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Article type : Letter to the Editor

Letter to the editor

Integrin $\beta 6$ microparticles in nasal lavage fluids; potential new biomarkers for basal cell activation in chronic rhinosinusitis

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To the editor

Microparticles (MP) are extracellular vesicles that are released by shedding from the plasma membrane of activated cells. Different from exosome, another type of extracellular vesicles which are released through exocytosis of multivesicular bodies, typically MP share the same membrane markers and intracellular proteins with their parent cells. We previously reported the successful use of MP analysis in nasal lavage fluids (NLFs) as a biomarker to evaluate activation of structural and immune cells in subjects with chronic rhinosinusitis (CRS) and aspirin-exacerbated respiratory disease (AERD)¹. Abnormal epithelial repair characterized by basal cell hyperplasia was recently reported², with evidence of epithelial-mesenchymal transition (EMT) in hyperplastic basal cells in nasal polyps³, suggesting that epithelial repair fails due to abnormal basal cell function or differentiation, accompanied by aberrant EMT in severe phenotypes of CRS with polyps (CRSwNP) with high risk of polyp recurrence. We report here that elevated levels of the putative biomarkers of activated basal cells undergoing EMT, MP expressing both the basal cell marker, integrin $\beta 6$ (ITGB6), and canonical EMT/mesenchymal markers, Snail (SNAI 1) or alpha-smooth muscle actin (ACTA2- α SMA) in NLF are associated with severe polyp phenotype which had previously necessitated revision surgery.

We enrolled 196 CRSwNP, 70 CRS without polyp (CRSsNP) and 31 AERD cases who had endoscopic sinus surgery (ESS) at Northwestern Memorial Hospital between January 2014 and December 2017 (Table 1). We also enrolled 47 control cases who underwent surgery other than ESS without sinus disease during the same period. NLFs were collected by spraying 8 mL of phosphate buffered saline toward the middle meatus, or polyps arising from the middle meatus and their surrounding mucosa, under direct visual guidance at the time of surgery, and MP were measured using a BD FACS LSRII flow cytometer (BD Biosciences, Erembodegem, Belgium) as previously described (Fig.E1A,B)^{1,4}. Three types of epithelial MP were defined according to the expression of epithelial specific antigens: EpCAM(+)MP, E-cadherin(+)MP and ITGB6(+)MP; we also tested the EMT markers, Snail, Slug (SNAI 2), and α SMA. After staining the MP surface with an epithelial antibody, permeabilization of MP was performed to allow intravesicular staining. We compared MP assay results with and without permeabilization to see localization of EMT/mesenchymal markers on the MPs (surface or inside of MP) (Fig.E1C), and decided to use the MP assay with permeabilization to detect α SMA but not Snail or Slug on epithelial MP.

The increase of epithelial MP subtypes varied according to phenotype of CRS (Fig.1A). Levels of E-cadherin(+)MP and EpCAM(+)MP were higher in CRSsNP and AERD than in CRSwNP, whereas ITGB6(+)MP were higher in CRSwNP and AERD than in CRSsNP. EpCAM and E-cadherin are constitutively expressed on epithelial cells and down-regulated during EMT³. The release of EpCAM(+)MP and E-cadherin(+)MP may thus reflect epithelial injury more than repair and EMT¹. In contrast, ITGB6 is rarely expressed on normal epithelial cells and is up-regulated on basal cells during epithelial repair^{5,6}. Thus, ITGB6(+)MP release may better reflect basal cell activation during repair. Our findings suggest that basal cells are more highly activated during epithelial repair in the nasal mucosa of patients with polypoid CRS than in patients with non-polypoid CRS.

Epithelial repair has been reported to occur actively in parallel with inflammation starting at an early stage of polyp formation⁷. Among CRSwNP cases, high ITGB6(+)MP levels were associated with those with the most severe phenotype, i.e. revision surgery, independent of asthma, atopy and steroid use (Fig.1C, Table E2). Conversely, ITGB6(+)MP were not increased compared with controls in CRSwNP cases with no history of prior surgery despite the need for surgery (Fig.1C). We speculated that ITGB6(+)MP may be released from the nasal epithelium on the polyp perimeter (where acanthosis is more frequently observed)⁷ rather than from the epithelium spanning the mature polyp itself (which is often either acanthotic or consists of only a simple layer of basal cells)^{3,7}. Our findings suggest that basal cells may be more highly activated in nasal epithelium of patients with recurrent CRSwNP.

We examined commonly used EMT markers to test level of expression on ITGB6(+)MP to evaluate the phenotype of basal cells undergoing EMT and/or epithelial repair in CRSwNP. All three MP subtypes expressing ITGB6 along with EMT markers were significantly higher in CRSwNP cases with history of prior surgery than those without; especially Snail(+)ITGB6(+)MP and α SMA(+)ITGB6(+)MP (Fig.1C). *In vitro* studies confirmed that these ITGB6(+)MP subtypes were all consistently released from A549 cells along with up-regulation of ITGB6 during TGF- β -induced EMT (Fig.E2). Because myoepithelial cells have not been identified in nasal mucosa of CRS, these results may indicate aberrant or excessive EMT and basal cell activation during repair in this subset of subjects with particularly severe disease. While snail, slug, and/or α SMA positive ITGB6(+)MPs did overlap significantly, they were not fully equivalent, implying that they may not control or be derived

from identical signaling pathways.

We classified CRSwNP cases into 4 groups according to asthma comorbidity and history of prior endoscopic sinus surgery (Table E3) and compared levels of ITGB6(+)MP subtypes. CRSwNP subjects with comorbid asthma have a higher recurrence rate after surgery than those without asthma and are regarded as a severe phenotype with high type 2 inflammation⁸. However, dual Snail(+)ITGB6(+)MP and α SMA(+)ITGB6(+)MP were elevated to the highest levels in patients with AERD (Fig.1B) and in patients with CRSwNP with history of prior surgery regardless of whether there was comorbid asthma or not (Fig.1D), and correlations between levels of the eosinophil cation protein (EPC) and levels of Snail(+)ITGB6(+)MP ($r_s=.20$, $p<.06$) or α SMA(+)ITGB6(+)MP ($r_s=.27$, $p<.02$) were weak. Assessment of epithelial repair using ITGB6(+)MP subtypes therefore appears not to represent a simple surrogate of type 2 inflammation. Biopsy tissue-based studies reported that higher counts of eosinophils, mast cells and basophils, and high levels of type 2 cytokines including EPC, were detected in mature polyps, rather than in tissues around polyps such as uncinate/ethmoid tissues^{8,9}. As mentioned above, the perimeter of polyps appears to have the highest level of acanthosis⁷; whether ITGB6(+)MP originate from nasal epithelium on the perimeter of nasal polyps is worthy of further study. Because the MP assay in NLFs is representative of the condition in the entire nasal cavity, it would be preferable to collect MPs released from nasal tissues selected for biopsy using a sponge-based approach in order to directly compare histological features in specific regions of the sinonasal cavity to the MP profiles.

There are limitations to this study. NLFs were only collected from surgical patients in the operating room before surgery and we were not able to follow their levels with subsequent measurements after surgery. The degree of basal cell activation and EMT might be elevated in response to prolonged chronic inflammation in severe CRSwNP endotypes with prior surgery. There was no significant difference in levels of ITGB6(+)MP subtypes between CRSsNP with and without prior surgery (Fig.E3). In addition, among 196 CRSwNP cases enrolled in the study, with the exception of one subject who had had a revision surgery 1.7 year after the prior surgery, no CRSwNP subjects had undergone surgery previously between January 2014 and December 2017. Therefore, the surgical procedure itself used during prior surgery may not directly influence ITGB6(+)MP levels in cases undergoing revision surgery.

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In conclusion and based on the results of our MP assays, basal cells are significantly activated with up-regulation of ITGB6 and markers of EMT in patients with AERD or a severe phenotype of CRSwNP which had previously necessitated revision surgery. Biopsy of nasal tissues is invasive and not generally practical in the clinic. Basal cells undergoing EMT are unevenly distributed in tissue and are difficult to quantify. While it is generally accepted that CRSwNP is mostly characterized by type 2 inflammation, inflammatory profiles are highly variable and polyp recurrence necessitating revision surgery occurs in diverse inflammatory endotypes, with or without comorbid asthma. We believe that ITGB6(+)MP subtypes in NLFs may help to clarify roles of basal cells in pathophysiology, provide new insights for CRS endotyping and play valuable roles in testing the link between status of basal cells and the natural history, comorbidities and long term outcomes of CRS.

Competing interests: The authors declare no conflict of interest as to the interpretation and presentation of this manuscript.

Table 1. Demographics of the cases

Characteristics	Control (n=47)	CRSsNP (n=70)	CRSwNP (n=196)	AERD (n=31)	<i>p value</i>
Gender, male/female	31/16	38/32	129/67	15/16	.13
Ethnicity, White/ non-White	39/8	54/16	150/46	20/11	.33
Age, mean $\pm$ Standard deviation	44.8 $\pm$ 15.6	46.4 $\pm$ 15.1	47.5 $\pm$ 13.1	47.5 $\pm$ 13.4	.63
Smoking status, never/prior /current smokers	34/12/1	51/16/3	133/53/10	19/12/0	.43
Comorbidity					
Asthma, yes (%)	-	17 (24%)	83 (42%)*	31 (100%)*#	<.0001
Atopy, yes (%)	- ¹⁾	40 (57%)	131 (67%)	22 (71%)	.27
Steroid use, yes (%)					
Oral	-	2 (3%)	6 (3%)	6 (19%)*#	<.006
Intranasal	-	21 (30%)	52 (27%)	10 (32%)	.73
Inhaled	-	4 (6%)	40 (20%)*	13 (42%)*#	<.0001
Preoperative oral steroid use	-	1 (1%)	29 (15%)*	11 (35%)*#	<.0001

Cases with autoimmune diseases, immunodeficiency, systemic coagulation disorders, cancer, metabolic diseases, cystic fibrosis, an antrochoanal polyp, or unilateral nasal polyp were excluded from the study. Control subjects had no comorbid asthma and no steroid therapy.

¹⁾; Thirteen out of 46 control cases had history of atopy, but none of them was on medication for atopy at the time of enrollment. The chi-squared test or Kruskal-Wallis test (Age) was used to compare demographics among the 4 groups. *, $p < 0.05$, vs. CRSsNP, #; $p < 0.05$, vs. CRSwNP

Figure Legends

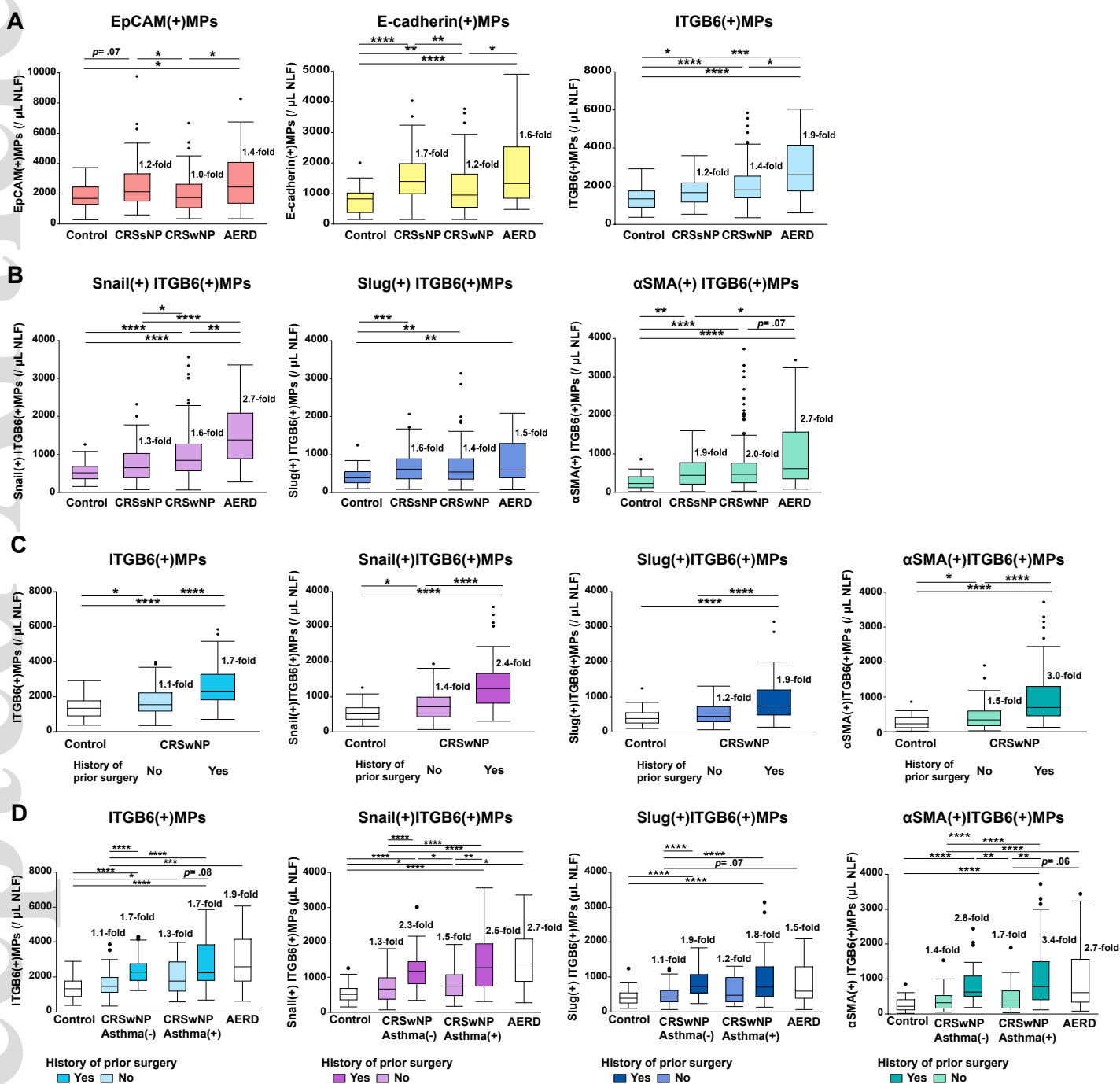
Figure 1. Comparisons of MP levels

(A) Comparison of epithelial MP among control, CRSsNP, CRSwNP and AERD.

(B-D) Comparison of ITGB6(+)MP subtypes (B) among control, CRSsNP, CRSwNP and AERD, (C) CRSwNP with history of prior surgery (cases undergoing surgery to treat polyp recurrence) vs. without prior surgery (primary surgical cases), (D) CRSwNP with asthma vs. without asthma (including those with history of prior surgery and those without prior surgery, as indicated). The Steel-Dwass test was used to analyze MP levels between each two groups.

*; $p < .05$, **; $p < .01$, ***; $p < .001$, ****; $p < .0001$, ##-fold; significantly higher by ##-fold vs control.

Figure 1.

