separated from PBMCs of healthy subjects (see Fig E3, *E* and *F*). According to additional analysis with $CD4^+CD45RA^+$ T cells from cord blood, which were induced to differentiate into T_H1 and T_H2 subsets, T_H1-dominant cells preincubated with sirtinol demonstrated no significant change in the ratios of IFN-γ to IL-4 and T-box transcription factor (T-bet) to GATA-3 when normalized against glyceraldehyde-3-phoshpahte dehydrogenase (GAPDH; data not shown). However, there was a highly significant increase in the ratio of IL-4 to IFN-γ in T_H2-dominant cells (see Fig E3, *G*). This was accompanied by a borderline significant ($P = .06$) increase in the ratio of GATA-3 to T-bet (see Fig E3, *H*). In addition, we have preliminary data that indicate sirtinol promotes T_H2-specific proliferation after 21 days of culture (data not shown). This is the first *in vitro* evidence in human cells that inhibition of SIRT1 promotes a T_H2-like phenotype in T cells.

The effect of sirtinol on the acetylation of GATA-3 was examined to identify the mechanism of increase in T_H2 cytokine expression by sirtuin inhibition. Sirtuin inhibition by 30 μmol/L sirtinol resulted in increased acetylation of GATA-3 in the nuclear protein of HUT78 T lymphocytes (Fig 2, *C*) without any increase in GATA-3 protein expression (data not shown). In addition, immunoprecipitated GATA-3 was confirmed to be highly acetylated when sirtuins were inhibited (see Fig E3, *I*). Binding of GATA-3 to its response element was also enhanced when treated with sirtinol at 30 μmol/L, suggesting that increased acetylation of GATA-3 directly affected its activity (Fig 2, *D*). In addition, GATA-3 overexpression increased IL-4 mRNA expression in HEK293 cells, which are largely GATA-3 deficient, and sirtuin inhibition induced IL-4 gene expression further (Fig 2, *E*). These data indicate that T_H2 development in human subjects is reliant on a relationship between SIRT1 and GATA-3. However, in this study we were unable to show a statistically significant correlation between IL-5 transcripts and SIRT1 activity in all subjects, as well as between SIRT1 activity and blood eosinophils (as a percentage) in patients with severe asthma (see Fig E1, *G* and *H*). GATA-3 is essential for initial expression of T_H2 cytokines, such as IL-4 and IL-5, but less important for the maintenance of IL-4 expression in mature cells.[7] Therefore other T_H2 transcription factors, such as signal transducer and activator of transcription 6, c-Maf, or nuclear factor of activated T cells, might be involved. It is also possible that acetylated GATA-3 preferentially associates with the IL-4 promoter. Even more importantly, a SIRT1 activator (SRT2172)[8] significantly reduced IL-4 mRNA expression in PBMCs from 10 patients with severe asthma without any significant change in IFN-γ expression (Fig 2, *F* and *G*). This supports the case that reduced SIRT1 activity causes increased IL-4 expression in patients with severe asthma.

Thus we demonstrate that SIRT1 plays a role in maintaining the T_H cell balance and preventing sustained cellular differentiation toward a T_H2 phenotype. This article provides new evidence that reduction in SIRT1 levels in patients with severe asthma contributes to increased IL-4 expression and that this would be a novel target for the treatment of severe asthma.

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Fcγ-receptor 3B (*FCGR3B*) copy number variations in patients with eosinophilic granulomatosis with polyangiitis

To the Editor:

Eosinophilic granulomatosis with polyangiitis (EGPA; Churg-Strauss syndrome) is a rare anti-neutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) characterized by asthma, rhinosinusitis, eosinophilia, and involvement of different organs.[1] Two major disease subsets, defined as vasculitic and eosinophilic, have been identified. In the former subset clinical manifestations caused by small-vessel vasculitis (eg, peripheral neuropathy, glomerulonephritis, and purpura) predominate, whereas the latter subset more commonly shows manifestations related to eosinophil infiltration (eg, cardiomyopathy and gastroenteritis).

