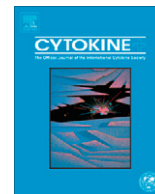




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Protective role of *IL-18* –137G/C polymorphism in a North Indian population with asthma: A pilot study

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ABSTRACT

Background: *IL-18*, a pleiotropic, pro-inflammatory cytokine that plays a major role in innate as well as acquired immunity, has been implicated in asthma etiology and this is the first study investigating the role of *IL-18* –137G/C (rs 187238) promoter polymorphism in asthma pathogenesis in a North Indian population.

Methods: A pilot study was conducted with a total of 824 subjects, out of which 410 were asthma patients including 323 patients suffering from allergic rhinitis and 414 healthy controls from regions of North India. Tetra-Primer Amplification Refractory Mutation System Polymerase Chain Reaction (Tetra-Primer ARMS PCR) was used for genotyping the *IL-18* –137G/C polymorphism.

Results: While the homozygous wild (GG) genotype was equally prevalent in asthma patients as well as control subjects (70.0%), the homozygous mutant (CC) genotype was more prevalent among the controls (8.0%) than in asthma patients (3.4%), which yielded a significant protection or decreased risk towards asthma. Statistical analysis revealed Odds Ratio (OR) = 0.43 (95% CI = 0.21–0.85), χ^2 (χ^2) = 6.93 and p -value = 0.008 ($p < 0.005$). Moreover, a few asthma phenotypic traits also revealed significant protective associations with the polymorphism.

Conclusions: The *IL-18* –137G/C polymorphism confers a significant protection from asthma in the studied North Indian population. This is the first study to report the protective association of the polymorphism with the disease.

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1. Introduction

The complex interplay of genetic as well as environmental factors has offered a great challenge to the researchers worldwide in understanding the mechanisms of onset and severity of allergy [1]. According to the second National Family Health Survey (NFHS 2), conducted in the year 1998–1999, asthma is one of the leading diseases in India [2]. Research conducted on asthmatic populations across the world have revealed different results, leading to a definite conclusion that asthma is a complex polygenic disease, which certainly does not follow classical Mendelian pattern of inheritance [3]. As a result, it makes it all the more crucial to identify the genetic makeup associated with the complexity of asthma.

Mature *IL-18* is an 18 kDa protein that is enzymatically cleaved from a 24 kDa inactive precursor expressed by the *IL-18* gene located on 11q 22.2–22.3. The *IL-18* gene has six exons and its expression is regulated by 2 promoters upstream of exon 1

(Promoter 1) and exon 2 (Promoter 2) [4,5]. *IL-18* –137G/C polymorphism located in the GATA 3 binding site, is the most important polymorphism in Promoter 1, as GATA3 is responsible for inducing the T_H2 phenotype [6,7]. *IL-18* binds to the *IL-18R* and activates the *IL-1R*-associated kinase/TNF-receptor-associated factor 6 signal transduction pathways, leading to the activation of protein-1 through c-Jun N-terminal kinase and nuclear translocation of nuclear factor (NF- κ B) which in turn upregulates cytokine production as its recognition sites are located in the promoters of various genes such as *IL-4*, *IL-13* and *IFN- γ* [5,8,9]. *IL-18*, a pro-inflammatory cytokine produced by monocytes, macrophages and dendritic cells, is pleiotropic and plays a dual role as it regulates both the innate and acquired immunity. T_H2 cytokines induce bronchial asthma while T_H1 cytokines inhibit asthma. *IL-18* is also known to regulate the IgE production by striking a balance between the T_H1 cell and T_H2 cell mediated immune responses [8]. *IL-18*, in synergy with *IL-12*, stimulates T_H1 mediated immune response by inducing IFN- γ expression and inhibiting IgE production [10–12]. But contrastingly, in the absence of *IL-12*, *IL-18* mediates T_H2 immune response and induces IgE production by enhancing the expression of *IL-4* and *IL-13* in mast cells and basophils [8,13,14].

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Several studies have evaluated the role of *IL-18* gene in allergies, eczema, rhinitis as well as asthma. *IL-18* stimulates IgE production, has been proved in murine models with helminth infection [9] and ragweed allergy [15]. It has also been observed that *IL-18* administration in normal murine models causes polyclonal IgE production [8]. Enhanced levels of *IL-18* have been detected in patients with allergic diseases such as asthma, allergic rhinitis and atopic dermatitis [16–19]. *IL-18* also leads to differentiation of naive T cells into T_H2 cells [9] and induces eosinophils to enhance *IL-8* production [20].

