Scores of asthma and asthma severity reveal new regions of linkage in EGEA families

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ABSTRACT

There is a debate on how asthma should be defined to progress in the understanding of underlying mechanisms. Our goal was to build quantitative scores of asthma and asthma severity and to assess whether refining disease phenotypes can facilitate the identification of chromosomal regions harboring susceptibility genes.

A genome-wide linkage scan was conducted in 110 families with ≥ 2 asthmatic sibs (n=508) from the French Epidemiological Study on the Genetics and Environment of Asthma (EGEA). Phenotypes studied were an asthma severity score assessed among asthmatics by combining clinical data and treatment, forced expiratory volume in 1 second (FEV_1) and an asthma score including both asthmatics and non-asthmatics and representing the whole disease spectrum.

Our analysis showed genome-wide suggestive evidence for linkage of asthma score to 18p11 (p=0.0004), a novel region undetected by our previous screen of dichotomous asthma. There was potential linkage of 2p23 to asthma severity score (p=0.002) and of three regions to FEV_1: 1p36 (p=0.004), 2q36 (p=0.003) and 6q14 (p=0.003). Moreover, FEV_1 appeared to have no genetic determinant in common with asthma severity and asthma scores.

Asthma and asthma severity quantitative scores revealed new regions of linkage and thus provides support for considering these phenotypes in future genetic studies.

INTRODUCTION

The general consensus now emerging is that asthma is unlikely to be a single disease but rather a collection of different phenotypes, as recently pointed out in the Lancet [1]. These phenotypes may be defined from various criteria including clinical and physiological characteristics (eg severity, resistance to treatment, chronic airflow restriction…) [2]. One strategy to determine whether these phenotypes represent different manifestations of a common underlying pathological process or are distinct disease entities is to refine phenotypic characterization and to search for the determinants of these phenotypes. Indeed, improving phenotype definition can increase the power to detect the underlying genetic mechanisms and, conversely, genetic analysis can provide support for new phenotype definitions and new phenotypic entities.

Regarding the asthma phenotype, it has been recently shown that use of quantitative scores based on symptoms of asthma [3] rather than simple dichotomous definitions can improve the identification of risk factors by reducing misclassification of the disease status. Definition of asthma severity depends on its primary purpose. The 2002 and 2004 Global initiative for asthma (GINA) guidelines, set up to improve asthma management, combine current symptoms, respiratory function and treatment [4]. However, it is now recognized that asthma severity is heterogeneous, being represented by different phenotypes which may result from different risk factors [2]. Low lung function is a marker of asthma severity. It antedates the occurrence of persistent wheeze, tracks over the life span, predicts in the general population the severity of asthma in adults [5], and exacerbations in clinical settings [6]. Asthma occurrence, asthma severity and forced expiratory volume in 1 second (FEV_1) may result from common determinants but also from specific ones. Analyzing these phenotypes separately rather than combining them in a single index makes it possible to discriminate between these hypotheses.

Evidence for familial resemblance for asthma severity and absence of significant relationship between asthma severity in probands and occurrence of disease in relatives have suggested that specific familial factors, possibly genetic, are involved in asthma severity [7]. Many genome-wide scans including the Epidemiological Study on the Genetics and Environment of Asthma (EGEA) have reported chromosomal regions linked to asthma, based on classical dichotomous definitions [8]. Whereas candidate genes for severe asthma have been investigated [9], there is limited information regarding the localisation of potential new genes. To our knowledge, no genome scan has yet been conducted for categorical phenotypes of asthma and asthma severity.

The EGEA study with detailed data enabling to construct scores for asthma and asthma severity in family members and the availability of linkage analysis methods for ordered categorical traits make it possible to conduct a genome-wide screen for the three following phenotypes: a categorical asthma score in order to reduce potential asthma misclassification and to investigate the whole spectrum of disease expression; an asthma severity score in order to investigate variation in disease severity; and a measure of ventilatory function, FEV_1, an objective marker of asthma severity. This genome screen will allow to evaluate whether examining more refined disease phenotypes can help identifying novel regions of linkage. It will also permit to assess whether the three phenotypes under study are more likely to share genes or to depend on different genetic factors.

METHODS AND MATERIALS

The protocol of the EGEA data collection has been described elsewhere [7, 8, 10]. Subjects answered a detailed questionnaire regarding respiratory symptoms and treatment based on international standardized questionnaires. FEV_1 was measured according to the ATS recommendations.

