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Original Article

MCP-1-2518 A>G and CCR2-V64I Polymorphism in Pediatric Asthma: A Single Egyptian Center Study

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Abstract:
Background: CCL2 is a chemokine; also known as monocyte chemoattractant protein 1 (MCP-1) influences inflammation severity and reactive airway response through its interaction with its receptor CCR2-V64I. Single nucleotide polymorphism of MCP-1-2518 A/G gene increases the level of MCP-1 expression in response to inflammatory stimuli, in addition its receptor CCR2-V64I polymorphism is more common among subjects with asthma.
Aim of the Work: To study genetic polymorphisms (MCP-1-2518) and (CCR2-V64I) in pediatric asthma and their effect on susceptibility and severity.
Methods: We conducted a prospective hospital-based case-control study that included 48 children with asthma and 23 healthy control children recruited from outpatient clinics of New Children Hospital, Cairo University Hospitals, Cairo University, Egypt. MCP- 1 and CCR2-V64I gene mutation were detected by polymerase chain reaction- restriction fragment length polymorphism (PCR- RFLP).
Results: A/G MCP-1-2518 polymorphism was significantly higher among asthma patients 23 (47.9%) versus 4 (17.4%) in controls (p = 0.01), Among patients A/G MCP-1-2518 polymorphism was present in 7 (30.4%), 10 (43.5%), 6 (26.1%) of cases of mild, moderate and severe asthma respectively with no significant difference (p = 0.46). G/A CCR2-V64I polymorphism was found in 18 (18.8%) of asthma patients versus 7 (30.4%) in controls with no significant difference (p = 0.27), among patients polymorphism was found in 3 (33.3%), 4 (44.4%), 2 (22.2%) of cases of mild, moderate and severe asthma respectively (p = 0.71).
Conclusion: In pediatric asthma MCP-1 (A/G -2518) polymorphism was significantly higher among asthma patients and might prove to increase asthma susceptibility, but its relation to asthma severity could not be confirmed. CCR2-V64I polymorphism had no relation to asthma susceptibility nor severity in our studied group of patients. Further analyses should be carried out on larger population-based studies.

Level of Evidence of Study: IIIA. (*1*)
Keywords: Asthma: MCP-1 (A/G -2518) polymorphism; CCR2-V64I polymorphism.
Abbreviations: BAL: Bronchoalveolar Lavage; EDTA: ethylene diamine tetra- acetic acid; CCL2: (C-C Motif) Ligand 2; CCR2: C-C Chemokine Receptor Type 2; FokI: Flavobacterium okeanokoites restriction endonuclease enzyme; GINA: Global Initiative for Asthma; MCP1: Monocyte Chemoattractant Protein 1; PCR- RFLP: polymerase chain reaction-restriction fragment length polymorphism; PvuII: Proteus vulgaris restriction endonuclease enzyme; SNP: single nucleotide polymorphism.

Introduction

Asthma is a common, serious pulmonary disease in which there is an increase in bronchial responsiveness to different stimuli. A lot of previous studies had suggested that asthma is influenced by genetic and environmental factors (*2*, *3*). Studying the genetics of asthma will lead to better diagnosis, treatment, and prevention as there are many proofs that genetic liability for asthma, airway responsiveness, and allergic traits are regulated through distinct loci (*4*). Certain chemokines have been identified in asthma whose production influences inflammation severity and reactive airway response (*5*) by attraction and stimulation of specific subsets of leukocytes (*6*) according to complementary nature of their receptors (*7*).

High levels of Specific chemokine; was detected in bronchoalveolar lavage (BAL) of asthma patients (*8*). One of the important chemokines is CCL2 which is also called MCP-1 (Monocyte chemoattractant protein 1), it belongs to a group of CC chemokines its coding gene is located on chromosome 17q11.2. MCP1 protein interacts with CCR2-V64I (chemokine C-C motif receptor 2) its gene is located on chromosome 3p21.3, these regions demonstrated linkage to asthma (*9*), and may contribute to hyperplasia of airway smooth muscles (*10*). In addition, by interacting with its receptor CCR2-V64I it can induce mast cell activation and release of leukotriene C4 into the airways, resulting in airway hyper-responsiveness (*11*).