While the studies conducted on *IL-18* gene SNPs in Japanese, Swiss, German and Australian populations have revealed positive associations with increased risk towards atopic eczema, allergic rhinitis and asthma [21–24], other studies on Jordanian, German, Czech and Singaporean populations have reported no association with asthma [25–28]. These findings suggest that the role of *IL-18* in the etiology of asthma remains controversial and varies, depending a lot on the study populations and ethnicities.

Since *IL-18* can have an inhibitory or a promoting role in asthma pathogenesis, this is the first study conducted in India, aimed at evaluating the role of *IL-18* –137G/C (rs 187238) polymorphism in asthma.

2. Materials and methods

2.1. Ethical clearance

Ethical Clearance for conducting the study on human blood samples was granted by the “Ethics Committee, PGIMER, Chandigarh” vide Ethical Approval Grant No. Micro 2007/30 dated 6.1.2007. The study was conducted strictly in accordance with the ethical guidelines for bio-medical research on human subjects proposed by the “Central Ethics Committee on Human Research (CECHR) ICMR-2000” and of those contained in the “Declaration of Helsinki”. The selection of asthma patients was based on physician's diagnosis. However, only the patients fulfilling the criteria of GINA (Global Initiative for Asthma) [29] guidelines for diagnosis of bronchial asthma were recruited in the study. The subject recruitment and sample collection was done during the period 2007–2010.

2.2. Inclusion criteria

This is the first case-control study conducted in India evaluating the role of *IL-18* –137G/C (rs 187238) polymorphism in asthma pathogenesis by recruiting a total of 824 adult subjects. The patients were recruited from different states of North India such as Punjab, Haryana, Chandigarh, Uttar Pradesh, Himachal Pradesh, Uttaranchal, Jammu & Kashmir, Rajasthan and New Delhi. A total of 410 asthma patients visiting the Out Patient Department, Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, were enrolled in the study, out of which, 323 subjects were asthma patients with allergic rhinitis. Informed Consent was duly obtained from the asthma patients participating in the study, and a detailed proforma of the asthma patients with a complete questionnaire regarding the clinical symptoms of the disease, i.e. wheeze/whistling, cough, shortness of breath (SOB), allergy, early morning or night symptoms, along with spirometry tests, etc. was assessed. Complete information of the patient regarding name, age, sex, history of the disease, occupation, etc. was taken into account (Table 1).

A total of 414 age-matched, normal and completely healthy controls were inducted in the study. Some of the healthy volunteers were blood donors at various blood donation camps, educational institutes, employee groups. Completely healthy control subjects with no history of asthma, rhinitis, eczema, allergic

Table 1
Characteristics of the study population.

	Asthma patients (n = 410)	Controls (n = 414)
Sex		
Males	183 (44.6) ^a	351 (60.3) ^a
Females	227 (55.4) ^a	231 (39.7) ^a
Age	38.11 ± 16.20	41.86 ± 16.57
Allergic rhinitis	323 (78.8) ^a	0
No rhinitis	87 (21.2) ^a	414
Allergic to atleast		
2 Provoking factors	366 (89.3) ^a	0
Non-allergic	44 (10.7) ^a	414
Ever-smoker	65 (15.9) ^a	0
Non-smoker	345 (84.1) ^a	414
Spirometry data	(n = 190) ^c	
FVC observed ^b	2.56 ± 0.96	0
FVC predicted	3.19 ± 0.73	0
FEV ₁ observed	1.94 ± 0.82	0
FEV ₁ predicted ^b	2.68 ± 0.77	0
FEV ₁ /FVC observed (%)	75.00 ± 13.71	0
FEV ₁ /FVC predicted (%)	83.12 ± 5.84	0
Weight (kg)	57.6	0
Height (cm)	158.7	0
BSA (m ²)	1.58	0
BMI (kg/m ²)	22.7	0
Underweight (<18.5)	17.1	0
Normal weight (18.5–24.9)	22.2	0
Over weight (25.0–29.9)	26.8	0
Obesity (30.0 or >30.0)	32.7	0
IgE (IU/ml) ^d	(n = 219) 4066.55	(n = 150) 2354.40

^a Percent values shown in parenthesis.

^b FVC, Forced Vital Capacity; FEV₁, Forced Expiratory Volume in 1 s.