TABLE I. Clinical characteristics of patients with EGPA with respect to their *FCGR3B* CNs

Clinical finding in patients with EGPA	FCGR3B CN = 1		FCGR3B CN ≥ 2				
	Patients with EGPA no. (%)	Control subjects no. (%)	Patients with EGPA no. (%)	Control subjects no. (%)	*P* value	P_{corr} value	OR (95% CI)
Lung involvement (n = 63)	7/63 (11.1)	19/249 (7.6)	56/63 (88.9)	230/249 (92.4)	.168	1.00	2.00 (0.74-5.42)
CV symptoms (n = 33)	7/33 (21.2)	19/249 (7.6)	26/33 (78.8)	230/249 (92.4)	**.011**	.088	3.26 (1.25-8.48)
GI symptoms (n = 24)	4/24 (16.6)	19/249 (7.6)	20/24 (83.4)	230/249 (92.4)	.129	1.00	2.42 (0.75-7.81)
Purpura (n = 20)	5/20 (25.0)	19/249 (7.6)	15/20 (75.0)	230/249 (92.4)	**.009**	.072	4.03 (1.32-12.31)
Impaired renal function (n = 21)	6/21 (28.6)	19/249 (7.6)	15/21 (71.4)	230/249 (92.4)	**.0009**	**.0072**	5.19 (1.79-15.04)
Peripheral neuropathy (n = 96)	17/96 (17.7)	19/249 (7.6)	79/96 (82.3)	230/249 (92.4)	**.006**	**.048**	2.60 (1.29-5.26)
Positive ANCA (n = 70)	11/70 (15.7)	19/249 (7.6)	59/70 (84.3)	230/249 (92.4)	**.041**	.328	2.26 (1.02-5.00)
Vasculitis on biopsy (n = 38)	8/38 (21.1)	19/249 (7.6)	30/38 (78.9)	230/249 (92.4)	**.008**	.064	3.23 (1.3-8.02)

ANCA levels were tested by using immunofluorescence). Impaired renal function was defined as a serum creatinine increase of greater than 30% or an estimated glomerular filtration rate decrease of greater than 25%. Values in boldface indicate statistical significance.
CV, Cardiovascular; *GI*, gastrointestinal; *OR*, odds ratio; P_{corr}, *P* values corrected by using Bonferroni correction for multiple testing.

ANCAs, which are detectable in 40% of patients, are associated with the vasculitic subset.[2]

The genetic basis of EGPA is poorly investigated. Genetic associations include *HLA-DRB4* and *IL10* single nucleotide polymorphisms.[3,4] In addition to single nucleotide polymorphisms, copy number variations (CNVs) represent a significant source of genetic heterogeneity. In patients with autoimmune diseases, CNVs involving Fcγ receptor (FcγR) genes were extensively investigated. FcγRs bind the IgG constant domain and regulate mobilization of macrophages, natural killer cells, and neutrophils to sites of immune complex deposition. Fcγ-receptor 3B (*FCGR3B*) deficiency (ie, low copy number [CN]) was shown to predispose to systemic autoimmune diseases.[5] We investigated whether *FCGR3B* CNVs confer susceptibility to EGPA and explored their associations with disease phenotypes.

We studied 126 patients with EGPA and 249 healthy white subjects. The diagnosis of EGPA fulfilled the American College of Rheumatology 1990 Criteria and the Chapel Hill Consensus Conference definition of EGPA.[6,7] The Ethics Committee of Parma Hospital approved the study; all participants signed a written informed consent form.

FCGR3B CNs were determined from genomic DNA by using a TaqMan CN real-time PCR assay (Life Technologies, Grand Island, NY); RNaseP (Life Technologies) was used as a reference assay, and both target and reference samples were run in a single tube. All samples were tested in triplicate. For further details, see the Methods section and Figs E1 and E2 in this article's Online Repository at www.jacionline.org.

Statistical analysis was performed with the package Coin of R software (http://www.r-project.org/). Differences in *FCGR3B* CN frequency among groups were compared by using Kruskal-Wallis and Mann-Whitney rank sum tests, where appropriate. Bonferroni correction for multiple testing was applied. The association between an *FCGR3B* CN of 1 and the number of vasculitic manifestations was explored through a model assigning a score of 1 to each vasculitic manifestation (purpura, renal involvement, and neuropathy); the significance of this variation across groups was tested by using the Cochrane-Armitage trend test (log additive model).

