



Opisthorchis felineus liver fluke invasion is an environmental factor modifying genetic risk of atopic bronchial asthma



Irina V. Saltykova^a, Ludmila M. Ogorodova^a, Elena Yu. Bragina^b, Valery P. Puzyrev^{a,b}, Maxim B. Freidin^{b,*}

^a Siberian State Medical University, 634050 Tomsk, Russia

^b Research Institute for Medical Genetics, Siberian Branch of Russian Academy of Medical Sciences, 634050 Tomsk, Russia

ARTICLE INFO

Article history:

Received 9 January 2014

Received in revised form 30 June 2014

Accepted 3 July 2014

Available online 11 July 2014

Keywords:

Opisthorchis felineus

Helminth invasion

Bronchial asthma

Allergy

Gene–environment interaction

SOCS5 gene

ABSTRACT

According to epidemiological observations, *Opisthorchis felineus* liver fluke invasion is negatively associated with the development and severity of allergic diseases in endemic regions of Russia. We hypothesized that the invasion is an important factor in gene–environmental interactions (GEI) underlying allergy. To prove this, we tested 10 single nucleotide polymorphisms of immune response modifying genes in 428 individuals stratified by atopic bronchial asthma presence and *O. felineus* invasion. Using regression models, a statistically significant interaction between the rs6737848 polymorphism of SOCS5 gene and *O. felineus* invasion was observed ($p_{\text{int}} = 0.001$, OR = 5.66, 95% CI 1.96–16.31 for dominant model; $p_{\text{int}} = 0.003$; OR = 4.38, 95% CI 1.68–11.45 for additive model). The interaction is based on the statistically significant association between the SOCS5 gene and atopic bronchial asthma in patients without *O. felineus* infection, while no such association is seen in patients infected by the helminth. These data confirm for the first time the importance of the helminth invasion as an environmental factor influencing the association between genetic factors and atopic bronchial asthma. In particular, *O. felineus* diminishes the risk of atopic bronchial asthma associated with the SOCS5 gene polymorphism.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Atopic bronchial asthma (BA) is a common multifactorial disorder caused by complex interplay between genetic and environmental factors. Gene–environmental interaction (GEI) studies of BA and other allergic diseases attract remarkable interest and infectious agents are considered as having an essential role in the GEI for asthma-related phenotypes (Vercelli, 2010). The growing body of evidence suggests that the helminth invasion influences the prevalence of asthma and allergy in endemic regions (Rodrigues et al., 2008; Leonardi-Bee et al., 2006). *Opisthorchis felineus* is a parasitic worm (type *Platyhelmintha*, class *Trematoda*, family *Opisthorchiidae*), which causes opisthorchiasis, a primarily liver infection in humans. Its major region of distribution is the Ob-Irtish rivers basin running from the south to north of West Siberia (Russia). People in this area are being infected by *O. felineus* through the eating of fresh or undercooked fish, the intermediate host of the

parasite (Mordvinov et al., 2012). According to the pilot screening, the prevalence of *O. felineus* invasion is 32.8% and 11.2% in rural and urban populations of West Siberia, respectively. It has been shown that *O. felineus* invasion negatively correlated with specific atopic sensitization by skin-prick tests (Ogorodova et al., 2007).

The studies of the GEIs are typically grounded on biological hypotheses about the role of functional SNPs in candidate genes that most likely interact with a given environmental agents and mediate the exposure effect (Vercelli, 2010). The genes regulating immune response, especially those influencing T-helper 2 (Th2) immune response, e.g. genes involved in Th2 cytokines signaling pathways, play the critical role in the protective host response against helminthes (Kouriba et al., 2005; Peisong et al., 2004). Notably, some of these genes are also candidates for BA and allergy (Quinnell, 2003).

Shared candidate genes and pathways as well as epidemiological evidences of the significance of helminthic invasion for the development of allergic diseases allowed us suggesting that *O. felineus* can be an important component of GEI underlying BA and other allergic diseases.

To test this hypothesis, we examined the impact of the interaction between the single nucleotide polymorphisms in genes

* Corresponding author at: Research Institute for Medical Genetics, 10 Naberezhnaya Ushaiki, 634050 Tomsk, Russia. Tel.: +7 3822 420956.

E-mail address: mfreidin@medgenetics.ru (M.B. Freidin).

Table 1
Patient demographics.