^c Spirometry test, weight, height, BSA and BMI have been recorded for 190 asthma patients and mean values in each category have been calculated.

^d IgE levels were confirmed for 219 asthma patients and 150 controls and given as average in IU/ml.

skin diseases or any other co-morbid illness were recruited in the study. Care was taken that both the asthma patients as well as the control subjects were free from any other systemic immune or inflammatory conditions.

2.3. Exclusion criteria

Asthma patients with history of any other pulmonary ailment such as tuberculosis, Chronic Obstructive Pulmonary Disease (COPD), and bronchitis were excluded from the study. No ABPA (Allergic Bronchopulmonary Aspergillosis) patients were taken in the study. Any subject having a first degree relative with asthma or allergy has not been recruited as a control in the present study. Not only the respiratory or allergic skin disorders, any subject with other diseases such as diabetes, and high blood pressure or with drinking and smoking habits have also not been included as controls in the study. Each control was first enquired for all of the above conditions at the time of taking their written informed consent and before the collection of blood samples.

2.4. Asthma diagnosis

Both allergic rhinitis as well as asthma often may co-exist. However, their unique symptomatic differences make them easily distinguishable. Allergic rhinitis results from IgE-mediated inflammation of the nasal mucosa on allergen exposure resulting in itchy ear, nose and throat (ENT), rhinorrhea, nasal obstruction, repeated

sneezing, usually after waking up in the morning, not to forget the characteristic watery and itchy eyes which may be allergic pinkeyes.

However, asthma symptoms include airflow obstruction in the lungs resulting in shortness of breath (SOB) on exposure to allergens. Lung function tests, such as spirometry, apart from other traits of the disease such as wheezing and cough help in distinguishing asthma from rhinitis. The diagnosis of asthma was based on history of asthma symptoms as well as improvement on use of anti-asthmatic medications.

2.5. Lung function test

Lung function measurements yield an assessment of the severity, reparability and variability of airflow limitation and help confirm the diagnosis of asthma. Spirometry measures the airflow limitation and its reversibility establishes the diagnosis of asthma. Spirometer device Spiro 232 (PK Morgan, Rainham, Kent, UK) was used for plethysmography which was performed strictly in accordance with the British Thoracic Society/Association of Respiratory Technicians and Physiologists (BTS/ARTP) guidelines [31]. The subjects were asked to relax, avoid any kind of exercise at least 30 min prior to the test and avoid smoking or using bronchodilator at least four hours prior to the test. After placing the mouth-piece in the subject's mouth, the procedure involved a maximal forced expiratory and then a forced inspiratory manoeuvre. Three acceptable manoeuvres, all within 5% of each other were recorded as flow-volume curve.

The frequency of patients both with 60–80% FEV₁/FVC observed% as well as 40–59% FEV₁/FVC observed% was the highest for the wild GG homozygous genotypes (57.1% and 54.7% respectively) and lowest for the CC mutant homozygous genotypes. But a limitation of the study is that as we do not have spirometry data for controls, statistics cannot be applied.

2.6. Immunological investigation

Total serum IgE concentration (IU/ml) was assessed for 219 asthma patients and 150 control subjects with an ELISA reader (Table 1). The BAL (bronchoalveolar lavage) fluid of subjects was examined for acid fast bacillus, aspergillosis and malignancy so as to distinguish the asthma patients from patients suffering from tuberculosis (TB), allergic bronchopulmonary aspergillosis (ABPA) and lung cancer, respectively. However, the T_H1/T_H2 cytokine profiling of BALF was not performed. No significant difference for IgE level among cases and controls was observed for the polymorphism.

2.7. Body Mass Index (BMI)

Body Mass Index (BMI), a measure of body fat based on height (cm) and weight (kg) that applies to adult men and women was calculated using the measures set by the U.S. National Heart Lung and Blood Institute (NHLBI) [32].

BMI of 190 asthma patients has been recorded in Table 1. The mean values have been calculated for 4 different categories: underweight (<18.5 kg/m²), normal weight (18.5–24.9 kg/m²), overweight (25.0–29.9 kg/m²) and obesity (30.0 or >30.0 kg/m²). It was observed that the wild G allele was most prevalent among the asthma patients with normal BMI while the mutant C allele was most prevalent among the asthma patients who were underweight.

Apart from BMI (kg/m²), height (cm), weight (kg) and Body Surface Area (BSA) (m²) have also been recorded for the patients and have been given as mean values in Table 1.