Expression of CCR2-V64I receptor largely regulates (MCP1) function, there are two RNA splice-variants of CCR2-V64I named CCR2-V64IA and CCR2-V64IB, both bind to MCP-1 and differ only in their carboxyl tails (*12*). Single nucleotide polymorphism of the MCP-1 -2518 A/G allele gene was found to be related to increase its expression (*13*). Also, single nucleotide polymorphism (G to A) at position 190 of the gene encoding the first CCR2-V64I transmembrane region of the protein, results in a change from valine to isoleucine at codon 64 (64I) of the polypeptide chain (*14*), it was found that the CCR2-64I allele to be more common among subjects with asthma (*15*). To the best of our knowledge previously mentioned polymorphisms were not studied among Egyptian children with asthma and due to ethnic genetic variability. We hypothesized that genetic polymorphisms in chemokines (MCP-1-2518) and their receptors (CCR2-V64I) influence the risk and severity of childhood asthma. We aimed to investigate whether pediatric asthma susceptibility and severity are related to genetic polymorphisms in (MCP-1-2518) and (CCR2-V64I).

Subjects and Methods

We conducted a prospective hospital-based case-control study comprising 48 children with asthma and 23 healthy control children recruited from new children hospital outpatient clinics. The study was approved by ethical committee. Patients were recruited from asthma clinic, new children hospital according to the following inclusion criteria: age 1-13 y with no other comorbidities or chronic diseases.

Participants

All cases were subjected to history taking and clinical examination. Asthma severity categorization was done according to (GINA 2019) guidelines (*16*). Controls have been recruited from age matched non-asthmatic children coming to hospital for minor surgeries.

Methods

Sample collection

Two milliliters (2ml) of venous blood have been withdrawn from each subject and placed in a sterile ethylene diamine tetra- acetic acid (EDTA) vacutainer tube, under aseptic conditions for detection of MCP- 1 and CCR2-V64I gene mutation by polymerase chain reaction- restriction fragment length polymorphism (PCR- RFLP). Samples were immediately stored at –20°C till time of DNA extraction and analysis.

MCP- 1 promotor genotyping (A/G -2518)

MCP-1 gene polymorphism was studied by PCR- RFLP.

DNA extraction was done by using (Sigma- Thermo Scientific spin columns, USA) according to the manufacturer's instructions followed by PCR which was carried out using ready to use master mix (Sigma Fast Start SYBR Green Master) in a final volume of 20 µL containing 80 ng DNA, 2 units of Taq DNA polymerase, 10 µL dNTPs mixture , 8.4 µL ddH2O, 0.4µL of forward primer (5'CCGAGATGTTCCCAGCCAG-3') and 0.4 µL reverse primer (5'-CTGCTTTGCTTGTGCCTCTT-3') spanning the promoter region of MCP -1 gene AT POSITION -2518 containing A/G site. Primers were supplied by Thermo Scientific.

PCR was run for 40 cycles using the following temperature profile: denaturation at 94°C for 60sec, annealing at 55°C for 60 sec, extension at 72°C for 90 sec followed by a single final extension step at 72°C for 10 min. The PCR products result in DNA fragment of 930 bp (*3*). PCR products (10 µL) were loaded on agarose gel (2%) and electrophoresis was carried out. The gel was visualized under ultraviolet light after staining with ethidium bromide. Ten microliters of the amplified PCR products were then digested using 1 µL of the restriction enzyme PvuII (Fast Digest- Thermo Scientific). The mixture was mixed gently and spined down. It had been incubated at 37°C for 15 minutes. The digestion products were run together with 100 base pairs ladder (Qiagen) on 2% agarose gel containing ethidium bromide and visualized under ultraviolet light. Samples showing only a 930 bp were assigned as A/A, samples showing two bands of 708

bp and 222 bp were considered G/G and samples showing three bands 930, 708 and 222 bp were typed A/G (*3*).

CCR2-V64I genotyping

CCR2-V64I gene polymorphism has been studied by PCR- RFLP using the following pair of primers; forward primer (5' –GGATTGAACAAGGACGCATTTCCCC-3') and reverse primer (5'-TTGCACATTGCATTCCCAAAGACCC-3')

DNA extraction was done by using (Sigma- Thermo Scientific spin columns) according to the manufacturer's instructions followed by PCR which was carried out using ready to use master mix (Sigma Fast Start SYBR Green Master) in a final volume of 20 µL containing 80 ng DNA, 2 units of Taq DNA polymerase, 10 µL dNTPs mixture, 8.4 µL ddH2O, 0.4µL of forward primer and 0.4 µL reverse primer, primers were supplied by Thermo Scientific, USA.

