luciferase activity was measured. In the meantime, the Stop & Glo Reagent (Promega) was prepared, just before use, by diluting 1 volume of the Stop & Glo Substrate to 50 volumes of Stop & Glo Buffer. After the first measure, 100 µL of Stop & Glo Reagent was dispensed in the corresponding wells. The plate was replaced in the luminometer, a second reading was initiated, and the Renilla Luciferase activity was recorded. Data are expressed as 2× GRE fold induction of luciferase relative to unstimulated cells.

In vitro stimulations

For the *in vitro* studies, primary NP epithelial cells or BEAS-2B cells were stimulated with the TLR2 agonist PGN (10 µg/mL; InvivoGen, San Diego, Calif) or the TLR4 agonist LPS (Ultrapure LPS, *E coli* 0111:B4; 1 µg/mL; InvivoGen) for the indicated times. Dexamethasone (1 µmol/L, Sigma) was added 2 hours before stimulus. In other experiments cells underwent knockout for the *MUC4* gene for 48 hours and stimulation, as described above, in the presence or absence of dexamethasone.

ELISA for IL-8 and GM-CSF

IL-8 and GM-CSF levels were determined in cell supernatants by using a commercially available ELISA kit for IL-8 and GM-CSF (catalog nos. DY208 and DGM00; R&D Systems, Nottingham, United Kingdom), according to the manufacturer's protocol.

Doxycycline-induced *MUC4* expression of the HEK293/Tet3G/TRE3G-*MUC4* stable cell line

The HEK293 cell line was acquired from the American Type Culture Collection and cultured in DMEM (Lonza, Madrid, Spain), with 10% FBS, 0.4 mg/mL G418 (Geneticin; Thermo Fisher Scientific, Madrid, Spain), and 0.8 µg/mL puromycin (Thermo Fisher Scientific) at 37°C in a 5% CO₂ atmosphere in humidified air.

Transfection of HEK293 cells with pEf-1a-Tet3G plasmid was performed, according to the manufacturer's protocol (Tet-On 3G Inducible Expression

System; catalog no. PT5148-1 [010814]; Clontech Laboratories, Mountain View, Calif).

HEK293 cells were seeded into 6-well plates with 2 mL of medium per well to make the cells 90% confluent. Two mix reactions were created: for mix A, 2.4 μ g of pEF-1a-Tet3G plasmid was diluted in 125 μ L of Opti-MEM medium, and for mix B, 5 μ L of Lipofectamine 2000 (Thermo Fisher Scientific) was diluted with 125 μ L of Opti-MEM medium and incubated at room temperature for 5 minutes. Mixes A and B were mixed and incubated at room temperature for 20 minutes. Mix solution was added to HEK293 cells with 2 mL of culture medium (DMEM + 10% FBS) and incubated in CO₂ incubator at 37°C for 40 hours. After the culture time period, cells were trypsinized, serially diluted in 10-cm dishes with culture medium (DMEM, 10% FBS, and 0.4 mg/mL G418), and cultured for 2 weeks until the colonies were visible. When clones were visible, a total of 80 clones were selected by using a clone cylinder. Clones were cultured in 96-well plates and split into 3 sets for each clone. One set of clones was kept in culture. The other 2 sets were transfected with pTRE3G-Luc reporter plasmid. Twenty-four hours after transfection, we induced the cells with 1 μ g/mL doxycycline (for each clone, one for uninduced and one for doxycycline induced) for another 24 hours. We then tested luciferase activity using the GeneCopoeia firefly luciferase kit (Luc-Pair Firefly Luciferase HS Assay Kit; catalog no. LF007; GeneCopeia, Rockville, Md). The clone showing the best doxycycline-induced luciferase activity was selected as the best clone for the HEK293/Tet3G stable cell line. We then went back to the set culture without transfection of pTRE3G-Luc and cultured it as the HEK293/Tet3G Cell Bank. From the HEK293/Tet3G cell line, we used the same transfection method to cotransfect CS-H2247-pTRE3G (2.4 μ g, Fig E5) and the puromycin linear marker (0.12 μ g) into 6-well plate. Forty hours after selection, we serially diluted the transfected cells with 0.4 μ g/mL G418 and 0.8 μ g/mL puromycin in 10-cm dishes. Using the same protocol, we then picked 20 single clones for clone screening.