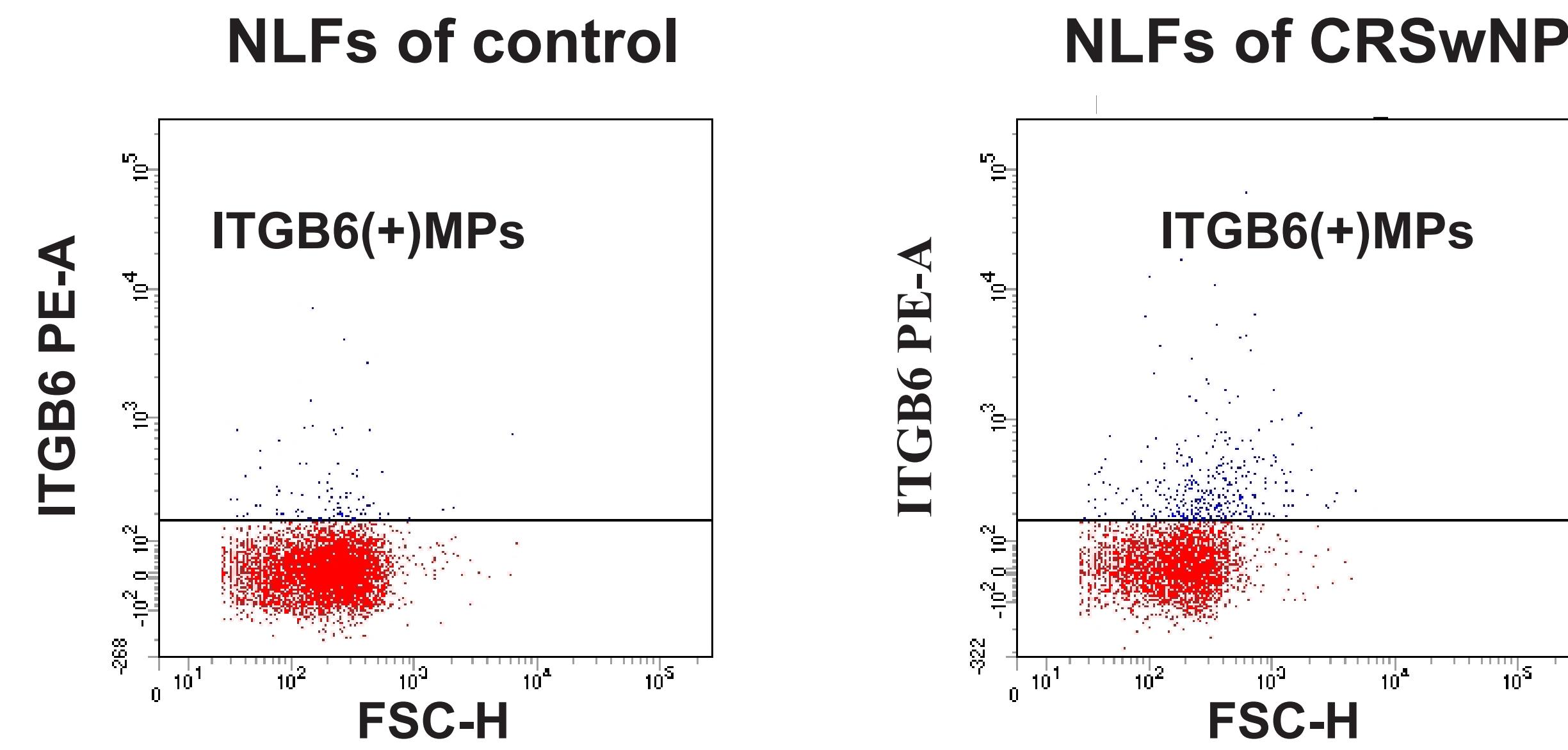


References

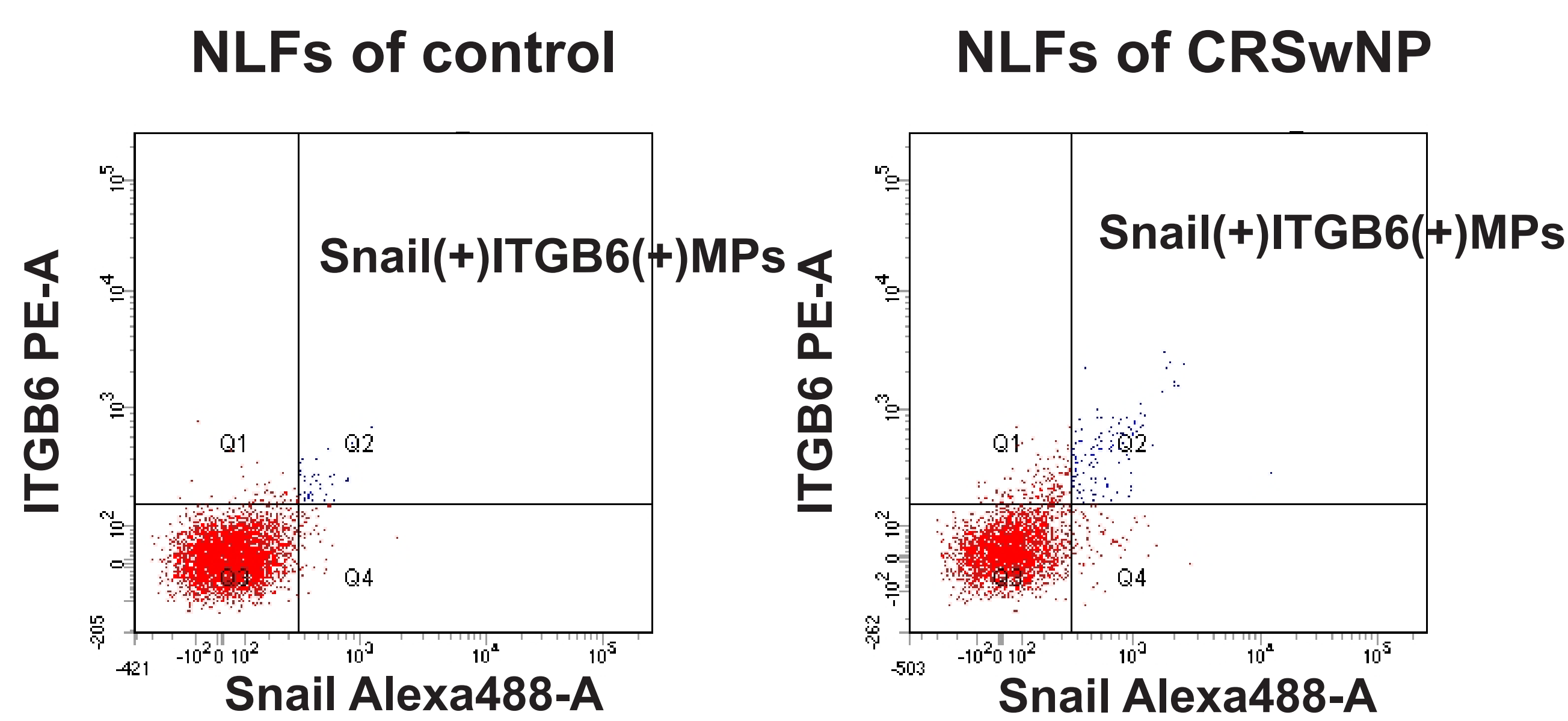
1. Takahashi T, Kato A, Berdnikovs S, et al. Microparticles in nasal lavage fluids in chronic rhinosinusitis: Potential biomarkers for diagnosis of aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol.* 2017;140(3):720-729.
2. Ordovas-Montanes J, Dwyer DF, Nyquist SK, et al. Allergic inflammatory memory in human respiratory epithelial progenitor cells. *Nature.* 2018;560(7720):649-654.
3. Schleimer RP. Immunopathogenesis of Chronic Rhinosinusitis and Nasal Polyposis. *Annu Rev Pathol.* 2017;12:331-357.
4. Takahashi T, Kobayashi S, Fujino N, et al. Increased circulating endothelial microparticles in COPD patients: a potential biomarker for COPD exacerbation susceptibility. *Thorax.* 2012;67(12):1067-1074.
5. Haapasalmi K, Zhang K, Tonnesen M, et al. Keratinocytes in human wounds express alpha v beta 6 integrin. *J Invest Dermatol.* 1996;106(1):42-48.
6. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest.* 2009;119(6):1420-1428.
7. Meng J, Zhou P, Liu Y, et al. The development of nasal polyp disease involves early nasal mucosal inflammation and remodelling. *PLoS One.* 2013;8(12):e82373.
8. Tomassen P, Vandeplas G, Van Zele T, et al. Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers. *J Allergy Clin Immunol.* 2016;137(5):1449-1456 e1444.
9. Mahdavinia M, Carter RG, Ocampo CJ, et al. Basophils are elevated in nasal polyps of patients with chronic rhinosinusitis without aspirin sensitivity. *J Allergy Clin Immunol.* 2014;133(6):1759-1763.