PCR was run for 40 cycles using the following temperature profile: initial denaturation at 94°C for 5 min followed by 40 cycles at 94°C for 30 seconds, 63°C for 30 s and 72°C for 1 min. A final extension step at 72°C for 7 min was also done. The PCR products resulted in DNA fragment of 380 bp [15]. PCR products (10 µL) were loaded on agarose gel (2%) and electrophoresis was carried out. The gel was visualized under ultraviolet light after staining with ethidium bromide. Ten microliters of the amplified PCR products were then digested using 1 µL of the restriction enzyme FOKI (Fast Digest- Thermo Scientific). The mixture was mixed gently and spinned down. It was incubated at 37°C for 15 minutes. The digestion products were run together with 50 base pairs ladder (Qiagen) on 2% agarose gel containing ethidium bromide and visualized under ultraviolet light. A single 380 bp fragment determines the wild type gene, while FOKI restriction enzyme produced two bands at 215 bp and 165 bp only when an ATC triplet coding for isoleucine was present. Three bands at 380 bp, 215 bp, and 165 bp were present for the heterozygotes (*17*).

Statistical Analysis

Statistical calculations were performed using 'SPSS software package/version 15.0' (SPSS Inc., Chicago, Illinois, USA). Differences between cases and controls were tested using Chi square test. The results were considered significant whenever P values less than 0.05 were observed.

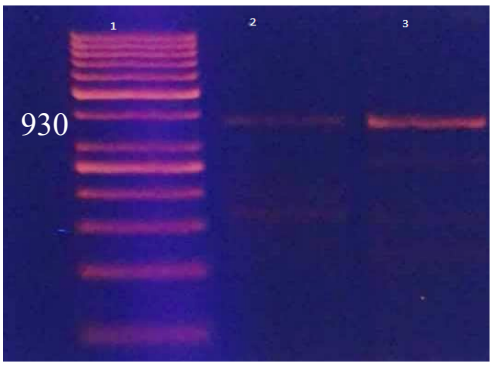
Results

This study included 17 (35.4%) females and 31(64.6%) males aged 1-13years (mean ± SD= 5.1± 2.53 year). According to GINA criteria 2019 (*16*), 16 (33.3%) patients had a severe disease while17 (35.4%) and 15 (31.3%) had moderate and mild disease respectively. Almost half 47.9% of patients had A/G MCP-1-2518 polymorphism while none had G/G MCP-1-2518 Polymorphism, but only nine (18.8%) of patients had CCR2-V64I Polymorphism. (Figures 1,2) MCP-1 (A/G -2518) polymorphism was significantly more frequent in patients than controls (p value 0.01). While in CCR2-V64I (+/64l) polymorphism distribution was no significantly differences between patients and controls. (Figures 1,2,3). (Table 1). MCP-1 (A/G -2518) polymorphism had no significant relation to severity of the disease among patients but was significantly higher in female patients with asthma than males (p= 0.000) (Table 2, 3 and 4) (Figure 4 and 5). The CCR2-V64I (+/64l) polymorphism, allele and genotype distribution were not significantly related to any of the above-mentioned variables.

Table 1. MCP-1-2518 and CCR2-V64I Polymorphism in patients and controls.

	Genotype	Grouping				Chi square	P value
		Cases		Control			
		No	%	No	%		
MCP-1-2518 polymorphism	A/G(Heterozygous)	23	47.9%	4	17.4%	6.14	0.01
	A/A (No mutation)	25	52.1%	19	82.6%		
CCR2-V64I (SNP) polymorphism	G/A (Heterozygous)	9	18.8%	7	30.4%	1.2	0.27
	G/G (No mutation)	39	81.3%	16	69.6%		

SNP: single nucleotide polymorphism



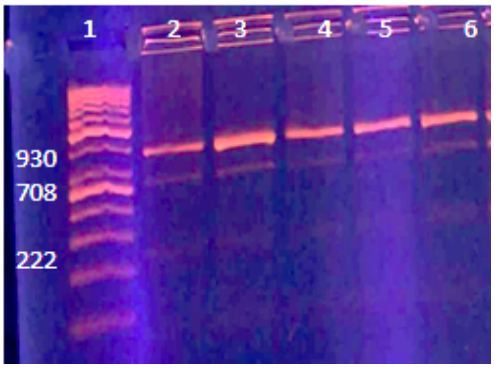


Figure 1 (a) Figure 1 (b)

Figure 1. shows the RFLP pattern obtained following digestion with restriction enzyme PVU II for detection of MCP-1-2518 Polymorphism and, Gel represents the RFLP pattern for detection of MCP-1 polymorphism, (a and b) lane 1 represents 100 bp DNA ladder, (a) lanes 2 and 3 represent wild type allele. (b) lanes 2 to 6 represent heterozygous mutant alleles

