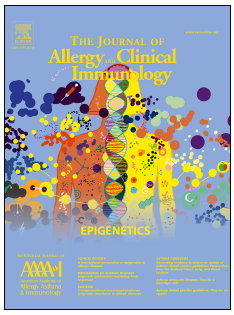
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Hypomethylation of *IL8* promoter in nasal epithelial cells of patients with chronic rhinosinusitis with nasal polyps

Jingyun Li, PhD, Jian Jiao, PhD, Ming Wang, PhD, Yunbo Gao, MD, Ying Li, BS, Yang Wang, BS, Yuan Zhang, MD, Xiangdong Wang, MD, Luo Zhang, MD, PhD

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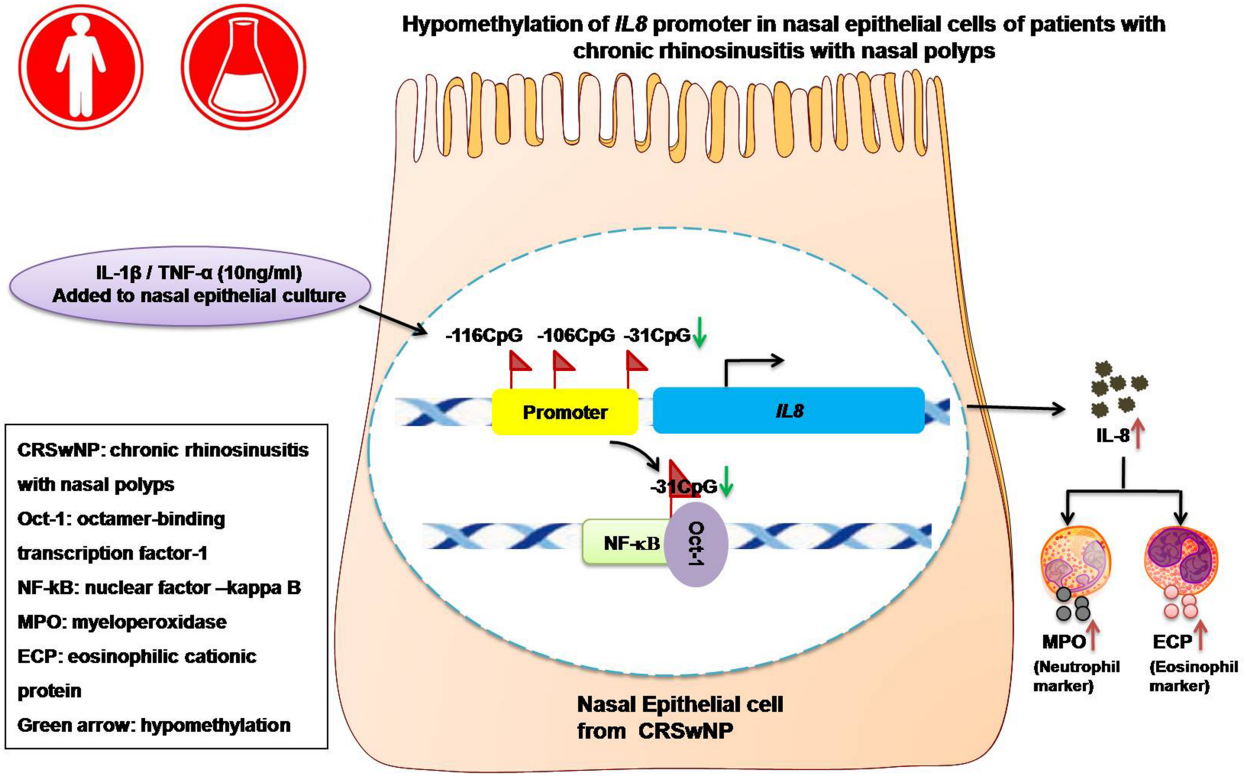
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Hypomethylation of *IL8* promoter in nasal epithelial cells of patients with chronic rhinosinusitis with nasal polyps

Jingyun Li, PhD[1, 2,*], Jian Jiao, PhD[1, 2,*], Ming Wang, PhD[1,2], Yunbo Gao, MD[1,2], Ying Li, BS[1,2], Yang Wang, BS[1,2], Yuan Zhang, MD[1, 2, 3], Xiangdong Wang, MD[1, 2, 3] and Luo Zhang, MD, PhD[1 ,2, 3]

1 Department of Otolaryngology Head and Neck Surgery, Beijing TongRen Hospital, Capital Medical University, Beijing 100730, China.

2 Beijing Key Laboratory of Nasal diseases, Beijing Institute of Otolaryngology, Beijing 100005, China.

3 Department of Allergy, Beijing TongRen Hospital, Capital Medical University, Beijing 100176, China.

* These authors contributed equally to this work

Corresponding authors:

Luo Zhang, M.D.,Ph.D.

Beijing Institute of Otolaryngology

No. 17, HouGouHuTong, DongCheng District,

Beijing, 100005. China

E-mail: dr.luozhang@139.com

Phone: +861065141136

Fax:+8610 85115988

Xiangdong Wang, M.D.Ph.D

Beijing Institute of Otolaryngology

No. 17, HouGouHuTong, DongCheng District,

Beijing, 100005. China

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E-mail: entwxd@vip.sina.com

Mobile phone: +86 13911189954

Yuan Zhang, M.D., Ph.D.

Beijing Institute of Otolaryngology

No. 17, HouGouHuTong, DongCheng District,

Beijing, 100005. China

E-mail: summer_zhang1211@126.com

Mobile phone: +86 15901566981

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ABSTRACT

Background: IL-8 is an important chemokine implicated in the pathogenesis of chronic rhinosinusitis (CRS), but little is known about epigenetic regulation of *IL8* in the pathogenesis of CRS.

Objective: To investigate the relationship between the DNA methylation level in *IL8* proximal promoter and CRS in Han Chinese.

Methods: CRS patients with (CRSwNP; n=187) and without nasal polyps (CRSsNP; n=89), and control subjects (n=57) were enrolled in two independent cohorts. Purified nasal epithelial cells (HNECs) from each participant were assessed for percentage % DNA methylation of CpG sites in *IL8* proximal promoter using bisulfite pyrosequencing; and for functional aspects of methylation status using in-vitro assays.

Results: DNA methylation of CpG sites 1, 2, and 3, respectively, in *IL8* proximal promoter was significantly decreased in HNEC of CRSwNP patients compared with CRSsNP patients ($P<0.001$) and control subjects ($P<0.001$). % DNA methylation of the CpG3 site was correlated negatively with both tissue eosinophilic cationic protein (ECP) ($P< 0.01$) and myeloperoxidase (MPO) ($P<0.05$) levels. IL-1β ($P<0.001$) and TNF-α ($P<0.01$) significantly increased *IL8* expression, accompanied by reduction in methylation at the CpG3 site ($P<0.001$). Electrophoretic mobility shift assays demonstrated that methylation status of CpG3 changed the binding of octamer-binding transcription factor-1 (Oct-1) and nuclear factor –kappa B (NF-κB).

Conclusion: Decreased DNA methylation of particularly CpG sites in *IL8* proximal promoter may play a role in pathogenesis of CRSwNP.

Key Messages

- CRSwNP patients have more significant decrease of DNA methylation of three CpG sites in the *IL8* proximal promoter than both CRSsNP and control subjects.
- The level of hypomethylation of IL8 proximal promoter CpG sites is associated with tissue IL-8 protein expression and granulocyte activation.

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The CpG3 site in *IL8* is further hypomethylated following IL-1β or TNF-α stimulation.

Hypermethylated CpG3 site in the *IL8* promotes the ability of Oct-1 and NF-κB to bind.

Capsule summary

CRSwNP patients demonstrated significantly decreased DNA methylation of CpG sites in *IL8* proximal promoter than CRSsNP patients and control subjects. Modulation of DNA methylation in *IL8* promoter may provide a novel way for managing CRS.

Keywords: Chronic rhinosinusitis; DNA methylation; Han Chinese; Human nasal epithelial cells; IL-8 proximal promoter, Nuclear factor –kappa B; Octamer-binding transcription factor-1.

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Abbreviations:

ALI: air-liquid interface

AP-1: activating protein-1

ECP: eosinophilic cationic protein

EMSA : Electrophoretic mobility shift assay

EPOS: European Position Paper on Rhinosinusitis and Nasal Polyps

CRS: Chronic rhinosinusitis

CRSwNP : CRS with nasal polyps

CRSsNP : CRS without nasal polyps

C/EBP : CCAAT/enhancer-binding protein

DNMT: DNA methyltransferase

GAPDH : glyceraldehyde-3-phosphate dehydrogenase

HNECs: human nasal epithelial cells

IT: inferior turbinate

MPO: myeloperoxidase

NF-κB: containing nuclear factor –kappa B

Oct-1: octamer-binding transcription factor-1

PCR: polymerase chain reaction

SNP: single nucleotide polymorphism

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INTRODUCTION

Chronic rhinosinusitis (CRS) is a common airway disease characterized by the presence of recurrent and spontaneous chronic inflammation in the nasal mucosa.[1] CRS can be differentiated into two major forms; CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). Although CRS is not a life-threatening disorder, the persistent inflammation can lead to perennial nasal congestion/obstruction, anosmia or hyposmia and facial pain/headache, as well as other problems that impact the quality of lives of affected individuals.[2, 3] A cross-sectional survey in seven major cities across China has reported CRS prevalence of 8%; with 107 million people affected.[4] Similarly, a more recent survey of residents in 18 major Chinese cities has reported an overall self-reported CRS prevalence of 2.2%,[5] indicating the serious burden associated with CRS in China.

Studies investigating genetic mechanisms in diseases underpinned by complex gene-environment risk have suggested that epigenetic or non-epigenetic mechanism/s are likely to be important;[6] with particularly DNA methylation modulating gene expression directly by affecting the binding of transcriptional factors and spatial organization of the chromatin. It has been suggested that hypermethylation of CpG sites in genes promotes gene silencing. Indeed, DNA methylation has been shown to regulate the expression of key pathways in the pathogenesis of immunologic and inflammatory disorders.[7] There is evidence that DNA methylation is also involved in the pathogenesis of CRS.[8,9] One study from Korea examined genome-wide DNA methylation levels in CRSwNP tissues and peripheral blood cells collected from aspirin-intolerant asthma and aspirin-tolerant asthma patients and demonstrated characteristic methylation patterns affecting 337 genes.[8] Another recent study employed DNA methylation microarray in a Chinese cohort and demonstrated a total of 198 genes, which had a methylated signal in the promoter region of these genes in nasal polyp tissue of CRSwNP patients compared with the genes in inferior turbinate mucosa from controls.[9]

Infiltration of the sinus mucosa with inflammatory cells is a hallmark of CRS; with IL-8 released by airway epithelial cells in response to inflammatory stimuli possibly playing an important role in attracting neutrophils to the site of inflammation.[10] Indeed, elevated levels of IL-8 have been detected

in significantly greater amounts in nasal mucosa of patients with CRS versus healthy control subjects.[11] We have recently studied $T_H1/T_H2/T_H17$ cytokine patterns in six cohorts of CRS patients from four different populations (two European, two Chinese, one Japanese and one Australian), and found that although there appeared to be an extreme diversity of the T_H cytokine profiles among these populations, the IL-8 levels were significantly upregulated in the CRSwNP groups in all populations compared to controls.[12] Moreover, IL-8 levels in the cohort from Beijing were much higher than in the other five cohorts. Similarly, other studies have demonstrated significantly increased levels of IL-8 in multidimensional endotypes of chronic rhinosinusitis.[13-15]

Although regulation of *IL8* gene expression in CRS has been reported to mainly involve a non-epigenetic mechanism,[16] relatively little is known about the role of epigenetic regulation of *IL8* in the pathogenesis of CRS. The sequences spanning 200bp within the *IL8* proximal promoter; which contains nuclear factor –kappa B (NF-κB), CCAAT/enhancer-binding protein (C/EBP), activating protein-1 (AP-1) and transcriptional binding elements; have been observed to be essential and sufficient for transcriptional regulation of *IL8* gene.[17-19] Thus, in the present study we aimed to identify the DNA methylation signal/s in *IL8* proximal promoter in human nasal epithelial cells (HNECs) from independent cross-sectional groups of CRSwNP and CRSsNP patients, and its relevance to the pathogenesis of CRS.