Among the whole EGEA family sample ascertained through one asthmatic proband, 119 nuclear families including at least two asthmatic sibs were selected for the present study. Asthma was defined using the same criteria as in previous EGEA linkage scans [8], associating report of asthma attacks or attacks of breathlessness with wheezing ever and either airway hyperresponsiveness or reversible obstruction or hospitalization for asthma or asthma treatment.

Phenotypes analyzed

Asthma severity

Based on extensively documented phenotypes in EGEA, two measures of asthma severity were considered in the present study:

1. Asthma severity score

This score ranging from 1 to 4 was built in asthmatic siblings in two steps, following the concept of combining treatment and symptoms according to the 2002 and 2004 GINA guidelines [4]. In step 1, the sum of the following clinical items regarding the last 12 months was computed: frequency of asthma attacks (< 1 attack/month (0), <1 attack/week (1), < 1 attack/day (2), $\geq$ 1 attack/day (3)); symptoms between asthma attacks (none (0), wheezing (1), wheezing and dyspnea (2), activities limited by dyspnea (3)); and hospitalisation for asthma (no(0)/yes(1)). This sum of clinical items was grouped into 4 classes: 0, 1, 2 and $\geq$3. In step 2, this 4-class variable built in step 1 was combined with the type of anti-asthmatic treatment in

the past 12 months as reported in the EGEA questionnaire (no treatment, treatment without inhaled steroids, treatment with inhaled steroids) resulting into the asthma severity score taking the values 1, 2, 3 or 4, as shown in table 1.

2. Forced expiratory volume in 1 second

The ventilatory function was assessed by FEV_1 measured before bronchodilator according to a protocol previously described [10] and expressed in percentage of predicted values (%FEV_1) based on age, height and gender [8].

Asthma score

An asthma score was built to represent the whole disease spectrum, encompassing non-asthmatics and asthmatics with varying asthma expression. This score ranged from 0 to 4: a score of zero was assigned to non-asthmatics and the scores of 1 to 4 represented the 4 classes of asthma severity as defined above.

Genotypes

Genotyping of 396 microsatellites, with an average spacing of 10 centimorgans (cM), was performed in EGEA families with at least two sibs with DNA, following a protocol described elsewhere [8]. After rigorous genotype quality control, the final sample for the present analysis included 110 families (508 subjects) with at least two asthmatic sibs, comprising 218 genotyped parents (99% of all parents) and 288 genotyped sibs.

Linkage analysis

Linkage analyses of the two ordered categorical phenotypes (asthma severity score and asthma score) were performed using the Maximum Likelihood Binomial method [11, 12]

implemented in MLB-GENEHUNTER [11]. This method is a unique linkage analysis method for categorical traits. The principle of this approach is to introduce a latent (unobserved) binary variable (Y={0;1}) which captures the linkage information between the observed categorical phenotype and the marker. For each phenotypic category, the latent (unobserved) variable Y can take the value of 1 (being affected) with probability p_i and the value of 0 (being unaffected) with probability $1-p_i$. Linkage is then investigated for all possible sets of Y values within sibships weighted by their probabilities (2^s sets for a sibship of size s). Choice of these probabilities is guided by the hypothesis one wants to test. The idea is that the probability of Y=1 increases as the severity of asthma increases. Different sets of probabilities were used to model the correspondence between the observed category and the unobserved latent variable. When analysing the asthma severity score, the four probabilities assigned to the 4 classes (1, 2, 3, 4) were chosen to provide maximal power to detect genes controlling the mildest forms (classes 1 and 2 having low probabilities of 0.05 and 0.25 for the latent variable Y to be one) or the most severe forms of asthma (classes 3 and 4 having high probabilities of 0.75 and 0.95 for Y to be one). When analysing the asthma score, the probabilities modeling the correspondence between this score and the latent variable were chosen to investigate a continuum of disease expression and thus varied from 0 for non-asthmatics to 1.0 for asthmatics belonging to severity class 4, intermediate probabilities of 0.25, 0.45 and 0.75 being assigned to classes 1, 2 and 3 respectively. The likelihood of the observations is written by use of a binomial distribution of parental marker alleles among offspring according to the value of the unobserved binary latent variable and depends on only one parameter, α (probability that sibs with Y=1 receive one of the two marker alleles with disease allele from his/her heterozygous parent, $1-\alpha$ being the corresponding probability among sibs with Y=0). The null hypothesis of no linkage ($\alpha = 0.5$) against the alternative hypothesis of linkage ($\alpha > 0.5$) is tested by a likelihood-ratio test. Note that contribution to linkage information

comes from sets of sibs having the same value of the latent variable (concordant sibs for Y=1 or Y=0) and from sets of sibs having opposite values of the latent variable (discordant sibs).