Characteristics	Patients with bronchial asthma		Patients without bronchial asthma	
	Positive for <i>Opisthorchis felineus</i> invasion	Negative for <i>Opisthorchis felineus</i> invasion	Positive for <i>Opisthorchis felineus</i> invasion	Negative for <i>Opisthorchis felineus</i> invasion
Number of subjects	102	104	102	120
Age ($\pm$ SD) ^a	44.3 $\pm$ 12.7	40.72 $\pm$ 12.9	37.4 $\pm$ 13.0	31.9 $\pm$ 11.1
Male/female	23/79	27/77	8/94	19/101
Median IgE levels, IU/ml (IQR) ^b	109.8 (85–180)	151.3 (107–360)	52.2 (18–234)	15.0 (1.8–38.57)

^a SD, standard deviation.^b IQR, inter-quartile range.

encoding interferon- γ (*IFNG*), interferon- γ receptor 2 (*IFNGR2*), interleukin-4 (*IL4*), interleukin-4 receptor subunit- α (*IL4RA*), GATA binding protein 3 (*GATA3*), T-box transcription factor 21 (*TBX21*), protein inhibitor of activated STAT 4 (*PIAS4*), protein inhibitor of activated STAT 3 (*PIAS3*), signal transducer and activator of transcription 5B (*STAT5B*), and suppressor of cytokine signaling 5 (*SOC5*) and *O. felineus* invasion on the risk of atopic BA in the city of Tomsk.

2. Material and methods

A total of 428 ethnically Russian people were enrolled into the study. The participants live in Tomsk Region (Russia), where *O. felineus* infection is endemic. Among them, there were 222 healthy volunteers without history of BA and allergy, including 102 with *O. felineus* invasion, and 206 individuals with atopic BA, including 102 individuals infected by *O. felineus* (Table 1).

The diagnosis of BA was defined according to the Global Initiative for Asthma criteria (Bateman et al., 2008), using standardized questionnaire, physical and laboratory examination. Atopic status was determined by skin-prick tests to the most common locally encountered allergens. Total serum immunoglobulin-E (IgE) levels were measured using ELISA (Vector-best, Russia). The invasion of the participants by *O. felineus* was established by Kato-Katz technique for stool examination and a custom-designed real-time polymerase chain reaction assay (Ogorodova et al., 2009).

The study was approved by the Bioethics Committee of the Siberian State Medical University. A full verbal explanation of the study aim and protocol was given to all the participants and written informed consent was obtained from them.

Ten SNPs in the genes involved in the regulation of the Th1/Th2 immune responses cytokine pathways were chosen for the study. All SNPs were selected based on the criteria of the frequency of a minor allele (at least 10% in Caucasians) and known or proposed functional significance. The selection of the SNPs in promoter regions of the *IFNG* (rs2069705) and *IFNGR2* (rs17880053) was based on the potential impact of the SNPs on binding sites for transcription factors. Using published data, we selected *IL4* rs2070874 (Gervaziev et al., 2006), *IL4RA* rs1805010 (Beghé et al., 2007), *GATA3* rs10905277 (Pykäläinen et al., 2005), which have been implicated into pathogenesis of asthma and allergy. The SNPs in the *TBX21* (rs11652969), *PIAS4* (rs3760903), *PIAS3* (rs12756687), *STAT5B* (rs16967593), and *SOC5* rs6737848 genes were chosen based on the suspected functional effect: the SNPs were in the Txn factor ChIP clusters or were tagSNPs for the region covering Txn factor ChIP clusters (ENCODE ChIP-seq data).

Total genomic DNA was isolated from peripheral blood of the participants by a standard phenol/chloroform extraction method. Genotyping was done by restriction fragment length polymorphism technique. The details of primers for polymerase chain reaction and restriction enzyme digestion are available on request.

Hardy–Weinberg equilibrium was assessed by the Pearson's χ^2 test. Each SNP was analyzed for association with phenotypes using

logistic regression models (dominant, recessive and log-additive). The model included the phenotypes as the dependant variables and genotypes as the predictor variables. To test gene–environmental interactions, SNP genotypes, *O. felineus* invasion and their interaction term were included into multiple logistic regression models. Sex and age were used as covariates in the regression models. The analysis was done using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at $p < 0.05$. To control for multiple testing, Bonferroni correction was applied.