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METHODS

Subjects

A total of 333 adult subjects of Chinese Han origin were enrolled over two specific periods as two independent cohorts (discovery and replication cohorts, respectively) from the Rhinology Department of Beijing Tongren Hospital. The discovery cohort comprised 20 patients with CRSsNP, 68 patients with CRSwNP and 23 control subjects recruited from February 2013 to February 2014, and the replication cohort comprised 69 patients with CRSsNP, 119 patients with CRSwNP and 34 control subjects recruited from November 2014 to November 2015. The study protocol was approved by the Ethics Committee of Beijing TongRen Hospital and all subjects provided written informed consent prior to recruitment.

Diagnosis of CRSsNP and CRSwNP was based on patients' histories, clinical examinations, nasal endoscopies and computed tomographic scans of the sinuses according to the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012 guidelines for CRS.[1] CRSwNP was classified as eosinophilic when percentage tissue eosinophils exceeded 10% of total infiltrating cells. Patients without any sinonasal disease who presented for septoplasty for anatomic variations were recruited as control subjects. Atopic status of each participant was evaluated by detection of IgE antibodies against various common inhalant allergens using the Phadiotop test (Phadia, Uppsala, Sweden). Diagnosis of co-morbid asthma in CRS patients was made by pulmonologists based on lung function and challenge tests. None of the subjects had used oral or nasal corticosteroids for 4 weeks or antibiotics for 2 weeks before their surgeries.

Tissue samples were obtained from the inferior turbinate (IT) mucosa of control subjects, the ethmoid mucosae of patients with CRSsNP, and the nasal polyps (NP) of patients with CRSwNP during surgery. The tissues were cut into small sections and some sections from each sample were processed for staining by haematoxylin-eosin (H&E) stain (Figure E1), some sections were frozen in liquid nitrogen and stored at -80□ until immunoassay for inflammatory mediators, and the remaining sections from each sample were used for preparation of primary HNECs as previously described.[20] In

the latter case, the tissue sample was dissociated enzymatically, by incubation overnight in medium containing 0.1% protease, and the isolated epithelial cells were washed and collected by centrifugation at 120g for 10 mins. The purified nasal epithelial cells were suspended in culture medium and small aliquots from each suspension were stored at -80□ until assay for DNA methylation. The remaining cells were cultured overnight and primary HNECs were assessed for morphological features by phase contrast microscopy and for presence of pancytokeratin (epithelium; Sigma-Aldrich, St.Louis, MO, USA), type □β-tubulin (ciliated cells;Sigma-Aldrich), and mucin (MUC)-5AC (goblet cells; Abcam, Cambridge, UK)(Figure E2) by immunofluorescence staining.

DNA methylation analysis by bisulfite pyrosequencing

Genomic DNA was extracted from primary HNECs of each participant using human tissue DNA kit (Omega Bio-tek, Georgia, CA, USA). The quality of the DNA was assessed by electrophoretic migration on 1% agarose gel, and the concentration of the DNA was determined by UV spectroscopy at 260 nm. A total of 500ng genomic DNA was bisulfite-treated with EZ DNA methylation-Gold™ kit (Zymo Research, Orange, CA, USA).

As the 200bp of the proximal promoter region of *IL8* gene contains only three CpG sites (-116bp, -106bp and -31bp) close to transcriptional binding sites (Figure 1), DNA methylation was investigated for these CpG1, CpG2 and CpG3 sites using the PyroMark Q96 ID system (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) and sequencing primers were designed using the PyroMark assay design software version 2.0 (Qiagen) (Table E1 provided in online repository for this manuscript). The reverse primer was biotinylated at its 5'end and the bisulfite-converted DNA was amplified by PCR using PyroMark PCR kit (Qiagen). Pyrosequencing was performed using PyroMark Gold CDT kit (Qiagen) according to the manufacturer's instructions. Data were analyzed using the Pyro Q-CpG software program.

SNP genotyping and selection

The rs4073, a known functional single nucleotide polymorphism (SNP) frequently located within the proximal promoter region of *IL8* and associated with allergic and immunological diseases, was

assessed to test for any association with methylation at the CpG sites in the *IL8* promoter. The rs4073 was genotyped by Sanger sequencing and direct PCR products were sequenced using Bigdye terminator v3.1 cycle sequencing kit (Applied Bigsystems, FosterCity, CA, USA).and the PCR products were analyzed using ABI 3700XL Genetic Analyzer.

Measurement of IL-8, ECP and MPO levels in tissue homogenates

Tissue homogenates from discovery cohort were prepared, as previously described.[21] Frozen samples of inferior turbinate tissue from control subjects, ethmoid tissue from CRSsNP patients and NP tissue from CRSwNP patients were weighed, mixed with 0.9% NaCl (1 ml of 0.9% NaCl per 0.1g of tissue) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich, St Louis, Mo), and homogenized for 2 minutes using an automated homogenizer (TissuesLyser LT; Qiagen). After homogenization, the samples were centrifuged at 3000 rpm for 10 minutes, and the supernatants were collected and stored at -80□ until analysis of IL-8, ECP and MPO by immunoassay.

The concentration of IL-8 and myeloperoxidase (MPO) were measured using commercially available ELISA kit (R&D Systems, Minneapolis, USA; Oxis International, Portland, Ore), and the concentration of eosinophilic cationic protein (ECP) was measured using the UniCAP system (Pharmacia, Uppsala, Sweden), according to the manufacturer's protocols.

Cell culture

NP tissues from 10 CRSwNP patients obtained at surgery were immediately placed in cold Hank's balanced salt solution and used for establishing ex-vivo air-liquid interface (ALI) cell cultures as previously described.[20] Two days-old ALI cultures were stimulated by addition of 10ng/ml each of IL-1β (R&D Systems, Minneapolis, USA), TNF-α (R&D, Minneapolis, USA)or IL-17 (Santa Cruz Biotechnology, Santa Cruz, Calif) to the culture medium in the basolateral compartments of separate cultures. The culture medium in the basolateral compartment was replaced with fresh medium containing the appropriate cytokines every other day for a total period of 14 days, and at the end of this treatment period the cultured cells were harvested for assessment of *IL8* gene expression,

methylation of the CpG sites in the IL-8 promoter and expression of three key DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*).

Real-time PCR to assess expression of *IL8, DNMT1, DNMT3A* and *DNMT3B* genes

Total RNA from HNECs and ALI cell cultures was extracted with TRIzol reagent (Ambion-Life Technologies, Carlsbad, Calif), and the purity and integrity of the total RNAs were determined by ultraviolet spectrometric measurements and agarose gel electrophoresis, respectively. Complementary DNA (cDNA) was synthesized in a 20 μl reaction volume from 1 μg total RNA using QuantscriptRT kit (Cwbio Co., Beijing, China). The primers employed for the RT-PCR were obtained from QuantiTect Primer Assays (Qiagen, Hilden, Germany). Expression of *IL8, DNMT1, DNMT3A* and *DNMT3B* mRNAs was measured using UltraSYBR Mixture (Cwbio Co.), and relative quantitative analysis was performed using ABI7500 Real-Time PCR Detection system (Applied Biosytems). Relative expression level of targeted genes was normalized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and quantified using the ΔΔCt method.

Electrophoretic mobility shift assay (EMSA)

A total of 3 transcriptional factors (NF-κB, C/EBP and octamer-binding transcription factor-1 (Oct-1)) were predicted to bind to the proximal promoter region where the CpG3 methylation site was located.. Nuclear protein extracts were prepared from 1.5×10^7 healthy HNECs stimulated with IL-1β (10ng/ml) for 5 h. Sequences of oligonucleotides for probe preparation were as follows:

CpG3_unmethylated: ggccatcagttgcaaatCGtggaatttcctctgac;

CpG_methylated: ggccatcagttgcaaat^{me}CGtggaatttcctctgac;

NF-κB consensus sites: AGTTGAGGGGACTTTCCCAGGC;

OCT1 consensus sites: TGTCGAATGCAAATCACTAGAA;

C/EBPB consensus sites: TGCAGATTGCGCAATCTGCA.

Double-stranded DNA probes 5'labelled with biotin were used. The oligonucleotides and nuclear extract were incubated at room temperature for 20 minutes using Lightshift Chemiluminescent EMSA kit (Thermofisher, Waltham, MA, USA). The reaction mixture was separated on 6% PAGE, and the products were detected as stabilized streptavidin-horseradish peroxidase conjugates. Unlabeled oligonucleotides at 100-fold excess were added to the reaction for cold competition. For supershift assays, 4μg IgG (Abcam), p65 (GeneTex, Irvine, USA) or Oct-1 (Abcam) antibodies were added to the reaction before the first incubation step.

Blockade of IL-8 activity in ex vivo NP explants model

Fresh NP tissues from 4 CRSwNP patients were cut into 1mm^{3}fragments, and suspended in RPMI 1640 culture medium supplemented with 10% fetal bovine serum at 0.2g fragments per 1 ml. 0.5ml aliquots of this suspension were placed into separate wells of a 24-well tissue culture plate and incubated at 37□for 48 h in the presence of 10μg/ml human IL-8 monoclonal IgG1 antibody (R&D, Minneapolis, MN, USA) or control IgG1 (R&D). At the end of this incubation the NP explant tissue and the culture medium were collected from each well. The tissue explants were homogenised and both the homogenates and culture medium were assayed for ECP (CUSABIO, Wuhan, China), MPO (eBioscience, San Diego, CA, USA) using commercial ELISA kits. The protein levels of 27 different cytokines were also analysed using commercial Bio-Plex Pro human cytokine standard 27-Plex kits (Bio-Rad, Hercules, CA, USA) on Luminex 100 analyzer (Luminex, Austin, Tex).

Statistical analysis

SPSS 22.0 software for Windows was used for data analysis. All data are expressed as means±SEMs, unless otherwise noted. Multiple comparisons between groups were analyzed using the Kruskal-Wallis test with the Dunn test or 1-way ANOVA with the post hoc Holm-Sidak test. The Mann-Whitney *U* test and paired t test were used for between-group comparison. Differences in proportions between groups were tested by the χ^2 or Fisher exact test. Linear regression modeling was used to assess the relationship between methylation ratios at CpG1, CpG2 and CpG3sites within the *IL8* promoter and disease status after adjustment for age, gender, allergic status, smoking and

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drinking, and the rs4073 genotype. Log transformation was investigated to stabilize variance. Relationships between variables were evaluated using Spearman correlation analysis. A value of $P < 0.05$ was considered statistically significant.

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RESULTS

Demographic and clinic characteristics of the subjects

The demographic and clinic characteristics of the subjects are summarized in Table 1. Both groups were well matched for ethnicity and geographical location. Groups were comparable in terms of age, female/male ratio, allergy (positive Phadiotop test), asthma, smoking and drinking status. Allergy was significantly more frequent in patients with CRSwNP and CRSsNP than control subjects in the two cohorts (P =0.005 and 0.05), and comorbid asthma was noted mostly in the CRSwNP patient group.

Histochemical features of nasal tissues and HNECs from the different patient groups

Tissue sections stained by Hematoxylin-eosin (H&E) demonstrated that the nasal mucosa in inferior turbinate tissue of control subjects, ethmoid tissue of CRSsNP patients, and NP tissue of CRSwNP patients was lined by similar epithelium and subepithelium (endothelium, gland and stroma), regardless of variable abnormalities of NP (Fig E1 provided in online repository for this manuscript).

Similarly, phase contrast microscopy demonstrated a cobblestone-like morphology, and immunofluorescence staining demonstrated the presence of pancytokeratin, type □β-tubulin and mucin (MUC)-5AC in HNEC cultures derived from inferior turbinate tissue of control subjects, ethmoid tissue of CRSsNP patients, and nasal polyp (NP) tissue of CRSwNP patients (Fig E2 provided in online repository for this manuscript).

