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Association Analysis between the Mast Cell Dipeptidyl Peptidase I(DPPI)-T153I Polymorphism and Asthma: A Family-based Association Test

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ABBREVIATIONS

DPPI: Dipeptidyl Peptidase I

CTSC : Dipeptidyl Peptidase I gene

HRM: High resolution melting curve method

FBAT: Family-based Association Test

ABSTRACT

Background*:* Dipeptidyl Peptidase I (DPPI) is one of the essential components which initiate chain reaction for triggering the inflammatory response mediated allergic diseases such as asthma. Previous studies have reported that several mutations have been detected within the dipeptidyl peptidase I gene (*CTSC*) cause Papillon-Lefévre syndrome. It has been suggested that a mutation in this gene could give protection against the inflammatory response in allergic diseases. The aim of this study was to investigate wither the polymorphism (rs217086 (T153I)) in the gene for DPPI may be protective in asthma. ***Methods****:* To examine possible associations of DPP1 polymorphism with asthma susceptibility, we performed this study. 341 families with at least two siblings with asthma were genotyped for a *CTSC* polymorphism using high resolution melting curve analysis. Genetic association analysis was analysed using family based association tests (FBAT) and resultant called genotypes were sequenced. ***Results:*** DNA sequence data confirmed all variant genotypes. Analysis by FBAT showed no significant association between this polymorphism and asthma susceptibility or with any of asthma-related phenotypes tested. ***Conclusions:*** Our data proved that DPPI does not contribute substantially to susceptibility for asthma. Further studies examining both genotypes and environmental factors will be necessary to elucidate the role of DPPI in the development of allergic diseases.

1. Introduction

Allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis, are growing major public health issues. Asthma affects nearly 155 million individuals worldwide [1]. Although environmental factors are important, there

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are strong genetic predispositions for the development of allergic diseases. It has been reported that more than 100 candidate genes in every chromosome which are identified to have a linkage with asthma and the strength of association of these SNPs with asthma varies in different parts of the world [2-6]. However, the precise functions of these genes are unclear, so more studies are needed to determine the exact function of these genes. A better knowledge of asthma susceptibility will hold promise for a better understanding of the pathology, diagnosis, prevention, treatment and management of this increasingly frequent disease. In this study we looked for the first time to the association of the dipeptidyl peptidase I or cathepsin C gene (*CTSC*) gene in asthma susceptibly as a new gene may be contributing in the pathophysiology of asthma.

The cathepsin C (*CTSC*) gene, or dipeptidyl-peptidase I (DPPI) (EC 3.4.14.1) encodes a cysteine lysosomal protease, it exert its functions by removing dipeptides from the amino terminus of the protein substrate. It has been reported that the *CTSC* gene spans approximately 4.7 kb long and consists of seven exons and six introns [7, 8]. Initially it was thought that the DPPI, 200 kDa tetramer protein, coded by two exons with one intervening intron on chromosome 11q14.1-q14.3. However recharacterision the genomic organization of *CTSC* revealed that this region actually contained 7 exons with two regions located in the previously described exon 2, although the same polypeptide was encoded from both transcripts [9]. Smaller alternatively spliced variant has also been described using only two exons with an addition of 31 amino acids [10]. The molecule DPPI is a tetramer that contains four identical subunits. Each subunit consists of three chains; heavy, light and pro-domains. Study of the pro-domain has suggested possible links for a sulphide bond and a site for glycosylation [9].

Dipeptidyl peptidase I (DPPI) is expressed in the cytoplasmic secretory granules of bone marrow-derived leukocytes, such as myelomonocytic, cytotoxic T and mast cells [8]. DPPI is released from these cells, and it has therefore been suggested that the enzyme acts extracellularly, it was suggested from this study that roles of DPPI, as found *in-vitro*, include degradation and turnover of proteins, and conversion of pro-enzymes to their active forms. Enzymes that are processed via this pathway include granzyme A and B, mast cell chymase,

granzyme K and thrombin-cleaved plasminogen. DPPI may also have a role in the growth and differentiation of mast cells [11]. It has been suggested that activation of mast cells, for example by IgE-bound antigen, may trigger the release of serine proteinases such tryptase and chymase from the secretory granules of these cells. It has been thought that proteinases have an important role in the process of inflammation and tissue remodelling in bronchial asthma [12]. The finding that DPPI interacts with tryptase and chymase which may support its role in allergy [12,13]. Other studies that have been conducted in dog models have suggested an extra-cellular function for this gene [13]. Also, it has been demonstrated that the inhibition of cathepsin C leads to a decrease in activity of neutrophil elastase, cathepsin G and proteinase-3 that are involved in inflammation. This suggests that high fractional inhibition is necessary to reach therapeutically significant effects. This study shows the importance of the *CTSC* gene in the initiation of the inflammatory pathway through cleavage of the nascent form of certain proteins to the active form [14].