Multipoint linkage analysis of the continuous phenotype (%FEV$_1$) was conducted using the Variance Components method [13] implemented in MERLIN [14]. The VC method separates the total variation of a trait into genetic and environmental components and evaluates linkage by comparing a model incorporating both a genetic additive variance at a putative quantitative trait locus (QTL) and a polygenic component with a purely polygenic model (QTL variance being set to zero) by a likelihood ratio test. Since VC method is known to be sensitive to departure from normality assumptions of the trait distributions, a probit transformation was applied prior to linkage analysis to %FEV$_1$ to normalize its distribution.

For either method, minus twice the natural logarithm of this likelihood ratio is assumed to follow a one-sided chi-square with one degree of freedom. This chi-square divided by 2ln10 can be considered as a LOD score.

Correlations among genome-wide LOD scores

To assess whether the asthma score, asthma severity score and %FEV$_1$ may share common genetic determinants, we computed the correlation matrix among the standardized genome-wide LOD scores obtained at 378 marker positions for these three phenotypes, using STATA 7.0.

RESULTS

Sample characteristics

The relationship between the asthma severity score and the sum of clinical items is presented in table 1. Taking treatment into account changed the scoring based on clinical items for 91 sibs (42.7%): three quarters of these sibs being reclassified as more severe and one quarter less severe as compared to step 1 where treatment was ignored.

The 288 genotyped siblings of the 110 nuclear families considered in the present analysis, were on average 15 years old (ranging from 3 to 43) and included 82% of asthmatics. The principal characteristics of the asthmatic and non asthmatic sibs are presented in table 2.

Regarding the asthma score that included asthmatic and non-asthmatic sibs, 18% of these sibs belonged to class 0, 38% to class 1, 19% to class 2, 12% to classes 3 and 4 each.

Genome-wide screen

The genome-wide linkage-test results for the asthma score, the asthma severity score, and %FEV$_1$ are shown in Figure 1 and table 3.

The most significant evidence for linkage was found on chromosome 18p11 for the asthma score where the maximum LOD score reached 2.40 at D18S53, with a p-value of 0.0004 being lower than the critical threshold of 7.10^{-4} for genome-wide suggestive evidence of linkage [15]. There was modest evidence of linkage (p < 0.01) to asthma score in two other regions: 5q15 (LOD = 1.22 at D5S428; p=0.009) and 14q13 (LOD = 1.20 at D14S70; p=0.009).

When considering the asthma severity score, a maximum LOD score of 1.80 (p=0.002) was obtained on chromosome 2p23 at marker D2S165. Additional potential linkage was observed in the 7q36 region (LOD = 1.00 at D7S2465; p=0.01).

Three regions were found linked to $\%FEV_1$ at $p \leq 0.005$: 1p36 (D1S468, LOD = 1.52 - p=0.004), 2q36 (D2S126, LOD = 1.59 - p=0.003) and 6q14 (D6S460, LOD = 1.64 - p=0.003). There were two additional linkage signals at the 1% significance level: 4p14 (D4S405, LOD = 1.21 - p=0.009) and 7p22 (D7S531, LOD = 1.19 - p=0.01).

Correlations among genome-wide LOD scores

Estimation of the correlations among genome-wide LOD scores for the three phenotypes showed non-significant correlations that were close to zero and with negative sign between LODs for $\%FEV_1$ and LODs for the asthma score ($r = -0.03$) and for the asthma severity score ($r = -0.07$) respectively. Thus, $\%FEV_1$ appears to have no genetic factor in common with the asthma score and the asthma severity score. There was a significant positive correlation, although not very high, between the LODs for the asthma score and the asthma severity score ($r = 0.13$; p=0.01), suggesting that these phenotypes may share a few genetic factors.