IL8 proximal promoter CpG sites are hypomethylated in HNECs from patients with CRSwNP

The sequences spanning 200bp within the *IL8* proximal promoter are important for transcriptional activity. Analysis of this region did not identify a CpG island, but 3 CpG sites were identified at positions -116bp, -106bp and -31 bp; which are referred to as CpG 1, CpG 2 and CpG3, respectively (Figure 1). The methylation status of each individual CpG sequence was determined by bisulfite pyrosequencing in the two cohorts. In the discovery cohort, the percentage methylation of all

three CpG sites in the promoter region was significantly decreased in the CRSwNP group, compared to the control group ($P < 0.001$); whereas in the larger replication cohort the percentage of methylation of all three CpG sites was significantly decreased in the CRSwNP group compared to both the control and CRSsNP groups ($P < 0.001$) (Figure 2). Similarly, a combined analysis of both cohorts also demonstrated significantly decreased methylation of all three CpG sites in the CRSwNP group compared to the control and CRSsNP groups.($P < 0.001$)

It is well recognized that the fine phenotyping of CRSwNP is a determinant of the underlying pathogenesis of CRS. In this regard evaluation of the percentage of methylation of the three CpG sites in eosinophilic and non-eosinophilic CRSwNP patients showed no significant differences between these sub-types (Figure E3 provided in online repository for this manuscript). Similarly, evaluation of CRSwNP patients with and without asthma demonstrated the CRSwNP with asthma subgroup to have a lower tendency for methylation of all three CpG sites than the CRSwNP without asthma subgroup, in both cohorts. However, analysis of combined data demonstrated a significant difference in CpG3 methylation levels ($P < 0.05$) (Figure E4).

In contrast, other factors, including allergic status, smoking and drinking, which might exert confounding effects on methylation, demonstrated no significant effect on the percentage of methylation of any of the three CpG sites in any of the three study groups in the two cohorts (Figures E5, E6 and E7 provided in online repository for this manuscript).

Assessment of the effect of the genetic variant SNP rs4073 was also not found to significantly influence the DNA methylation at *IL8* CpG sites (Figure E8 provided in online repository for this manuscript).

To determine if a combination of the above-mentioned factors might confound the association between methylation levels at CpG sites of *IL8* promoter and disease status, we performed linear regression model analysis and found that the relationship between hypomethylated *IL8* promoter and NP susceptibility remained robust even after controlling for these potential confounders (Table E2 provided in online repository for this manuscript).

Hypomethylated levels of *IL8* proximal promoter CpG sites associate with IL-8 protein expression and granulocytes activation

ELISA analysis showed that tissue IL-8 concentrations were significantly higher in patients with CRSwNP compared with patients in the CRSsNP and control groups ($P < 0.001$). Moreover, a significant negative correlation was observed between tissue IL-8 levels and the percentage of methylation of all three CpG sites (Figure 3).

We next evaluated the effect of activated eosinophils and neutrophils, based on levels of ECP for eosinophils and MPO for neutrophils, on DNA methylation at the three CpG sites of *IL8* promoter. In this respect tissue ECP and MPO levels were found to be significantly increased in patients with CRSwNP compared with the CRSsNP and control groups ($P < 0.001$) (Figure 4A). Moreover, ECP levels were significantly negatively correlated with the percentage of methylation of all three CpG sites and MPO levels moderately negatively correlated with only CpG3 methylation levels (Figures 4B,C, D).

Increased *IL8* gene expression and hypomethylated DNA methylation at CpG3 site after stimulation ex vivo

Considering that the percentage methylation of the three CpG sites displayed high correlations with each other (Figure E9 provided in online repository for this manuscript) and the CpG3 site was located closer to the transcriptional binding sites in *IL8* gene promoter than the CpG 1 and CpG 2sites (Figure 1), we further investigated the methylation at specifically the CpG3 site in nasal epithelial ALI cell cultures. These experiments demonstrated that *IL8* gene expression was significantly decreased from baseline levels after 14 days in control unstimulated cultures (Figure 5A, $P < 0.01$), and that there was a significant hypermethylation of the CpG3 site (Figure 5B, $P < 0.01$). In contrast, *IL8* gene expression was significantly increased from baseline levels in cultures incubated for 14 days with IL-1β ($P < 0.001$), TNF-α ($P < 0.01$) and IL-17 ($P < 0.05$) (Figure 5A). Furthermore, pyrosequence analysis showed that nasal cultures stimulated with IL-1β and TNF-α, but not with IL-17, displayed significant reduction in methylation of the CpG3 site compared to control cultures ($P <$

0.001)(Figure 5B). Assessment of the effect of stimulation with IL-1β , TNF-α and IL-17 on the expressions of the DNA methyltransferases *DNMT1*, *DNMT3A* and *DNMT3B* showed that these were not affected (Figure 5C).

DNA methylation at the CpG3 site in *IL8* influences transcription factor binding

EMSA was performed to examine the effects of CpG methylation on the binding of transcription factors, particularly NF-κB, C/EBP and Oct-1. Assessment of nuclear extracts from HNECs of healthy individuals showed that the DNA-protein complexes had higher binding affinity for the non-methylated status of the CpG3 site (Figure 6A lane 3) than the methylated status of the CpG3 site (Fig 6A lane 5).

Binding specificity was further validated by competitive assays for non-methylated status for the CpG3 site. Addition of a 100-fold molar excess of unlabelled CpG3-unmethylated oligonucleotides prevented DNA-protein complexes formation (Figure 6A lane 6), whereas a 100-fold molar excess of OCT1 consensus sequence completely abolished the formation of the high-molecular-weight complex (Figure 6A lane 9). Similarly, addition of anti-oct1 antibody abolished the formation of a high molecular weight complex (Figure 6B lane 4). In contrast, a 100-fold molar excess of NF-κB site had no effect on the high-molecular-weight complex, but abolished the NF-κB-specific complexes (Figure 6A lane 8). Addition of antibody against NF-κB p65 also had no effect on the high-molecular-weight complex, but resulted in the clearance of NF-κB complex (Figure 6B lane3). Similar competition experiments for probe of CpG3-methylated oligonucleotides and the consensus site for C/EBP showed no effect (Figure 6A lanes 7 and 10).

Blockade of IL-8 reduces NP tissue inflammation

The effect of incubation with anti-IL-8 monoclonal antibody on the expression of several inflammatory mediators in ex vivo NP explants from CRSwNP patients is demonstrated in Figure 7 and Figure E10 provided in online repository for this manuscript). Anti-IL-8 monoclonal antibody efficiently decreased the level of IL-8 in tissue homogenates and culture medium (Figure 7A). The

levels of ECP and IL-17A in tissue homogenates (Figure 7B), as well as the levels of MPO and IL-13 in culture medium (Figure 7C) were also significantly decreased in NP explants treated with anti-IL-8 monoclonal antibody compared to control IgG. However, no significant changes were noted in the levels of another 23 inflammatory mediators and growth factors in tissue homogenates or culture medium collected from NP explants treated with anti-IL-8 monoclonal antibody or control IgG (Figure E10 provided in online repository for this manuscript).

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DISCUSSION

The *IL8* gene is located on chromosome 4q12-4q21 and exhibits dysregulated methylation of CpG sites that have previously been associated with inflammatory phenotypes. We and others have previously indicated that elevated IL-8 protein concentrations in patients with CRSwNP appear to be the only common feature of T_H cytokine profiles in different ethnicities, and that appropriate modulation of the overproduced IL-8 leads to the attenuation of airway inflammation.[12, 13, 15] However, little is known about the effects of epigenetic regulation of *IL8* gene in the pathogenesis of CRS. To our knowledge, this is the first study to compare DNA methylation of proximal promoter of the gene encoding *IL8* in nasal epithelial cells of patients with CRSsNP, CRSwNP and the control subjects in two independent cohorts. We found that three CpG sites (CpG 1, 2 and 3) of the *IL8* gene proximal promoter exhibited significantly decreased DNA methylation in the HNECs from patients with CRSwNP from both cohorts. Furthermore, combined analysis of the two cohorts identified significantly lower levels of DNA methylation at the CpG3 site in *IL8* gene promoter of CRSwNP patients with comorbid asthma compared to patients without comorbid asthma. In contrast, no evidence was found of the influence of other potentially confounding factors, including CRSwNP phenotypes, allergic status, smoking, drinking or the genetic variant SNP rs4073. The percentage methylation of the three CpG sites was negatively correlated with tissue IL-8 concentrations, while tissue ECP (eosinophils) and MPO (neutrophils) levels were correlated negatively with the levels of CpG3 site methylation. Furthermore, increased expression of *IL8* in primary HNECs was accompanied by reduced methylation of the CpG3 site in cells treated with IL-1β and TNF-α, and the methylation status of the CpG3 site seemed to change the binding of transcription factors Oct-1and NF-κB.

The epithelial mucosa plays a critical role in the recruitment of inflammatory cells, because it is a major source of chemoattractant cytokines, including IL-8 in the pathogenesis of CRS. CpG hypomethylation of the IL-8 promoter has been investigated in osteoarthritis[22] several carcinomas,[23] and aggressive and chronic periodontitis[24, 25], with some studies showing distinct hypomethylation pattern of the *IL8* to be correlated with an increase in *IL8* expression. One recent study reported that

DNA methylation level at *IL8* promoter in bronchial epithelial cells from cystic fibrosis patients was lower than that in bronchial epithelial cells from control subjects.[26] However, no differences in *IL8* DNA methylation were observed in airway smooth muscle cells from asthmatic donors compared with non-asthmatic donors in the other study.[27] In the present study, methylation levels of *IL8* appeared to be down regulated, particularly in CRSwNP patients from the two independent cohorts, suggesting that local changes in methylation of *IL8* may contribute to the progression of chronic inflammation in the sinus mucosa.

Although previous studies have shown different immunologic features in eosinophilic and non-eosinophilic CRSwNP and different inflammatory patterns between European and Chinese patients with CRSwNP, [28, 29] in the present study we found that these phenotypes have similar methylation ratios in *IL8*. This is consistent with the findings from our previous multicenter study, which showed tissue IL-8 level to be the only cytokine that was elevated in CRSwNP patients from all 6 cohorts from four different populations (two European, two Chinese, one Japanese and one Australian).[12] Although it is well recognized that IL-8 is capable of eliciting neutrophilic inflammation in the airways, several studies have shown IL-8 to also be a potent mediator for eosinophil chemotaxis and transendothelial migration in vitro.[30, 31] Indeed, blockade of IL-8 in the present study was found to effectively decrease the levels of the Th2 and eosinophil-related mediators IL-13 and ECP, neutrophil-related mediator MPO, and Th17-related cytokine IL-17A in our NP explant model. Furthermore, the present study also demonstrated that tissue concentrations of ECP and MPO measured as markers of severity of local eosinophil and neutrophil activation, respectively, correlated negatively with the level of methylation of the CpG3 site in *IL8* promoter. Thus, these findings further support the notion that IL-8 can mediate activation of both eosinophils and neutrophils in eosinophilic and non-eosinophilic CRSwNP. However, the role of methylation in *IL8* promoter in the HNECs in activation of different granulocytes in CRSwNP requires further investigation.

The pro-inflammatory cytokines IL-1β and TNF-α as well as IL-17 have been shown to play a role in the development of CRS.[28] These cytokines have also been shown to regulate inducible expression of *IL8* gene. While *IL8* is barely expressed at the resting stage, *IL8* expression is

436 exponentially elevated by more than 100-fold in response to pro-inflammatory cytokines, particularly
437 IL-1β and TNF-α, which in turn rapidly stimulates recruitment of neutrophils from the blood to the
438 site of inflammation.[10] Similarly, IL-17 has also been demonstrated to induce *IL8* production for the
439 chemotaxis of neutrophils.[32] The present study has demonstrated that stimulation of nasal epithelial
440 cells with IL-1β or TNF-α, but not with IL-17, leads to hypomethylation of the CpG3 site in *IL8*
441 promoter suggesting that these cytokines regulate the expression of *IL8* gene via epigenetic
442 mechanisms. A recent study by Caradonna and colleagues[33] has shown that IL-1β-modulated changes
443 in the DNA methylation pattern of cytokine gene promoters may result in transcriptional changes of
444 pro-inflammatory genes. In accordance with the findings of the present study, these authors also
445 showed that exposure to IL-1β did not affect the expression of maintenance DNA methyltransferases.
446 However, little is known about the effect of TNF-α on DNA methylation pattern in cytokine gene
447 promoters. In recent years, DNA methyltransferase activity and protein stability has been shown to be
448 regulated by post-translational modification of the DNA-binding subunits by phosphorylation and
449 acetylation.[34] Thus, it is possible that IL-1β and TNF-α could affect DNA methylation patterns in *IL8*
450 promoter via post-translational regulation of the DNA methyl transferases.

