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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Metabolomics to identify omalizumab responders among children with severe asthma: A prospective study

To the Editor,

In this study, we aimed to assess the potential value of baseline urinary metabolomic profiles in predicting the response to omalizumab in children with severe asthma. Although most children show a good response to this drug,¹ 10–15% are partial or non-responders.² Identifying the patients most likely to benefit from

omalizumab is crucial to personalise the treatment and optimise cost-effectiveness. So far, clinical or biochemical predictors of response to omalizumab have not been definitively recognised. Metabolomic analysis, using an untargeted approach, has the potential to enable the identification of metabolic features associated with relevant clinical outcomes.³

*See Acknowledgement for all the members of IPSAN.

In this multicentre prospective study (Ethics Committee approval 4329/AO/17), after obtaining informed consent, we consecutively recruited patients 6–17 years old with severe asthma participating in a national database of the Italian Society for Paediatric Respiratory Diseases, who were candidates for omalizumab. Children had been treated for at least 4 months with a daily ICS dose ≥ 500 mcg of fluticasone propionate or equivalent. A urine sample was collected before starting omalizumab. After 52 weeks of follow-up, or sooner if omalizumab was stopped before, children were classified as responders or not, using a multidimensional approach.⁴ We considered the variation from baseline of GINA score (which evaluates asthma control in the last 4 weeks) and CASI⁵ score (which evaluates

control in the last 2 weeks together with lung function, exacerbations and treatment). We defined as responders children with at least a 1-point reduction in CASI score⁶ and controlled or partially controlled according to GINA. Since QoL is significantly affected in severe asthma,⁷ in case of inconsistency, we considered as responders those with an increase of at least 0.5 points⁸ in Pediatric Asthma Quality of Life Questionnaire (PAQLQ) score.⁹

Urine analysis was performed through high-resolution mass-spectrometry (Q-ToF Synapt G2, Waters, Milford, USA) interfaced with the chromatography Acquity UPLC using the reverse phase column HSS T3 (Waters, Milford, USA)

TABLE 1 Children's characteristics, clinical variables and identified metabolites

Baseline characteristics	Responders (n = 42)		Non-responders (n = 10)	
Males (n, %)	23 (55%)		4 (40%)	
Age (mean, SD)	12.2 (2.7)		10.9 (2.3)	
BMI (mean, SD)	20.7 (4.2)		19.8 (2.9)	
Total IgE (IU/ml) (mean, SD)	701.5 (516.3)		494.1 (472.8)	
Allergic comorbidities ^a (n, %)	26 (62%)		5 (50%)	
Sensitised to perennial allergens (n, %)	41 (98%)		8 (80%)	
Parental smoking (n, %)	29 (69%)		7 (70%)	
Parental asthma (n, %)	19 (45%)		4 (40%)	
Clinical variables	Baseline	End of follow-up ^b	Baseline	End of follow-up ^b
Number of steroid courses in the previous 12 months (mean, SD)	4.2 (4.2)	0.7 (1.2)	4.5 (3.3)	3.4 (4.0)
LABA (n, %)	40 (95%)	32 (76%)	9 (90%)	9 (90%)
Montelukast (n, %)	29 (69%)	9 (21%)	4 (40%)	4 (40%)
FEV1 (% pred) (mean, SD)	91 (19)	96 (16)	91 (14)	85 (11)
GINA score U/P/C (n)	37/5/0	5/9/28	8/2/0	8/2/0
CASI score (mean, SD)	9.8 (3.8)	4.7 (1.7) ^c	9.6 (4.1)	8.8 (4.6) ^c
PAQLQ (mean, SD)	4.7 (1.3)	6.5 (0.5) ^c	4.7 (1.0)	4.1 (0.7) ^c
Identified metabolites at baseline	Responders (n = 42) Median [IQR]		Non-responders (n = 10) Median [IQR]	
L-Histidine (178.0590 m/z; RT 0.523) [ID: POS413]	0.23 [0.20–0.34]		0.13 [0.10–0.19] ^d	
Uric acid (169.0361 m/z; RT 0.827) [ID: POS1370]	0.22 [0.10–0.30]		0.42 [0.35–0.58] ^d	
L-Kynurenine (209.0930 m/z; RT 1.885) [ID: POS 2148]	0.05 [0.03–0.13]		0.018 [0.007–0.037] ^d	
3-Dimethylallyl-4-hydroxyphenylpyruvate (249.1139 m/z; RT 5.582) [ID: POS7858]	0.12 [0.10–0.15]		0.18 [0.16–0.22] ^e	
Aspartylglycosamine (316.1141 m/z; RT 0.677) [ID: NEG3634]	0.28 [0.18–0.35]		0.13 [0.07–0.22] ^e	
Aspartyl-Threonine (215.0661 m/z; RT 0.789) [ID: NEG3420]	0.21 [0.15–0.27]		0.12 [0.02–0.15] ^e	

Abbreviations: BMI, body mass index; GINA, Global Initiative for Asthma; U, uncontrolled asthma; P, partially controlled asthma; C, Controlled Asthma; CASI, Composite Asthma Severity Index; RT, retention time; ID, variable identifier coded as [ionization mode][identifier].