A

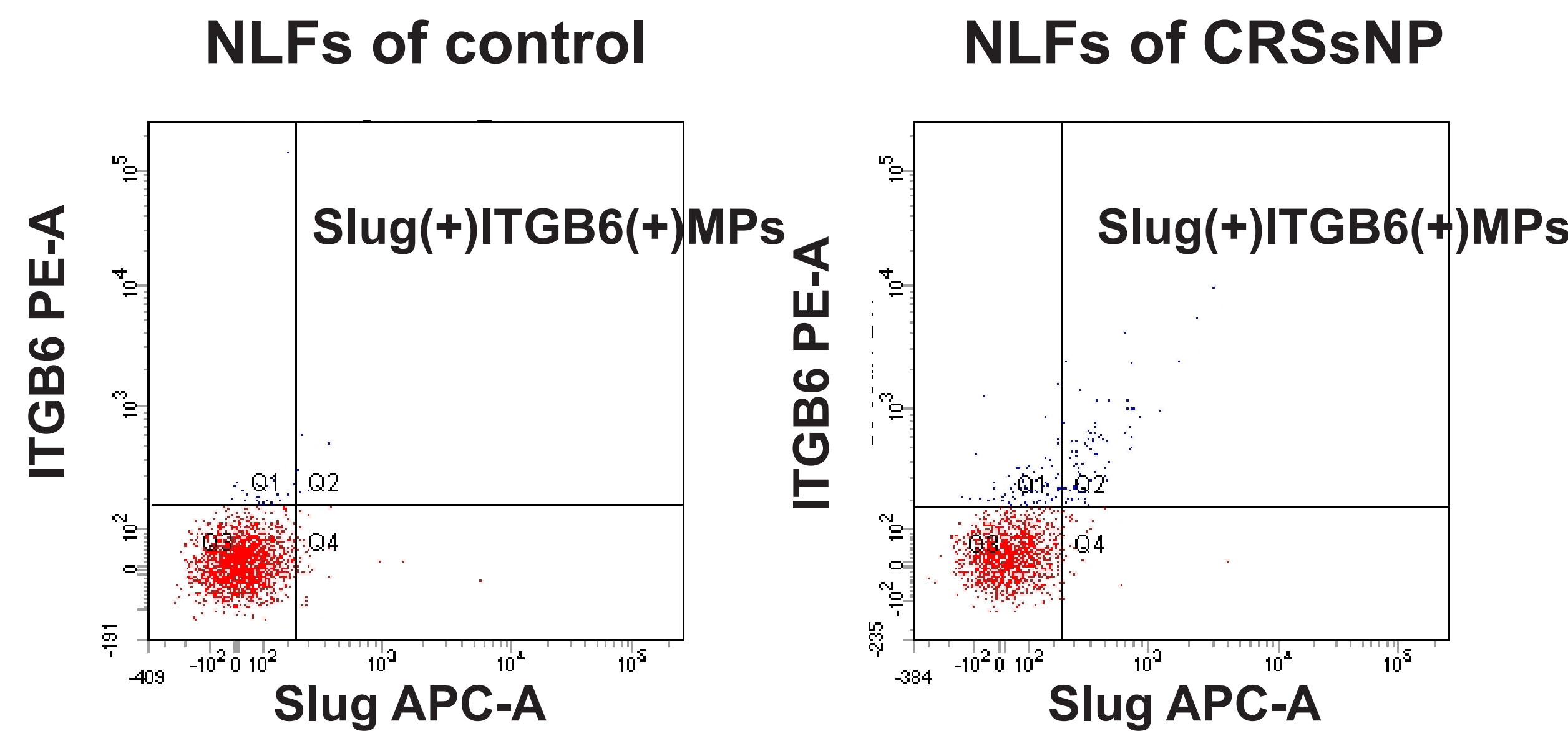
ITGB6(+) MPs



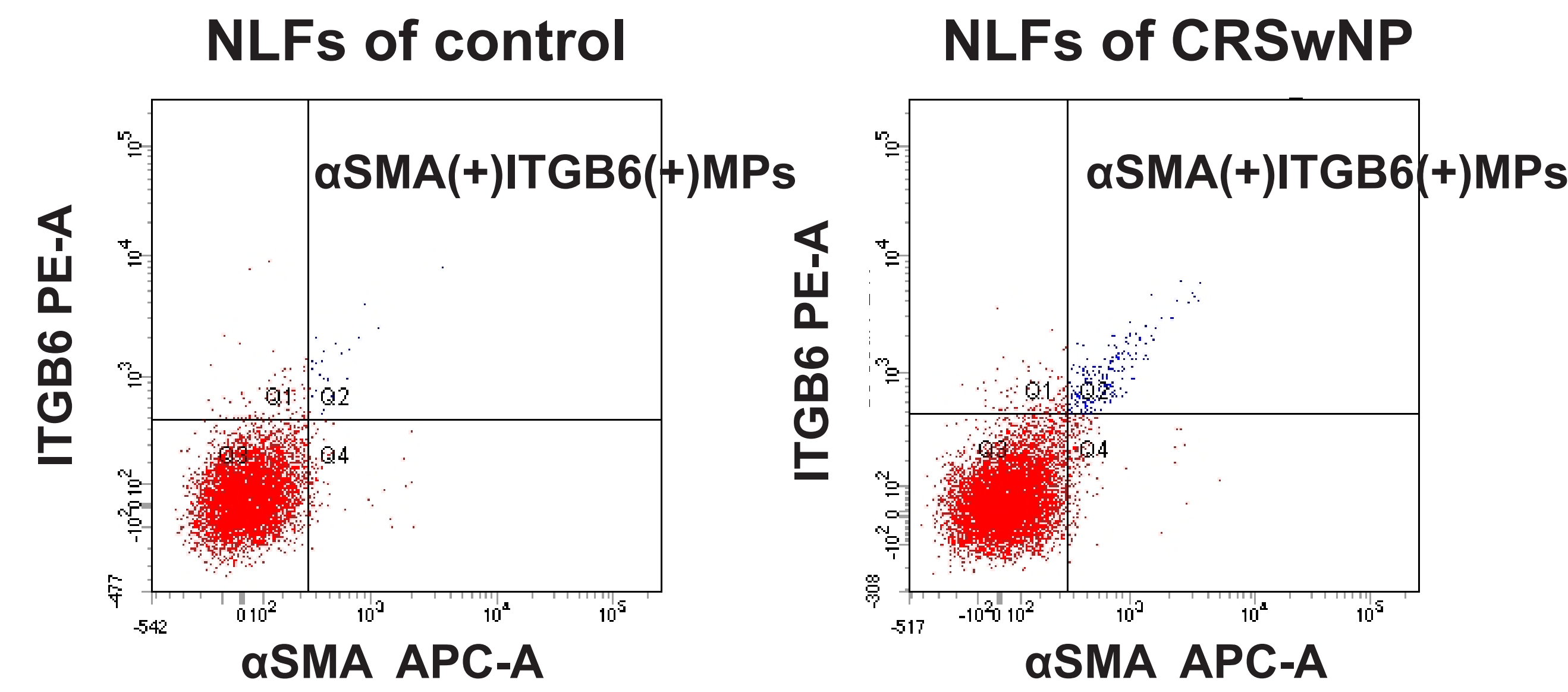
Snail(+) ITGB6(+) MPs



Slug(+) ITGB6(+) MPs



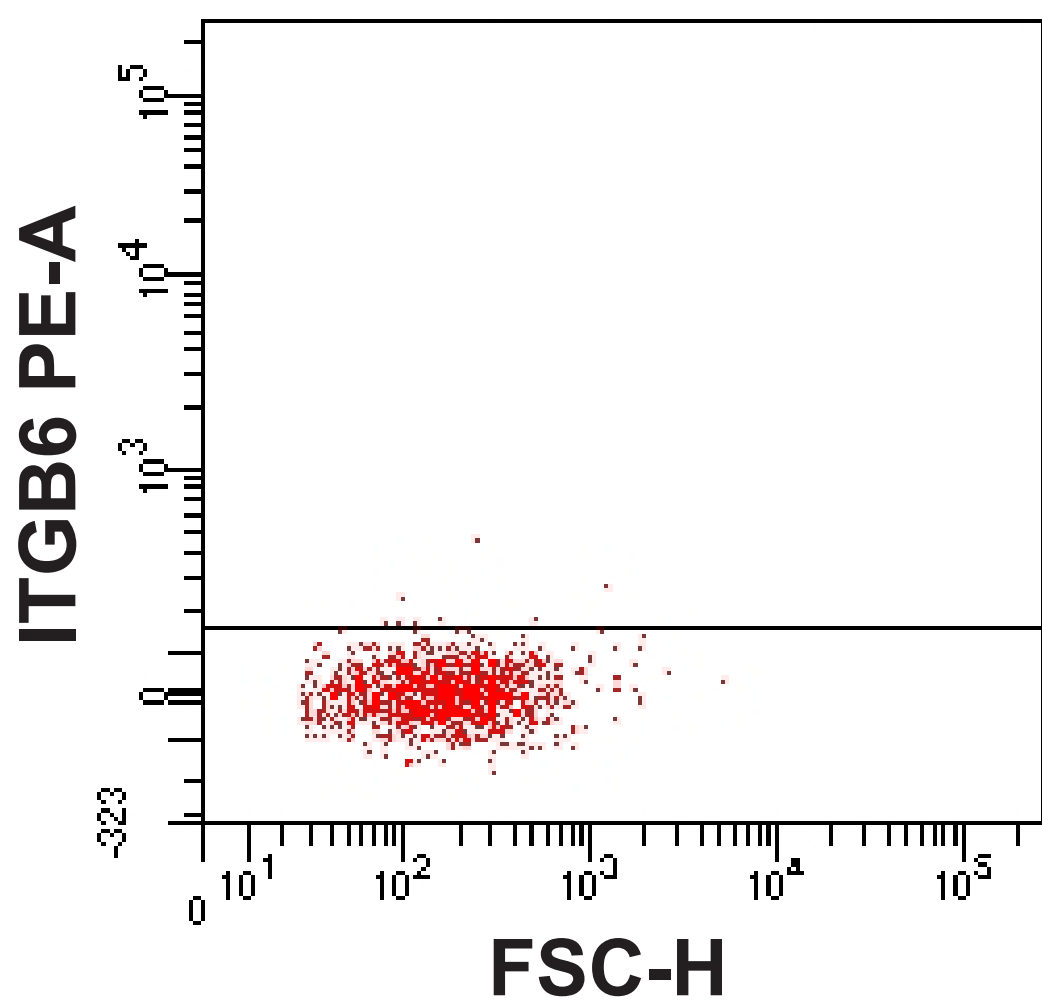
α SMA(+) ITGB6(+) MPs



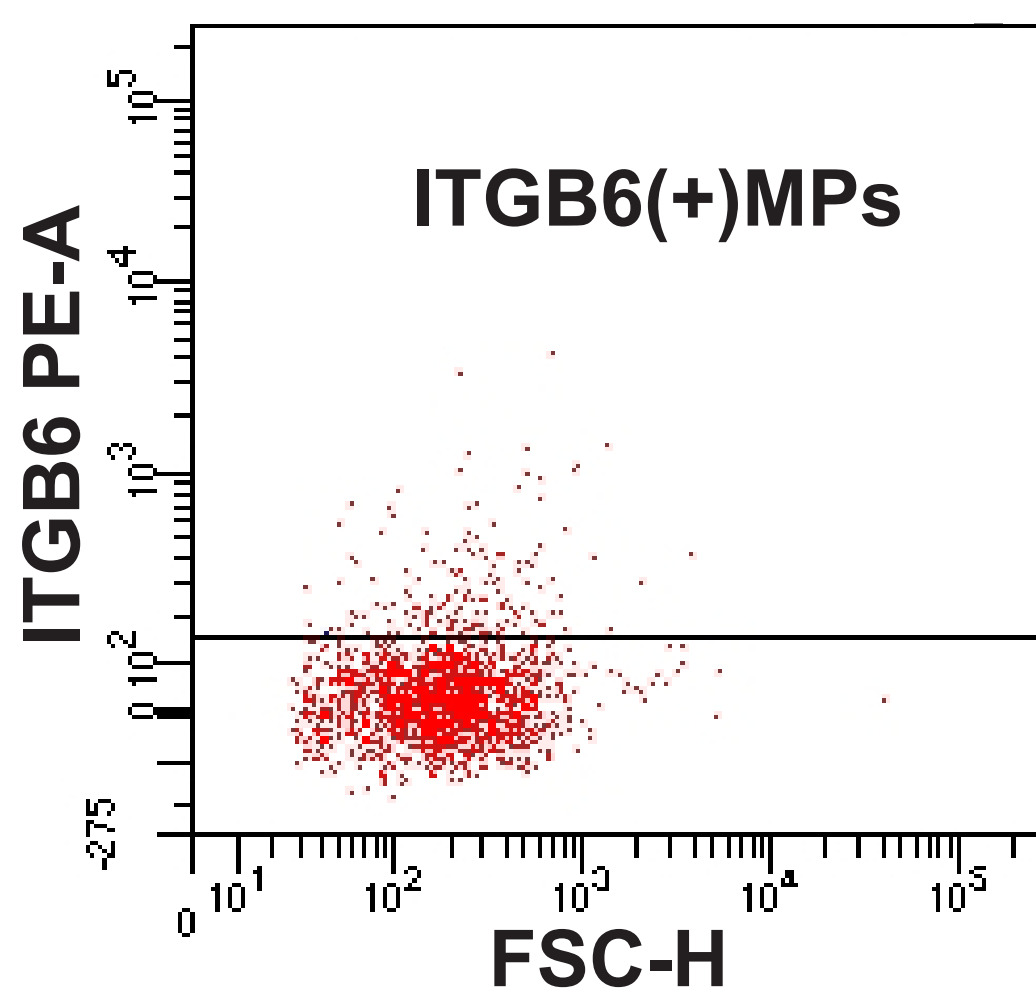
B

ITGB6-PE single staining

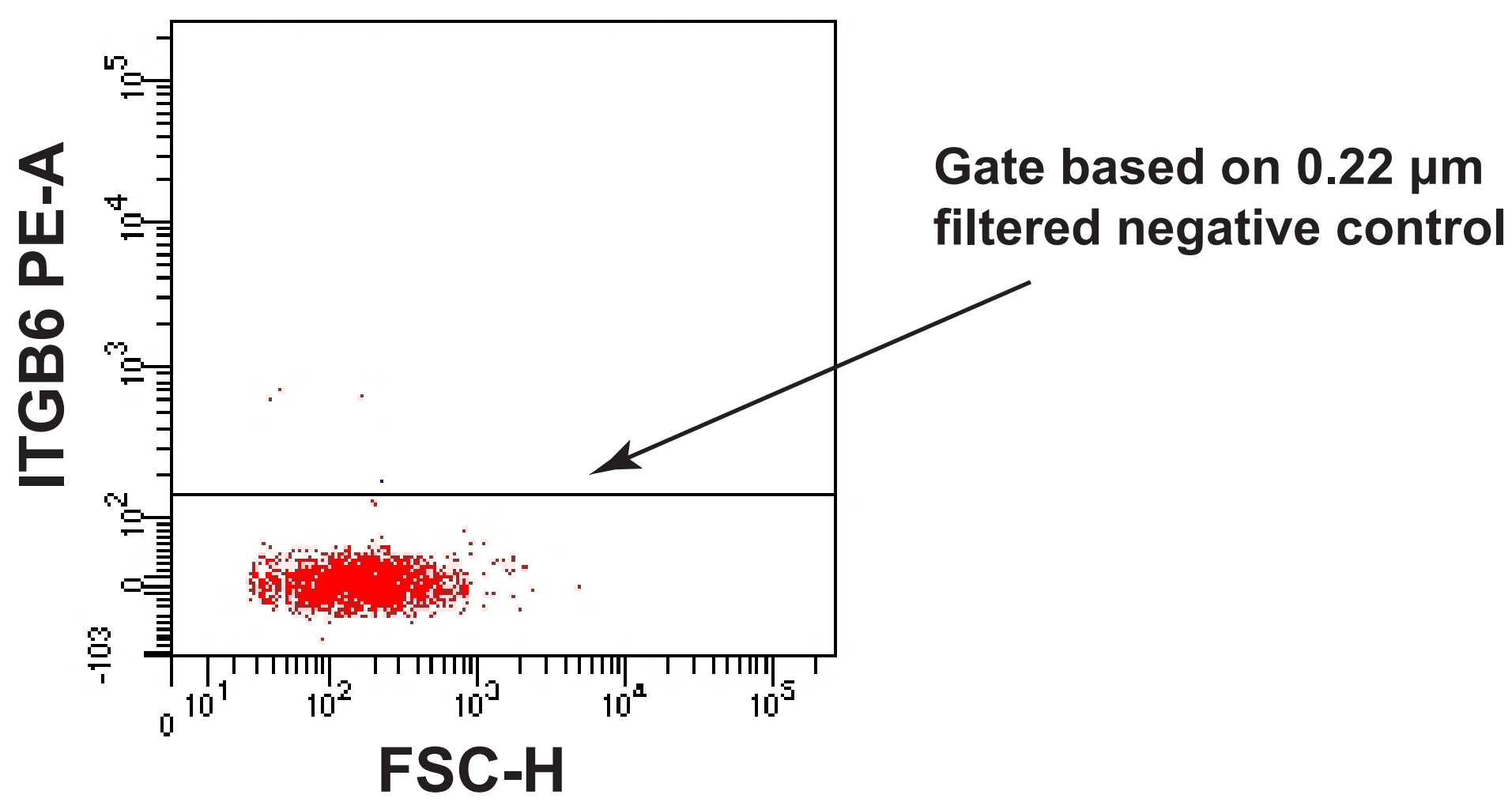
0.22 μ m filtered negative control (ITGB6-PE single staining)



ITGB6-PE single staining

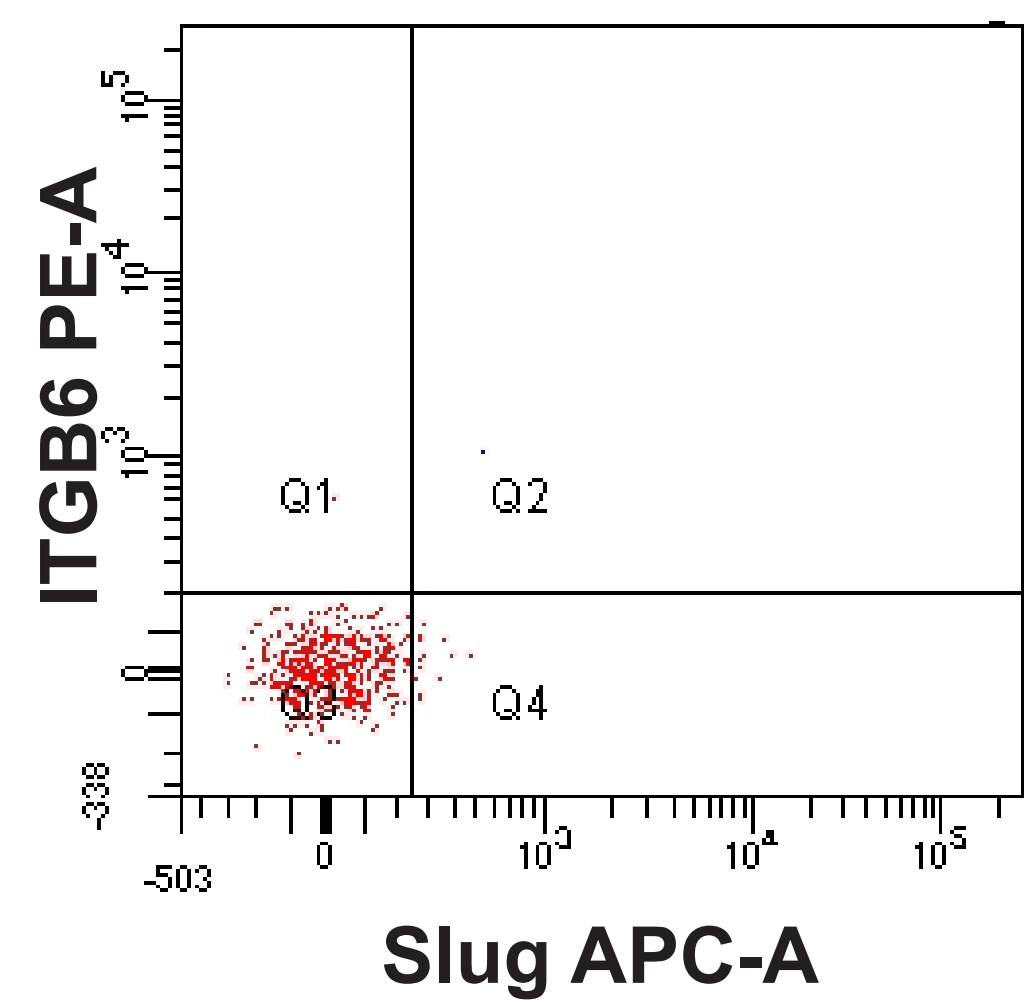


Unstained control

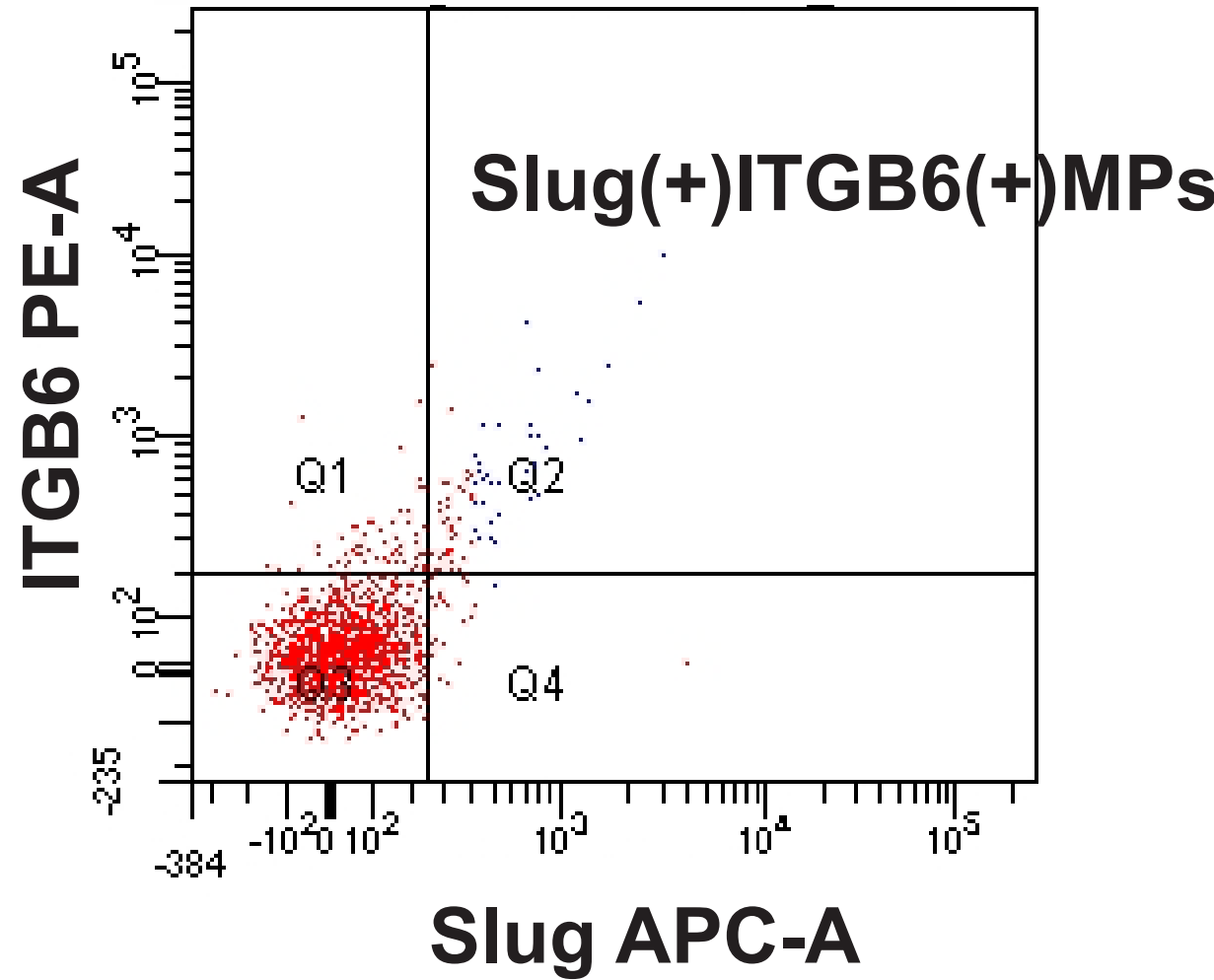


ITGB6-PE Slug-APC double staining

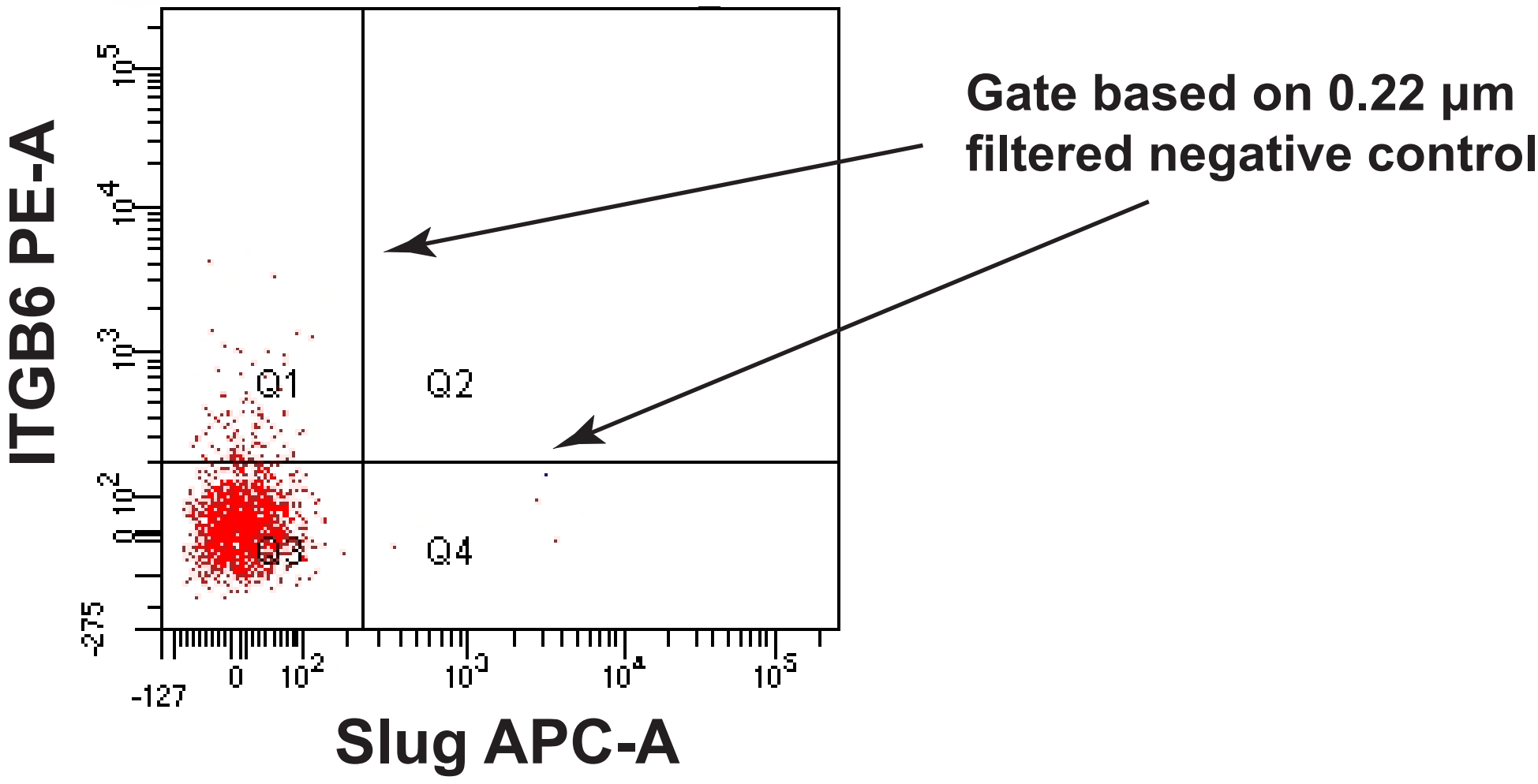
0.22 μ m filtered negative control (ITGB6-PE Slug-APC double staining)