3. Results

The baseline characteristics of studied participants are presented in Table 1. The frequencies of the genotypes for all the SNPs in healthy control group did not deviate significantly from the expectation under Hardy–Weinberg equilibrium. There were no statistically significant differences in either alleles or genotypes prevalence between *O. felineus* invaded and non-invaded individuals (data not shown), which suggests that the analyzed polymorphisms are not essential for the genetic risk of *O. felineus* invasion. Univariate logistic regression revealed an association between the rs12756687 polymorphism of the *PIAS3* gene and BA ($p = 0.01$; OR = 1.99; 95% CI 1.18–3.39); however, it was non-significant after Bonferroni correction for multiple testing. No other statistically significant associations between BA and the studied polymorphisms were found.

While no statistically significant main effect of the *SOC5* genotypes on asthma risk was found, a statistically significant interaction between the rs6737848 polymorphism of the *SOC5* gene and *O. felineus* invasion was observed for dominant model (genotypes encoding: C/C-0; G/C-1; G/G-1; $p_{\text{int}} = 0.001$, OR = 5.66, 95% CI 1.96–16.31) and for additive model (genotypes encoding: C/C-0; G/C-1; G/G-2; $p_{\text{int}} = 0.003$; OR = 4.38, 95% CI 1.68–11.45) for BA risk (Table 2, Supplementary Table 1). This association remains significant after Bonferroni correction.

Supplementary Table S1 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actatropica.2014.07.004>.

The interaction is explained by the statistically significant association between the rs6737848 polymorphism and BA in patients without *O. felineus* invasion, with the minor allele G associated with the decreased risk of BA in a dominant fashion ($p = 0.002$, OR = 0.28, 95% CI = 0.13–0.64) (Fig. 1), whereas no significant association is present between the polymorphism and BA in individuals infected by *O. felineus* ($p = 0.170$, OR = 1.60, 95% CI = 0.81–3.17). Thus, the rs6737848 polymorphism of the *SOC5* gene is an inherited factor underlying the development of atopic BA in the context of absence *O. felineus* invasion.

4. Discussion

To the best of our knowledge, this is the first report of the significant interaction between a genetic factor and a helminth invasion

Table 2

Odds ratios (95% CI) and *p*-values for coefficients of univariate and multivariate logistic regression models for the analysis of association between the SOCS5 rs6737848 polymorphism and atopic bronchial asthma.

Model	Univariate logistic regression	Multivariate logistic regression without interaction	Multivariate logistic regression with interaction ^d
Additive			
SOCS5 ^a	OR = 0.82 (0.53–1.27); <i>p</i> = 0.370	OR = 0.82 (0.53–1.26); <i>p</i> = 0.361	OR = 0.34 (0.16–0.72); <i>p</i> = 0.005
<i>O. felineus</i> infection ^b	–	OR = 1.05 (0.71–1.56); <i>p</i> = 0.370	OR = 0.79 (0.51–1.21); <i>p</i> = 0.277
Interaction between SOCS5 and <i>O. felineus</i>	–	–	OR = 4.38 (1.68–11.45); <i>p</i> = 0.003
Dominant			
SOCS5 ^c	OR = 0.72 (0.44–1.19); <i>p</i> = 0.200	OR = 0.75 (0.46–1.22); <i>p</i> = 0.245	OR = 0.28 (0.13–0.64); <i>p</i> = 0.002
<i>O. felineus</i> infection ^b	–	OR = 1.05 (0.71–1.56); <i>p</i> = 0.788	OR = 0.77 (0.49–1.19); <i>p</i> = 0.231
Interaction between SOCS5 and <i>O. felineus</i>	–	–	OR = 5.66 (1.96–16.31); <i>p</i> = 0.001

^a Genotypes coding, CC – 0, GC – 1, GG – 2.

^b Status coding, positive – 1, negative – 0.

^c Genotypes coding, CC – 0, GC – 1, GG – 1.

^d Overall significance for additive model, *p* = 0.013; for dominant model, *p* = 0.006.

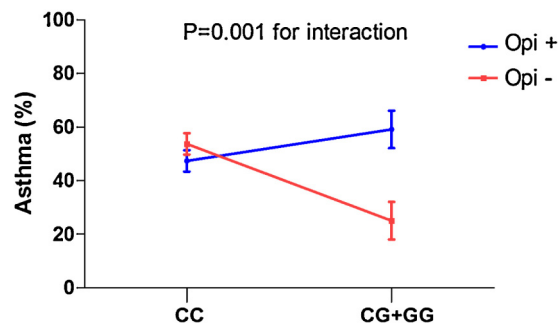


Fig. 1. The prevalence of atopic bronchial asthma according to the SOCS5 genotype in patients with or without *O. felineus* invasion. The figure shows the prevalence of the atopic asthma among the studied individuals according to whether they are invaded or non-invaded by *O. felineus* liver fluke. For dominant regression model, *p* = 0.002 for the main effect of the SOCS5 rs6737848 in non-invaded individuals; *p* = 0.170 in invaded individuals; *p* = 0.001 for the interaction between the SOCS5 rs6737848 and *O. felineus* invasion with respect to the development of atopic bronchial asthma. Opi+, Opi–, patients with or without *O. felineus* invasion, respectively.

for the risk of atopic BA. Our results suggest that *O. felineus* diminishes the risk of BA associated with the SOCS5 gene polymorphism.