Each stable clone was duplicated, one set kept in culture as a cell bank and the other set used to test MUC4 expression by using RT-PCR. For each clone to be tested, cells were split into 2 wells equally. One well was induced by 1 μ mol/L doxycycline for 24 hours. The other was not induced with doxycycline. Gene sequence and CS-H2247-pTRE3G MUC4 full-length plasmid is shown in Fig E4 and online repository data 2 (full sequence). Doxycycline did not induce changes in cell viability at the concentrations used in this work, as assessed by using the trypan blue assay (data not shown).

Statistical analysis

Statistical analysis was carried out by using parametric or nonparametric analysis, as appropriate. A *P* level of less than .05 was considered to indicate statistical significance. In the NP tissue experiments, when the comparisons involved only 2 groups, between-group differences were analyzed by using the Mann-Whitney test. When comparisons involved several groups (NPwA, NP-ATA, NP-AIA, and NP-CR), a nonparametric 1-way ANOVA (Kruskal-Wallis test) was first performed in the case of a global significant difference; between-group comparisons were assessed by using the Dunn *post hoc* test, which generalizes the Bonferroni adjustment procedure.

In vitro mechanistic cell experiments were performed in primary NP epithelial cells or BEAS-2B cells. In these cases results were expressed as means (SEMs) of *n* experiments because a normal distribution for each data set was confirmed by means of histogram analyses and the Kolmogorov-Smirnov test. Statistical analysis was carried out by using parametric analysis. Two-group comparisons were analyzed with the 2-tailed Student paired *t* test for dependent samples and the unpaired *t* test for independent samples. Multiple comparisons were performed by using 1- or 2-way ANOVA, followed by the Bonferroni *post hoc* test. The percentage of inhibition of IL-8 and GM-CSF secretion was calculated, considering the increment between control (basal release) and stimulus (PGN or LPS) to be 100% of response, and the percentage inhibition of dexamethasone was calculated respect the 100% of response.

The CS-H2247-pTRE3G (with G63074) plasmid sequence is as follows:

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gccaccATGAAGGGGG
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REFERENCES

- E1. Milara J, Peiro T, Armengot M, Frias S, Morell A, Serrano A, et al. Mucin 1 downregulation associates with corticosteroid resistance in chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2015;135:470-6.
- E2. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012;50:1-12.
- E3. Casadevall J, Ventura PJ, Mullol J, Picado C. Intranasal challenge with aspirin in the diagnosis of aspirin intolerant asthma: evaluation of nasal response by acoustic rhinometry. *Thorax* 2000;55:921-4.
- E4. Milara J, Armengot M, Mata M, Morcillo EJ, Cortijo J. Role of adenylate kinase type 7 expression on cilia motility: possible link in primary ciliary dyskinesia. *Am J Rhinol Allergy* 2010;24:181-5.
- E5. Ortiz JL, Milara J, Juan G, Montesinos JL, Mata M, Ramon M, et al. Direct effect of cigarette smoke on human pulmonary artery tension. *Pulm Pharmacol Ther* 2010;23:222-8.