The main demographic and clinical characteristics of patients and control subjects are summarized in Table E1 in this article's Online Repository at www.jacionline.org. We examined the association between *FCGR3B* CNs and susceptibility to EGPA. Most published studies dichotomizing *FCGR3B* CNs (1 vs ≥2) found associations between autoimmune diseases and an *FCGR3B* CN of 1.[5] An *FCGR3B* CN of 1 was significantly enriched in patients with EGPA (P_{corr} = .026; odds ratio, 2.284; 95% CI, 1.17-4.46; see Fig E3 in this article's Online Repository at www.jacionline.org), whereas the overall *FCGR3B* CN distribution did not significantly differ between patients and control subjects (P_{corr} = .166; see Fig E4 in this article's Online Repository at www.jacionline.org). We also assessed differences in frequencies of CNs of 1 and 2 or greater considering the main features of the eosinophilic (ie, lung, gastrointestinal, and cardiac involvement) and vasculitic (ie, peripheral neuropathy, purpura, and renal function impairment) subsets (Table I). No significant differences in CN frequency were found with respect to lung (*P* = .168), gastrointestinal (*P* = .129), and cardiovascular (*P* = .011, P_{corr} = .088) involvement. Conversely, a CN of 1 was enriched in patients with peripheral neuropathy (P_{corr} = .048), impaired renal function (P_{corr} = .0072), and purpura, although the latter finding was of borderline significance after correction (P_{corr} = .072). Patients with histologic evidence of vasculitis also had a higher frequency of a CN of 1, as did ANCA-positive patients (Table I). Eosinophil counts and C-reactive protein levels were comparable in patients with a CN of 1 versus those with a CN of 2 or greater (see Figs E5 and E6 in this article's Online Repository at www.jacionline.org). Finally, we assessed the percentage of an *FCGR3B* CN of 1 across subgroups of patients with different numbers of vasculitic manifestations and observed a higher frequency of an *FCGR3B* CN of 1 in patients with increasing numbers of vasculitic manifestations (*P* = .0037, Table II).

TABLE II. Distribution of the CNs of *FCGR3B* with respect to the number of vasculitic manifestations in patients with EGPA

	FCGR3B CN = 1, no. (%)	*FCGR3B* CN ≥ 2, no. (%)
Control subjects (n = 249)	19 (8)	230 (92)
Patients with EGPA (n = 126)		
Vasculitic manifestations = 0 (n = 30)	3 (10)	27 (90)
Vasculitic manifestations = 1 (n = 65)	10 (15)	55 (85)
Vasculitic manifestations ≥ 2 (n = 31)	7 (29)	24 (71)

Cochrane-Armitage test for trend (log additive model): χ^2 = 8.41, *P* = .0037. The vasculitic manifestations include glomerulonephritis, purpura, and peripheral neuropathy.

FCGR3B CNVs are associated with autoimmunity. A recent meta-analysis showed that *FCGR3B* deficiency (CN ≤ 1) predisposes to systemic but not organ-limited autoimmune diseases (systemic lupus erythematosus and rheumatoid arthritis),[5] which might indicate that *FCGR3B* gene dosage influences the risk of systemic autoimmunity. An early study on AAV showed that both low and high *FCGR3B* CNs predispose to granulomatosis with polyangiitis and microscopic polyangiitis,[8] whereas a subsequent study found no association,[9] a discrepancy probably caused by different detection and analytic approaches. In our study we found an association between *FCGR3B* deficiency and risk of EGPA. Intriguingly, *FCGR3B* deficiency was particularly associated with vasculitic manifestations (renal involvement, neuropathy, and purpura); an increased proportion of *FCGR3B* deficiency was found in patients with increasing numbers of vasculitic manifestations, with approximately 30% of patients with 2 or more vasculitic manifestations carrying *FCGR3B* deficiency.

The ANCA-related vasculitic-eosinophilic dichotomy of EGPA was demonstrated by several studies, although clinical manifestations of the 2 subsets often overlap.[1,2] Interestingly, the genetic associations of EGPA with *HLA-DRB4* and *IL10* variants were also influenced by disease phenotype because *HLA-DRB4* frequency correlated with the number of vasculitic manifestations,[3] and the *IL10.2* haplotype was associated only with the ANCA-negative subset.[4] Our results further suggest that these 2 EGPA subsets are genetically distinct.