According to the hygiene hypothesis, based on epidemiological observations, the infection exposure reduces risk of allergic disorders (Strachan, 2000). A number of genes related to bacterial infection have been studied in the context of the gene–environment interactions in allergy. Pattern recognition receptors, such as CD14 and Toll-like receptor (TLR) 4 are involved in the recognition and clearance of bacterial endotoxin by activating a cascade of host innate immune responses (Haziot et al., 1996; Poltorak et al., 1998). The results of the CD14/-159 polymorphism studies suggest that the C allele is a risk factor for allergic phenotypes at low levels of microbial or house-dust endotoxin exposure, whereas the T allele is a risk factor at high levels of exposure. These findings were discussed as genetically determined heterogeneity for the protective effect of microbial exposure on allergic phenotypes (Martinez, 2007). In case–control and family based studies, it was found that SNPs in CD14, TLR2, TLR4, and TLR9 genes modified associations between country living and asthma (Smit et al., 2009). There is evidence of the joint effects of genetic factors and virus infection on the development of asthma. The variants at the 17q21 locus were associated with asthma in children with human rhinovirus wheezing illness, but not in children without human rhinovirus wheezing illness (Caliskan et al., 2013). The interaction of six SNPs of innate immunity genes with early life viral infections and asthma risk were shown (Daley et al., 2012).

In the current study, we provide first proof that helminth invasion can also play an important role as the environmental factor in GEI interface for asthma and, therefore, can modify risk of the

disease in endemic regions. These results agree with the previous report suggesting the negative correlation between *O. felineus* invasion and the specific allergic sensitization in urban population (Ogorodova et al., 2007).

The molecular mechanism underlying the GEI between *O. felineus* invasion and the rs6737848 polymorphism of the SOCS5 remain unknown; however, it can be explained by the important role of the SOCS5 protein in regulation of Th1/Th2-immune responses. In an experimental model, it was shown that the interaction of the SOCS5 protein with IL-4R inhibits IL-4-mediated activation of STAT6 and differentiation of Th2-helpers (Seki et al., 2002). The expression of SOCS5 gene was negatively correlated with IL4 mRNA and positively correlated with *Tbet* in blood of children (Daegelmans et al., 2008). Based on these observations, we can assume that the C allele of the rs6737848 polymorphism of the SOCS5 is associated with the decreased gene expression, which, in turn, is associated with the increased risk of IL-4-mediated allergic response and the development of asthma. At the same time, according to our unpublished data, *O. felineus* suppresses IL-4 release by PBMCs of children with asthma, thus acting as a suppressor for the development of allergy in predisposed individuals. This can explain the absence of association between the SOCS5 gene and asthma in people infected by the helminth in our study.

This proposed mechanism requires further experimental confirmation. Also little is known about the associations between the SOCS5 polymorphisms with human allergic diseases, future studies are needed to validate our findings in different populations.

5. Conclusions

In conclusion, our findings suggest that *O. felineus* invasion is an important factor that diminishes the risk of atopic BA associated with the rs6737848 SOCS5 gene polymorphism. This observation can be taken into consideration while planning genetic studies for asthma and allergic diseases in regions endemic for helminths.

Acknowledgments

We thank Levko A., Fedosenko S., Cherevko N., and Vasiljeva M. for the help with the organization of sample collection for the current study. The work was supported in part by the Russian Foundation for Basic Research grant # NK 13-04-98056\13.