ITGB6-PE Slug-APC double staining

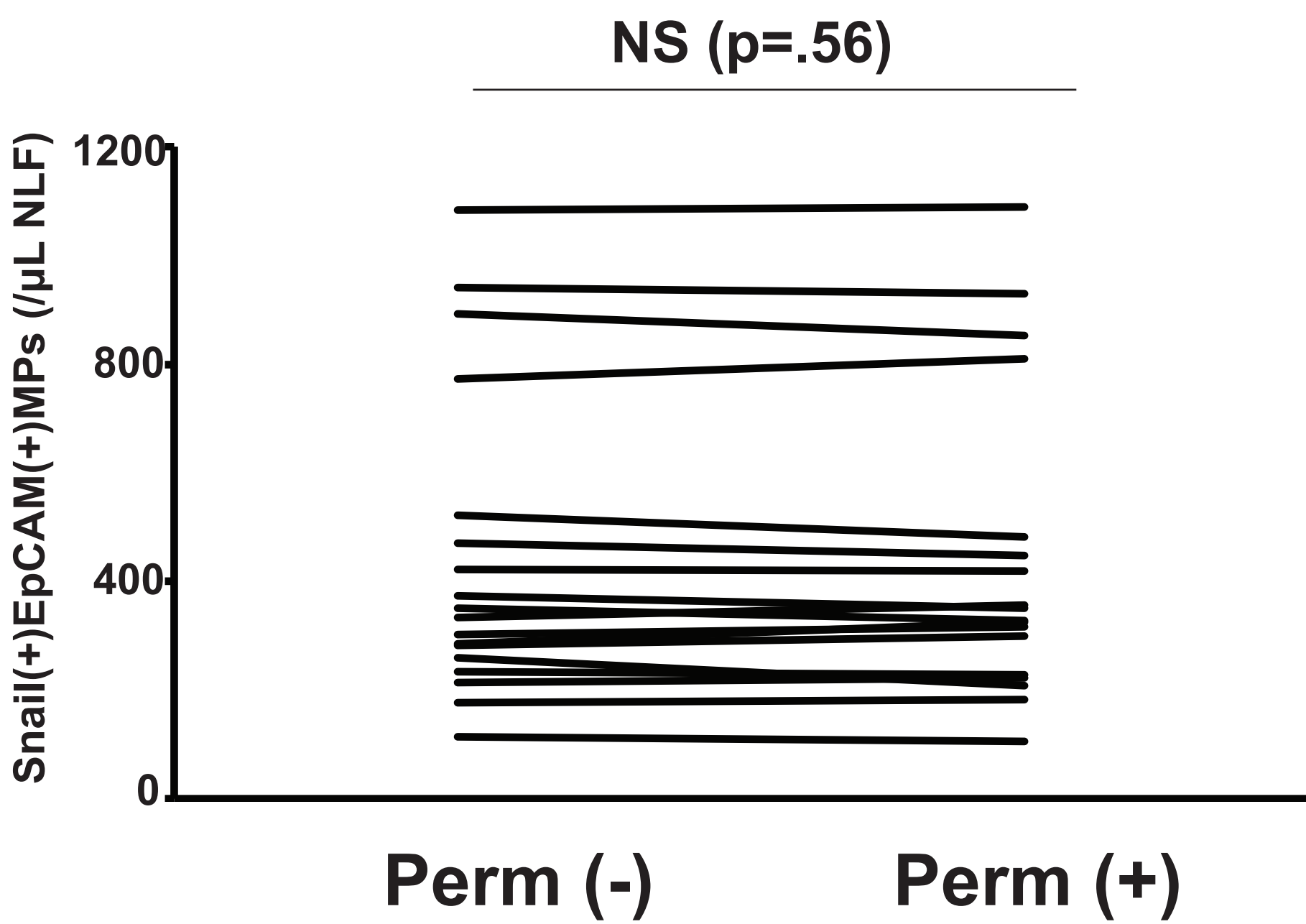


Fluorescence Minus One (ITGB6-PE single staining)



C

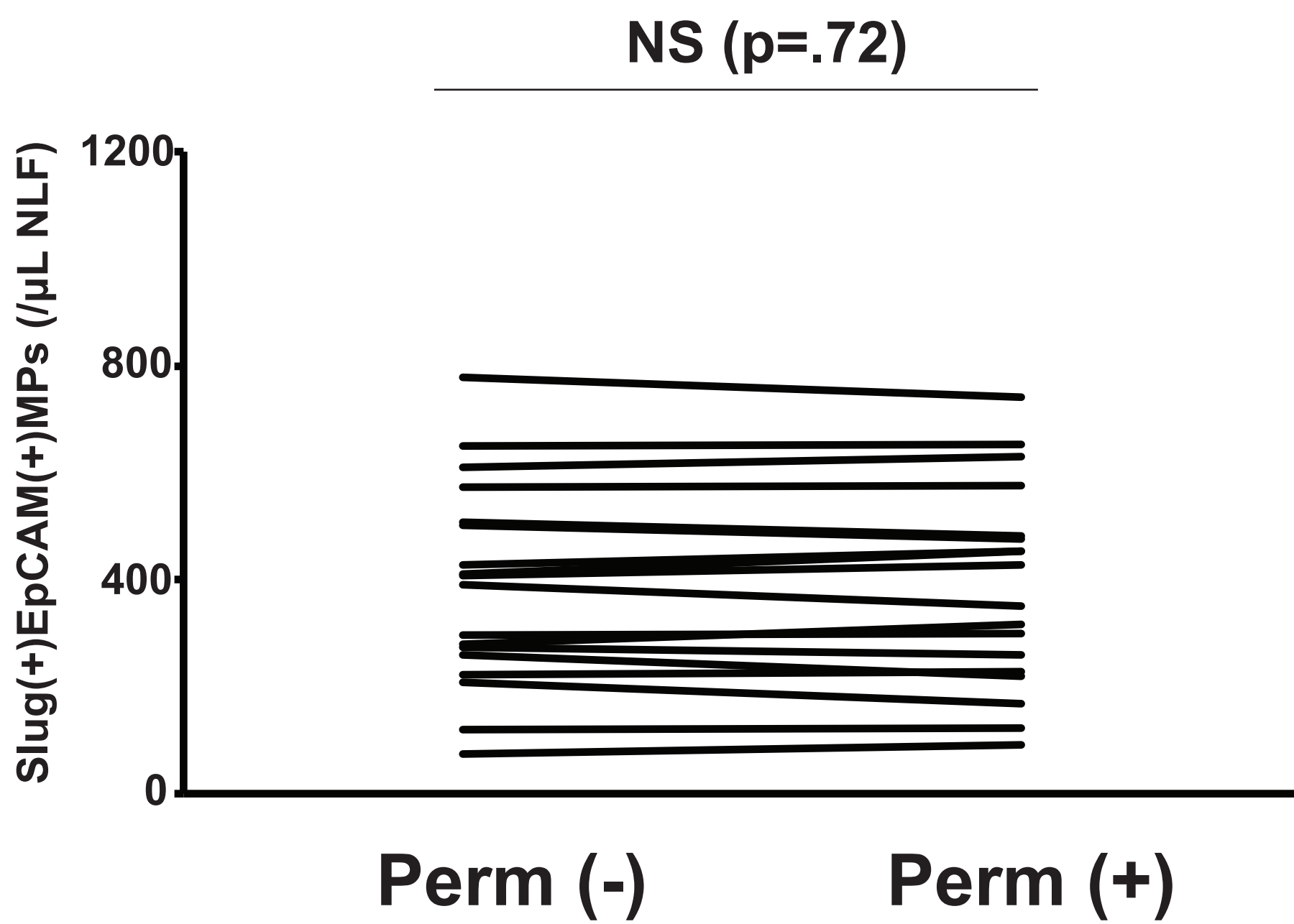
Snail(+)EpCAM(+)MPs



“Perm(+)” - “Perm(-)”

Mean: -3.65 (95% CI: -16.7 to 9.36)
Range: -53.2 to 40.4

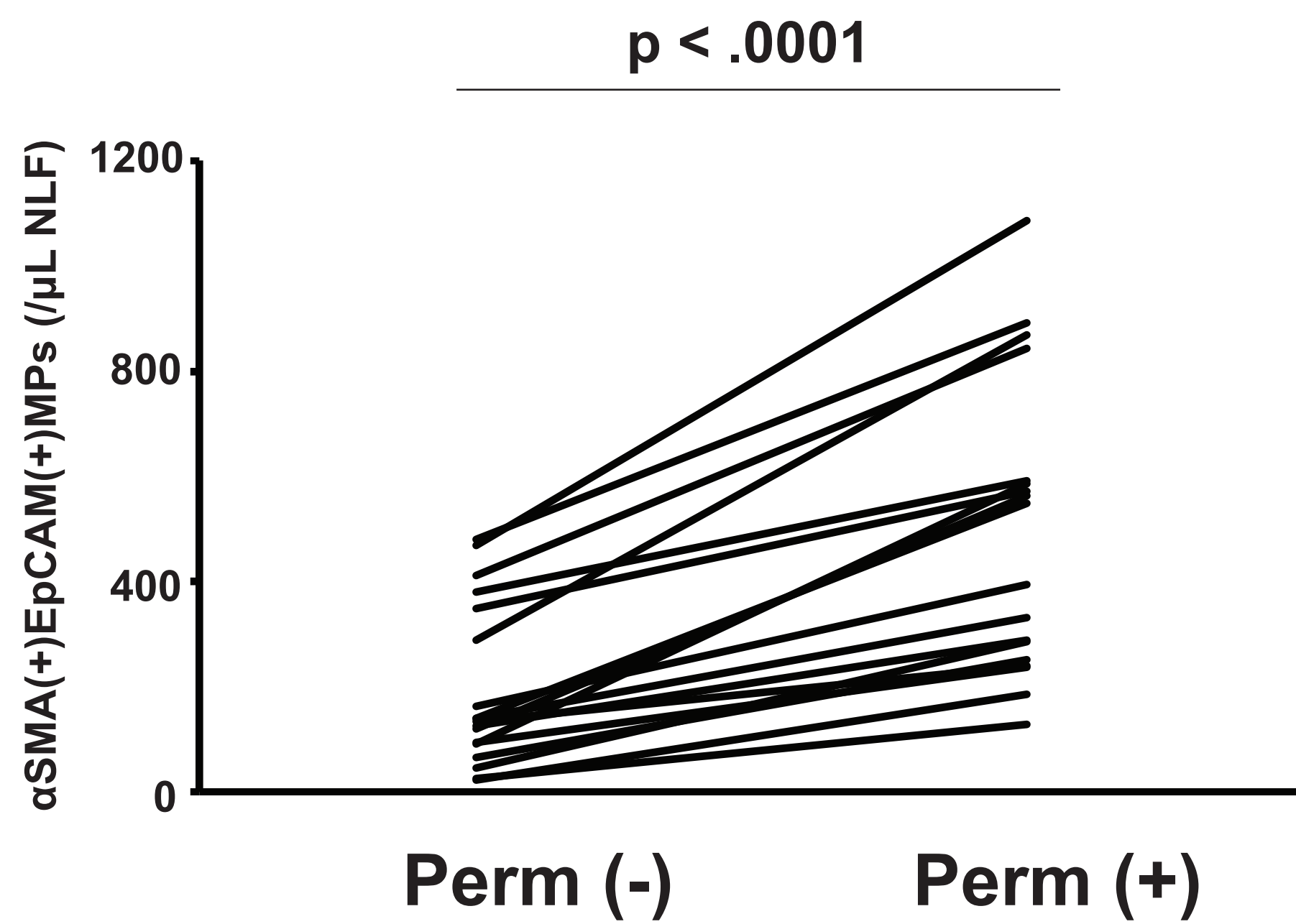
Slug(+)EpCAM(+)MPs



“Perm(+)” - “Perm(-)”