451 Modification of methylation at specific CpG sites could potentially influence the affinity of
452 transcription factors for cis-elements. In the present study, the EMSA results showed that in IL-1β
453 stimulated HNECs, non-methylated CpG3 in *IL8* gene promoted the ability of Oct-1 and NF-κB to
454 bind the gene. Oct-1 is a crucial member of the POU family transcription factor that is ubiquitously
455 expressed across cell types and recognizes the octamer DNA element. Depending on the nuclear
456 environment of certain cell types and the context of regulatory sequence, Oct-1 can positively or
457 negatively regulate transcription activity.[35] Oct-1 is implicated in promoting initiation, progression
458 and maintenance of epithelial tumors,[36, 37] and also has a role in regulating immunity by targeting
459 production of cytokines and pro-inflammatory mediators.[38] In tumor cell lines, Oct-1 can bind the cis-
460 element of *IL8* promoter and repress the basal transcriptional activity.[39, 40] Similarly, NF-κB also plays
461 an important role in the inflammatory response and cytokine production. While IL-8 production is
462 mainly dependent on activity of NF-κB,[17] two studies have reported that Oct-1 can serve as an

463 alternative to NF-κB in activating *TNF*-α and *HLA-C* expression.[41, 42] Thus, hypomethylation at CpG3
464 in *IL8* promoter raises the possibility that Oct-1 may play a role in controlling NF-κB-mediated
465 effects on *IL8* expression in nasal epithelial cells.

466 In conclusion, whilst other cytokines, such as IL-5, IL-4, IL-13, IL-33 and TSLP, are no doubt
467 important in pathogenesis of CRSwNP, epigenome-wide association studies (EWAS) of CRS are still
468 lacking due to high costs and inadequate coverage of many candidate genes in the detection arrays.
469 Thus, in the present study we employed candidate gene strategy using quantitative pyrosequencing,
470 which is suitable for analysis of only short DNA segments (< 60 bp) and often limited to one gene per
471 study, to investigate epigenetic mechanism/s in the pathogenesis of CRSwNP using the *IL8* gene
472 promoter as an example, based on available data on the role of this mediator in CRS. However,
473 further studies investigating the role of drugs in modifying methylation in the *IL8* gene promoter and
474 studies determining the role of epigenetic modifications in other relevant cytokine genes are required
475 to fully elucidate the role of epigenetic mechanisms in the pathogenesis of airway inflammation in
476 CRS.

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TABLE

TABLE 1. Demographic and clinical characteristics of subjects in two independent cohorts

Characteristic	Control subjects	Patients with CRSsNP	Patients with CRSwNP	*P*
Discovery cohort				
No.	23	20	68	
Sex (female/male)	10/13	7/13	30/38	0.76
Age (y), median (range)	49 (19-68)	47 (18-72)	47 (18-70)	0.97
Allergy, no (%)	1 (4.3)	2 (10.0)	23 (33.8)	0.005
Asthma, no (%)	0 (0)	1 (5.0)	8 (11.8)	0.17
Smoking, no (%)	6 (26.1)	10 (50.0)	25 (36.8)	0.27
Drinking, no (%)	4 (17.4)	7 (35.0)	19 (27.9)	0.42
Replication cohort				
No.	34	69	119	
Sex (female/male)	13/21	24/45	42/77	0.94
Age (y), median (range)	40 (18-61)	44 (18-72)	45 (18-75)	0.05
Allergy, no (%)	2 (5.9)	18 (26.1)	24 (20.2)	0.05
Asthma, no (%)	0 (0)	1 (1.4)	9 (7.6)	0.06
Smoking, no (%)	11 (32.4)	18 (26.1)	29 (24.4)	0.64
Drinking, no (%)	10 (29.4)	18 (26.1)	29 (24.4)	0.83

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598 **FIGURE LEGENDS**

599 **FIGURE 1.** Genomic localization of CpG sites, binding sites of transcription factors and the SNP
600 rs4073 within the IL-8 proximal promoter. TSS: transcription start site.

601

602 **FIGURE 2.** Comparison of methylation ratios at CpG1 (A), CpG2 (B) and CpG3 (C) sites within
603 IL-8 proximal promoter in two independent cohorts.
604 *Dot plots* illustrate individual data points and solid lines represent means with SEMs. ***, $P < 0.001$.

605

606 **FIGURE 3.** Concentrations of IL-8 in tissue homogenates from discovery cohort. (A) and
607 relationship between concentrations and the methylation ratio of CpG1 (B), CpG2 (C) and CpG3 (D)
608 sites within IL-8 proximal promoter. *Dot plots* illustrate individual data points and solid lines
609 represent means with SEMs. ***, $P < 0.001$.

610

611 **FIGURE 4.** Concentrations of ECP and MPO in tissue homogenates from discovery cohort (A) and
612 relationship between concentrations and the methylation ratio of CpG1 (B), CpG2 (C) and CpG3 (D)
613 sites within IL-8 proximal promoter in CRSwNP. *Dot plots* illustrate individual data points and solid
614 lines represent means with SEMs. ***, $P < 0.001$.

615

616 **FIGURE 5.** Effect of IL-1β (10 ng/ml), TNF-α (10 ng/ml) and IL-17 (10 ng/ml) on CpG3
617 methylation and gene expression of IL-8 and DNA methyl transferases (DNMTs) (n = 10) in human
618 nasal epithelial cells (HNECs). A, IL-8 mRNA levels against *GAPDH* in pre-culture, unstimulated
619 (u.s) and stimulated cultures of HNECs. B, CpG3 methylation ratios in pre-culture, unstimulated (u.s)
620 and stimulated cultures. C, *DNMT1*, *DNMT3A* and *DNMT3B* mRNA levels against *GAPDH* in pre-
621 culture, unstimulated (u.s) and stimulated cultures.
622 Results are expressed as means ± SEMs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

623

624 **FIGURE 6.** Impact of methylation status of CpG3 site of IL-8 proximal promoter on binding Oct-
625 1and NF-κb. A, EMSA analysis with CpG3 site probe either unmethylated (lane 2, 3) or methylated
626 (lane 4, 5) was performed with nuclear extract from the human nasal epithelial cell cultured for 5
627 hours in presence of IL-1β (10 ng/ml). Lane 1 added the EBNA control. Competitor, 100-unlabed
628 probe; U, unmethylated probe; (lane 6) M, methylated probe (lane 7); NF-κb, NF-κb consensus probe

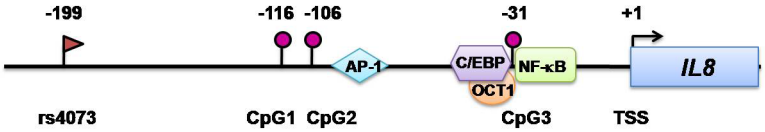
629 (lane8); OCT1, OCT1 consensus probe (lane 9); C/EBPB, C/EBPB consensus probe (lane 10). B,
630 EMSA supershift experiments with antibody aginst IgG (lane 2), p65 (lane 3) and Oct-1 (lane4).

631

632 **FIGURE 7.** The effect of IL-8 neutralization on the levels of IL 8 in tissue homogenates and culture
633 medium (A); and the levels of inflammatory mediators and growth factors in homogenates (B) and
634 culture medium (C) in ex vivo nasal polyp (NP) explants (n=4). NP explants were treated with
635 control IgG or human anti-IL-8 monoclonal antibody (10 μg/ml) for 48h. # ECP was not detectable in
636 culture medium and IL-13 was not detectable in tissue homogenates. Results are expressed as means
637 ± SEMs. *, $P < 0.05$; ***, $P < 0.001$.

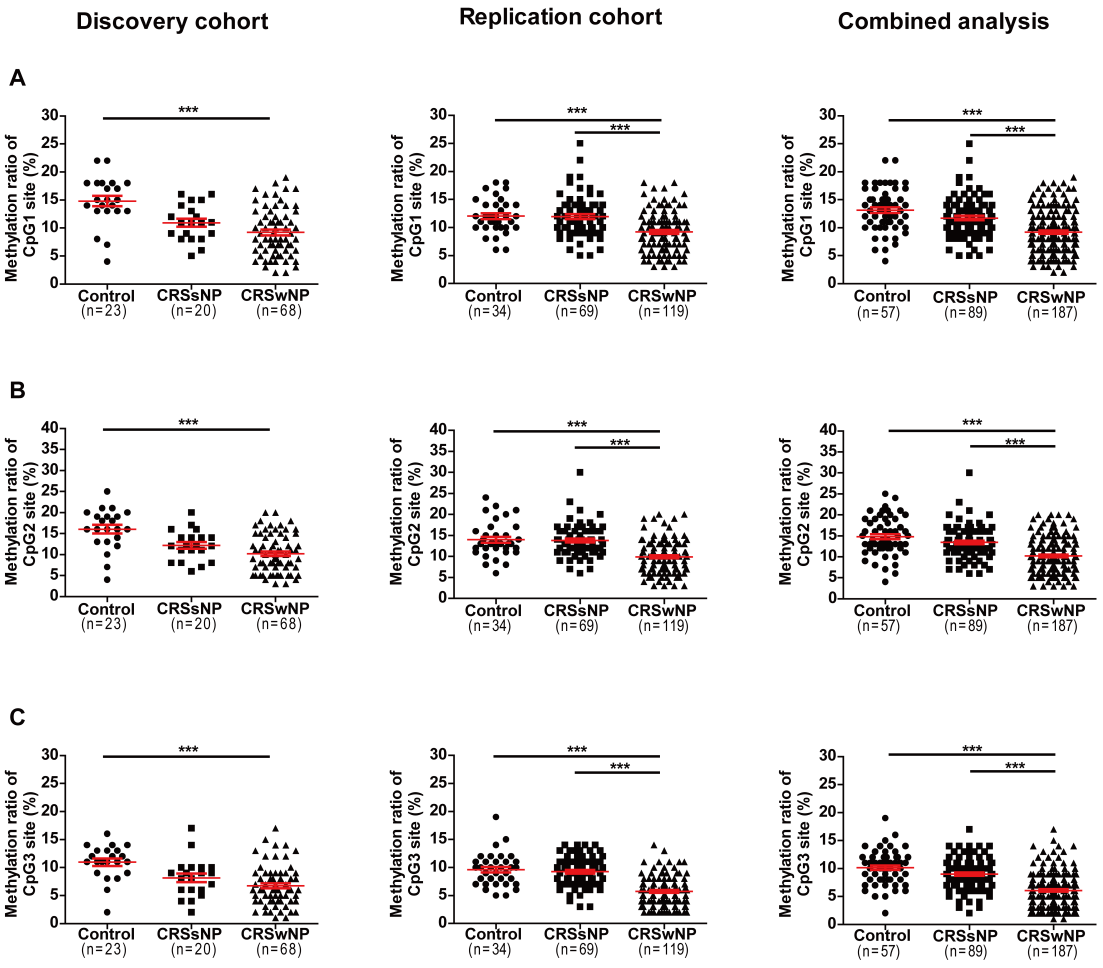
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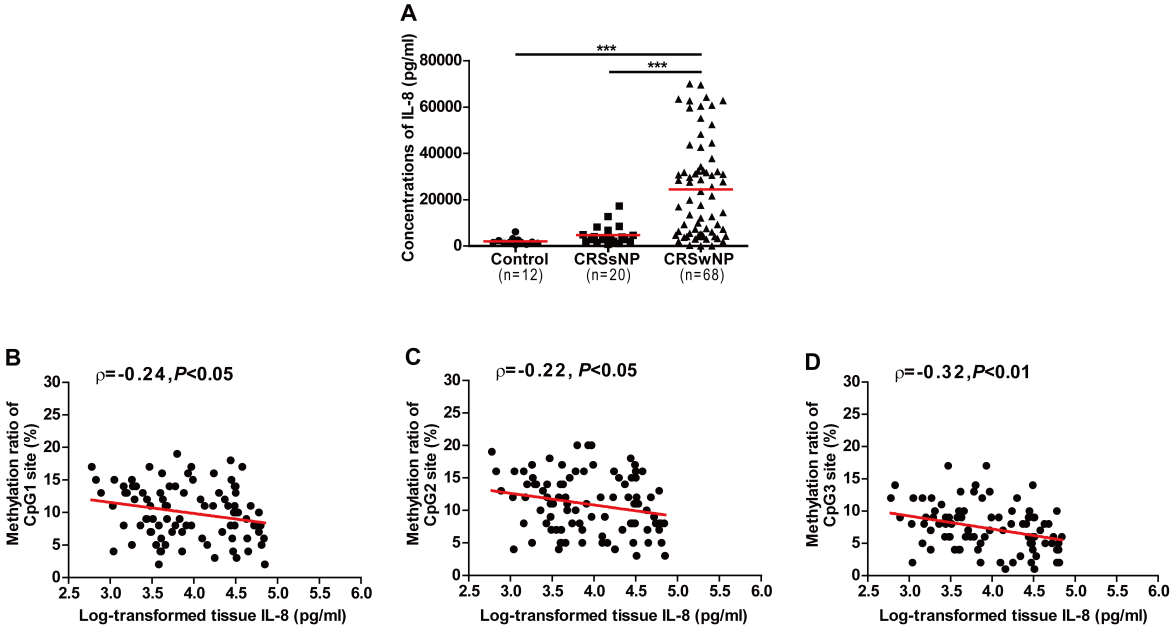