References

- Bateman, E.D., Hurd, S.S., Barnes, P.J., 2008. Global strategy for asthma management and prevention: GINA executive summary. Eur. Respir. J. 31, 143–178.
- Beghé, B., Barton, S., Rorke, S., Peng, Q., Sayers, I., Gaunt, T., Keith, T.P., Clough, J.B., Holgate, S.T., Holloway, J.W., 2007. Polymorphisms in the interleukin-4 and

- interleukin-4 receptor alpha chain genes confer susceptibility to asthma and atopy in a Caucasian population. *Clin. Exp. Allergy* 4, 221–225.
- Calışkan, M., Bochkov, Y.A., Kreiner-Möller, E., Bonnelykke, K., Stein, M.M., Du, G., Bisgaard, H., Jackson, D.J., Gern, J.E., Lemanske Jr., R.F., Nicolae, D.L., Ober, C., 2013. Rhinovirus wheezing illness and genetic risk of childhood-onset asthma. *N. Engl. J. Med.* 368, 1398–1407.
- Daegelmann, C., Herberth, G., Röder, S., Herbarth, O., Giese, T., Krämer, U., Behrendt, H., Borte, M., Heinrich, J., Emmrich, F., Lehmann, I., 2008. Association between suppressors of cytokine signalling, T-helper type 1/T-helper type 2 balance and allergic sensitization in children. *Clin. Exp. Allergy* 8, 438–448.
- Daley, D., Park, J.E., He, J.Q., Yan, J., Akhabir, L., Stefanowicz, D., Becker, A.B., Chan-Yeung, M., Bossé, Y., Kozyskyj, A.L., James, A.L., Musk, A.W., Laprise, C., Hegele, R.G., Paré, P.D., Sandford, A.J., 2012. Associations and interactions of genetic polymorphisms in innate immunity genes with early viral infections and susceptibility to asthma and asthma-related phenotypes. *Allergy Clin. Immunol.* 130, 1284–1293.
- Gervaziev, Y.V., Kaznacheev, V.A., Gervazieva, V.B., 2006. Allelic polymorphisms in the interleukin-4 promoter regions and their association with bronchial asthma among the Russian population. *Int. Arch. Allergy Immunol.* 141, 257–264.
- Haziot, A., Ferrero, E., Köntgen, F., Hijiya, N., Yamamoto, S., Silver, J., Stewart, C.L., Goyert, S.M., 1996. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity* 4, 407–414.
- Kouriba, B., Chevillard, C., Bream, J.H., Argiro, L., Dessein, H., Arnaud, V., Sangare, L., Dabo, A., Beavogui, A.H., Arama, C., Traoré, H.A., Doumbo, O., Dessein, A., 2005. Analysis of the 5q31-q33 locus shows an association between IL13-1055C/T IL-13-591A/G polymorphisms and *Schistosoma haematobium* infections. *J. Immunol.* 174, 6274–6281.
- Leonardi-Bee, J., Pritchard, D., Britton, J., 2006. Asthma and current intestinal parasite infection: systematic review and meta-analysis. *Am. J. Respir. Crit. Care Med.* 174, 514–523.
- Martinez, F.D., 2007. CD14, endotoxin, and asthma risk: actions and interactions. *Proc. Am. Thorac. Soc.* 4, 221–225.
- Mordvinov, V.A., Yurlova, N.I., Ogorodova, L.M., Katokhin, A.V., 2012. *Opisthorchis felinus* and *Metorchis bilis* are the main agents of liver fluke infection of humans in Russia. *Parasitol. Int.* 61, 25–31.
- Ogorodova, L., Petrova, I., Sazonov, A., Cherevko, N., Sakharovskaia, Z., 2009. Genetic diagnostic technique for opisthorchiasis. *Klin. Lab. Diagn.* 7, 37–39.
- Ogorodova, L.M., Freidin, M.B., Sazonov, A.E., Fedorova, O.S., Gerbek, I.E., Cherevko, N.A., Lebedeva, N.Y., 2007. A pilot screening of prevalence of atopic states and opisthorchosis and their relationship in people of Tomsk Oblast. *Parasitol. Res.* 101, 1165–1168.
- Peisong, G., Yamasaki, A., Mao, X.Q., Enomoto, T., Feng, Z., Gloria-Bottini, F., Bottini, E., Shirakawa, T., Sun, D., Hopkin, J.M., 2004. An asthma-associated genetic variant of *STAT6* predicts low burden of ascaris worm infestation. *Genes Immun.* 5, 58–62.
- Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B., 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* 282, 2085–2088.
- Pykäläinen, M., Kinoshita, R., Valkonen, S., Rydman, P., Kilpeläinen, M., Laitinen, L.A., Karjalainen, J., Nieminen, M., Hurme, M., Kere, J., Laitinen, T., Lahesmaa, R., 2005. Association analysis of common variants of *STAT6*, *GATA3*, and *STAT4* to asthma and high serum IgE phenotypes. *J. Allergy Clin. Immunol.* 115, 80–87.
- Quinnell, R.J., 2003. Genetics of susceptibility to human helminth infection. *Int. J. Parasitol.* 33, 1219–1231.
- Rodrigues, L.C., Newcombe, P.J., Cunha, S.S., Alcantara-Neves, N.M., Genser, B., Cruz, A.A., Simoes, S.M., Fiaccone, R., Amorim, L., Cooper, P.J., Barreto, M.L., 2008. Early infection with *Trichuris trichiura* and allergen skin test reactivity in later childhood. *Clin. Exp. Allergy* 38, 1702–1704.
- Seki, Y., Hayashi, K., Matsumoto, A., Seki, N., Tsukada, J., Ransom, J., Naka, T., Kishimoto, T., Yoshimura, A., Kubo, M., 2002. Expression of the suppressor of cytokine signaling-5 (SOCS5) negatively regulates IL-4-dependent *STAT6* activation and Th2 differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13003–13008.
- Smit, L.A., Siroux, V., Bouzigon, E., Oryszczyn, M.P., Lathrop, M., Demenais, F., Kauffmann, F., 2009. CD14 and toll-like receptor gene polymorphisms, country living, and asthma in adults. *Am. J. Respir. Crit. Care Med.* 179, 363–368.
- Strachan, D.P., 2000. Family size, infection and atopy: the first decade of the hygiene hypothesis. *Thorax* 55 (Suppl. 1), S2–S10.
- Vercelli, D., 2010. Gene–environment interactions in asthma and allergy: the end of the beginning? *Curr. Opin. Allergy Clin. Immunol.* 10, 145–148.