FCGR3B deficiency might contribute to EGPA pathogenesis through different potential mechanisms: FcγRIIIb is mainly expressed on neutrophils, and therefore its deficiency might delay clearance of immune complexes by neutrophils and promote a proinflammatory status.[9] This hypothesis is apparently in contrast with the pathogenesis of EGPA, which is considered, as the other AAVs, a pauci-immune disease. However, this view has recently been revised because a significant proportion of patients with AAV exhibit tissue deposition of immune complexes and complement.[10] In addition, EGPA is characterized by high levels of IgG_4, which typically form immune complexes with other immunoglobulins.[11] Alternatively, *FCGR3B* deficiency might alter the balance between activating and inhibitory *FCGRs*, causing differential expression of other genes in linkage disequilibrium with *FCGR3B*.[5,9]

Although our findings cannot have diagnostic implications, they can inform future mechanistic studies on humoral autoimmunity and FcγR-mediated responses in patients with EGPA, with the ultimate goal of manipulating this pathway for therapeutic purposes.

Our study has limitations essentially related to the small sample size and lack of a replication cohort. Additionally, CNV genotyping/assignment is exposed to variations because of different factors, such as DNA quality and type of data analysis.

In conclusion, *FCGR3B* deficiency might predispose to EGPA, particularly the vasculitic disease subset. Our results warrant validation in larger studies.

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METHODS

Nucleic acid isolation

Genomic DNA was extracted from EDTA-treated peripheral blood samples (5 mL of whole blood) by using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, Calif) and stored at −20°C until use. DNA concentrations were determined with a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Mass), and all DNA concentrations were adjusted to 5 ng/μL with sterile water.

Determination of *FCGR3B* CNs

FCGR3B gene CN determinations were performed by using quantitative real-time PCR on an ABI PRISM 7700 Sequence Detector (Life Technologies, Foster City, Calif). The *FCGR3B* CN was determined from genomic DNA by using a predesigned TaqMan CN real-time, quantitative PCR assay (Hs04211858, FAM-MGB dual-labeled probe, Life Technologies); RNaseP (4401631, VIC-TAMRA dual-labelled probe; Life Technologies) was used as a reference assay, and both target and reference samples were run in a single tube. All samples were tested in triplicate. The PCR amplifications were performed in a volume of 10 μL containing 5 μL of 2× TaqMan Universal PCR Master Mix, 0.5 μL of 20× TaqMan Copy Number Assay, 0.5 μL of 20× TaqMan Copy Number Reference Assay, 2 μL of Nuclease-Free water, and 2 μL of template DNA (5 ng/μL). Thermal cycling included an initial denaturation step of 10 minutes at 95°C, followed by 40 cycles each of 15 seconds at 95°C and 1 minute at 60°C. All quantitative PCR amplification plots constructed with 7 dilutions of input DNA fitted a straight line (R^2 = 0.99, Fig E1). The relative *FCGR3B* CN for each subject was assigned with the Δ cycle threshold cycle (ΔCt) method by using Copy Caller v2.0 software (Life Technologies), which calculates the probability that the observed data point represents an integer value.

The quantitative PCR efficiency for *FCGR3B* and the endogenous control RNaseP was calculated for the 3 samples by using the following formula:

$$E=10(-1/m)-1,$$

where m is the slope of the function derived from the Ct versus dilution plot (0.3125-20 ng input DNA with 7 different dilutions) of a DNA sample. Samples with Ct values of greater than 35 or Ct replicates with differences of greater than 0.5 were excluded from the analysis. Overall, this previously reported methodology[E1] allowed a reliable assignment of FCGR3B CNs (Fig E1).