DISCUSSION

Currently, there is a debate on how asthma should be defined to progress in the understanding of the underlying mechanisms involved [1]. Up to now, genetic studies have used dichotomous definitions, often established from questionnaires. The present genome-wide scan is the first one that has considered varying levels of asthma expression (from unaffecteds to severely affecteds). The asthma spectrum phenotype, encompassing non asthmatics and various classes of asthma, showed genome-wide suggestive evidence for linkage to the 18p11 region. The asthma severity score, combining clinical items and treatment, showed linkage to the 2p23 region while other linkage signals were detected for FEV_1 on chromosomes 1p36, 2q36 and 6q14. None of the regions detected for either one of these three phenotypes was revealed for the other two phenotypes. Moreover, estimation of the correlations among genome-wide LOD scores showed no evidence for any common genetic determinant between $\%FEV_1$ and either the asthma score or the asthma severity score while there was suggestion of sharing a few genetic factors by these two scores. These potential common genetic determinants are likely to have weak effects since no region common to the two latter phenotypes was detected at the 1% significance level.

Phenotype definition and linkage analysis outcomes

The strongest evidence for linkage was found for the asthma score on 18p11. Previous analysis of asthma in the same EGEA sample, using a dichotomous definition, revealed a single linkage signal in the 1p31 region (p=0.005) [8]. Further analysis led to increased evidence for linkage to this region when the affection status was defined by presence of both asthma and allergic rhinitis and showed that 1p31 is likely to contain a gene specific of this co-morbidity [16]. Alternatively, the 18p11 region revealed by the present analysis of the categorical asthma score was not detected by our previous analysis of dichotomous asthma

and seven other asthma-related phenotypes [8]. This shows that considering a more refined phenotype including various classes of asthma expression can lead to detect new genes influencing variation in disease expression. Finding new regions by considering a categorical definition for asthma raises several hypotheses. First, these results may be due to reducing asthma misclassification. Whereas asthmatic probands were defined with a very strict procedure and recruited in chest clinics [10], the definition of asthma in siblings was less specific although attention was given to include items such as BHR, treatment or hospitalization to confirm asthma. In that context, a categorical variable may decrease such potential misclassification bias. A second possibility is that asthma indeed is not a dichotomous phenotype, but, as airflow limitation, may occur on a rather continuous way. A similar approach of building continuous scores for asthma has been proposed by Pekkanen et al [3] and was shown to increase power of detecting risk factors of disease. The present score for asthma was constructed upon the asthma severity score itself defined from clinical symptoms and treatment but other types of continuous scores may be built. Interestingly, Pekkanen et al [3] found that a simple sum of positive answers to eight questions regarding clinical symptoms and taking medication for asthma, thus based on the same principles as our grading of asthma severity, was as effective for detecting risk factors as a score computed from a more sophisticated principal component analysis of 12 questions on asthma symptoms. Thus, our results and those reported by Pekkanen et al [3] provide strong support for the usefulness of examining quantitative scores of asthma in etiological research.

We chose not to include FEV_1 in the severity score, despite the fact that this physiological measure may be helpful to assess asthma severity in clinical settings, since our aim was to investigate whether the ventilatory function and the asthma severity score result from common or distinct genetic determinants. Our results show that $\%FEV_1$ and the asthma severity score do not share any genetic determinant. This is in agreement with phenotypic

studies which have shown that FEV_1 does not systematically strongly correlate with disease symptoms [2, 17]. Moreover, none of the published genome-wide screens conducted for lung function in asthmatic families was reported linked to the 2p23 region detected here for the asthma severity score [18-20]. Altogether, these results indicate that %FEV_1 is more likely to be controlled by specific genetic determinants. Thus, in the search for genetic determinants of asthma severity, it may be worthwhile to consider lung function separately from clinical symptoms and treatment. We should also note that the 2p23 region found linked to asthma severity score was not revealed by our previous scan of dichotomous asthma and seven asthma-associated phenotypes [8] and thus appears as a new and specific region of asthma severity in the present sample. Among the three linkage signals revealed for FEV_1, only the 6q14 region was detected by our previous scan of the whole EGEA sample with at least one asthmatic proband and even more significantly (p=0.001), suggesting genes in this region may rather control physiological variation of this phenotype.