To date several mutations more than 55 have been detected within the *CTSC* gene in individuals from diverse ethnic groups cause the monogenetic disease Papillon-Lefévre syndrome (PLS) [15,16] (table 1). This disease is characterised by symptoms of palmoplantar keratoderma, periodontitis and premature loss of dentition [17]. Those patients are not known to be unusually susceptible to inflammation and bacterial infection [36]. This most likely due to one of the loss-of function mutations within CSTC to some extent weakened innate immune response. An important function of the DPPI class of proteins is their role in degradation as well as being a coordinator of activation of serine proteases within immune and inflammatory cells [37]. Moreover, it has been demonstrated that PLS patients had no elastase, cathepsin G and proteinase 3 activities in their polymorphonuclear leukocytes (PMNs) [38]. This finding suggests that in humans as well as in mice a similar activation of serine proteinases by cathepsin C occurs.

It has been reported that the absence of dipeptidyl peptidase I (DPPI) dampens the acute inflammatory response and the subsequent mucous cell metaplasia that accompanies the asthma phenotype induced by Sendai virus infection in mice model [39]. However, the asthma development in mice is not certain issue to rely on it. DPPI may also be involved in other inflammatory states, for example

in asthma. Cathepsin C was identified as differentially up-regulated gene in bronchial biopsy specimens taken from patients with asthma compared with control subjects by using oligonucleotide microarrays technique [40]. Its potential role in asthma has been supported by other expression profiling studies [41-45]. The precise role of this gene in asthma pathogenesis is unknown; however, it may exert a protective role by inhibiting endogenous proteases associated with the inflammatory response [10, 46]. The results of the expression studies suggest that the role of this protease in asthma pathogenesis need further study.

The aim of this study was to investigate whether the polymorphism (rs217086 (T153I)) in the gene for *DPPI* may be protective in asthma. This polymorphism was chosen for this study for the following reasons. Firstly, it produces an alteration in the amino acid sequence from therionine to isoleucine residue and hence while not lying in an evolutionarily conserved domain of the protein, was more likely to have an effect. Secondly, information submitted in dbSNP have been validated, i.e. had been genotyped in with viral respiratory infection in young children, only subjects older than 3 years of age were evaluated for the asthma phenotypes. The baseline Forced a number of individuals and hence was real, not an arte-factual error in sequence.

2. MATERIALS AND METHODS

2.1. SUBJECTS

Caucasian asthma affected sib-pair families (n=341) were recruited from the Pediatric Allergy Clinic of our University Hospital. A full verbal and written explanation of the study was given to all family members interviewed and informed consent as well. Informed consent for subjects younger than school age was provided by their parents. Each family member was questioned regarding allergic symptoms and underwent a physical examination by a pediatrician. Asthma was diagnosed according to the criteria established by the National Institutes of Health (USA) with minor modifications (National Heart Blood and Lung Institute, 1995). Criteria included the following: two or more episodes of wheezing and shortness of breath during the past year and reversibility of the wheezing and dyspnea, either spontaneously or by bronchodilator treatment. Patients treated with systemic steroids were excluded from this study. Because wheezing is often associate Expiratory Volume in one second (FEV1) was obtained from pulmonary function testing. Bronchial hyper-responsiveness (BHR) to methacholine, total serum IgE

and specific serum IgE were measured and severity scores for atopy and asthma were generated as previously described by Sayers et al. [36]. Caucasian 'hyper-normal' controls (n = 184) were also recruited from the same area. These individuals had no diagnosis of asthma, and no family history of asthma. Phenotypic measurements were also collected and available from this cohort.