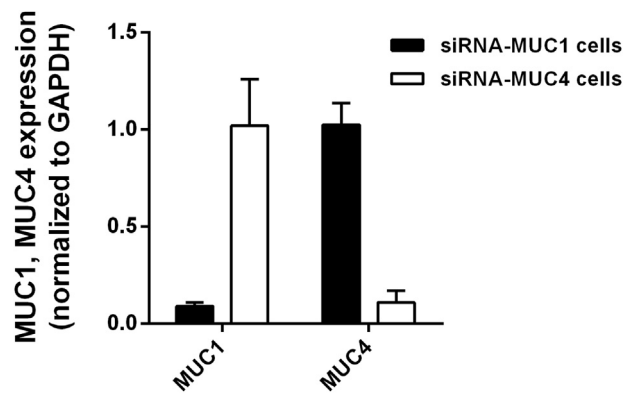


FIG E1. Silencing of MUC1 and MUC4 does not modify the other's expression. mRNA expression of *MUC1* or *MUC4* was measured in siRNA-MUC1 and siRNA-MUC4 BEAS-2B cells. Results are expressed as means (SEMs) of 3 independent experiments run in triplicate.

Beas2B epithelial cells

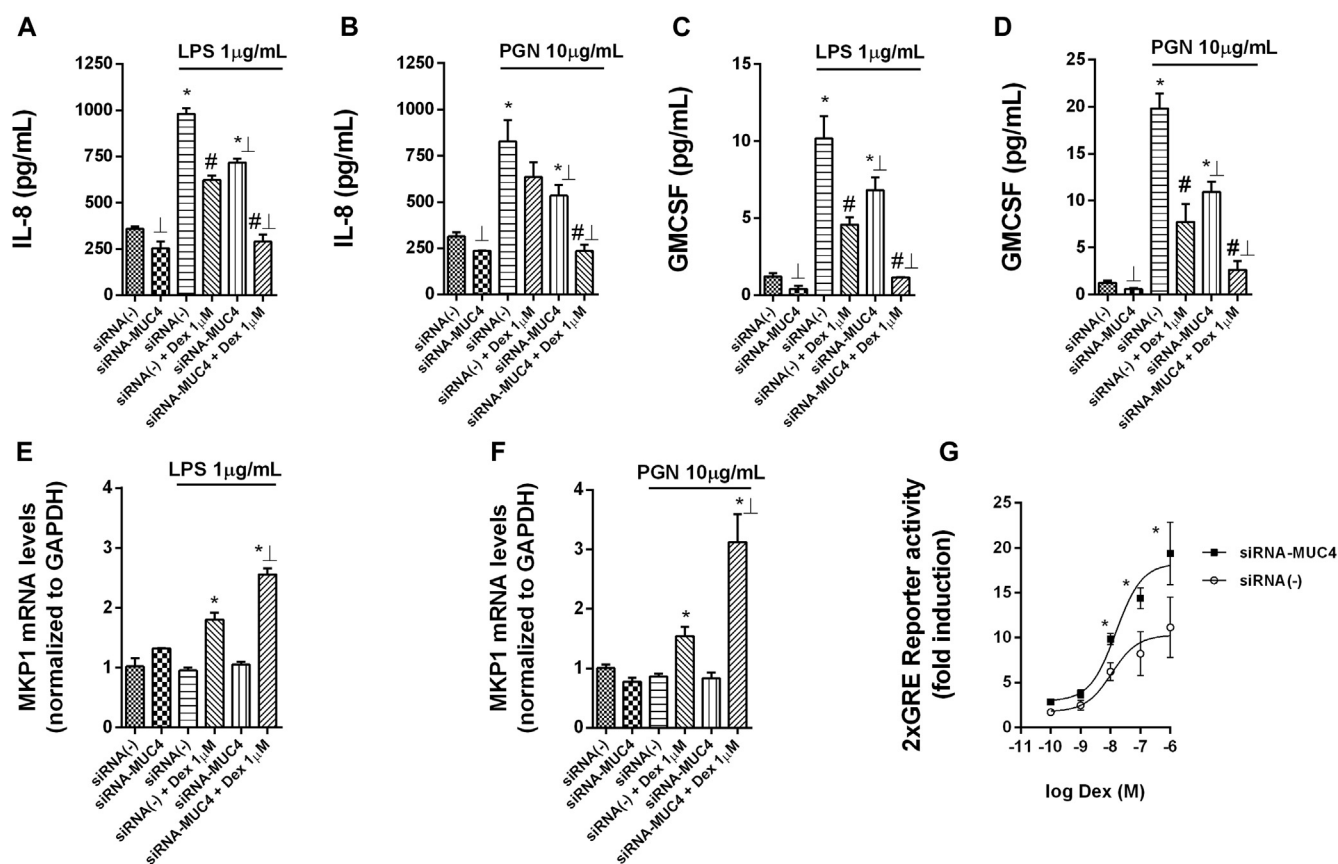


FIG E2. MUC4 knockdown improves the anti-inflammatory effects of dexamethasone. MUC4 knockdown by RNA interference (siRNA-MUC4) or negative control (siRNA(-)) was assayed in BEAS-2B cells. Cells were then incubated with or without 1 μ mol/L dexamethasone for 2 hours before LPS (1 μ g/mL) or PGN (10 μ g/mL) stimulation for 24 hours. **A-D**, IL-8 and GM-CSF concentrations were measured by using ELISA. **E** and **F**, MKP1 gene expression was quantified by using real-time PCR. **G**, GRE nuclear activation by dexamethasone (0.1 nmol/L-1 μ mol/L) in siRNA-MUC4 or siRNA(-) BEAS-2B cells. Results are expressed as means (SEMs) of 3 independent experiments run in triplicate. One-way ANOVA was followed by the *post hoc* Bonferroni test. Fig E2, A-F: * P < .05 related to control release in the same siRNA group; # P < .05 related to the stimulus LPS or PGN; $\perp$ P < .05 related to the siRNA(-) group. Fig E2, G: * P < .05 related to the siRNA(-) group.