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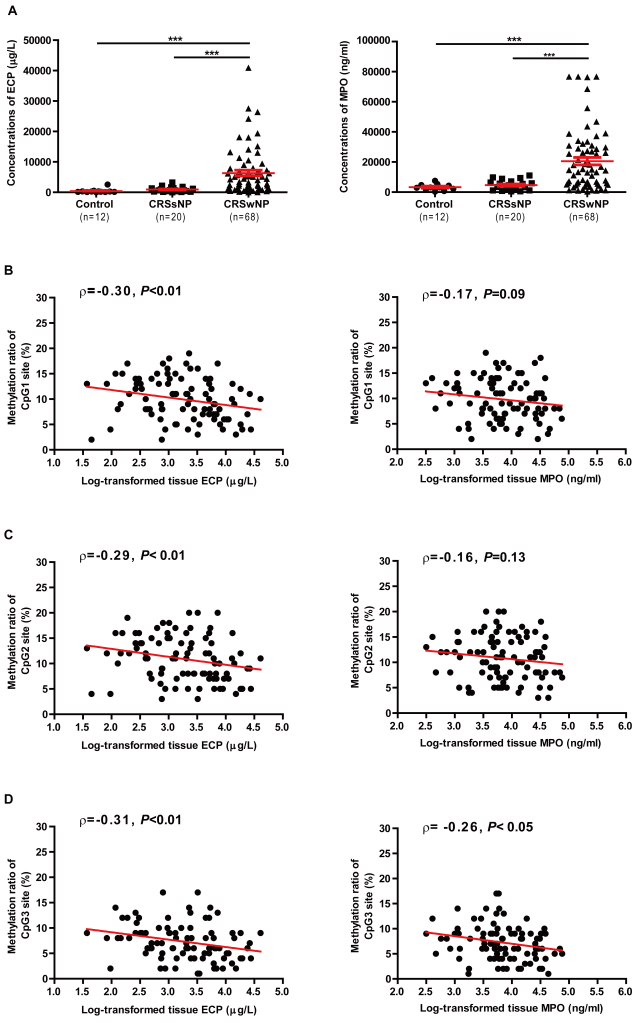
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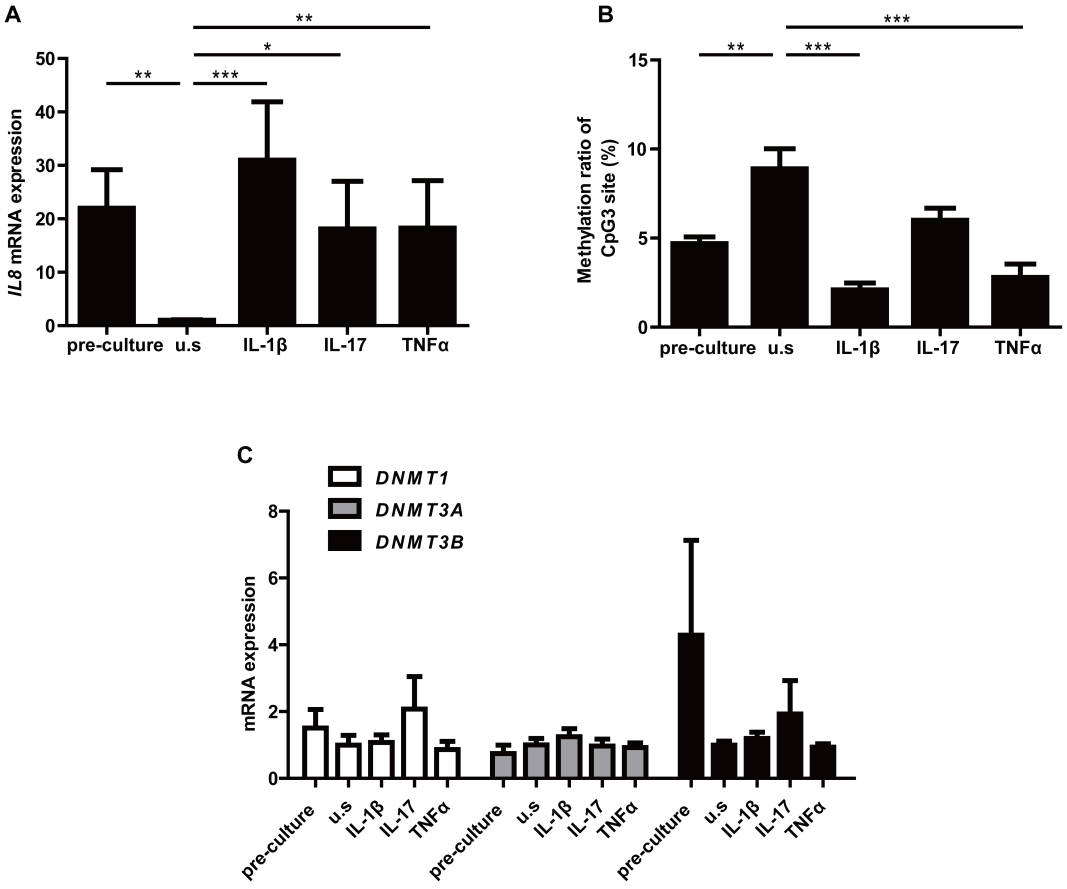
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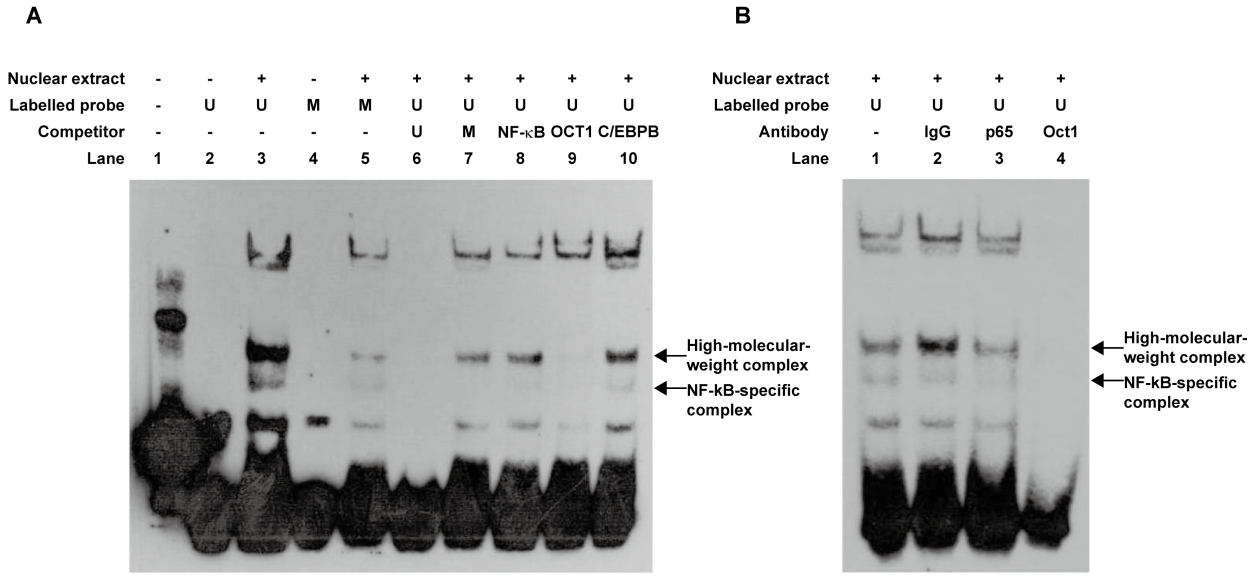
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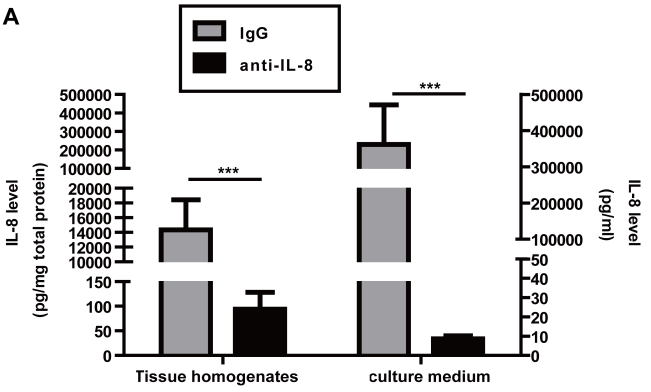
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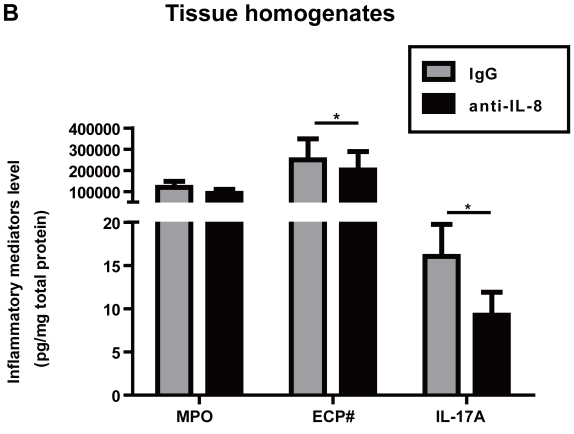


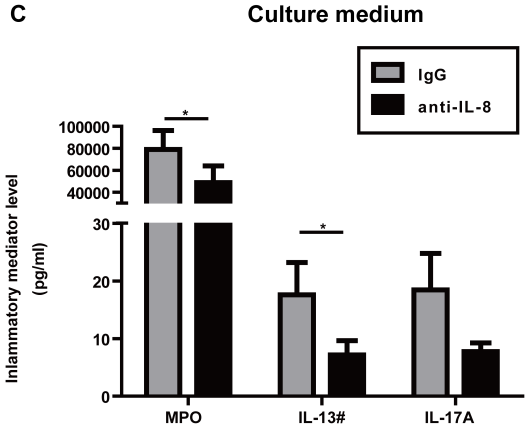
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A
IgG
anti-IL-8
IL-8 level (pg/mg total protein)
IL-8 level (pg/ml)
***
***
500000
400000
300000
200000
100000
20000
18000
16000
14000
12000
10000
150
100
50
0
50
40
30
20
10
0
Tissue homogenates
culture medium


B
Tissue homogenates
IgG
anti-IL-8
Inflammatory mediators level (pg/mg total protein)
*
*
400000
300000
200000
100000
20
15
10
5
0
MPO
ECP#
IL-17A


C
Culture medium
IgG
anti-IL-8
Inlammatory mediator level (pg/ml)
*
*
100000
80000
60000
40000
30
20
10
0
MPO
IL-13#
IL-17A

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Hypomethylation of *IL8* promoter in nasal epithelial cells of patients with chronic rhinosinusitis with nasal polyps

Jingyun Li, PhD[1, 2,*], Jian Jiao, PhD[1, 2,*], Ming Wang, PhD[1,2], Yunbo Gao, MD[1,2], Ying Li, BS[1,2], Yang Wang, BS[1,2], Yuan Zhang, MD[1, 2, 3], Xiangdong Wang, MD[1, 2, 3] and Luo Zhang, MD, PhD[1 ,2, 3]

1 Department of Otolaryngology Head and Neck Surgery, Beijing TongRen Hospital, Capital Medical University, Beijing 100730, China.