2.8. Sample collection

Blood samples were collected in EDTA coated vials, and stored at –80 °C until genomic DNA extraction was done. Genomic DNA was isolated from the thawed blood samples by the Sodium Saline Citrate Buffer Method [30] and checked for DNA on 0.8% agarose gel by electrophoresis.

2.9. Genotyping of the IL-18 –137G/C polymorphism

Identification of the IL-18 –137G/C genotypes was done by the Tetra-Primer ARMS PCR method as described previously [33,34]. It is a rapid and sensitive high-throughput assay for the simultaneous detection of both the alleles in a single PCR using a set of four primers to amplify a larger fragment of DNA with the SNP and the amplicon representing each of the two alleles of the gene,

forward outer (FO) 5'-AGATGCTTCTAATGGACTAAGGAG-3',
reverse outer (RO) 5'-GGCAAATGCACTGGGAGACAAT-3',
forward inner (FI) 5'-GCCCAACTTTTACGGAAGAATAG-3' and
reverse inner (RI) 5'-ATGTAATATCACTATTTTCATGAAGT-3'.

PCR was carried out in a thermal cycler, in a total volume of 25 µl containing: 10X PCR Buffer, 3 mM MgCl₂, 1 mg/ml nuclease free BSA, 50 pmol of each set of primers, 10 mM of each dNTP, 0.125 U Taq polymerase and 2 µl genomic DNA. The PCR conditions were: initial denaturation at 94 °C for 5 min, followed by 36 cycles at 94 °C for 30 sec, 56 °C for 30 sec, 72 °C for 1 min, and final extension step at 72 °C for 10 min. The results were observed by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized by UV transillumination. The 342 and 257 bp products in a lane indicated the presence of the wild (G) allele, while 342 and 135 bp products marked the presence of the mutant (C) allele. Heterozygotes were observed as 342, 257 and 135 bp products in a lane (Fig. 1).

The European Molecular Genetics Quality Network (EMQN) good practice guidelines have been followed. A few PCR vials with all the PCR contents except the DNA, were also included per PCR batch as “negative controls”. No contamination was observed and there were no “false positives”. To minimize the risk of contamination, sterilized and autoclaved solutions and equipment were used during DNA isolation. The ingredients for PCR were well stored at –20 °C and were thawed just before use. The patients and controls were not genotyped in separate batches but were randomly analyzed [35]. Retyping of samples was done at random to check for the homology of results.

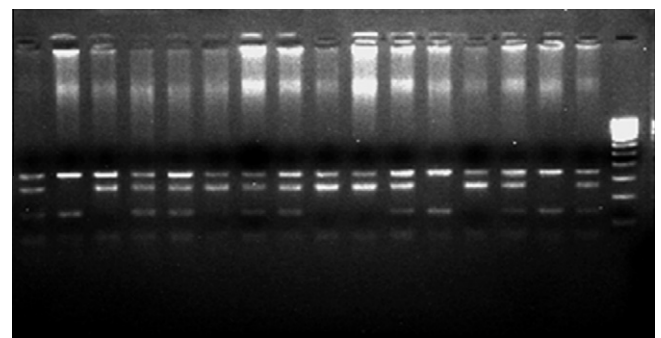


Fig. 1. PCR products of IL-18 –137G/C polymorphism on 2% agarose gel. Lanes 3, 6, 9, 10, 13: homozygous wild GG (342 and 257 bp), lanes 1, 4, 5, 7, 8, 11, 14, 16: heterozygous GC (342, 257 and 135 bp), lanes 2, 12, 15: homozygous mutant CC (342 and 135 bp), lane 17: ladder 100 bp.

Table 2Distribution of *IL-18* –137G/C allele and genotype frequencies in asthma patients and controls.

Frequencies	Asthma patients (n = 410)	Controls (n = 414)	χ^2	OR (95% CI)	p-Value
Allele frequencies					
G	683 (83.3) ^b	671 (81.0) ^b	1.43	Ref (1.0)	0.232
C	137 (16.7) ^b	157 (19.0) ^b		0.86 (0.66–1.11)	
Genotype frequencies					
GG ^a	287 (70.0) ^b	290 (70.0) ^b	1.35	Ref (1.0)	0.246
GC ^a	109 (26.6) ^b	91 (22.0) ^b		1.21 (0.87–1.69)	
CC ^a	14 (3.4) ^b	33 (8.0) ^b	6.93	0.43 (0.21–0.85)	0.008

^a GG, homozygous wild; GC, heterozygous; CC, homozygous mutant.^b Percent values shown in parenthesis.**Table 3**Phenotypic characteristics and *IL-18* –137G/C polymorphism.