Replication of linkage results

It has been often advocated that replication of linkage results across studies provides support for the actual involvement of linkage regions. We have carried out an exhaustive compilation of linkage results from published genome scans of asthma and asthma-associated phenotypes performed to date in 16 different populations (without counting the EGEA study). We considered all previously reported linkage peaks at $p \le 0.01$, spanning a 20 cM region on either side of each of our five main linkage peaks. Among the 16 asthma genome scans, the 18p11 region, detected for the asthma score, was reported once in Australian twins for asthma analyzed as a binary trait [18]. The linkage of the asthma severity score to 2p23 was detected for eosinophils in Australian Twins [18, 21, 22] and for severe asthma in German data [23]. Note the latter study examined dichotomous subtypes of asthma which were defined from the

three following items using broader categorization than ours: asthma attack frequency, receiving or not asthma treatment and having or not experienced at least one overnight hospital stay. Since %FEV_1 was studied in only four asthma genome-screens, we considered linkage scans conducted for lung function phenotypes in families with early onset chronic obstructive pulmonary diseases (COPD) and from the general population [24]. Among the three linkage signals detected for FEV_1, 1p36 was reported linked to asthma in two scans [22, 25] and 6q14 to eosinophils in one scan [26] while linkage to 2q36 was observed for atopy phenotypes in two asthma scans [18, 21, 22] and for lung function phenotypes in asthmatic families [19] and in early-onset COPD families [27, 28]. Regarding this latter result, longitudinal studies have shown that asthma is associated with accelerated lung function decline and is a risk factor for COPD [29]. Thus, genetic factors on chromosome 2q might participate to the common determinants of asthma and COPD.

Limitations/Strengths/Conclusion

A major strength of our study was the availability of extensively documented phenotypes for all subjects of the EGEA sample ascertained through asthmatic cases followed in chest clinics. This allowed to study a wide range of asthma severity, but we acknowledge that one limitation of the study is the small number of severe asthmatics, such as those patients included in specific studies of this asthma sub-phenotype [2]. Examination of the families that contributed to linkage to 2p23 came mainly from sibs affected with mild asthma as well as from discordant sets of sibs with mild and severe asthma, suggesting this region may harbor genes underlying mild asthma and possibly protecting from severe asthma.

Assessing asthma severity remains a difficult issue because of the paucity of knowledge and the heterogeneity of this condition, and the rapidly evolving concept regarding its measurement. Here, we chose to combine clinical items and treatment in order to follow the

same principle as recommended in the 2002 and 2004 GINA guidelines. Despite the fact that the dose of treatment was not available in our data, we could assess the use of inhaled corticosteroids (ICS) in the past 12 months. ICS are the main controller medication, and they differentiate intermittent from persistent asthma in the GINA guidelines. Moreover, the asthma severity classification presently used has already been used to examine the relationship between occupational exposure and asthma severity in the EGEA data [30].

We are aware that the last GINA recommendations (2006) have evolved and shifted from severity to control. However, if this approach is adapted to clinical practices for management of individual asthmatic patients, it may not be the best approach for epidemiological studies, aimed at determining risk factors for asthma severity in populations. In cross-sectional surveys, the level of asthma symptoms may not be interpreted in the same way in asthmatic populations with or without controller medication. Taking medication into account may provide useful additional information in populations, as underlined by Liard et al [31] who showed that a classification of asthma severity combining treatment to symptoms and FEV_1 had a better correlation with emergency visits or hospitalization for asthma than the classification without treatment. The degree of airway hyperresponsiveness could be also considered as an objective marker of asthma severity, but specific criteria for the test in the EGEA study led to a large number of missing data in asthmatics, and thus to a too small number of sibs available for linkage analysis.

Another strength of our study was the use of a unique method for categorical phenotypes that is very flexible since one can choose the probabilities modeling the correspondence between the observed scores and the underlying latent variable to increase power for testing a given hypothesis. Moreover, this method takes into account, in the same analytical framework, sets of sibs with similar forms and opposite forms of the disease spectrum (including possibly unaffecteds) that both contribute to linkage (concordant sibs sharing an excess and discordant

sibs sharing a lower proportion of marker alleles identical by descent than the distribution expected under the null hypothesis of no linkage) and can thus increase power to detect linkage signals.