2 Beijing Key Laboratory of Nasal diseases, Beijing Institute of Otolaryngology, Beijing 100005, China.

3 Department of Allergy, Beijing TongRen Hospital, Capital Medical University, Beijing 100176, China.

* These authors contributed equally to this work

Corresponding authors:

Luo Zhang, M.D., Ph.D.

Beijing Institute of Otolaryngology

No. 17, HouGouHuTong, DongCheng District,

Beijing, 100005. China

E-mail: dr.luozhang@139.com

Mobile phone: +86 13910830399

Fax:+86 85115988

Xiangdong Wang, M.D., Ph.D.

Beijing Institute of Otolaryngology

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No. 17, HouGouHuTong, DongCheng District,

Beijing, 100005. China

E-mail: entwxd@vip.sina.com

Mobile phone: +86 13911189954

Yuan Zhang, M.D., Ph.D.

Beijing Institute of Otolaryngology

No. 17, HouGouHuTong, DongCheng District,

Beijing, 100005. China

E-mail: summer_zhang1211@126.com

Mobile phone: +86 15901566981

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45 **TABLES**

46 **TABLE E1.** Primer sequences employed for pyrosequencing and Sanger sequencing

Sites	Direction	Sequence (5' to 3')
CpG1 and CpG2	Forward	AGGGGATGGGTTATTAGTTG
	Reverse	Biotin -ACTTATACACCCTCATCTTTTCATT
	Sequencing	GGATGGGTTATTAGTTGTA
CpG3	Forward	GGTTTATTTTTTTAGGGTAAATTTGAGTTA
	Reverse	Biotin -AATTCACCAAATTATAAAACTTCAATATT
	Sequencing	ATATTTTTTATTTGTTTTTTATTAA
rs4073	Forward	TCCTCTATTGAAGCCCTCC
	Reverse	ATCAGGAAGGCTGCCAAG

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49 **Table E2.** Multivariate analysis of potential factors influencing methylation ratios at CpG1,
50 CpG2 and CpG3sites within *IL8* promoter in combined populations.

Factors	Standardized Beta	95%CI	*P*
CpG1			
Disease status	-0.362	-2.530 – -1.360	**2.600E-10**
Age	-0.049	-0.049 – 0.018	0.368
Gender	-4.531E-4	-1.071 – 1.063	0.994
Allergic status	-0.003	-1.146 – 1.077	0.952
Smoking	0.010	-1.059 – 1.236	0.880
Drinking	-0.019	-1.333 – 0.970	0.756
rs4073	-0.088	-1.207 – 0.122	0.109
CpG2			
Disease status	-0.404	-3.065 – -1.776	**1.491E-12**
Age	-0.068	-0.060 – 0.013	0.209
Gender	0.010	-1.080 – 1.287	0.864
Allergic status	0.004	-1.168 – 1.270	0.935
Smoking	-0.011	-1.384 – 1.153	0.858
Drinking	-0.033	-1.622 – 0.925	0.591
rs4073	-0.067	-1.196 – 0.265	0.211
CpG3			
Disease status	-0.473	-2.667 – -1.732	**3.851E-18**
Age	-0.090	-0.051 – 0.002	0.075
Gender	-0.003	-0.871 – 0.821	0.854
Allergic status	-0.005	-0.922 – 0.834	0.922
Smoking	0.010	-0.848 – 1.011	0.172
Drinking	-0.023	-1.116 – 0.748	0.698
rs4073	-0.036	-0.716 – 0.337	0.479

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FIGURE LEGENDS

FIGURE E1. Representative H&E staining of nasal mucosa from inferior turbinate tissue from a control subject, ethmoid tissue from a CRSsNP patient and nasal polyp (NP) tissue inform a CRSwNP patient. Scale bar = 50 μm.

FIGURE E2. Characteristics of HNECs derived from inferior turbinate tissue of control subjects, ethmoid tissue of CRSsNP patients, and nasal polyp (NP) tissue of CRSwNP patients. (A) Representative phase contrast micrographs and (B) Immunofluorescence staining for pancytokeratin (epithelium, green), type □β-tubulin (ciliated cells, green) and mucin (MUC)-5AC (goblet cells, red); nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Scale bar = 50 μm.

FIGURE E3. Comparison of methylation ratios at CpG1 (A), CpG2 (B) and CpG3 (C) sites within *IL8* promoter between eos CRSwNP and non-eos CRSwNP.

FIGURE E4. Comparison of methylation ratios at CpG1 (A), CpG2 (B) and CpG3 (C) sites within *IL8* promoter between patients with CRSwNP plus asthma and CRSwNP alone (without comorbid asthma). *, $P < 0.05$.

FIGURE E5. Comparison of methylation ratios at CpG1 (A), CpG2 (B) and CpG3 (C) sites within *IL8* promoter between allergic and non-allergic subjects.

FIGURE E6. Comparison of methylation ratios at CpG1 (A), CpG2 (B) and CpG3 (C) sites within *IL8* promoter between smoking and non-smoking subjects.

FIGURE E7. Comparison of methylation ratios at CpG1 (A), CpG2 (B) and CpG3 (C) sites within *IL8* promoter between drinking and non-drinking subjects.

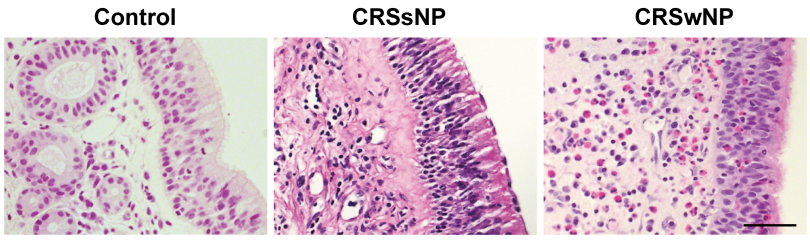
FIGURE E8. Comparison of methylation ratios at CpG1 (A), CpG2 (B) and CpG3 (C) sites within *IL8* promoter between rs4073 genotypes.

FIGURE E9. Correlation between methylation ratios at CpG1, CpG2 and CpG3 sites within *IL8* promoter.

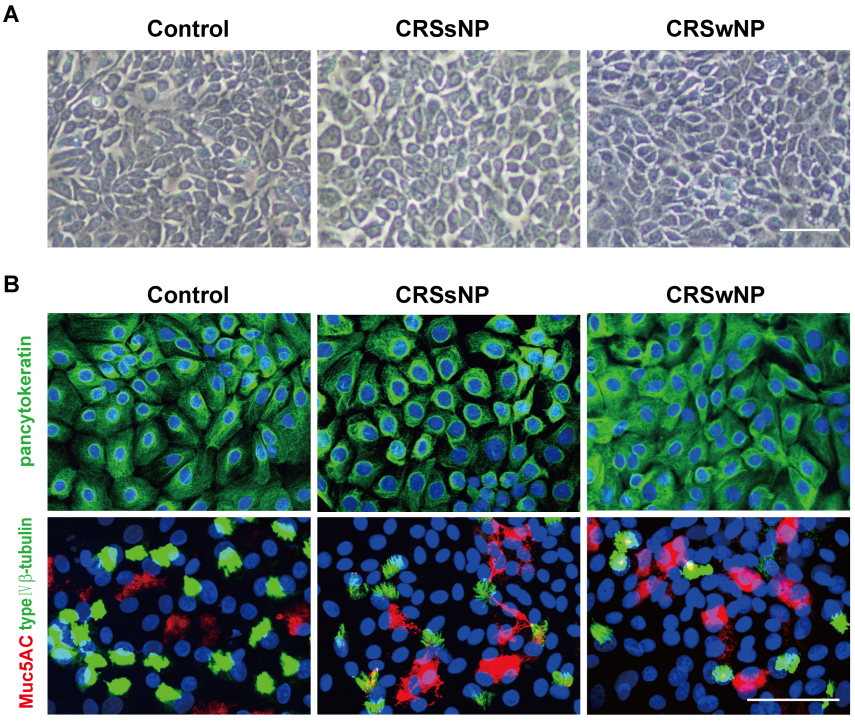
FIGURE E10. The effect of IL-8 neutralization on the inflammatory mediators and growth factors in tissues homogenates (A) and culture medium (B) from individual NP explant (n=4). NP explants were treated with control IgG or human anti-IL-8 monoclonal antibody (10 μg/ml) for 48h. # IL-10 was not detectable in tissues homogenates. Results are expressed as means ± SEMs.

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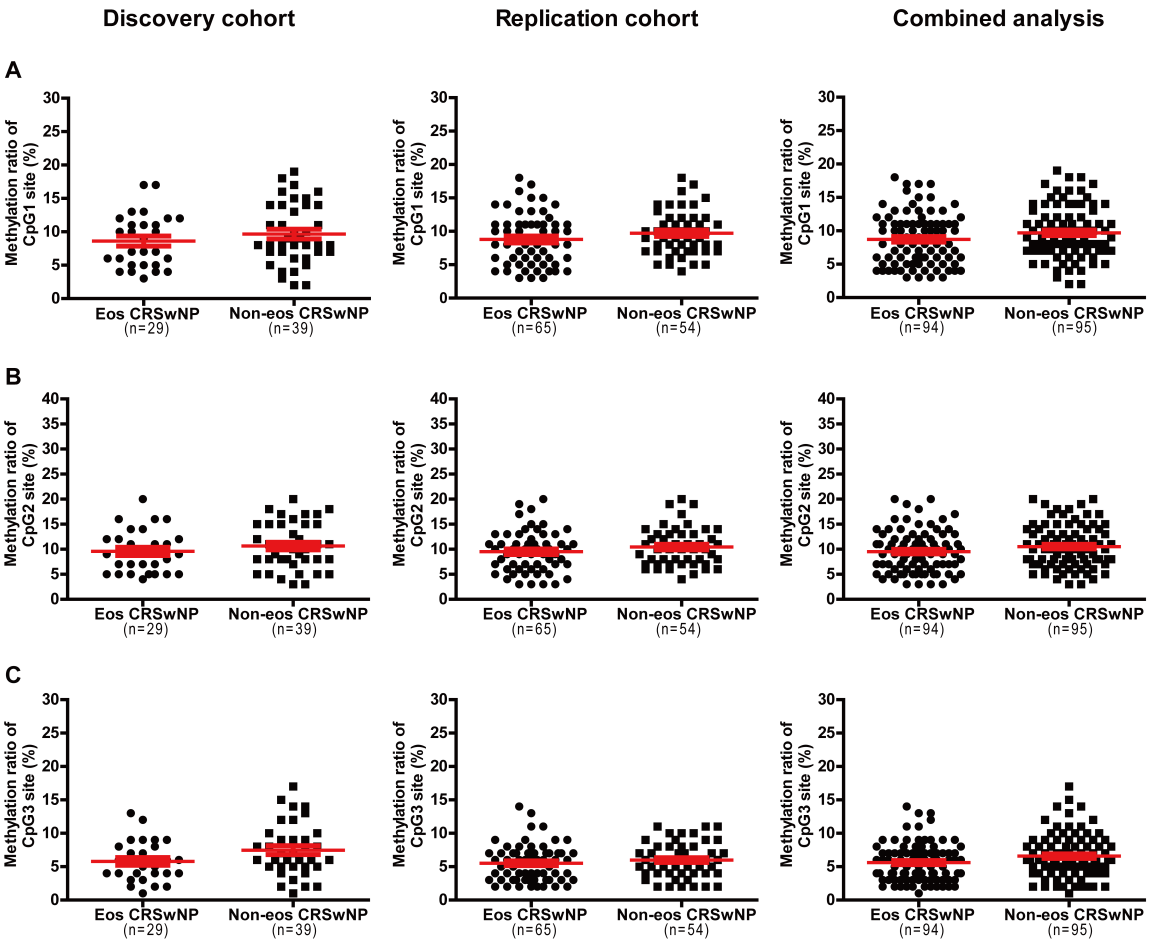