2.2 Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the salting-out procedure modified from Miller et al [47]. The T153I SNP was detected by qPCR amplification of 20 ng of genomic DNA with 10 pmol of primers (Eurogentec; Belgium (forward: 5'-TGTAGCAACTCACTTTTCCTGAG AATT-3' and reverse: 5'-TGCCTCTGA GAATGTGTATGTCAAC-'3) in a 10 µL reaction mixture containing SYTO9 (Invitrogen; UK) and 1x qPCR master mix as supplied by the manufacturer (Eurogentec). qPCR primers were designed using Primer3 software. The reaction mixture was subjected to an initial 15-minute denaturation period at 95°C followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, 72°C for 1 minute, and a final 1-minute extension at 72°C. Genotyping was determined using the melt curve functionality with the LightCycler® 480 real-time PCR detection system (Roche, Germany). Data is collected on fluorescence (proportional to the amount of DNA present as a double helix) at 0.25 second intervals as temperature is changed from 40oC to 90oC. The genotype calls for each plate were stored in an excel spreadsheet ready to be converted for use in the the Family Based Association Tests (FBAT) statistics programme. Identification of T153I SNP was confirmed by sequence analysis of 5 µL of PCR product after purification, using QIAquick kit, using BigDye Terminator V3.1 chemistry (Applied Biosystems) for some selected samples. Two samples of each genotype call were sequenced to ensure correct calling of genotypes. For each reaction plate genomic control DNA samples and non-template controls (water) were included. Genotyping was repeated for 96 samples (6% of cohort) to check for genotyping accuracy.

3. Statistical analysis

Association of CTSC polymorphism with asthma and atopy phenotypes was evaluated using the Family Based Association Tests (FBAT v1.7.4. software: http://www.biosat.Harvard.edu /_fbat.htm) using the additive model and bi-allelic tests [48]. The association

analysis investigated the relationship between the CTSC genotypes with asthma affection status and with related intermediate variables (FEV1% pred: forced expiratory volume in one second % predicted; BHR: bronchial hyperresponsiveness (using the least square slope method as described before [49]), serum total IgE levels and atopy and asthma score). Genotype data from the all the groups were tested for concordance with the Hardy–Weinberg equilibrium law using a Chi-squared test with one degree of freedom. A P-value of 0.05 or less was considered as significant.

4. RESULTS&DISCOUSION

4.1Genotyping and allele frequencies

The phenotypic characteristics of the study cohorts are summarized in Table 2. Genotype frequencies were determined for each polymorphism and the minor allele frequency were as fellows: The rs217086, isoleucine allele (G) was found at a frequency of 0.154. Allele frequency did not differ significantly from Hardy–Weinberg equilibrium assessed using Chi-square analysis ($p > 0.05$). The genotyping success rates were between 98 – 99.1 %. It has been detected ten Mendelian errors in 341 families and those families were excluded from further analysis.

4.2Genetic association analysis

Family based association tests showed that none of the genotyped CTSC polymorphisms to be significantly associated with asthma affection status or with any of the asthma related phenotypes (atopy, FEV1%, BHR, IgE level and asthma severity score) (table 3). Furthermore, Case–control analyses: Using asthmatic parents, sibling 1, sibling 2 and non-asthmatic adult control populations did not reveal any significant differences in the distribution of any of the polymorphisms analyzed between the subject groups for asthma diagnosis ($p = 0.671 – 0.923$).

In the present study we have studied the association of the CTSC ((T153I)) polymorphism with a number of atopy and asthma phenotypes in a large well-defined white family cohort. No significant associations were observed between these polymorphisms and any asthma related phenotypes examined. So, according to this data, these polymorphisms do not alter the risk or severity of asthma. However as with all analysis there are areas which can affect outcome, giving false positives or reporting no association where there is one. These factors have been reviewed by Cardon and Bell [50]. As the minor allele frequency of the SNP is low, at 3%, it

may be that given population size, our study was underpowered to detect an association with alleles of small effect. A larger population size could be used if repeated. This result also could be due to a random error that had not been perceived.