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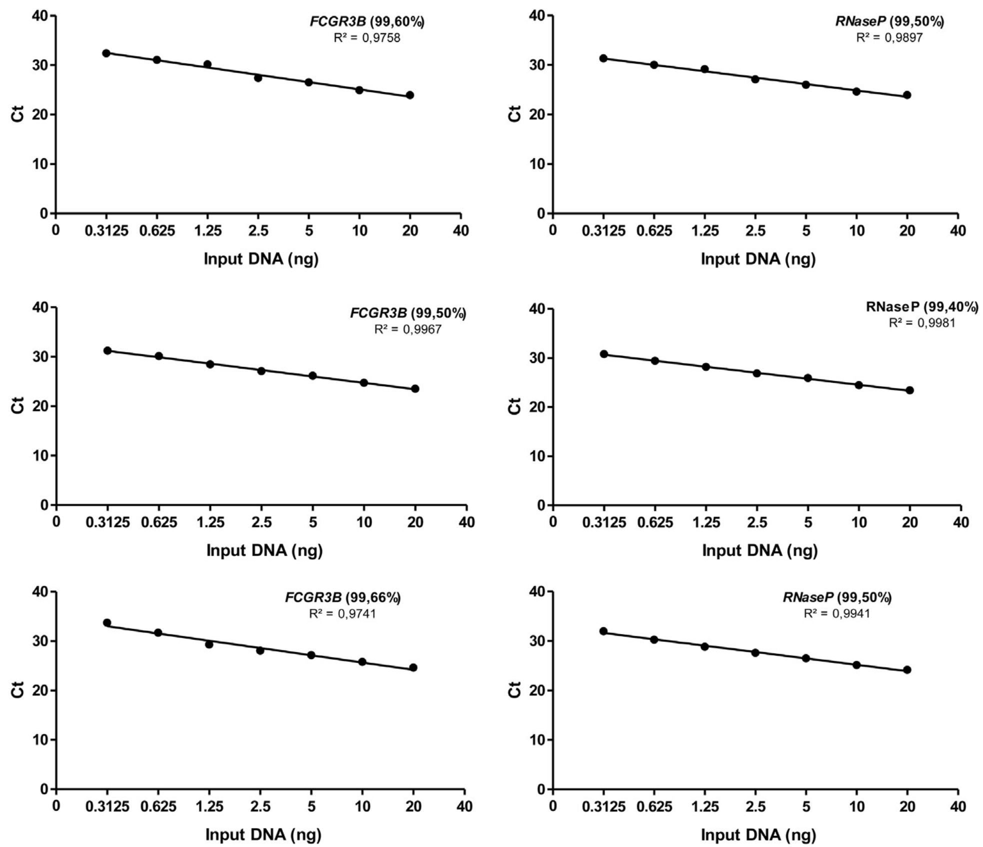

FIG E1. Three representative examples of amplification efficiency plots for CN assays (*FCGR3B* target and *RNaseP* reference) calculated with input DNA concentrations of 7 different dilutions (0.625-40 ng DNA) per reaction.

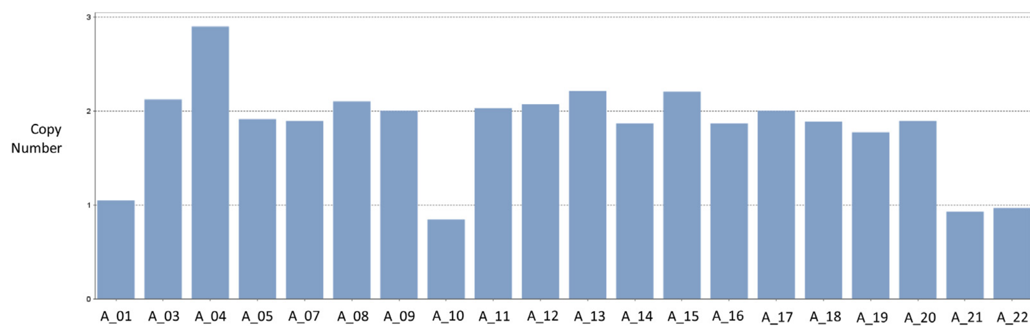

FIG E2. Histograms of *FCGR3B* CNV analysis with the CopyCaller v2.0 program. The software displays 1 bar for each analyzed sample. The *y-axis* displays the CN for each sample, and the *x-axis* displays the samples. For example, sample A_01 has 1 copy of *FCGR3B*, sample A_03 has 2 copies of *FCGR3B*, and sample A_04 has 3 copies of *FCGR3B*.