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A
Control
CRSsNP
CRSwNP
B
Control
CRSsNP
CRSwNP
pancytokeratin
Muc5AC type \\/ β-tubulin

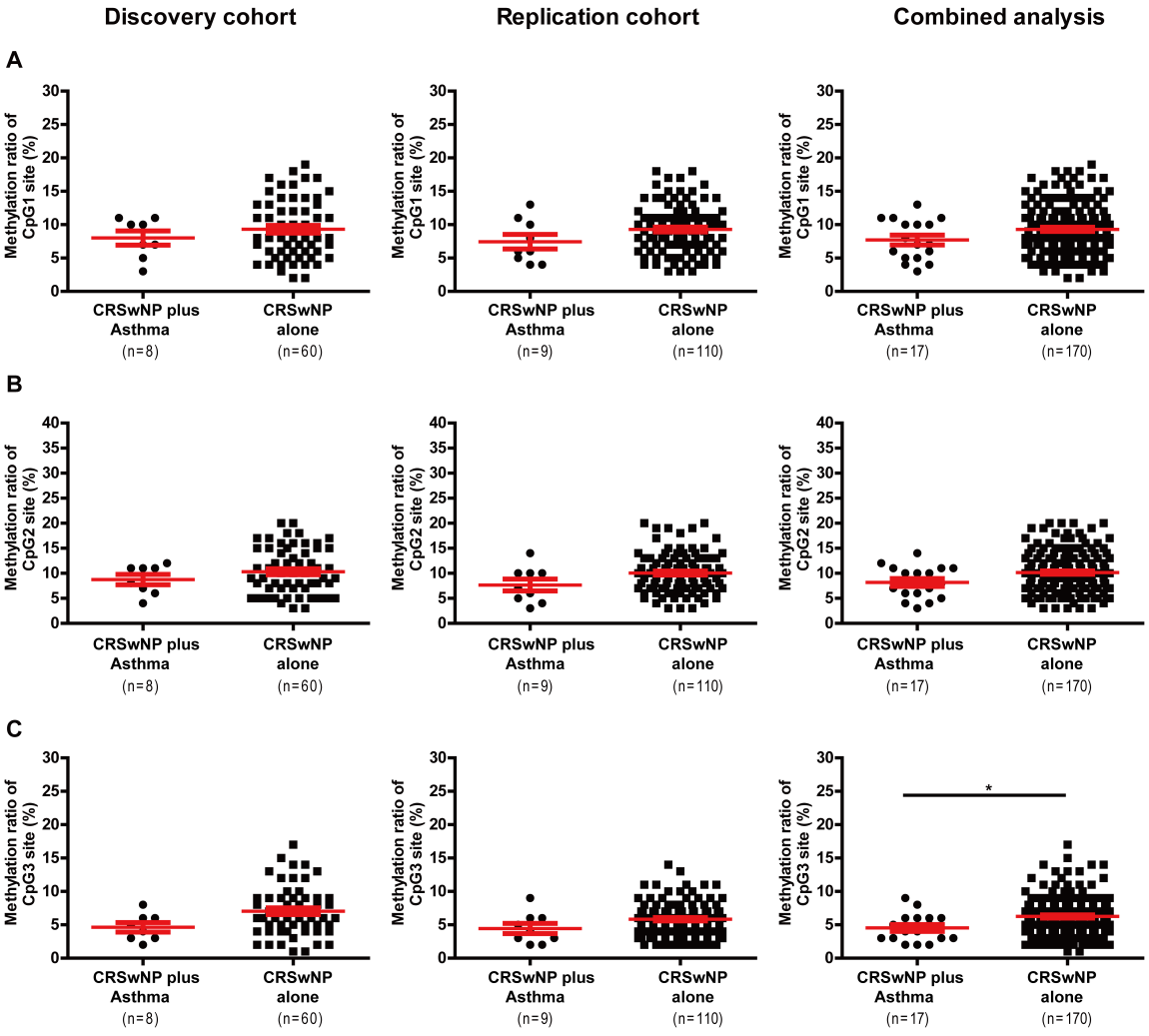
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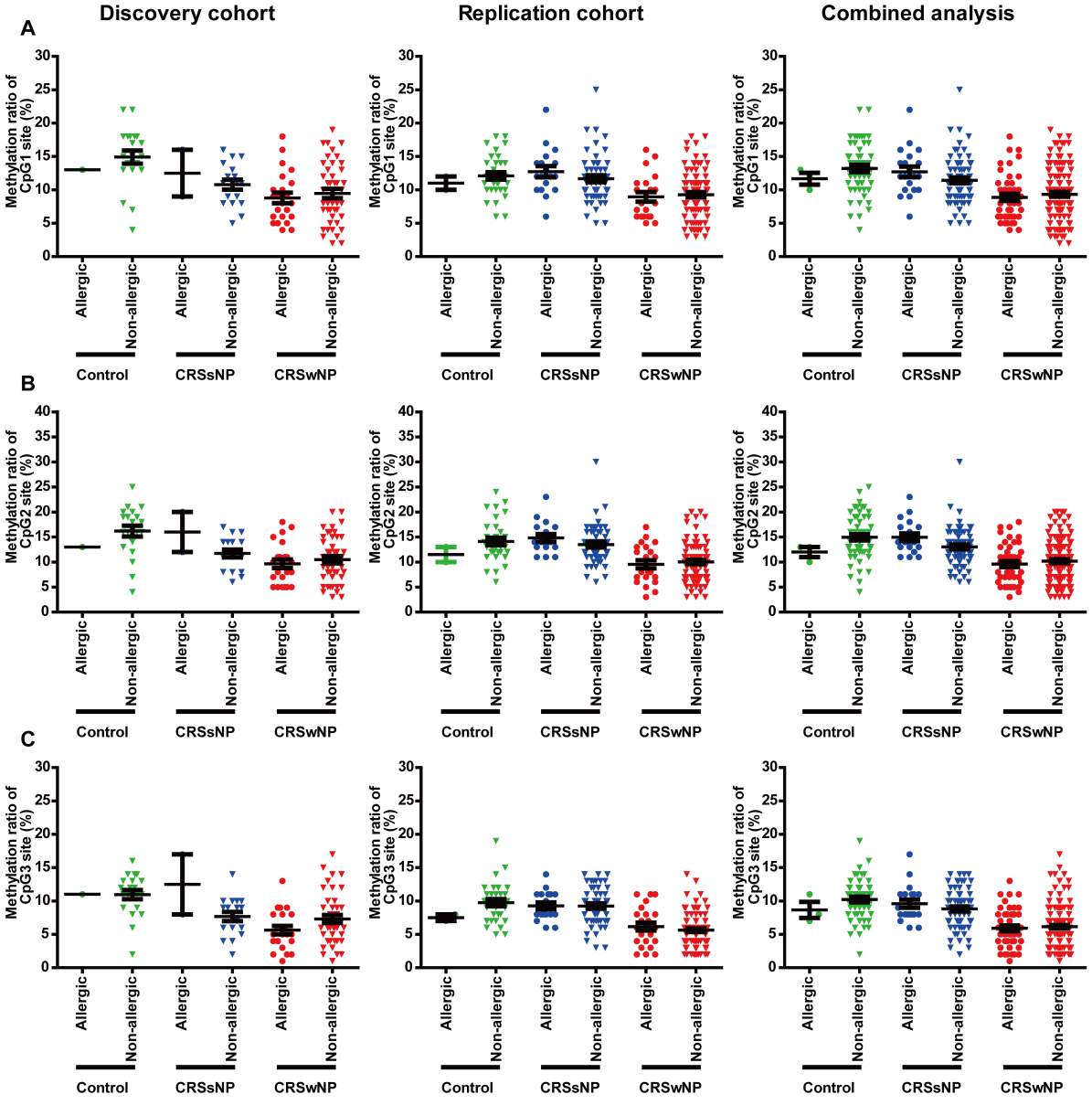