It has been believed that asthma is a complex genetic disease and a number of studies have shown an increased prevalence of asthma, and phenotypes associated with asthma, among the relatives of asthmatic subjects compared with non-asthmatic subjects [51]. Genome screens for asthma and asthma-associated phenotypes have identified a number of regions of linkage. The most consistent and strong linkages have been with regions on chromosomes 5, 6, 12, and 13 , although linkage to the chromosome 11q markers has also been reported in a genome scan of African-American families where *CTSC* gene is located [52]. Moreover, mutations in the *CTSC* gene have been associated with a several other non-allergic disease phenotypes including Papillon Lefevre syndrome (PLS) reflecting the wide range of physiological action of dipeptidyl peptidase I (DPPI).

In the immune cells, the protease plays a role in removing the activation dipeptide from many of the leukocyte and mast cell granule-associated proteinases, including human cathepsin G, leukocyte elastase, mast cell chymase and tryptase, and lymphocyte granzymes B and H. This is considered an essential step for the polymorphoneuclear leukocytes mediated killing of pathogens. For a number of inflammatory diseases, DPPI activated neutrophil and mast cell-derived serine proteases (e.g. elastase, cathepsin G, tryptase, etc.) are part of disease progression and studies in DPPI knockout mice have linked activity of DPPI to a range of inflammatory diseases, including COPD, Asthma, Rheumatoid Arthritis, Myocardial Infarction/Heart Failure, Diabetic Cardiomyopathy and Sepsis **[53-55]**. Other studies indicate that inhibition of DPPI also represent a therapeutic strategy for treating additional inflammatory diseases, such as Psoriasis, Multiple Sclerosis and Inflammatory Bowel Disease.

It has been reported that DPPI may be considered as a single important molecule involved in the recruitment of neutrophils following a respiratory viral infection, without being critical for the clearance of virus [39]. As this neutrophil-associated DPPI exacerbates asthmatic phenotype by amplifying the production of chemokines and cytokines. And the absence of DPPI diminishes chronic asthma phenotype.

Therefore, the possibility of delivering a DPPI inhibitor directly to the lung may be effective in limiting airway inflammation precipitated by respiratory viral infections while minimizing systemic side effects.

In the course of this study we examined a range of SNP databases for other possible polymorphic sites in the *CTSC* gene that might alter gene function/or expression. This SNP (rs217086 (T153I)) was chosen for this study as it produces an alteration in the amino acid sequence from therionine to isoleucine. In the first time the role of this SNP in the genetic suitability to asthma have been predicated by looking for the conserved regions for *CTSC* gene in different species. Multiple amino acid sequence alignment for DPPI proteins in different species for identification of conserved regions has showed that this therionine and isoleucine amnio acids are highly conserved points which indicate this change in these points could have effect on the DDPI function. Further more, information submitted in dbSNP should that SNP has been validated. This implies that this SNP had been genotyped in a number of individuals and hence was real, not an artefactual error in sequence deposited to Genbank. This polymorphism analyzed in this study have not been investigated previously in relation to asthma. However, it has been reported that the missense change p.I43V was observed in child with prepubertal periodontitis, which is characterized by periodontitis similar to that observed in PLS, but without the palmoplantar keratosis seen in PLS [31]. In the same study it has benn suggested that the p.I453V mutation affects I453, whose side chain forms part of the surface of the S2 substrate binding site [56]. They conceder this change as a benign mutation because, it is unlikely that deletion of a methyl group (replacing isoleucine with valine) would have any significant effect on CTSC function. Consistent with this, p.I453V mutation has been previously reported as a polymorphism with allele frequency 0.953/0.047, respectively [21].

Potential limitation of this study should be addressed. As families in this cohort can be considered as small number and would be increased to a suitable number with the aid of a power calculation. This will give the population for size of the effect to be detected given the frequency of the SNP and the number of families that would need to be available during analysis.

CONCLUSION

Considering the size of the group analyzed in this study, our data would indicate that polymorphisms in the *CTSC*

gene are unlikely to play a major role in determining susceptibly to asthma. However, this does not imply that DPPI itself is not an important mediator in asthma pathogenesis. In this regard, it has been reported that DPPI and neutrophils play a critical role in Sendai virus-induced asthma phenotype as a result of a DPPI-dependent neutrophil recruitment and cytokine response. This suggesting that DPPI molecule may explain the relationship between virues infection stress and airway function in asthma. Further studies examining both environmental endotoxin exposure and DPPI genotypes will be necessary to elucidate the role of DPPI in allergic diseases

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Table.1: PLS-causing mutations in the CTSC gene