Beas2B epithelial cells

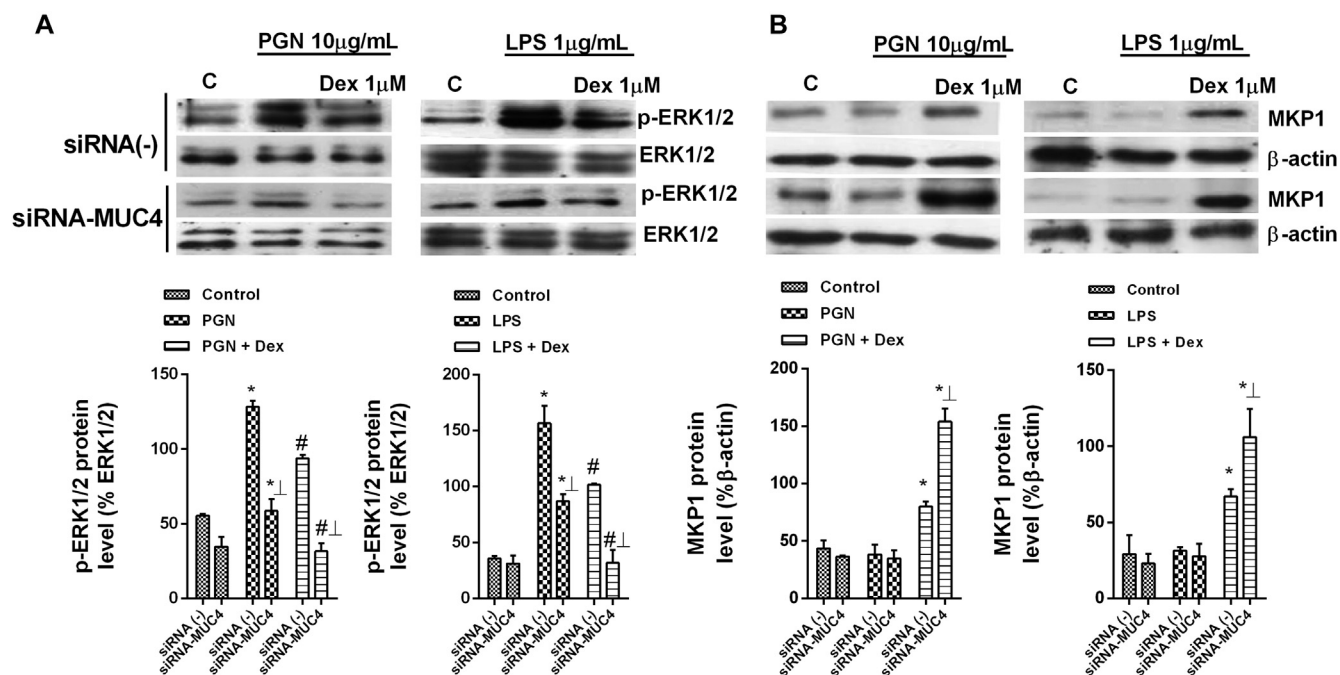


FIG E3. MUC4 knockdown improves the inhibitory effect of dexamethasone on ERK1/2 phosphorylation and MKP1 induction. MUC4 knockdown by means of RNA interference (siRNA-MUC4) or negative control (siRNA(-)) was assayed in BEAS-2B cells after stimulation with LPS or PGN in the presence or absence of 1 μ mol/L dexamethasone (added 2 hours before stimulus). After 20 minutes (**A**) or 4 hours (**B**) of stimulation, phospho-ERK1/2 or MKP1 protein levels, respectively, were