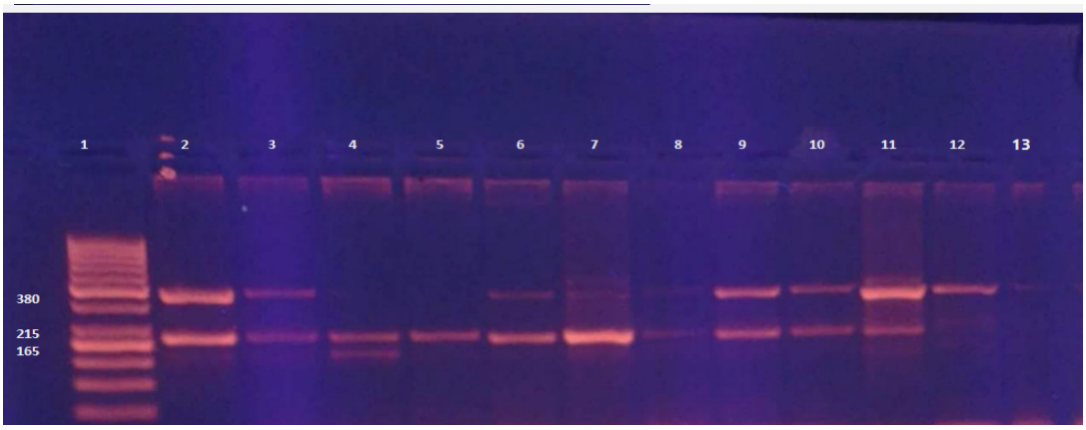


Figure 2. Gel represents RFLP pattern for detection of CCR 2 polymorphism following digestion with the restriction enzyme FOKI for the detection of CCR2-V64I polymorphism; lane 1 represents 50 bp DNA ladder, lanes 2, 3, 6, 7, 8, 9, 10 and 11 represent heterozygous mutant, lanes 12 and 13 represents wild type allele.

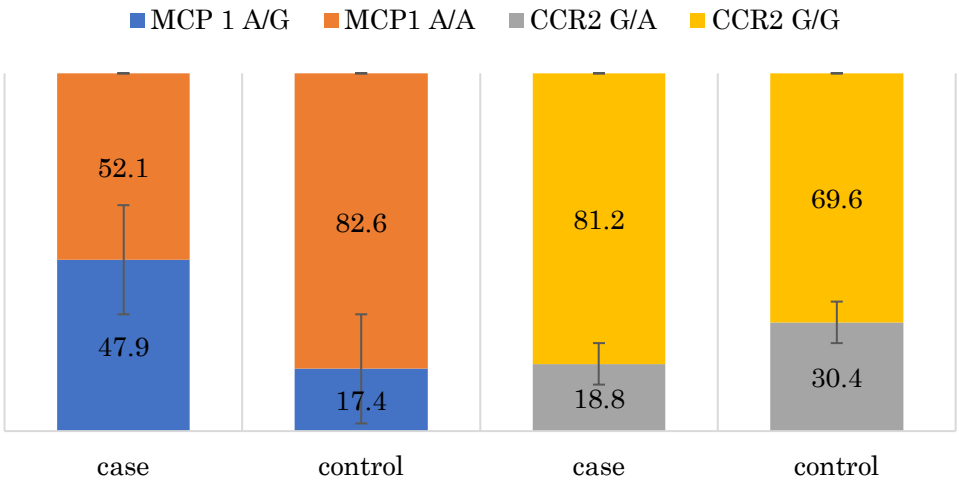


Figure 3. Comparison of MCP-1-2518 and CCR2-V64I Polymorphism frequencies between patients and controls.

Table 3. Correlation between MCP-1 polymorphism with gender and disease severity of asthma.

		MCP1(A/G -2518) genotype				Chi Square	P value
		A/G		A/A			
		No	%	No	%		
Gender	Male	10	43.5%	21	84.0%	8.6	0.000
	Female	13	56.5%	4	16.0%		
Severity	Mild	7	30.4%	8	32.0%	1.5	0.46
	Moderate	10	43.5%	7	28.0%		
	Severe	6	26.1%	10	40.0%		

Table 4. Correlation between CCR2-V64I polymorphism with gender and disease severity of asthma.