Id	Exon	Type	Mutation	Effect	Population description
1	1	Nonsense	c.72C>A (p.C24X)	Premature stop	Moroccan[18]
2	1	Nonsense	c.96T>G (p.Y32X)	Premature stop	Mexican[19], Caucasian[19], French[18]
3	1	Nonsense	c.145C>T (p.Q49X)	Premature stop	Indian[20]
4	1	Missense	c.116G>C (p.W39S)	AHCR**	Puerto Rican[21]
5	2	Nonsense	c.205C>T (p.Q69X)	Premature stop	Indian[20]
6	2	Deletion	c.199_222del (p. 67_74)	Frameshift	Chinese[22]
7	3	Missense	c.380A>C (p. H127P)	AHCR**	French[18]
8	3	Missense	c.415G>A (p.G139R)	AHCR**	Caucasian[19]
9	3	Insertion	c.445_446insATGT (p.V149fsX157)	Frameshift & premature stop	Indian[22]
10		Splice site (Intron 3)	IVS3_1G>A	Altered splicing	Egyptian[9], Jordanian[23]
11	4	Nonsense	c.545G>A (p.W185X)	Premature stop	Brazilian[24]
12	4	Missense	c.587T>C (p.L196P)	AHCR**	Brazilian[25]
13	4	Insertion	622_623insC (p.H208fsX223)	Frameshift & Premature stop	Turkish[9]
14	4	Nonsense	c.628C>T (p.R210X)	Premature stop	Lebanese[9], Algerian[18]
15	5	Nonsense	c.704G>A (p.W235X)	Premature stop	Iranian[22]
16	5	Missense	c.706G>T (p.D236Y)	AHCR**	Spanish[26]
17	5	Deletion	c.711del14	Frameshift & Premature stop	Algerian[18]
18	5	Missense	c.745G>T (p.V249F)	AHCR**	Indian-Pakistani[9]
19	5	Nonsense	c.748C>T (p.R250X)	Premature stop	Turkish[22]
20	5	Missense	c.755A>T (p.Q252L)	AHCR**	Egyptian[9]
21	6	Missense	c.815G>C (p.R272P)	AHCR**	Lebanese[9], Turkish[22], Caucasian[27], Saudi[28], Holland[18], French[18]
22	6	Nonsense	c.856C>T (p.Q286X)	Premature stop Truncated protein = 286 aa	Turkish[28,7,29]
23	6	Missense	c.857A>G (p.Q286R)	AHCR**	Indian[28], Spanish[26]
24	6	Missense	c.872G>A (p.C291Y)	AHCR**	Spanish[26]
25	7	Missense	c.898G>A (p.G300S)	AHCR**	Vietnamese[22]
26	7	Missense	c.899G>A (p.G300D)	AHCR**	Saudi[29]
27	7	Missense	c.901G>A (p.G301S)	AHCR**	Indian-Pakistani[9], Iranian[22], Japanese[21]
28	7	Missense	c.902G>T (p.G301V)	AHCR* *	Iranian[22]
29	7	Missense	c.910T>A (p.Y304N)	AHCR**	Panamanian[22]
30	7	Nonsense	c.912C>A (p.Y304X)	Premature stop	Indian[20]
31	7	Missense	c.956A>G (p.E319G)	AHCR**	Iranian[22]
32	7	Deletion	c.984del7	Frameshift & Premature stop	French[30]
33	7	Missense	c.1015C>T (p.R339C)	AHCR**	Egyptian[9,22], Turkish[27], Martinique[19]
34	7	Deletion	c.1028_1029delCT (p.S343X)	Frameshift & Premature stop	Turkish[7]
35	7	Missense	c.1040A>G (p.Y347C)	AHCR**	Indian-Pakistani[9], Jordanian[27]/ PLS, PPP
36	7	Deletion	c.1047delA (p.G349fsX359)	Frameshift & Premature stop	Turkish[7]
37	7	Deletion	c.1056delT	Frameshift & Premature stop	French[30]
38	7	Deletion	c.1141delC (p.L381fsX393)	Frameshift & Premature stop	Caucasian[19], French[30]
39	7	Nonsense	c.1286G>A (p.W429X)	Premature stop Truncated protein = 428 aa	Turkish[7,22]
40	7	Missense	c.1287G>C (p. W429C)	AHCR**	French[30]
41	7	Missense	c.1360A>G (p.E447G)	AHCR**	Vietnamese[22]
42	7	Missense	c.386T>A/p.V129E	Alteration of highly conserved residue	British[31]
43	7	Missense	c.935A>G/p.Q312R	Alteration of highly conserved residue	British[31]
44	7	Missense	c.1235A>G/p.Y412C	Alteration of highly conserved residue affect structure function relationship affect activity	British[31]
45	7	Missense	c.851G>A		* Chinese[32]
46	7	Deletion	c.112delCCTG		* Chinese[32]
47	3	Nonsense	c.322A4T (K108X)	Premature stop Truncated protein	Φ German[33]
48	3	Nonsense	c.436delT (S146fs153X)	Premature stop Truncated protein	Φ German[33]
49	7	Nonsense	c.1269 G4A (W423X)	Premature stop Truncated protein	German[33]
50	6	Missense	c.854C>T (p.P285L) homozygous	Alteration of highly conserved residue Activity loss	German[33]
51	4	Deletion	c.566-572del)	Premature stop Truncated protein	Germany[34]
52	7	Missense	c.947T>G, (p.L316R) GenBank accession no. X87212	Alteration of highly conserved residue	Germany[34]
53	7	Missense	c.1268G>C ((p.W423S)	Alteration of highly conserved residue	Germany[34]
54	1	Deletion	c.21delG (p.Leu7PhefsX57)	frameshift and premature termination codon	Pakistani[15]
55	7	Missense	c.1156G4C (p.G386R)	Alteration of highly conserved residue	French[35]
56	5	Splice site (Intron 5)	c.757G4A (p.A253SfsX30)	Altered splicing site & Premature stop	French[35]
57	3-7	intragenic deletion	exons 3–7 deletion	Premature termination codon	Frensh[35]
58	2	Misence	c.203 T > G (p.Leu68Arg	Alteration of highly conserved residue loss-of function mutation	Mexican[16]