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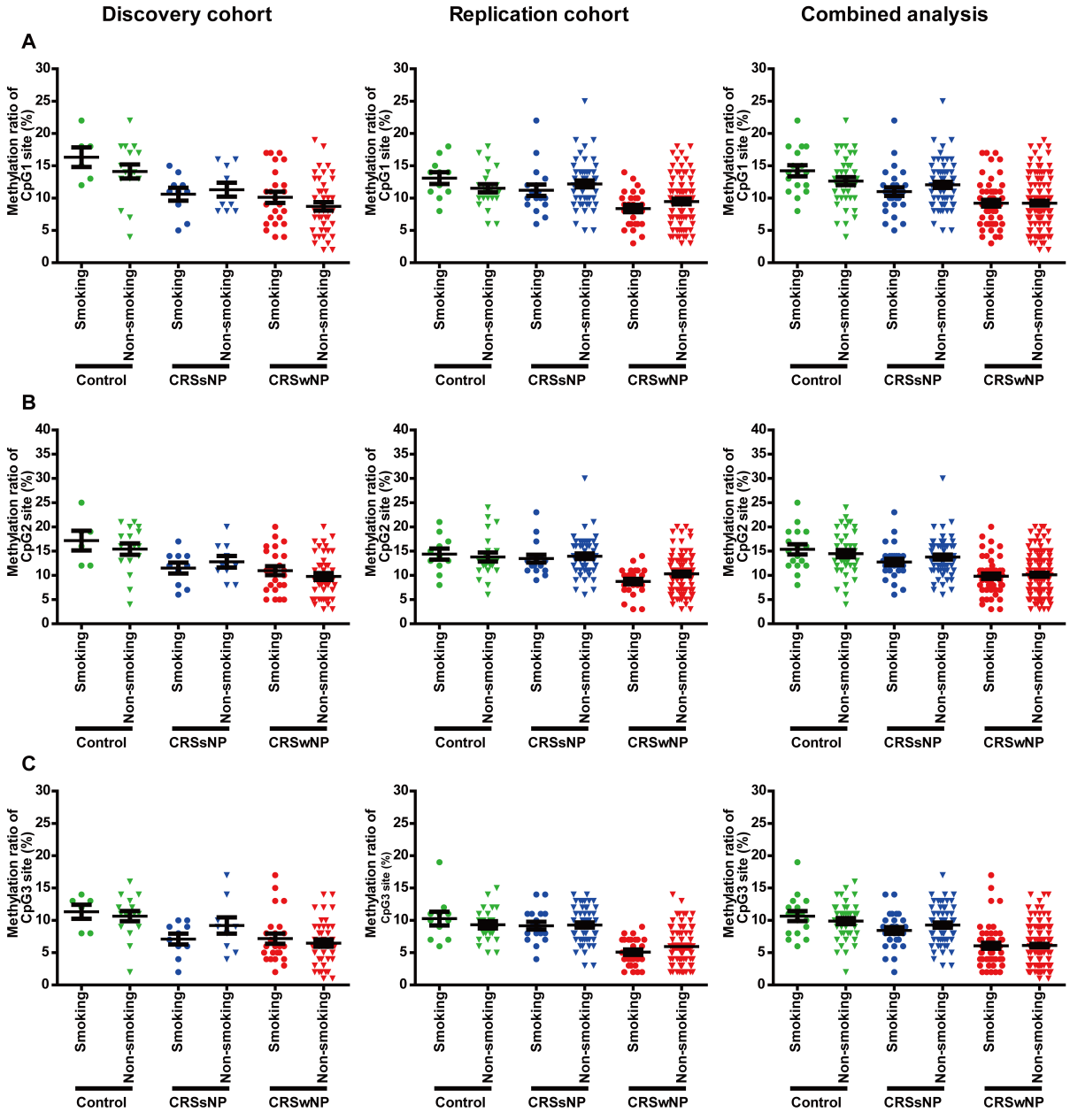
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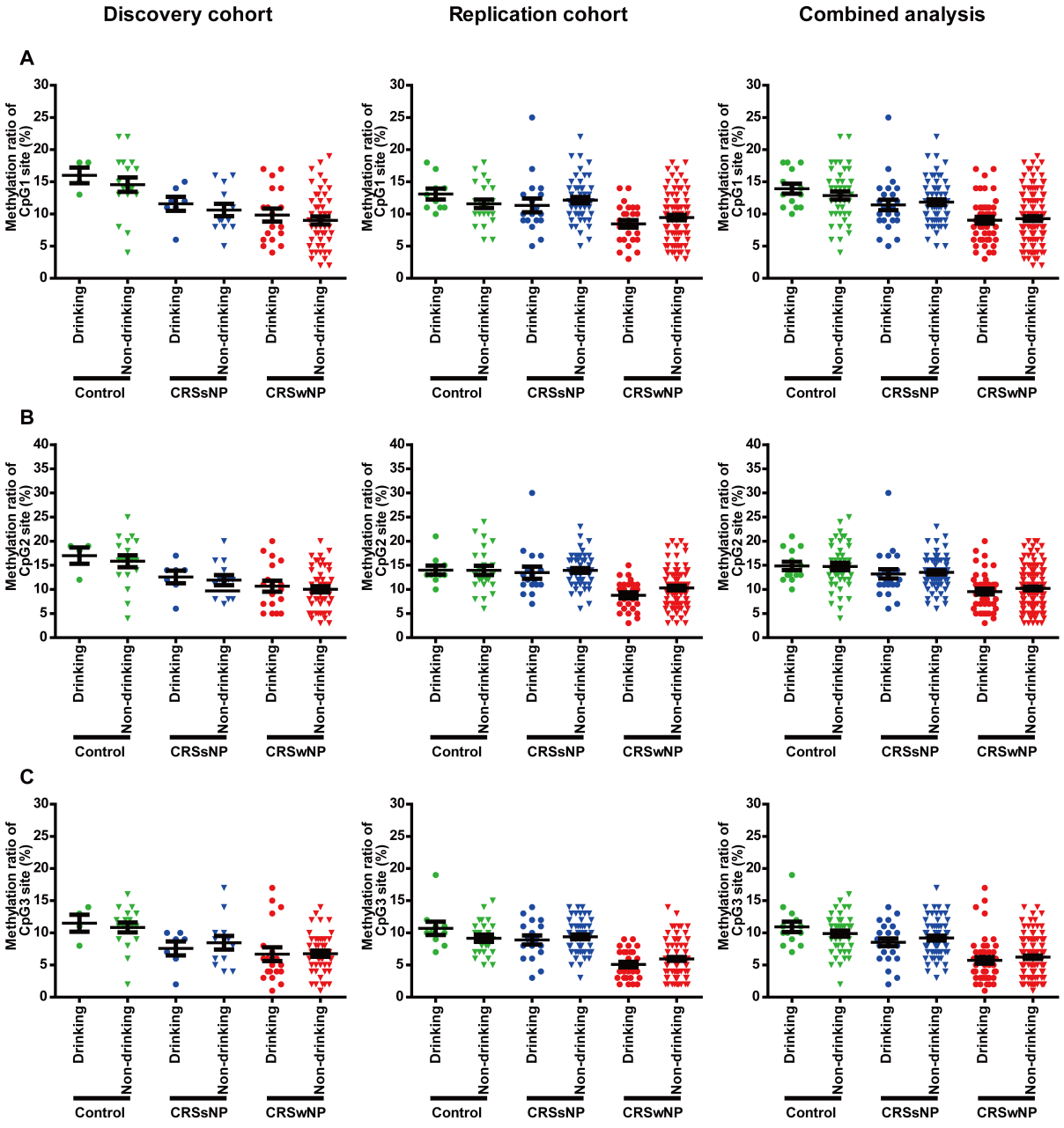
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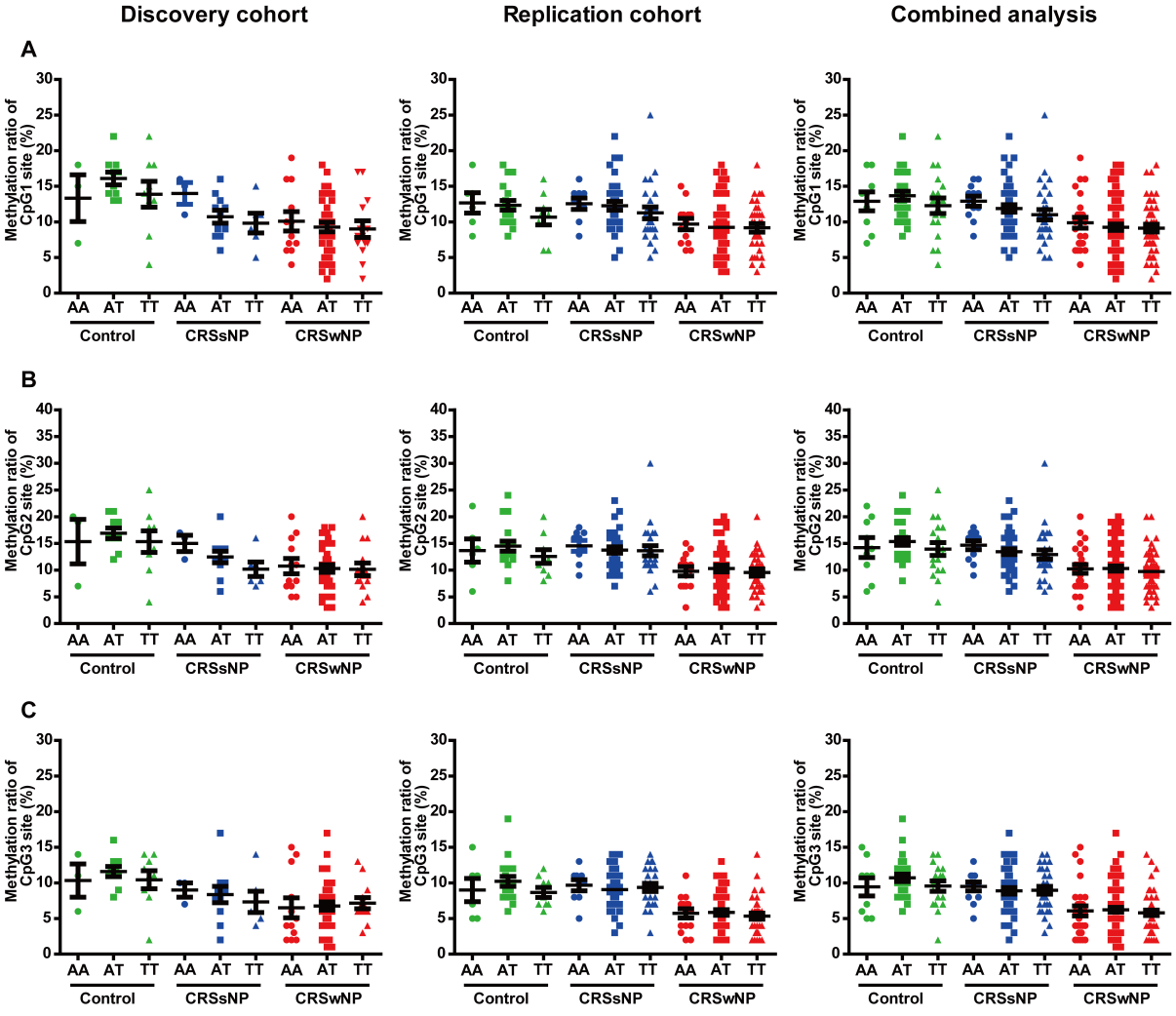
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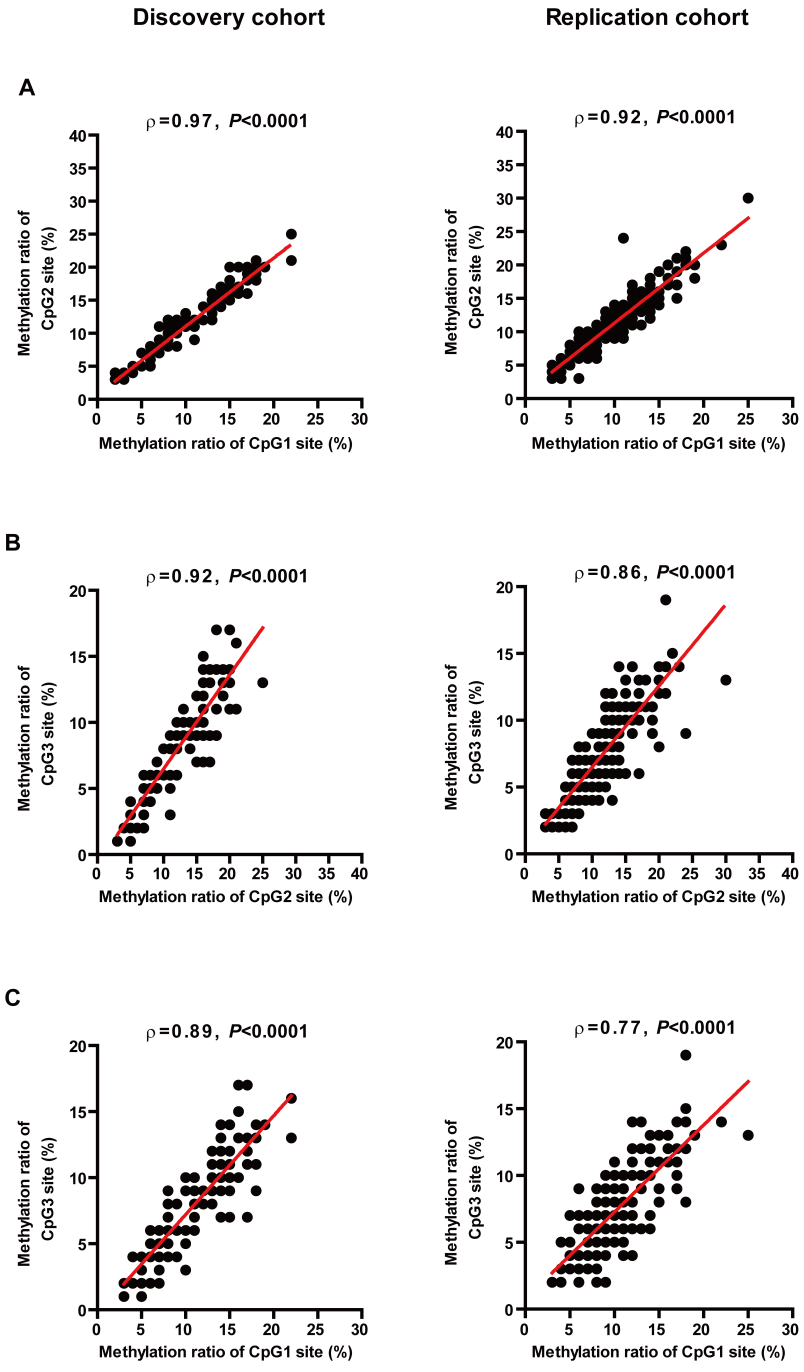
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