Phenotypic traits	n	GG ^a	GC ^a	CC ^a	G	C	χ^2	OR (95% CI)	p-Value
Controls	414	290	91	33	671	157	Ref (1.00)		
Males	271	189	64	18	442	100			
Females	143	101	27	15	229	57			
Asthmatics									
Males	183	135	39	9	309	57	1.26	0.82 (0.56–1.18)	0.260
Females	227	152	70	5	374	80	0.62	0.86 (0.58–1.28)	0.431
Occurrence									
Seasonal	128	112	14	2	238	18	20.56	0.32 (0.19–0.55)	0.000
Throughout	282	175	95	12	445	119	0.96	1.14 (0.87–1.51)	0.326
Severity									
Wheeze on exertion	216	163	48	5	374	58	6.15	0.66 (0.47–0.93)	0.013
Wheeze at rest	194	124	61	9	309	79	0.33	1.09 (0.80–1.49)	0.565
Rhinitis									
Rhinitis (Nil)	87	52	28	7	132	42	2.42	1.36 (0.90–2.04)	0.119
Rhinitis (+ve)	323	235	81	7	551	95	4.64	0.74 (0.55–0.98)	0.031
Sputum production									
Sputum (Nil)	95	57	33	5	147	43	1.32	1.25 (0.84–1.86)	0.250
Sputum (+ve)	315	230	76	9	536	94	4.10	0.75 (0.56–1.00)	0.042
Family history									
Family history (Nil)	285	200	73	12	473	97	0.86	0.88 (0.66–1.17)	0.354
Family history (+ve)	125	87	36	2	210	40	1.13	0.81 (0.55–1.21)	0.288
Allergy									
Allergy (Nil)	44	34	9	1	77	11	2.22	0.61 (0.30–1.22)	0.136
Allergy to at least 2 provoking factors	366	253	100	13	606	126	0.80	0.89 (0.68–1.16)	0.371
Smoking status									
Non smoker	345	238	94	13	570	120	0.62	0.90 (0.69–1.18)	0.430
Ever smoker	65	49	15	1	113	17	2.62	0.64 (0.36–1.13)	0.105
Cough									
Cough (Nil)	74	45	26	3	116	32	0.57	1.18 (0.75–1.85)	0.450
Longstanding cough (+ve)	336	242	83	11	567	105	2.86	0.79 (0.60–1.05)	0.090
Pattern of daily symptoms									
Morning SOB	20	8	9	3	25	15	8.25	2.56 (1.25–5.20)	0.004
Night SOB	292	206	77	9	489	95	1.70	0.83 (0.62–1.11)	0.192
Anytime SOB	98	73	23	2	169	27	2.89	0.68 (0.43–1.08)	0.890

^a GG, homozygous wild; GC, heterozygous; CC, homozygous mutant.

2.10. Statistical analysis

The genotypic as well as the allelic distribution of the *IL-18* –137G/C (rs 187238) polymorphism, between the asthma patients and control subjects, were analyzed statistically using Chi² (χ^2) test. The data was analyzed with SPSS 17.0 software and Epi Info version 3.4.3. Statistical significance was assumed for *p*-values < 0.05.

3. Results

In the present study, a total of 824 subjects, including 410 adult asthma patients and 414 adult healthy controls were genotyped for

the *IL-18* –137G/C (rs 187238) polymorphism. Comparing the allelic frequencies in both the groups (Table 2), the wild (G) allele was marginally more prevalent among the asthma patients (83.3%) than in the controls (81.0%), while the mutant (C) allele was slightly more prevalent among the controls (19.0%) as compared to the asthma patients (16.7%), with a Chi² (χ^2) = 1.43, Odds Ratio (OR) = 0.86, 95% CI (0.66–1.11) and *p*-value = 0.232.

The homozygous genotype (GG) was present in equal percentage (70.0%) in both asthma cases as well as control subjects. The heterozygous genotype (GC) was prevalent more among the asthma patients (26.6%) as compared to the controls (22.0%). However, the homozygous mutant genotype (CC) was more prevalent in the control subjects (8.0%) than in the asthma patients (3.4%), with a

protective Odds Ratio (OR) = 0.43, 95% CI (0.21–0.85) and a highly significant p -value = 0.008 ($p < 0.05$).