Supplementary

Table S1 – Prevalence of the genotypes and the rare allele ($\pm$ SE) of the *SOCS5* rs6737848 polymorphism in patients stratified by the diagnosis of atopic bronchial asthma and *Opisthorchis felineus* invasion

Genotypes	Asthma		No asthma	
	<i>O. felineus</i> negative	<i>O. felineus</i> positive	<i>O. felineus</i> negative	<i>O. felineus</i> positive
CC	95 (0.913)	71 (0.732)	80 (0.748)	80 (0.816)
CG	8 (0.077)	23 (0.237)	26 (0.243)	16 (0.163)
GG	1 (0.010)	3 (0.031)	1 (0.009)	2 (0.020)
G	0.952 $\pm$ 0.066	0.851 $\pm$ 0.061	0.869 $\pm$ 0.059	0.898 $\pm$ 0.064

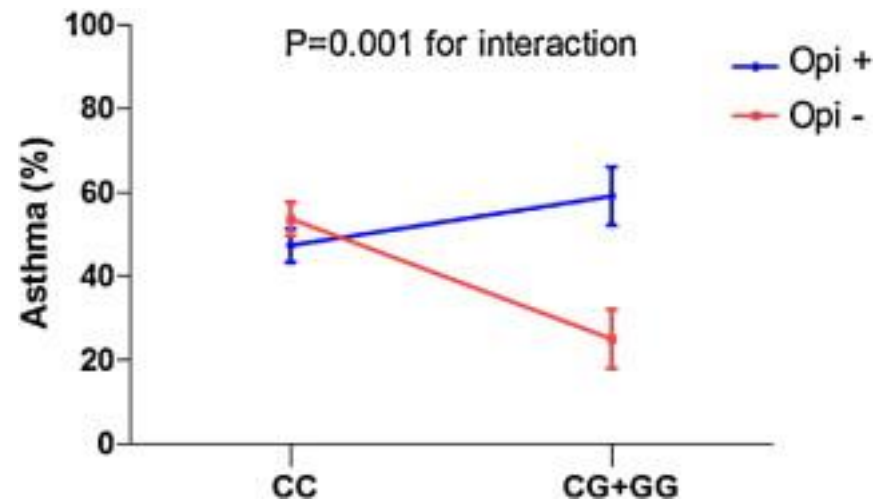


Fig. 1. The prevalence of atopic bronchial asthma according to the SOCS5 genotype in patients with or without *O. felineus* invasion. The figure shows the prevalence of the atopic asthma among the studied individuals according to whether they are invaded or non-...

Irina V. Saltykova, Ludmila M. Ogorodova, Elena Yu. Bragina, Valery P. Puzyrev, Maxim B. Freidin

Opisthorchis felineus liver fluke invasion is an environmental factor modifying genetic risk of atopic bronchial asthma

Acta Tropica, Volume 139, 2014, 53–56

<http://dx.doi.org/10.1016/j.actatropica.2014.07.004>