measured by means of Western blotting. Representative Western blots are shown. Data are expressed as the densitometry of percentage ratios to β -actin protein levels. Results are expressed as means (SEMs) of 3 independent experiments. One-way ANOVA was followed by the *post hoc* Bonferroni test. * $P < .05$ related to the control group. # $P < .05$ related to the stimulus. $\perp P < .05$ related to the siRNA(-) group.

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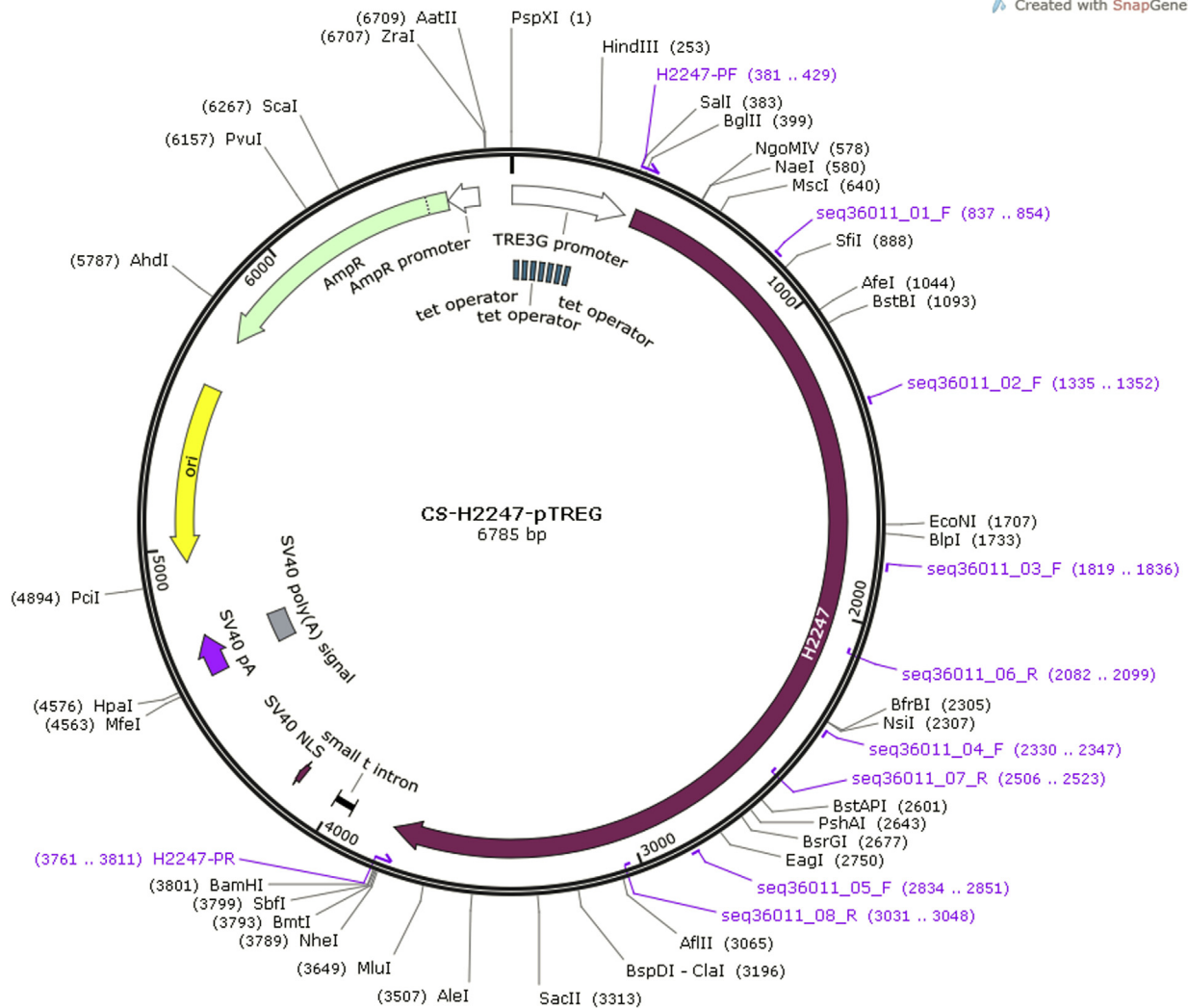