Further categorizing the asthma patients on the basis of the phenotypic characteristics of the disease (Table 3), as obtained from their detailed proforma, such as sex (male/female), occurrence (seasonal/throughout), severity (wheeze on exertion/wheeze at rest), rhinitis (positive/nil), sputum production (positive/nil), family history (positive/nil), allergy to at least 2 provoking factors (positive/nil), smoking status (non-smoker/ever-smoker), long-standing cough (positive/nil), pattern of daily symptoms (morning SOB/nocturnal SOB/anytime during day SOB), significant protective association was observed between the *IL-18* –137G/C polymorphism and seasonal occurrence with OR = 0.32, 95% CI (0.19–0.55) and $p = 0.000$, wheeze on physical exertion with OR = 0.66, 95% CI (0.47–0.93) and $p = 0.013$, rhinitis positive with OR = 0.74, 95% CI (0.55–0.98) and $p = 0.031$ as well as sputum positive with OR = 0.75, 95% CI (0.56–1.00) and $p = 0.042$, however a significant risk was observed for morning SOB asthma phenotype with OR = 2.56, 95% CI (1.25–5.20) and $p = 0.004$.

4. Discussion

This is the first study conducted in India evaluating the role of *IL-18* –137G/C (rs 187238) polymorphism in asthma etiology, revealing a significant protective role or decreased risk towards asthma in the studied North Indian population. Even though the allelic frequencies do not show any significant difference in the asthma and control groups, the genotypic frequencies reveal significant differences as the mutant genotype is more prevalent among the controls, yielding an overall protective effect (Table 2). Moreover, the phenotypic traits of seasonal occurrence, wheeze on physical exertion, rhinitis positive, sputum positive as well as morning SOB asthma phenotypes also reveal significant protective association with the polymorphism (Table 3).

IL-18 has been reported as a candidate gene for atopy and in a genome wide research in a Dutch study and evidence for linkage of the chromosomal region 11q 22 with atopy has been revealed [36]. Enhanced *IL-18* levels have been detected in studies on atopic diseases including asthma, dermatitis and rhinitis [16–19].

In a German study, a population suffering with atopic eczema was genotyped for *IL-18* –137G/C (rs 187238) promoter 1 polymorphism revealing significant association, with the highest allelic frequency in patients with intrinsic and non-atopic eczema, especially in patients with low serum IgE levels [24]. A German study conducted on a population with allergic rhinitis, the SNPs in exon 1 (+133T/G and +127C/T), promoter 1 (–137G/C) and in promoter 2 (–133C/G), showed significant associations with high IgE levels ($p < 0.005$) conferring increased risk towards allergic rhinitis [23]. Also in a Japanese population, –137G/C (rs 187238) was positively associated with asthma and it was inferred that there was genetic tendency to produce higher levels of *IL-18* cytokine by monocytes with –137G/C genotype, which may contribute to the development of asthma and allergic phenotypes [21]. The association between –137G/C polymorphism has also been confirmed with atopic asthma in a Swiss study [22].

A study conducted in the Czech Republic reports no association of the –137G/C (rs 187238) polymorphism with allergic rhinitis [27]. No significant association has been observed for –137G/C polymorphism in a study conducted in Singaporean Chinese, Malays and Indian population [28]. Further, a meta-analysis conducted in the People's Republic of China on five studies including 1883 cases and 6645 controls reported no significant association between asthma risk and rs 187238 polymorphism [37]. Moreover Jordanian and German population studies also report no association of –137G/C polymorphism with asthma [25,26]. However,

IL-18 production in lungs of Japanese patients with COPD was examined using immunohistochemical techniques and it was concluded that overproduction of *IL-18* in the lungs may be involved in pathogenesis of COPD [38].

The differences in the results of the studies conducted across the world can be attributed to the multiple ethnicities of the study populations, not to forget the complex interplay of genes and environmental factors.

5. Conclusions

The findings of this study aim to shed some light on the role of the *IL-18* –137G/C (rs 187238) polymorphism in asthma. Moreover, this is the first Indian study conducted on *IL-18* –137G/C (rs 187238) polymorphism which also reveals a first time ever protective effect or decreased risk towards asthma in any of the study populations till date.

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