		CCR2 -V64I genotype				Chi Square	P value
		G/A (+/64I)		G/G (+/+)			
		No	%	No	%		
Gender	Male	6	66.7%	25	64.1%	0.02	0.88
	Female	3	33.3%	14	35.9%		
Severity	Mild	3	33.3%	12	30.8%	0.68	0.71
	Moderate	4	44.4%	13	33.3%		
	Severe	2	22.2%	14	35.9%		

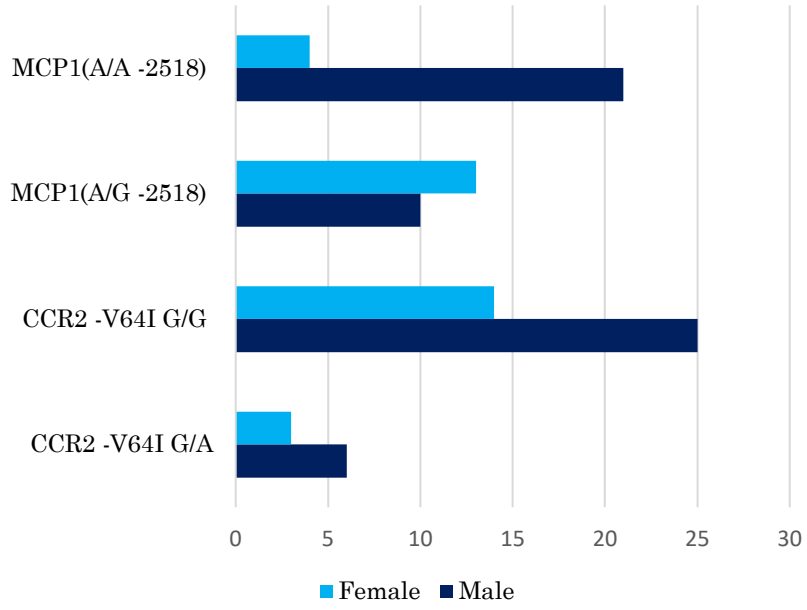


Figure 4. Gender distribution of MCP-1 and CCR2 polymorphisms.

Multinomial logistic regression with severity of disease as the dependent variable and its reference category is mild severity with independent variables as gender, MCP-1 and CCR2 it was found not predictive and the model was non-significant as the whole. (Table 5 and Figure 5).

Table 5. Multinomial logistic regression Predictive Value of MCP-1 and CCR2 mutations.

		Severity of Asthma						P value
		Mild		Moderate		Severe		
		No	%	No	%	No	%	
MCP-1	A/G	7	46.7	10	58.8	6	37.5	0.395
	A/A	8	53.3	7	41.2	10	62.5	
CCR2	G/A	3	20.0	4	23.5	2	12.5	0.783
	G/G	12	80.0	13	76.5	14	87.5	

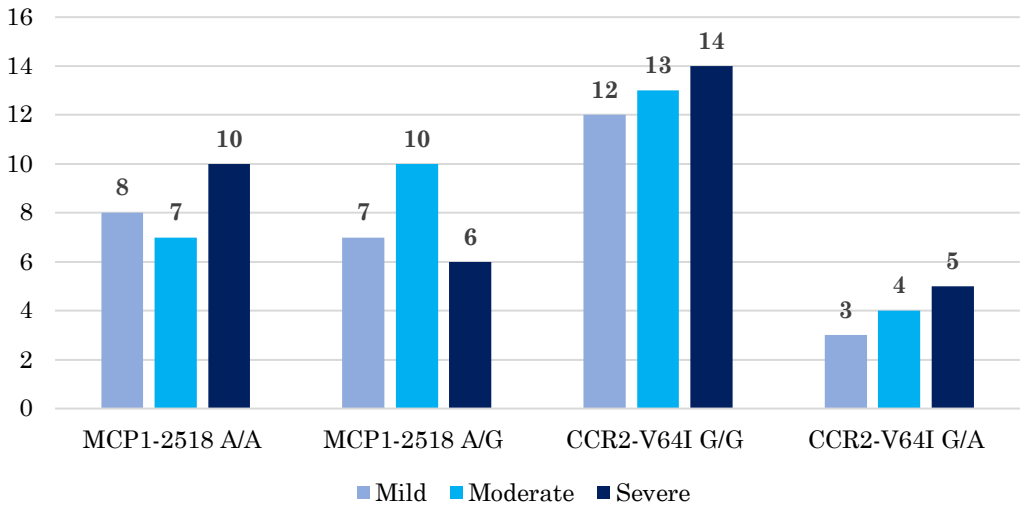


Figure 5. MCP-1 and CCR2 gene polymorphism in relation to asthma severity.