FIG E4. CS-H2247-pTREG full-length MUC4 map.

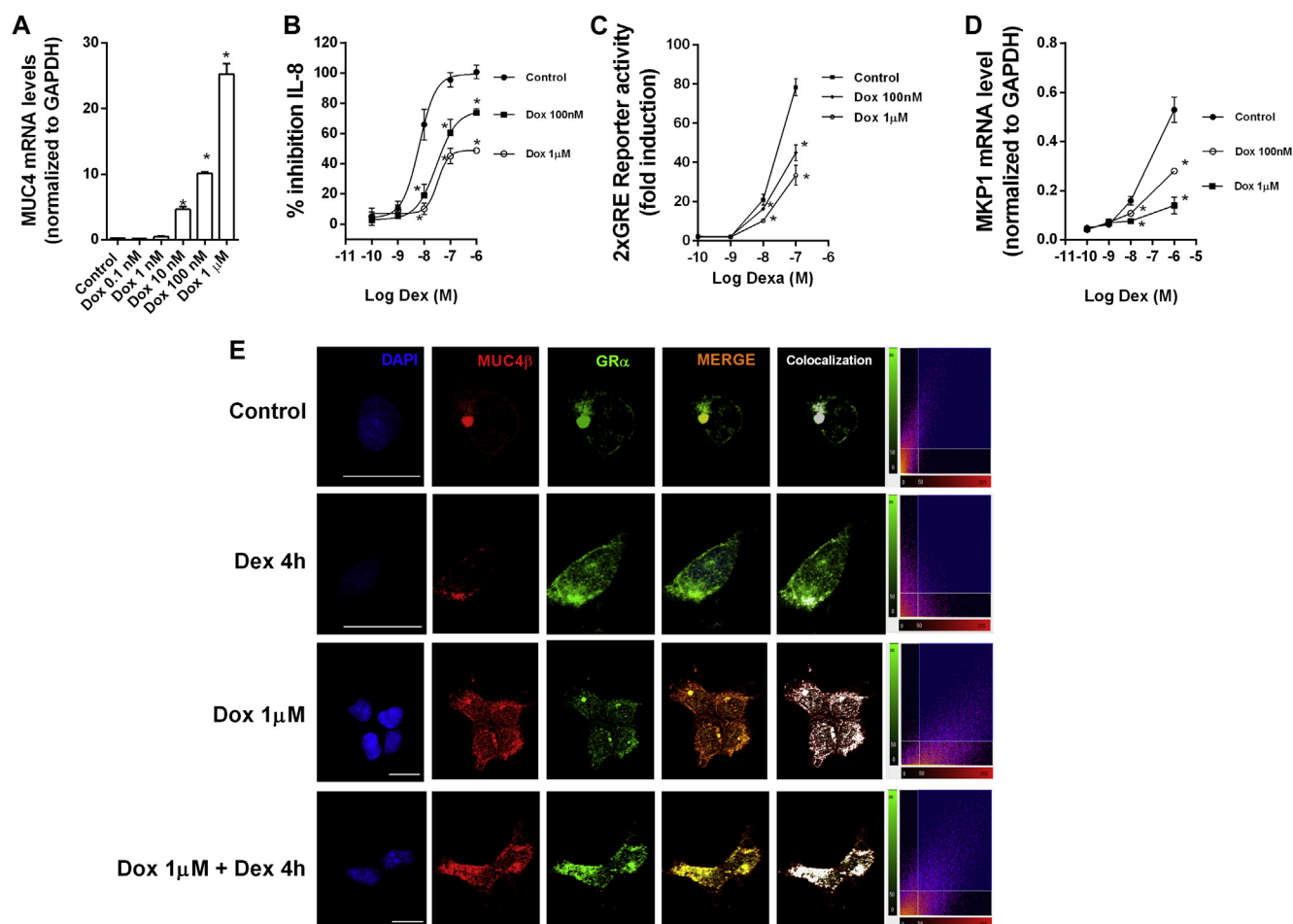


FIG E5. Effect of MUC4 overexpression on anti-inflammatory effects of dexamethasone in the HEK293/Tet3G/TRE3G-MUC4 stable cell line. **A**, The HEK293/Tet3G/TRE3G-MUC4 stable cell line was stimulated with 1 μ mol/L doxycycline (Dox) for 24 hours, and MUC4 mRNA expression was measured by using RT-PCR. **B**, The HEK293/Tet3G/TRE3G-MUC4 stable cell line was incubated with or without doxycycline (100 nmol/L, 1 μ mol/L) for 24 hours, followed by addition of dexamethasone (Dex; 0.1 nmol/L-1 μ mol/L) for 2 hours and later stimulation with LPS (1 μ g/mL) for 24 hours. Supernatants were collected to measure IL-8 levels by means of ELISA, and percentage IL-8 inhibition was calculated with respect to LPS (1 μ g/mL stimulus). **C**, GRE nuclear activation by dexamethasone (0.1 nmol/L-1 μ mol/L) in the HEK293/Tet3G/TRE3G-MUC4 stable cell line pretreated with or without doxycycline (100 nmol/L, 1 μ mol/L) for 24 hours. **D**, The HEK293/Tet3G/TRE3G-MUC4 stable cell line was incubated with or without doxycycline (100 nmol/L, 1 μ mol/L) for 24 hours, followed by addition of dexamethasone (0.1 nmol/L-1 μ mol/L) for 24 hours. At the end