In conclusion, the present study shows that use of a categorical phenotype to represent the whole spectrum of disease expression instead of a simple dichotomous phenotype can increase power to detect new genetic regions. It has also evidenced a specific genetic component involved in asthma severity and that different genetic components underlie the various dimensions of asthma severity represented on the one hand by combination of clinical symptoms and treatment and on the other hand by FEV_1. These findings have important implication for considering more refined disease phenotypes in future genetic studies of asthma.

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Table 1. Asthma severity score (1-4) based on the combination of the sum of clinical items (Step 1) and the type of treatment (Step 2) in 213 genotyped asthmatic siblings

Treatment level [*] / Sum of clinical items [†]	No treatment (n = 70)	Treatment without inhaled corticosteroids (n = 64)	Treatment with inhaled corticosteroids (n = 79)
0 (n = 124)	1 (n = 48)	1 (n = 42)	2 (n = 34)
1 (n = 36)	1 (n = 9)	2 (n = 9)	3 (n = 18)
2 (n = 33)	2 (n = 8)	3 (n = 8)	4 (n = 17)
$\geq$ 3 (n = 20)	3 (n = 5)	4 (n = 5)	4 (n = 10)

[*] The clinical items and treatment were both defined for the last 12 months;

[†] The clinical items include the following: frequency of asthma attacks; symptoms between attacks and hospitalization (see table 2)

Table 2. Phenotypic characteristics of genotyped siblings in the set of 110 families with at least two asthmatic sibs

Asthmatic sibs (n=237)		
Sex, n (%male)	142	(59.7)
Age (years), mean (SD)	14.7	(7.3)
FEV$_1$% predicted, mean (SD)	95.1	(13.4)
Age at asthma onset, mean (SD)	5.9	(5.5)
Frequency of asthma attack (last 12 months), n (%)		
None	43	19.0
< 1 attack / month	104	46.0
≥1 attack / month and < 1 attack / week	45	19.9
≥ 1 attack / week and <1 attack / day	27	12.0
≥1 attack / day	7	3.1
Persistent symptoms between attacks, n (%)	32	15.0
Hospitalization (last 12 months), n (%)	14	6.3
Sum of clinical items (Step 1), n (%)*		
0	124	(58.2)
1	36	(16.9)
2	33	(15.5)
≥ 3	20	(9.4)
Treatment (last 12 months), n (%)		
Without any treatment	80	33.7
With inhaled corticosteroids	86	36.3
Without inhaled corticosteroids	71	30.0
Asthma severity score, n (%)		
1	100	(46.7)
2	51	(23.8)
3	31	(14.5)
4	32	(14.9)
Non asthmatic sibs (n=49)		
Sex, n (%male)	26	(53.1)
Age (years), mean (SD)	15.4	(10.1)
FEV$_1$% predicted, mean (SD)	100.7	(16.2)

*One of the clinical items (persistent symptoms between attacks) was available on 213 siblings and thus the sum of clinical items was determined in 213 among the 237 genotyped asthmatic sibs.

Table 3. Chromosomal regions showing multipoint LOD scores with P values ≤ 0.01 with at least one phenotype in 110 EGEA families.

Markers	Position[*]	Asthma score		Asthma severity		%FEV$_1$	
		LOD	P	LOD	P	LOD	P
D1S468	4.20					1.52	0.004
D1S214	14.00					1.16	0.01
D2S305	38.90			1.46	0.005		
D2S165	47.40			1.80	0.002		
D2S126	221.10					1.59	0.003
D4S405	56.95					1.21	0.009
D5S428	95.40	1.22	0.009				
D6S460	89.80					1.64	0.003
D7S531	5.28					1.19	0.01
D7S517	7.14					1.14	0.01
D7S2465	180.4			1.00	0.01		
D14S70	40.11	1.20	0.009				
D18S464	31.17	2.06	0.001				
D18S53	41.24	2.40	0.0004				
D18S478	52.86	1.37	0.006				

[*] cM position of linkage peak based on Marshfield map

Legends to Figures:

Figure 1: Multipoint results of the genome-wide linkage scan for asthma score, asthma severity score and %FEV$_1$ conducted in 110 EGEA families. Multipoint LOD scores are shown on the vertical axis and map distances (in cM) on the horizontal axis. LOD scores of asthma score, asthma severity score and %FEV$_1$ are represented with pink, yellow and cyan lines respectively.