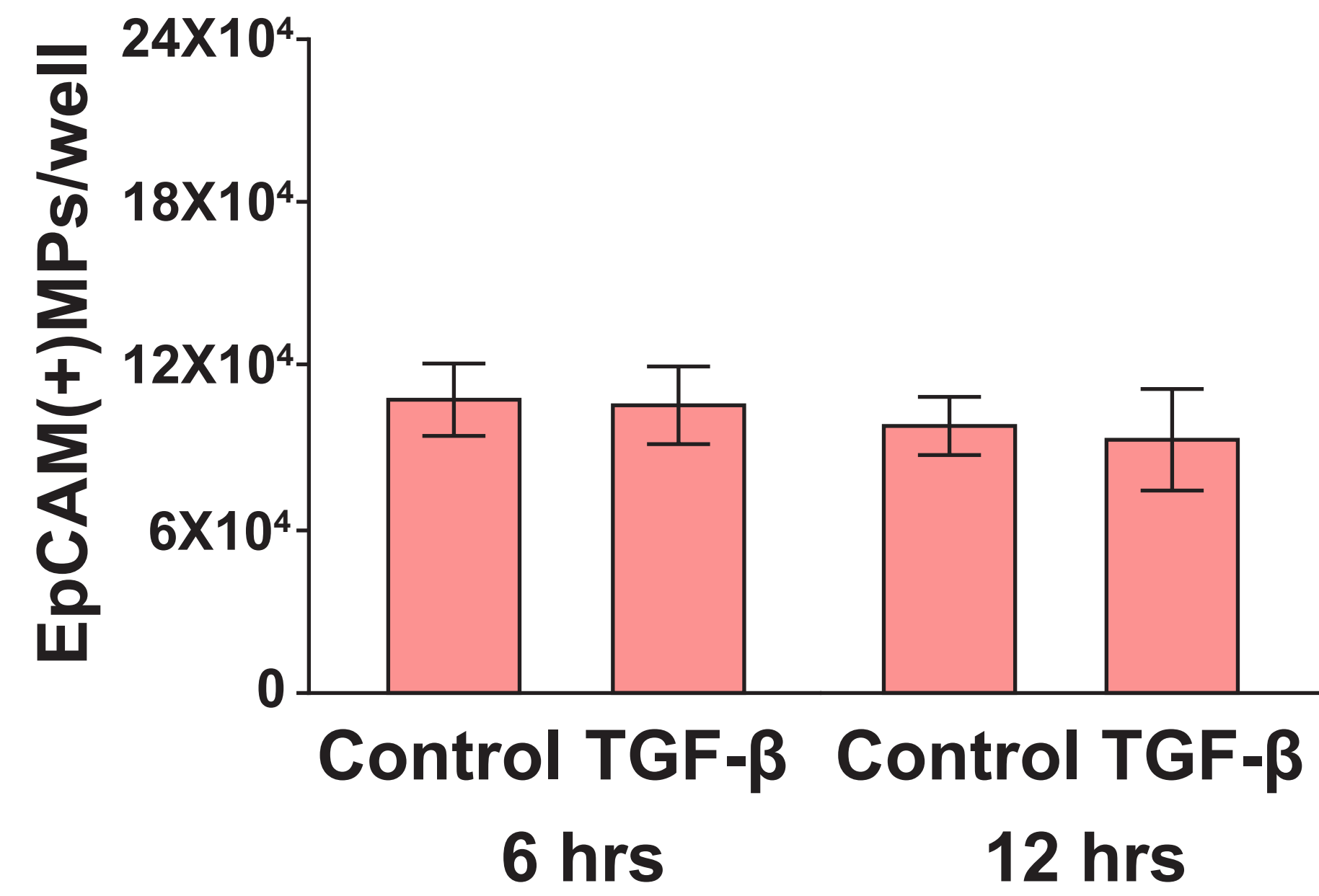
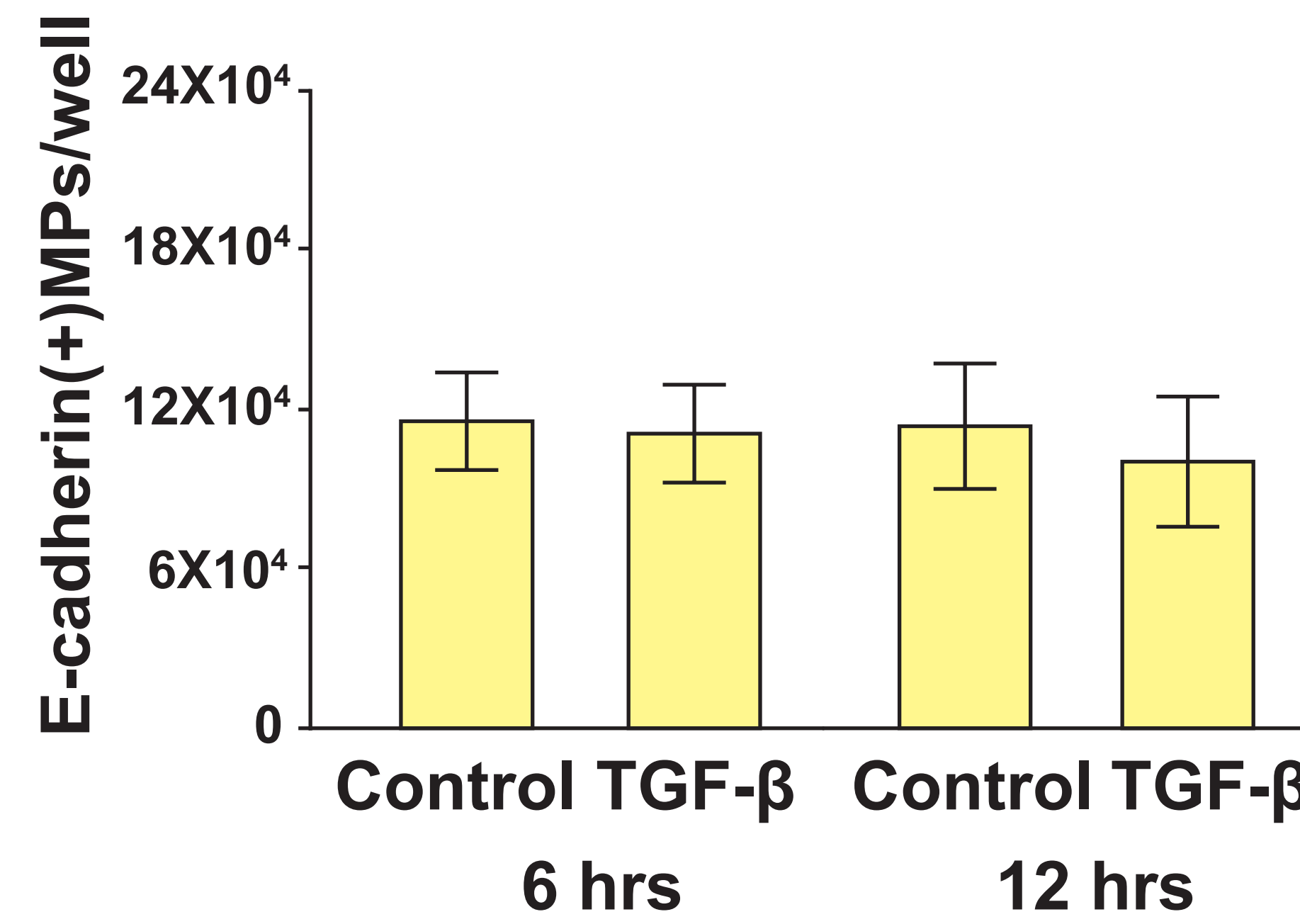
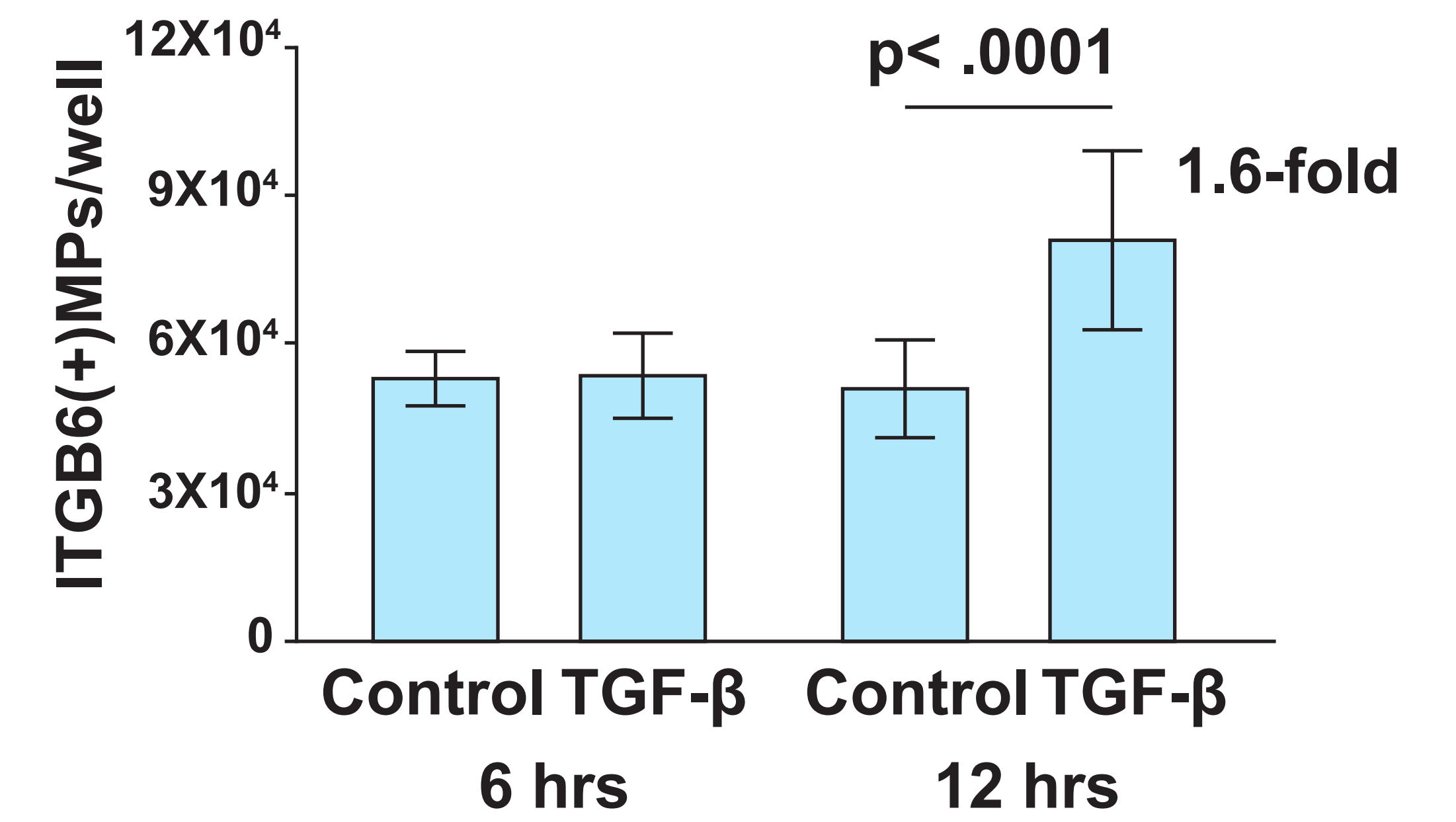
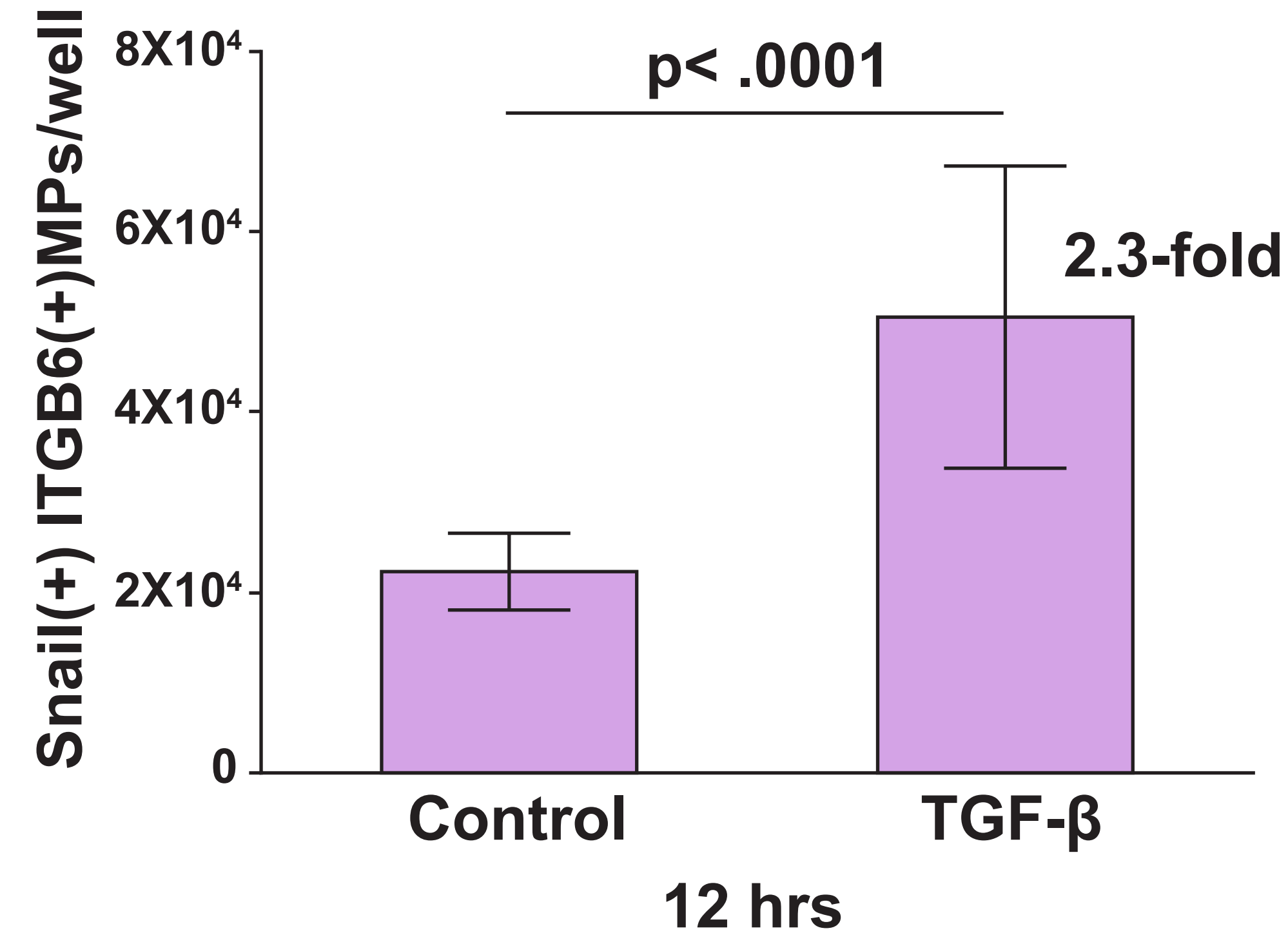
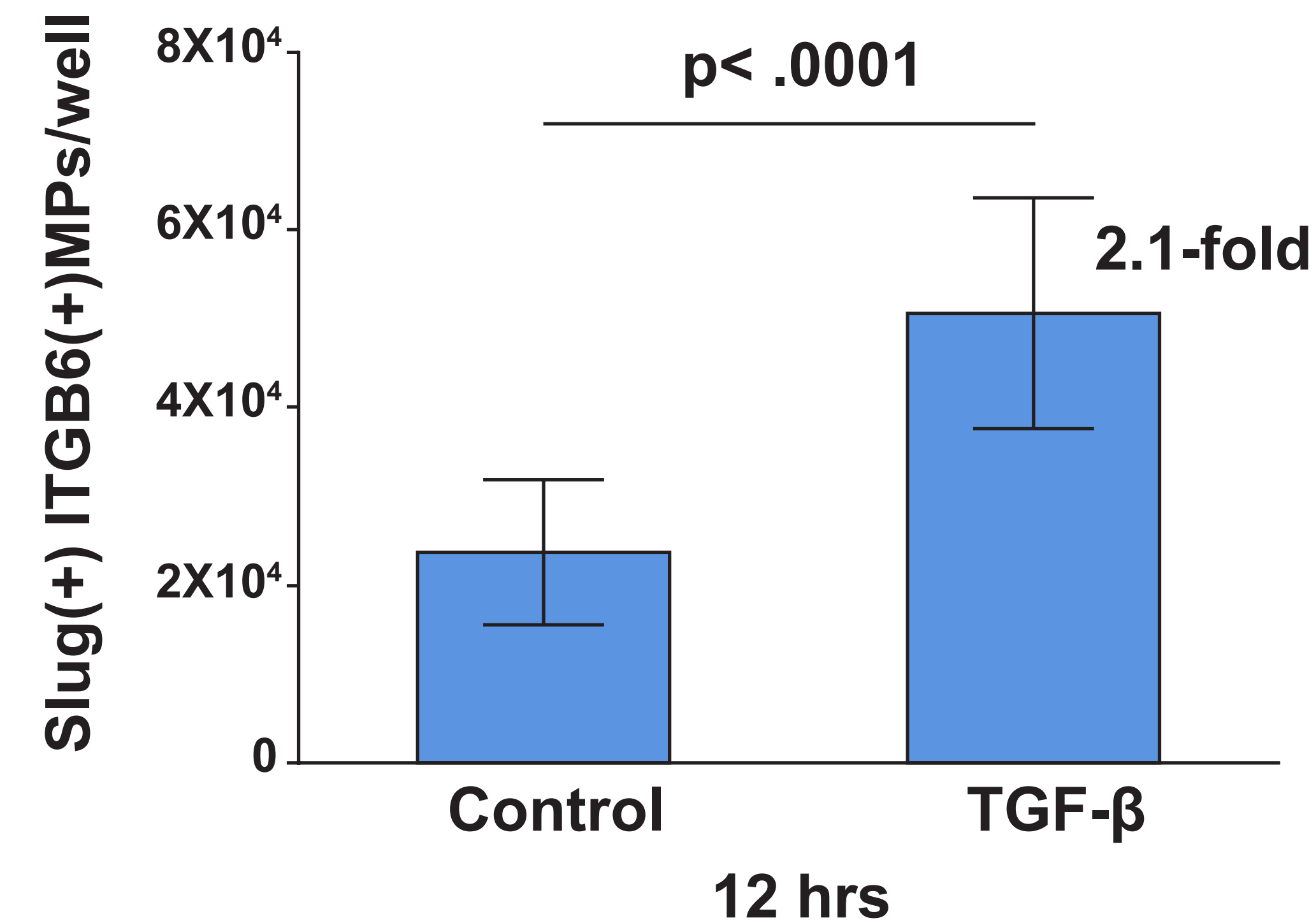
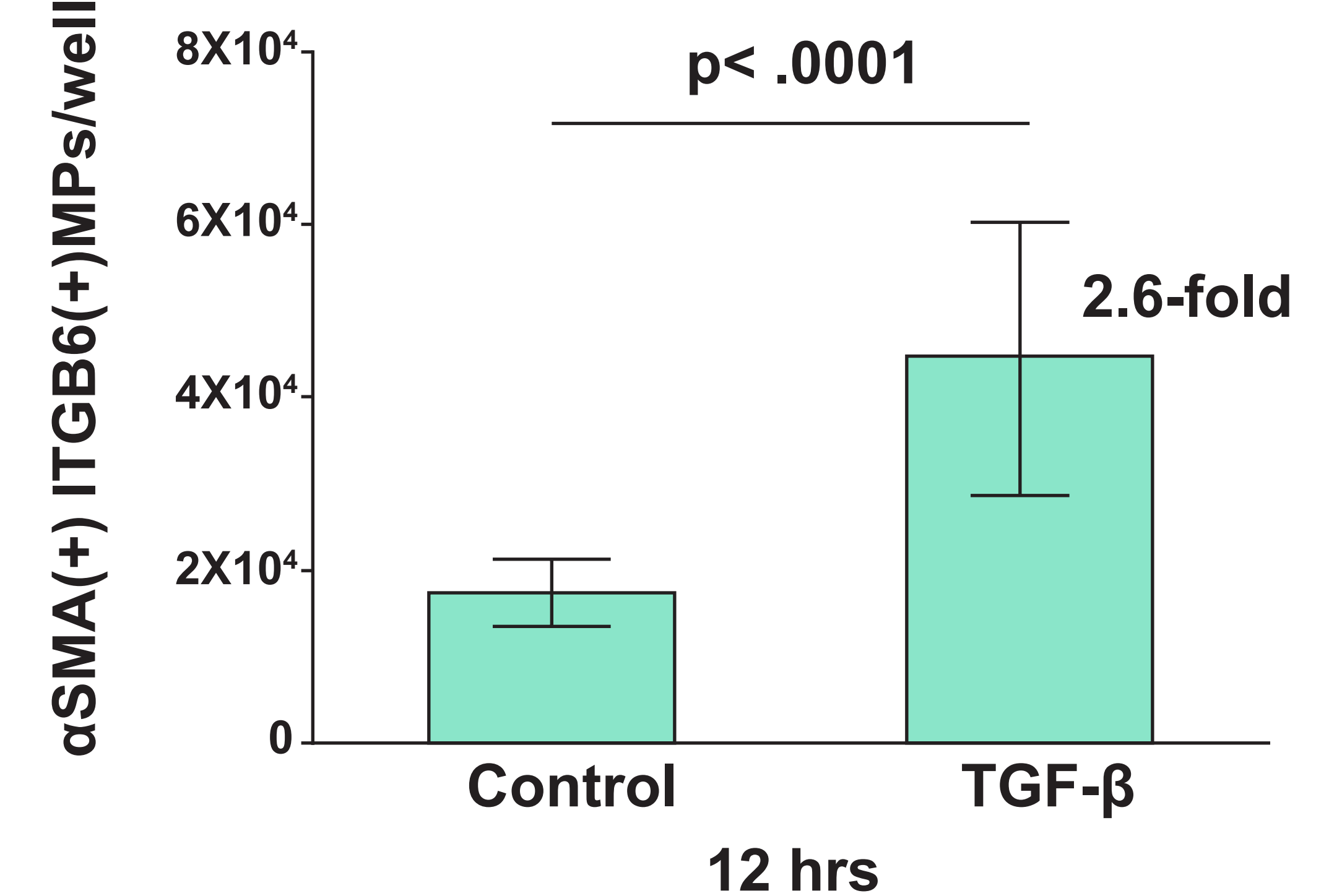
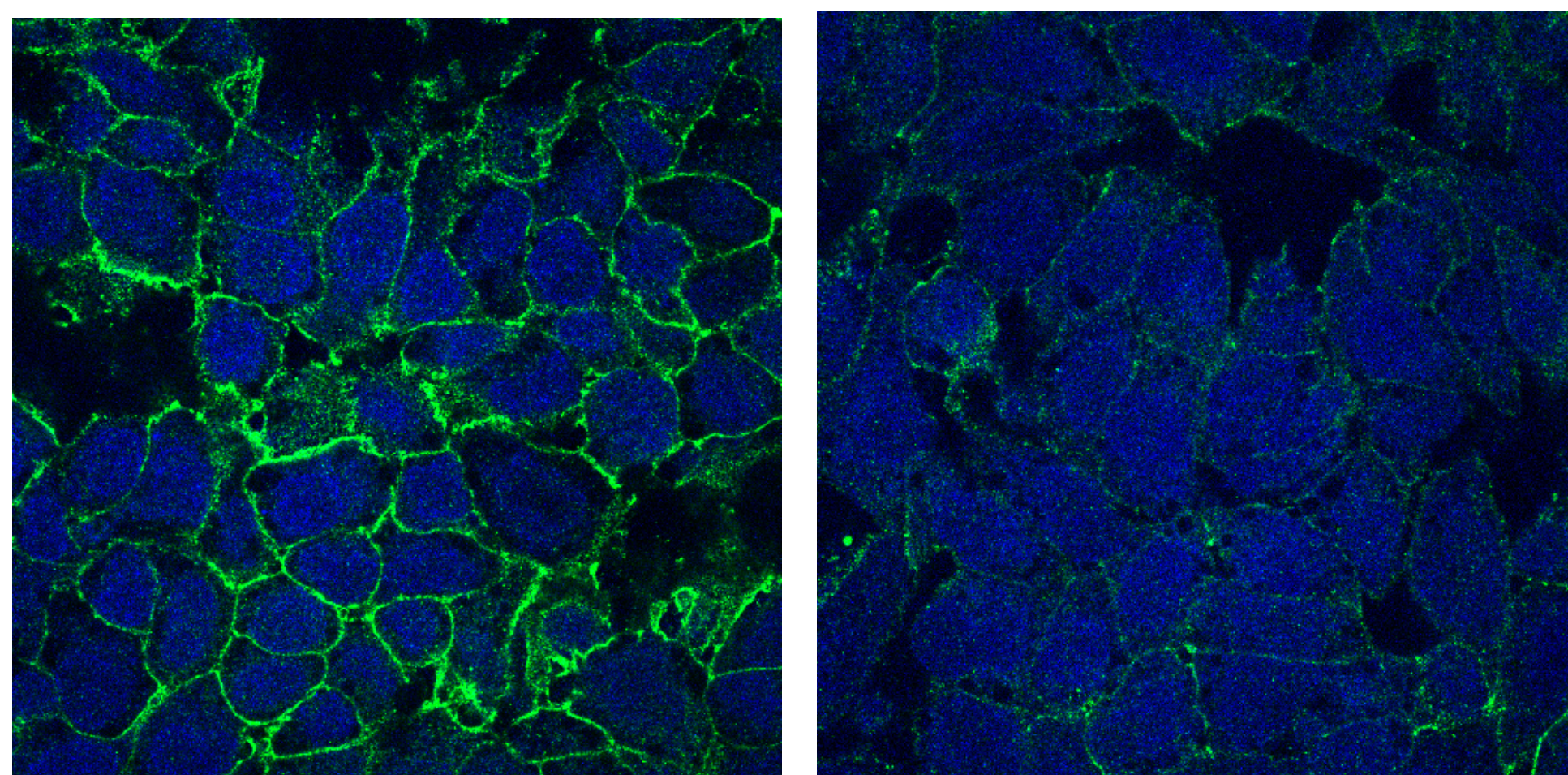
Mean: -2.30 (95% CI: -15.7 to 11.1)
Range: -39.8 to 42.3