*a compound heterozygous mutation (c.415 G>A and c.778 T>C) in one patient, and two novel compound heterozygous mutations (c.851G>A and c.112delCCTG) in the other patient. **Φ** Out of four novel mutations, three results in protein truncation and are thus considered to be pathogenic. The homozygous c.854C>T nucleotide exchange (p.P285L) was associated with an almost complete loss of enzyme activity. **AHCR: atteration of highly conserved residue

Table 2. Phenotypic characteristics of the study group

			Asthmatic	Non-asthmatic	Asthmatic	Asthmatic
	Pedigrees	**Parents**				
			parents	**Parents**	**Sibling1**	**Sibling 2**
Subjects n	1456	664	185	489	341	341
Mean age yrs	24.6	40.5	40.2	40.7	13.0	9.9
Gender (% male)	51.8	49.9	47.1	51.0	56.9	53.6
FEV1 % pred BHR#	98.05	100.81	94.12	103.39	94.74	95.62
(% PC20 <4)	32.4	15.3	34.3	7.9	43.7	56.0
Median total serum IgE (IU/mL)	107	56	107	43	249	307

All of the asthmatic family members had a current physician's diagnosis of asthma.

FEV1: forced expiratory volume in one second; % pred: % predicted; BHR: bronchial hyperresponsiveness ; Ig: immunoglobulin; #: FEV1 response to methacholine & PC20 <4.

Table 3: Family based association test results between DPP1 SNP rs217086 and Asthma and allergy phenotypes

Trait	Allele	Number of families	P value	Z score
Asthma	1	146	0.512691	-0.655
Asthma	2	146	0.512691	0.655
Asthma + Total IgE	1	146	0.896946	-0.130
Asthma + Total IgE	2	146	0.896946	0.130
Asthma + FEV1% predicted	1	146	0.899594	-0.126
Asthma + FEV1% predicted	2	146	0.899594	0.126
Asthma + Slope	1	145	0.986549	-0.017
Asthma + Slope	2	145	0.986549	0.017
Asthma + Atopy severity score	1	135	0.662427	-0.437
Asthma + Atopy severity score	2	135	0.662427	0.437
Asthma + Asthma severity score	1	144	0.592062	-0.536
Asthma + Asthma severity score	2	144	0.592062	0.536