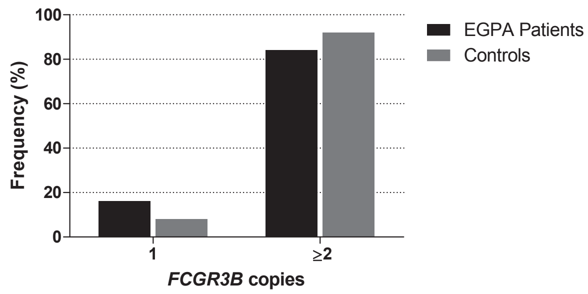

FIG E3. Distribution of *FCGR3B* CNs of 1 versus 2 or greater in patients with EGPA and healthy control subjects.

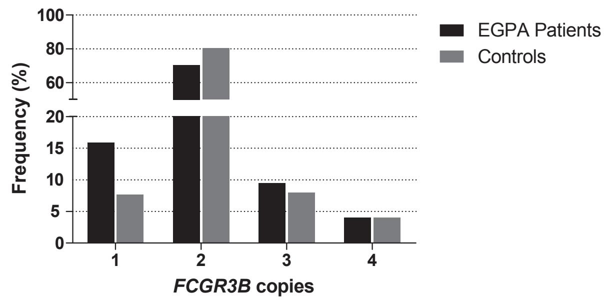

FIG E4. Distribution of each CN of the *FCGR3B* gene in patients with EGPA and healthy control subjects.

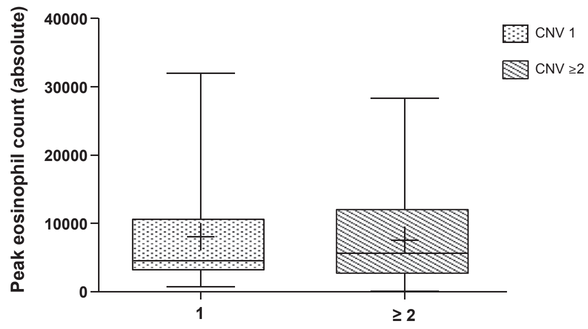

FIG E5. Eosinophil counts in patients with EGPA with *FCGR3B* CNVs of 1 versus 2 or greater ($P = .77$).

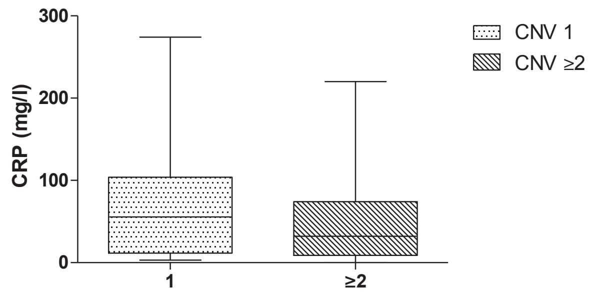

FIG E6. C-reactive protein *(CRP)* levels in patients with EGPA with *FCGR3B* CNVs of 1 versus 2 or greater ($P = .46$).

TABLE E1. Demographic and clinical characteristics of the study participants

Patients with EGPA (n = 126)	
Mean (SD) age at diagnosis (y)	55 (11.6)
Male sex, no. (%)	63 (50)
White ethnicity, no. (%)	126 (100)
Asthma, no. (%)	118 (93.6)
Ear-nose-throat and paranasal sinus involvement, no. (%)	104 (82.5)
Lung involvement, no. (%)	63 (50.0)
Cardiovascular symptoms, no. (%)	33 (26.2)
Gastrointestinal symptoms, no. (%)	24 (19.0)
Skin manifestations, all kinds, no. (%)	53 (42.1)
Purpura, no. (%)	20 (15.9)
Renal abnormalities, all kinds, no. (%)	38 (30.2)
Impaired renal function, no. (%)	21 (16.7)
Peripheral neuropathy, no. (%)	96 (76.2)
Positive ANCA result determined by using IIF, no. (%)	70/120 (58.3)
Positive ANCA result determined by using ELISA, no. (%)	65/120 (54.2)
Vasculitis on biopsy, no. (%)	38/51 (74.5)
Healthy control subjects (n = 249)	
Mean (SD) age (y)	54 (7.6)
Male sex, no. (%)	132 (53.0)
White ethnicity, no. (%)	249 (100)

IIF, Indirect immunofluorescence.