α SMA(+)EpCAM(+)MPs



“Perm(+)” - “Perm(-)”

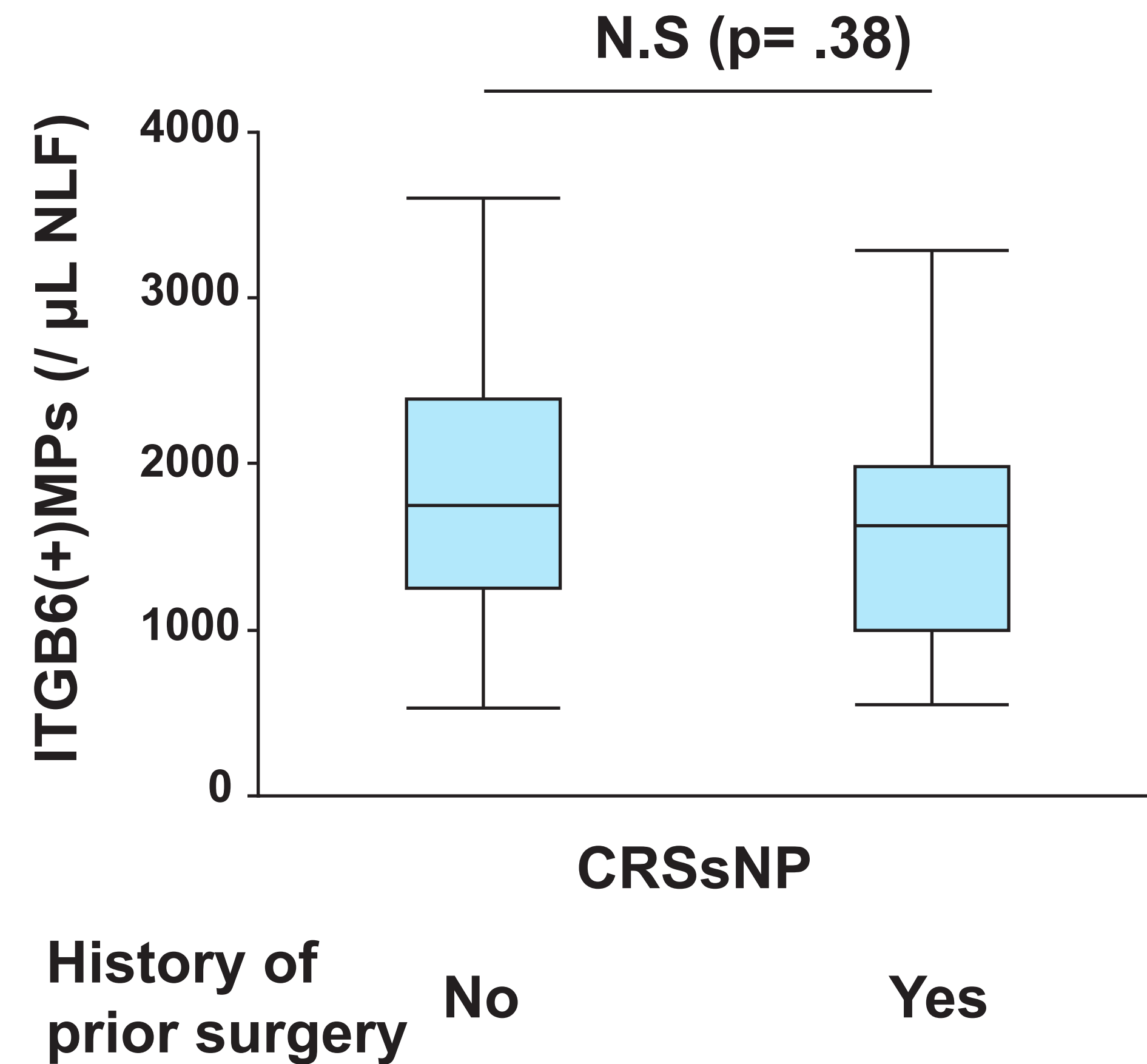
Mean: 296.5 (95% CI: 214.3 to 378.6)
Range: 102.8 to 616.7

A**EpCAM(+)MPs****E-cadherin(+)MPs****ITGB6(+)MPs****B****Snail(+) ITGB6(+)MPs****Slug(+) ITGB6(+)MPs****αSMA(+) ITGB6(+)MPs****C****A549 cells****Control****TGF-β****48 hrs****Expression of EMT markers on A549 cells (n=5)**

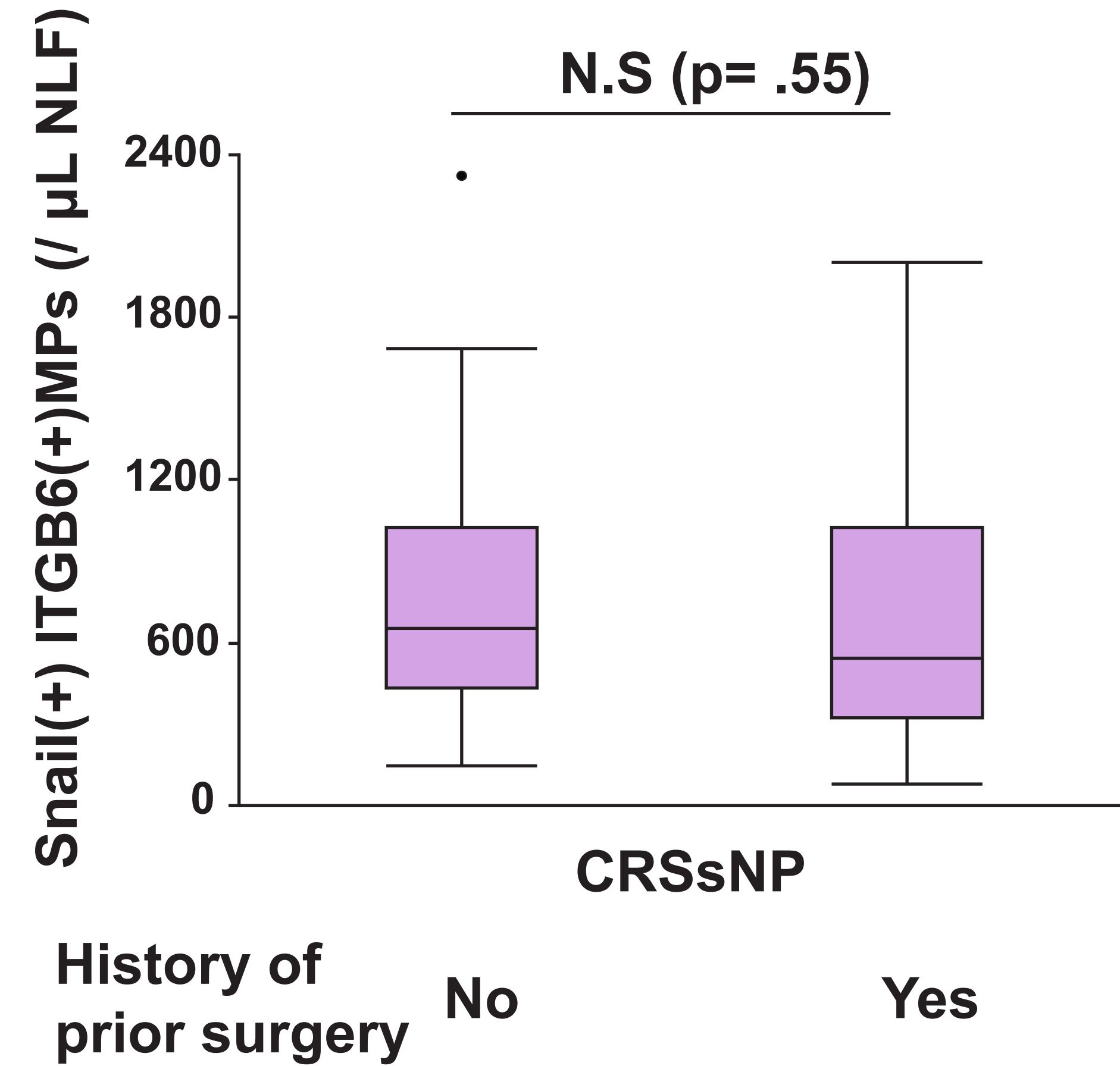
EMT Marker	Control	TGF-β1 10ng/mL for 24 hours
ITGB6	1536.2 ± 201.7	2093.6 ± 328.3*
Snail	5114.8 ± 1287.8	6770.8 ± 2185.5
Slug	1104.0 ± 188.8	1460.4 ± 245.6*
αSMA	1405.2 ± 184.2	2016.6 ± 248.8*

Values are expressed as the geometric mean fluorescence intensity of each antigen-positive cell. *, p< .05 vs. Control