of the incubation period, MKP1 mRNA expression was measured by using RT-PCR. Results are expressed as means (SEMs) of 3 independent experiments run in triplicate. One-way ANOVA was followed by the *post hoc* Bonferroni test. Fig E5, A-D: * $P < .05$ related to control group. **E**, The HEK293/Tet3G/TRE3G-MUC4 stable cell line was incubated with or without doxycycline (1 μ mol/L) for 24 hours, followed by 1 μ mol/L dexamethasone for 4 hours. Cells were fixed, permeabilized, and used for MUC4 β (red) and GR α (green) immunostaining. Under basal control conditions, MUC4 β colocalized with GR α in the cytoplasm in absence of dexamethasone. After dexamethasone stimulation, GR α dissociated from MUC4 β and translocates to the nucleus. In HEK293/Tet3G/TRE3G-MUC4 cells preincubated with doxycycline, MUC4 β colocalized with GR α throughout the cell and remained colocalized with MUC4 β in cytoplasm and nucleus after dexamethasone stimulation. Colocalization was analyzed with a confocal spectral microscope (Leica TCS SP2) that generated a bidimensional cytofluorogram that selected common localized points of both antibodies (white color). 4'-6-Diamidino-2-phenylindole dihydrochloride, blue nuclei. Scale bars = 10 μ m.