Discussion

In the current study it was found that MCP-1 (A/G -2518) polymorphism occurrence was significantly higher among asthmatic children which suggests that it increases asthma susceptibility, another similar study was done in Hungary by Szalai et al. (*5*) including 160 asthmatic subjects 3-18 years old indicated that children carrying G allele at –2518 of the MCP-1 gene regulatory region were at increased risk for asthma, they also found an association between asthma severity and homozygosity for the G allele but the current study did not confirm this as none of our patients was homozygous for G allele. Both MCP-1 and its receptor CCR2-V64I have been demonstrated to be involved in various diseases (*18*). Increased levels of MCP-1 in the asthmatic airways suggest that they may play a role in macrophage recruitment and activation and thereby contribute to the inflammatory pathology of bronchial asthma (*19*).

According to the best of our knowledge, in 2019 the first meta-analysis to assess the association between MCP-1 -2518 A>G gene polymorphism and asthma risk was conducted in different countries. The analysis was stratified by ethnicity. Caucasian population showed an increased risk of asthma with MCP-1 -2518 A>G polymorphism, African population showed a protective effect and no significant association was found in the Asian population (*20*).

Being an African country, our contradicting results may be explained by a study done in 2014 which discussed gene - gene interactions among MCP genes polymorphisms in asthma, which suggested that there was no single polymorphism of MCP-1 or MCP-2 gene that was associated with asthma but rather a nonindependence of effect or a gene - gene interaction is often described as "epistasis" (*20*), that concept was not investigated in the current study which was conclusively investigating a single polymorphism regardless its interaction with possible other existing polymorphisms. Furthermore, we can consider asthma genetic heterogeneity with respect to regulation of MCP-1 expression to explain our result (*21*). In addition, one of the two African studies which was evaluated in that meta-analysis was conducted on adult patients (*3*).

It was found that incidence of MCP-1 -2518 A>G gene polymorphism was higher in female patient may be due to small sample size and unequal gender distribution. Or it may be related to the scientific speculations about the long reach of the maternal intrauterine imprint searching in the power of maternal effects to disrupt genes (*22*).

Our results did not show a significant correlation with MCP-1 -2518 A>G gene polymorphism and asthma severity while another study suggest a significant association between asthma severity and homozygosity for the MCP 1-2518G allele, whereas the lack of the G allele seems to be beneficial with respect to the symptoms of asthma (*5*), this contradiction may be due to different ethnicity. It was found that incidence of MCP-1 -2518 A>G gene polymorphism was higher in female patient may be due to small sample size and unequal gender distribution.

Our results did not support that polymorphism of CCR2-V64I had an association with asthma susceptibility or severity. Another study was conducted in Tunisia on adult asthmatic patients concluded that CCR2-V64I Allele and genotype distribution was quite similar in patients and controls (*3*), but a population-based case– control study in a Korean population, the CCR2-V64I allele was more common among asthmatic than non-asthmatic subjects (*15*), this might be explained by that; although levels of mRNA seem to be very similar in peripheral blood in both mutated and homozygous wild-type allele subjects, cell surface expression is considerably decreased in case of a mutated allele and might be due to post-transcriptional regulatory mechanisms that influence the expression of CCR2-V64I (*3*).

Upon previously mentioned data MCP-1 gene might belong to the predictors of asthma and could be used in genomic testing. And if these relations can be proved in other groups of patients, and if the higher level of MCP-1 is truly responsible for these effects, evolution of therapeutic drugs for asthma precisely targeted against MCP-1 and/or its receptor, CCR2, might be useful in the deterrence of the disease and/or in the mitigation of its symptom. Our results were limited by the small patient number and we did not measure monocyte count nor the level of monocyte chemoattractant protein 1, as they were beyond the scope of our study.

Conclusion

In pediatric asthma among our population MCP-1 (A/G -2518) polymorphism may increase asthma susceptibility, but its relation to asthma severity could not be established. Polymorphism of CCR2-V64I had neither effect on asthma susceptibility nor severity in the same group of patients. Further analyses should be conducted on larger population-based studies with wider geographical area. Studies of gene - gene interactions among MCP genes polymorphisms are needed.

Author Contributions: All authors searched medical literature, databases, conceptualized, conducted the case review and reviewed the final manuscript. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in connection with the reported study. Authors declare veracity of information.

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