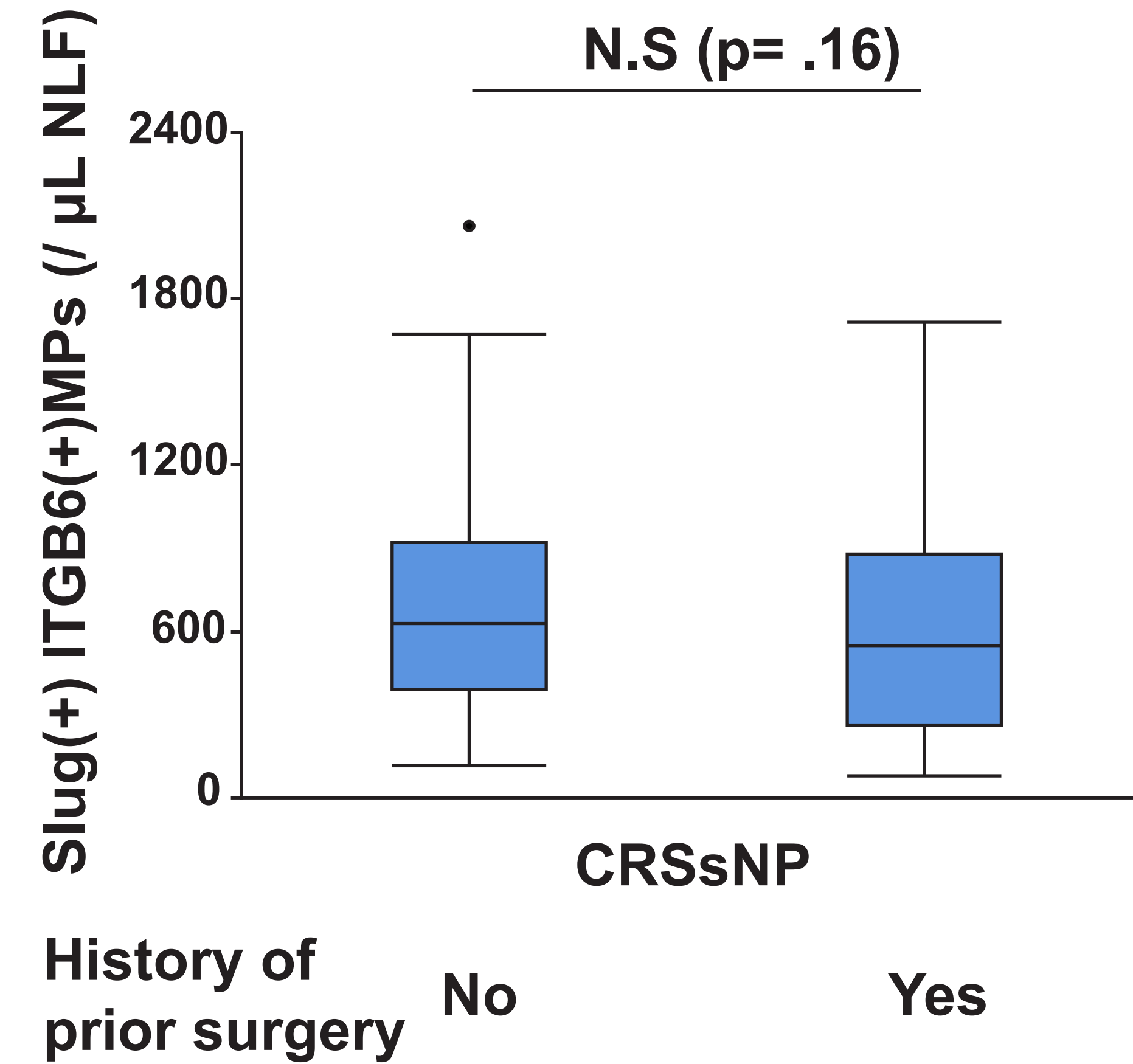
ITGB6(+)MPs



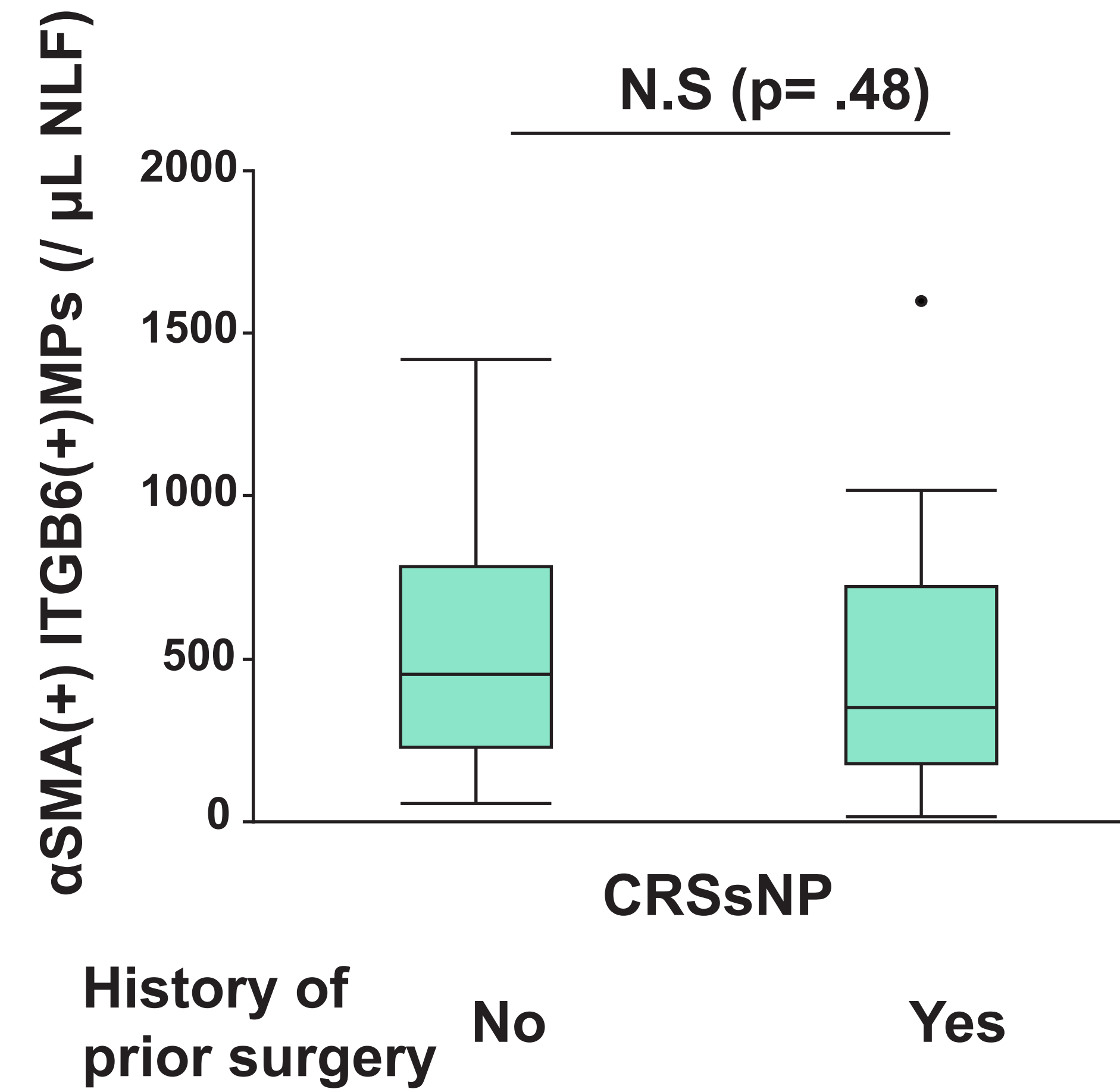
Snail(+)ITGB6(+)MPs



Slug(+)ITGB6(+)MPs



α SMA(+)ITGB6(+)MPs



Online Repository materials

Integrin $\beta 6$ microparticles in nasal lavage fluids: potential biomarkers for basal cell activation in chronic rhinosinusitis

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Methods

Study sample

The Northwestern CRS repository has enrolled cases of chronic rhinosinusitis (CRS), aspirin-exacerbated respiratory disease (AERD), other nasal diseases and control subjects who have had surgery at Northwestern Memorial Hospital (NMH) since 2007. For this study, we enrolled 196 CRS with polyps (CRSwNP) cases who were 80 years old or less and had endoscopic sinus surgery (ESS) at NMH between January 2014 and December 2017. In addition, 47 control subjects whose age, sex and smoking status were matched to the CRSwNP group were enrolled. Finally, 70 CRS without polyp (CRSSNP) and 31 AERD cases who had ESS were also enrolled from the same years. All subjects provided written informed consent, and the Institutional Review Board of the Northwestern University Feinberg School of Medicine approved this study. The presence of rhinosinusitis or bilateral nasal polyposis was confirmed in cases with CRS and AERD by endoscopy or computed tomography imaging. CRS cases met the criteria as previously defined by the American Academy of Otolaryngology–Head and Neck Surgery Chronic Rhinosinusitis Task Force^{1,2}. AERD was diagnosed based on physician-diagnosed asthma, CRSwNP, and history of respiratory reaction (at least once) following ingestion of a non-steroidal anti-inflammatory drug. Cases with autoimmune diseases, established immunodeficiency, a systemic coagulation disorder, cancer, metabolic diseases, hormonal disorder, cystic fibrosis, acute phase of pneumonia, an antrochoanal polyp, or unilateral nasal polyp were excluded from the study. The use of steroids (oral, intranasal and/or inhaled), history of asthma and atopy, and smoking status were also documented. Among 196 CRSwNP cases enrolled in the study, with the exception of one subject who had had a revision surgery 1.7 year after the prior surgery, no CRSwNP subjects had undergone surgery previously between January

2014 and December 2017. Control subjects underwent surgery other than ESS, such as skull base surgery without either sinus disease or pathological lesions in the nasal cavity. All control subjects had no history of sinonasal inflammation, asthma or use of steroid. Thirteen out of 47 control subjects had history of atopy without medication at enrollment.

Nasal lavage fluid sampling and processing.

Nasal lavage fluids (NLFs) were collected at the time of surgery according to the protocol previously described^{3,4}. After secretions were removed from the nasopharynx, 8 mL of phosphate buffered saline was sprayed with a syringe towards the middle meatus under direct visual guidance to minimize influence of anatomic alterations of the nasal cavity on the NLF collection area in cases undergoing revision surgery. Lavage fluid was collected with a suction collector connected to a 15-mL conical tube. The collected NLF was centrifuged for 10 minutes at 3000 rpm to remove cells and debris, and then centrifuged in an Amicon Ultra-4 10K Centrifugal Filter Unit (EMD Millipore, Billerica, MA) at 3,000 rpm for 10 minutes at 4°C. The resulting retentate was concentrated 2-fold, collected, divided into small aliquots, and stored at -80°C.

Measurement of microparticles (MPs) in NLFs by flow cytometry.

MPs in NLFs were measured as previously described^{4,5}. Ten microliters of NLFs was incubated with each specific antibody at room temperature for 30 min. After staining the MP surface with an epithelial specific antibody, permeabilization was performed with the Transcription Factor Buffer Set (BD Pharmingen™, San Diego, USA) when α SMA was examined on epithelial MPs. Samples were then diluted in 300 μ l of a filtered 0.9% saline salt solution. Equal volumes of sample and flow-count beads (Beckman-Coulter) were then added, and analyzed using a BD

FACS LSRII flow cytometer. The MP gate was set using Megamix plus SSC beads (BioCytex, Marseille, France) as previously reported^{4,6-8}. Antibodies used in this study are summarized in Table E1.

Characterization of epithelial MPs.

Three types of epithelial MPs were defined in NLFs according to the expression of the following epithelial specific antigens: EpCAM(+)MPs, E-cadherin(+)MPs and ITGB6(+)MPs. In addition, it has been reported that epithelial cells pass through intermediate states of EMT during which they can manifest both epithelial and mesenchymal traits (partial EMT) after injury^{9,10}. ITGB6 is up-regulated during EMT, in contrast to the majority of epithelial markers such as EpCAM and E-cadherin that are down-regulated. Thus, we simultaneously examined EMT markers, Snail, Slug, and α SMA, carried on ITGB6(+)MPs to evaluate status of EMT during epithelial repair. Because myoepithelial cells have not been identified in nasal mucosa of CRS¹¹, we used the working definition of MPs released from activated basal cells undergoing EMT as: Snail(+)ITGB6(+)MPs, Slug(+)ITGB6(+)MPs, and α SMA(+)ITGB6(+)MPs.

77 **Table E1. Antigens and antibodies used for MP identification**

Differentiation cluster	Antigen	Fluorescence	Clone	Manufacture
CD324	E-cadherin	PE	67A4	BioLegend
CD326	EpCAM	PE	1B7	Thermo Fisher
Not applicable	ITGB6	PE	REA748	Miltenyi Biotec
Not applicable	Snail	Alexa Fluor 488	20C8	Thermo Fisher
Not applicable	Slug	APC	REA404	Miltenyi Biotec
Not applicable	α SMA	APC	1A4	R&D Systems

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81 **Table E2. Multivariable logistic regression analysis for independent variables of CRSwNP**
 82 **cases significantly associated with having history of prior surgery**

Variables	Odds ratio (95% confidence interval)	p value
Gender, male/female	0.844 (0.400- 1.784)	.657
Ethnicity, White/ non-White	1.623 (0.664- 3.965)	.288
Age, per year, [Range: 18- 80]	1.035 (1.004- 1.066)	< .03
Smoking status		
Never	Reference	
Prior	0.996 (0.457- 2.330)	.914
Current	1.117 (0.247- 5.054)	.885
Atopy, yes/no	2.285 (0.995- 5.247)	< .06
Asthma, yes/no	2.749 (1.270- 5.948)	< .02
Oral steroid, yes/no	0.428 (0.053- 3.471)	.427
Intranasal steroid, yes/no	0.652 (0.292- 1.452)	.295
Pre-operative oral steroid, yes/no	2.087 (0.780- 5.584)	.143
ITGB6(+)MPs, per 100 MP counts [Range: 339.5- 5846.5]	1.109 (1.062- 1.158)	< .0001

83 Inhaled steroid treatment for asthma was excluded from the analysis.

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86 **Table E3. Demographics of the 196 CRSwNP cases**

	CRSwNP (n=196)				
Asthma	(-)	(-)	(+)	(+)	
Prior surgery	(-)	(+)	(-)	(+)	
	(n=84)	(n=31)	(n=35)	(n=46)	<i>p values</i>
Gender, male/female	65/19	23/8	19/16*	22/24*#	<.003
Ethnicity, White/ non-White	65/19	27/4	26/9	32/14	.32
Age, mean± Standard deviation	47.1±13.9	49.7±11.2	43.3±13.5	50.0±12.1§	
Smokers, never/ prior/ current	53/26/5	22/8/1	25/9/1	33/10/3	.87
Comorbidity					
Atopy, yes (%)	47 (56%)	19 (61%)	23 (66%)	42 (91%)*#§	<.0003
Steroid use, yes (%)					
Oral	0 (0%)	0 (0%)	4 (11%)*#	2 (4%)*	<.007
Intranasal	19 (23%)	4 (13%)	12 (34%)#	17 (37%)#	<.06
Inhaled	0 (0%)	0 (0%)	14 (40%)*#	26 (57%)*#	<.0001
Pre-operative oral steroid use	5 (6%)	3 (10%)	7 (20%)*	14 (30%)*#	<.002

87 Prior surgery (+); CRSwNP with history of prior endoscopic sinus surgery (Revision surgery
88 case), Prior surgery (-); CRSwNP without history of prior endoscopic sinus surgery (primary
89 surgery case). The chi-squared test or Kruskal-Wallis test (Age) was used to compare
90 demographics among the 4 groups. *; $p < .05$, vs. CRSwNP without asthma without prior
91 surgery, #; $p < .05$, vs. CRSwNP without asthma with prior surgery; §; $p < .05$, vs. CRSwNP with
92 asthma without prior surgery

Figure Legends

Figure E1. MP assay in NLFs using flow cytometry

A. Representative image of scatter gram of ITGB6(+)MP subtypes.

Blue dots indicate target MP and Red dots indicate other MP or background noise (Nonspecific fluorescence background)^{7,12,13}. MPs were treated with the permeabilization protocol optimized for epithelial MP when α SMA was analyzed.

B. Gating strategy of single ITGB6(+)MP and double EMT marker(+)ITGB6(+)MP.

Although unstained control and fluorescence minus one controls are used to evaluate auto-fluorescence and set compensation in MP analysis, they are often not suitable to set fluorescence positive gate as shown in the figure. Thus, we used the 0.22 μ m filtered negative samples to exclude background noise, and set fluorescence positive gate of ITGB6(+)MP subtypes. Before the stained NLF samples were analyzed by flow cytometry, they were filtered using 0.22 μ m membrane filters to exclude MPs in them. The filtered samples were used as negative MP control.

C. Comparison of MP assay with and without permeabilization.

Localization of intracellular proteins carried on MP has not been fully clarified yet. α SMA and cytokeratin have been reported to exist both inside MP as well as on the surface of MP¹⁴, and permeabilization of MPs may increase sensitivity of their detection in the MP assay¹⁵⁻¹⁸. DNA and chromatin were reported to exist on surface rather than inside of MP¹⁹, and Slug and Snail might exist on surface of MP. Thus, we perform preliminary experiments to compare EpCAM(+)MPs using the MP assay either with or without permeabilization using an independent set of NLFs collected from 18 subjects. Levels of Snail(+)EpCAM(+)MP and Slug(+)EpCAM(+)MP were not changed by permeabilization. On the other hand, α SMA(+)EpCAM(+)MP were increased by permeabilization ($p < .0001$). We therefore elected to

use permeabilization for detection of α SMA on epithelial MP and omitted permeabilization for detection of Snail or Slug. Perm (+): MP protocol with permeabilization, Perm (-): MP protocol without permeabilization. “Perm(+)” – “Perm(-)”: Difference of MP levels between the MP assay with and without permeabilization.