TABLE E1. Polyp scoring system

Polyp score	Polyp size
0	No polyps
1	Small polyps in the middle meatus not reaching below the inferior border of the middle concha
2	Polyps reaching below the lower border of the middle turbinate
3	Large polyps reaching the lower border of the inferior turbinate or polyps medial to the middle concha
4	Large polyps causing almost complete congestion/obstruction of the inferior meatus

TABLE E2. Characteristics of patients with NPs at baseline and after 15 days of oral corticosteroids

	NPwA group	NP-ATA group	NP-AIA group	NP-CR group
No.	14	12	33	22
Age (y), mean (range)	57.1 (39-71)	55 (41-68)	49.1 (23-73)	54.2 (26-71)
Sex (male/female)	9/5	5/7	9/24	10/12
Asthma, no. (%)	0 (0)	12 (100)	33 (100)	16 (72.7)
Aspirin-intolerant asthma, no. (%)	0 (0)	0 (0)	33 (100)	14 (63.6)
Atopic, no. (%)	5 (35.7)	4 (33.3)	15 (45.5)	8 (36.4)
Intranasal corticosteroid, no. (%)	12 (100)	12 (100)	33 (100)	22 (100)
Current smokers, no. (%)	3 (25)	2 (16.6)	6 (18.2)	3 (13.6)
Bilateral NP endoscopic score before treatment, mean (SEM)	5.52 (0.41)	5.72 (0.51)	5.82 (0.27)	5.58 (0.52)
Bilateral NP endoscopic score after treatment, mean (SEM)	4.01 (0.10)*	4.11 (0.22)*	4.31 (0.12)*	5.49 (0.24)†
Change in bilateral NP endoscopic score, Δ mean (SEM)	−1.51 (0.12)	−1.61 (0.30)	−1.51 (0.28)	−0.09 (0.02)†
<i>MUC4</i> mRNA tissue expression before treatment ($2^{-\Delta Ct}$), mean (SEM)	0.483 (0.25)	0.570 (0.25)	0.964 (0.49)	12.83 (4.6)†
<i>MUC4</i> mRNA tissue expression after treatment ($2^{-\Delta Ct}$), mean (SEM)	0.501 (0.220)	0.578 (0.47)	0.495 (0.31)	10.76 (3.12)†

* $P < .05$ versus before treatment.† $P < .05$ versus the NPwA, NP-ATA, and NP-AIA groups.

TABLE E3. Characteristics of patients resistant to oral corticosteroid therapy

Total no.	NPwA group (n = 20)	NP-ATA group (n = 14)	NP-AIA group (n = 47)
NP-CR group, no. (%)	6 (30)	2 (14.3)	14 (29.8)
Change in bilateral NP endoscopic score, Δ mean (SEM)	−0.11 (0.1)	−0.09 (0.04)	−0.07 (0.05)
Age (y), mean (range)	50.13 (26-58)	66.5 (60-71)	55.2 (34-68)
Sex (male/female)	4/2	1/1	4/10
Asthma, no. (%)	0 (0)	2 (100)	10 (100)
Aspirin-intolerant asthma, no. (%)	0 (0)	0 (0)	10 (100)
Atopic, no. (%)	2 (9.1)	1 (50)	5 (35.7)
Current smokers, no.	2	0	1
<i>MUC4</i> mRNA tissue expression before oral treatment ($2^{-\Delta Ct}$), mean (SEM)	9.95 (2.24)	12.91 (4.31)	12.42 (5.13)
<i>MUC4</i> mRNA tissue expression after oral treatment ($2^{-\Delta Ct}$), mean (SEM)	10.80 (3.61)	10.29 (5.71)	11.57 (4.19)