Figure E2. *In vitro* experiments assessing release of ITGB6(+)MP subtypes from A549 cells stimulated with TGF- β .

Confluent A549 cells in 12 well tissue plates were incubated in the absence or in the presence of TGF- β 1 (10 ng/ml) for 6 or 12 hours. The culture supernatants were submitted to ultracentrifugation and the pellets were resuspended and analyzed by flow-cytometry^{20,21}.

A. Release of epithelial MPs from TGF- β stimulated A549 cells at 6 and 12 hours.

B. Release of ITGB6(+)MP subtypes from TGF- β stimulated A549 cells at 12 hours.

MP data are mean $\pm$ standard deviation from 6 consecutive, independent experiments. ##-fold, significantly higher by ##-fold vs control.

C. TGF- β induced changes of expression of epithelial and EMT markers.

Left confocal microscopy image: E-cadherin is stained in green, and cell nuclei are stained in blue (n=3). Right: Flow cytometry analysis of EMT markers (n=5)

Figure E3. Comparison of ITGB6(+)MP subtypes between CRSsNP with and without a history of prior surgery.

136 **Figure E1.**
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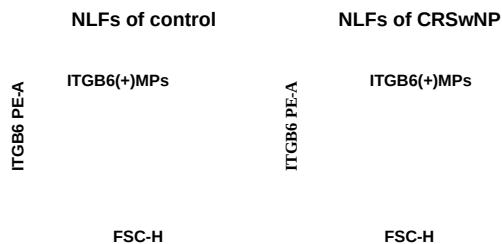
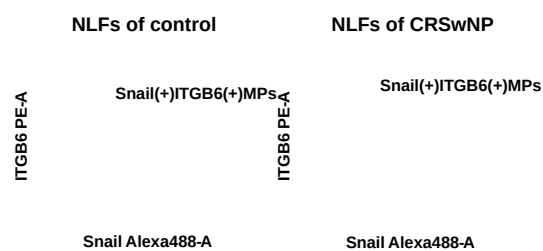
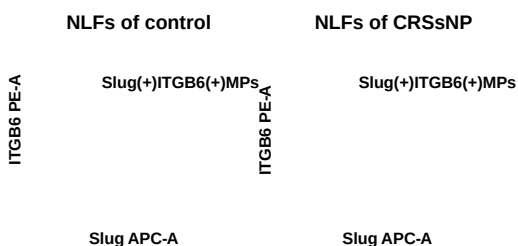
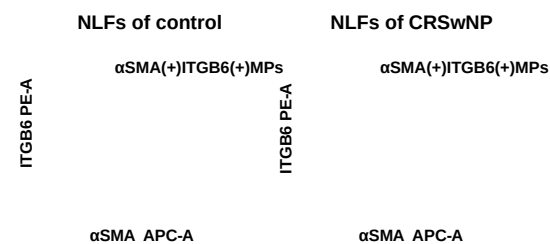
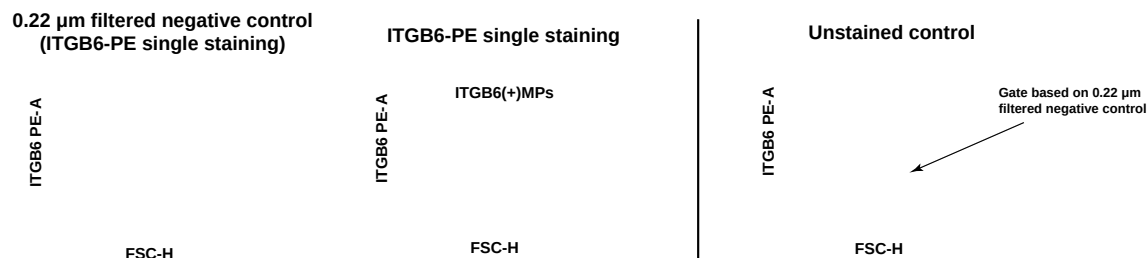
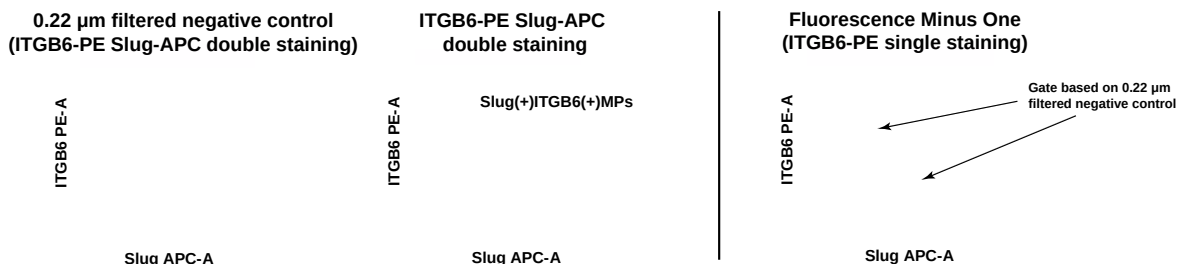
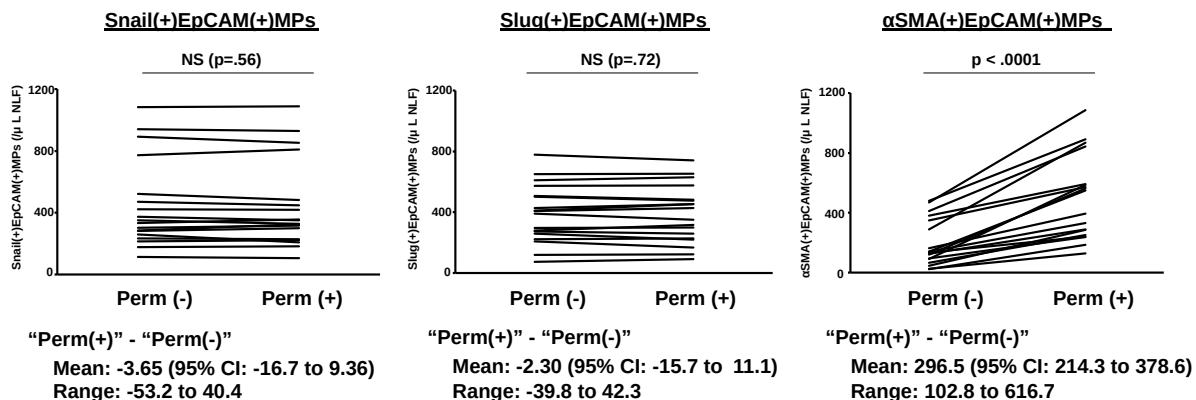
A**ITGB6(+) MPs****Snail(+) ITGB6(+) MPs****Slug(+) ITGB6(+) MPs** **α SMA(+) ITGB6(+) MPs****B****ITGB6-PE single staining****ITGB6-PE Slug-APC double staining****C**

Figure E2.

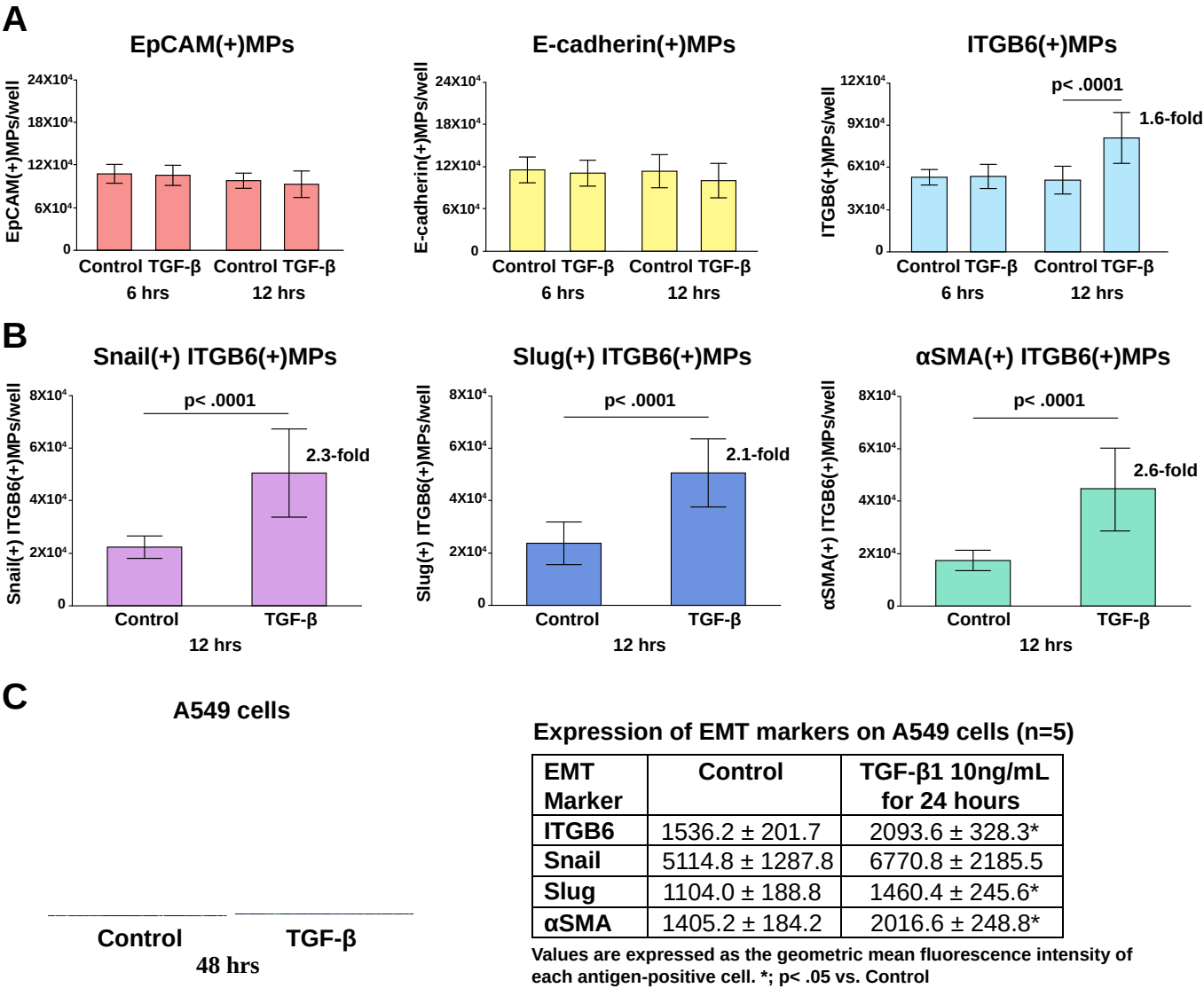
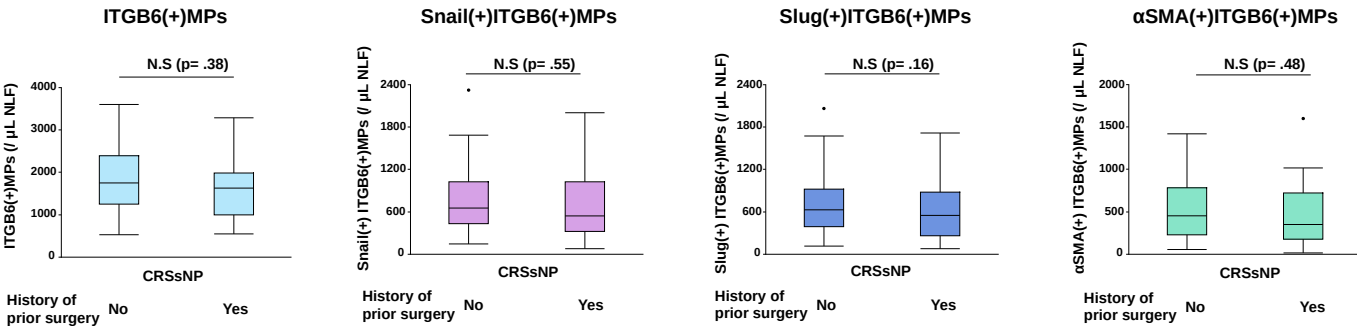


Figure E3.



References

1. Meltzer EO, Hamilos DL. Rhinosinusitis diagnosis and management for the clinician: a synopsis of recent consensus guidelines. *Mayo Clin Proc.* 2011;86(5):427-443.
2. Meltzer EO, Hamilos DL, Hadley JA, et al. Rhinosinusitis: establishing definitions for clinical research and patient care. *J Allergy Clin Immunol.* 2004;114(6 Suppl):155-212.
3. Stevens WW, Ocampo CJ, Berdnikovs S, et al. Cytokines in Chronic Rhinosinusitis. Role in Eosinophilia and Aspirin-exacerbated Respiratory Disease. *Am J Respir Crit Care Med.* 2015;192(6):682-694.
4. Takahashi T, Kato A, Berdnikovs S, et al. Microparticles in nasal lavage fluids in chronic rhinosinusitis: Potential biomarkers for diagnosis of aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol.* 2017;140(3):720-729.
5. Takahashi T, Kobayashi S, Fujino N, et al. Increased circulating endothelial microparticles in COPD patients: a potential biomarker for COPD exacerbation susceptibility. *Thorax.* 2012;67(12):1067-1074.
6. Boilard E, Nigrovic PA, Larabee K, et al. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science.* 2010;327(5965):580-583.
7. Poncelet P, Robert S, Bailly N, et al. Tips and tricks for flow cytometry-based analysis and counting of microparticles. *Transfus Apher Sci.* 2015;53(2):110-126.
8. Amabile N, Cheng S, Renard JM, et al. Association of circulating endothelial microparticles with cardiometabolic risk factors in the Framingham Heart Study. *Eur Heart J.* 2014;35(42):2972-2979.
9. Nieto MA. Epithelial plasticity: a common theme in embryonic and cancer cells. *Science.* 2013;342(6159):1234850.
10. Loffredo LF, Abdala-Valencia H, Anekalla KR, Cuervo-Pardo L, Gottardi CJ, Berdnikovs S. Beyond epithelial-to-mesenchymal transition: Common suppression of differentiation programs underlies epithelial barrier dysfunction in mild, moderate, and severe asthma. *Allergy.* 2017;72(12):1988-2004.
11. Ozolek JA, Barnes EL, Hunt JL. Basal/myoepithelial cells in chronic sinusitis, respiratory epithelial adenomatoid hamartoma, inverted papilloma, and intestinal-type and nonintestinal-type sinonasal adenocarcinoma: an immunohistochemical study. *Arch Pathol Lab Med.* 2007;131(4):530-537.

12. van Ierssel SH, Van Craenenbroeck EM, Conraads VM, et al. Flow cytometric detection of endothelial microparticles (EMP): effects of centrifugation and storage alter with the phenotype studied. *Thromb Res.* 2010;125(4):332-339.
13. Robert S, Poncelet P, Lacroix R, et al. Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies? *J Thromb Haemost.* 2009;7(1):190-197.
14. Rautou PE, Bresson J, Sainte-Marie Y, et al. Abnormal plasma microparticles impair vasoconstrictor responses in patients with cirrhosis. *Gastroenterology.* 2012;143(1):166-176 e166.
15. Leroyer AS, Isobe H, Leseche G, et al. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. *J Am Coll Cardiol.* 2007;49(7):772-777.
16. Timar CI, Lorincz AM, Csepanyi-Komi R, et al. Antibacterial effect of microvesicles released from human neutrophilic granulocytes. *Blood.* 2013;121(3):510-518.
17. Dieker J, Hilbrands L, Thielen A, Dijkman H, Berden JH, van der Vlag J. Enhanced activation of dendritic cells by autologous apoptotic microvesicles in MRL/lpr mice. *Arthritis Res Ther.* 2015;17:103.
18. Chiva-Blanch G, Suades R, Crespo J, et al. CD3(+)/CD45(+) and SMA-alpha(+) circulating microparticles are increased in individuals at high cardiovascular risk who will develop a major cardiovascular event. *Int J Cardiol.* 2016;208:147-149.
19. Sisirak V, Sally B, D'Agati V, et al. Digestion of Chromatin in Apoptotic Cell Microparticles Prevents Autoimmunity. *Cell.* 2016;166(1):88-101.
20. Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res.* 2003;109(4):175-180.
21. Takahashi T, Kobayashi S, Fujino N, et al. Differences in the released endothelial microparticle subtypes between human pulmonary microvascular endothelial cells and aortic endothelial cells in vitro. *Exp Lung Res.* 2013;39(4-5):155-161.