^aAllergic rhinitis and/or atopic dermatitis and/or food allergy.

^bEnd of follow-up: evaluation at 52 weeks or when omalizumab was stopped because of poor asthma control (5/10 non responders stopped omalizumab after 4 to 9 months).

^cVariation from baseline in responders versus non-responders: $p < .01$ for CASI; $p < .001$ for PAQLQ.

^dResponders versus non-responders $p = .01$.

^eResponders versus non-responders $p < .001$.

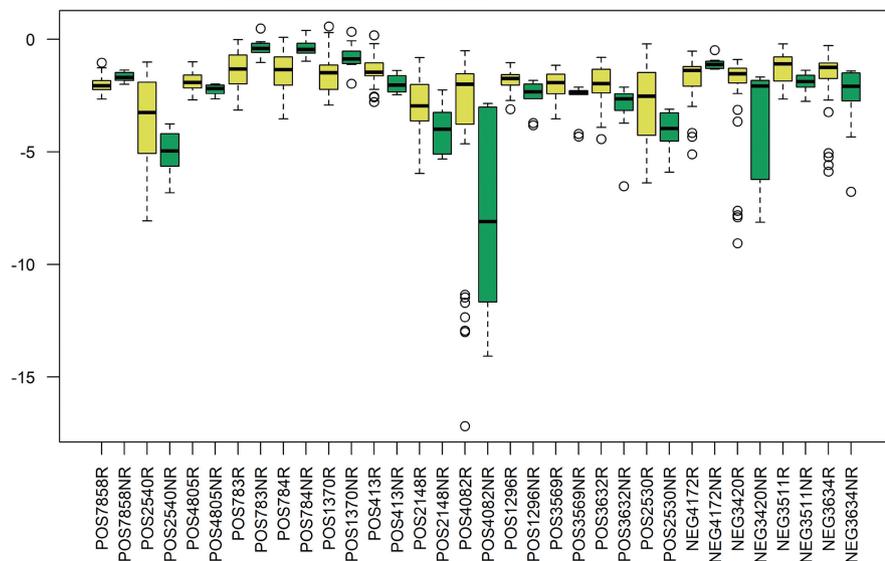


FIGURE 1 Boxplots showing the distributions of the features selected by Mood's test controlling the false discovery rate by Storey method (adjusted- $p < .15$); green bars: non-responders (NR); yellow bars: responders (R); variables are indicated as [ionization mode][ID][group]

Children's baseline characteristics were compared by *t*-test, Mann-Whitney test and Fisher's exact test; in case of differences in their distribution, sub-sampling was performed to match responders and non-responders (a standard procedure in metabolomic studies to avoid false discoveries due to experimental design bias).

For metabolomic analysis, a two-group comparison and a one-class modelling approach were applied. The former assumes that responders and non-responders belong to two different groups, each metabolically well-defined, and it was based on univariate methods (Mood's median test controlling the false discovery rate by Storey method, adjusted $p < .15$). The one-class approach assumes that responders belong to a metabolically homogeneous group while non-responders are scattered around it, and it was based on multivariate (Principal Component Analysis) and univariate methods for outlier detection. With the univariate approach, the distribution of each metabolic variable in responders was modelled by kernel density estimation; the probability of each non-responder being an outlier for that variable was estimated and false discovery rate was controlled by Storey method (adjusted $p < .15$).

The relevant variables were annotated searching our database of commercial standards, METLIN database and Human Metabolome Database.

52 children were included in the analysis (10 non-responders) (Table 1).

Metabolic features were differently expressed in responders and non-responders.

The two-class comparison approach highlighted 17 discriminating features (Figure 1); among these, six metabolites were identified (putative biomarkers), four higher in responders and two in non-responders (Table 1).

Biomarkers increased in responders were dipeptides and amino acids, in keeping with previous studies that suggest an altered amino acid metabolism in asthma.¹⁰ The better characterised were the

histamine precursor L-Histidine, a possible marker of an altered histamine pathway and L-Kynurenine.

On the other hand, uric acid, a metabolite with a possible role in innate and adaptive T2 response amplification,¹¹ was a putative biomarker in non-responders.

Moreover, the one-class modelling approach highlighted 100 features differently expressed in at least three non-responders with respect to responders (annotated metabolites in Table S1) and the Q-chart built by PCA was promising for non-responder detection (Figure S1).

Details of methods and results are reported in Appendix S1.

In conclusion, we found that children with severe asthma responding to omalizumab showed a different metabolomic urinary profile at baseline compared to non-responders. A metabolotype enriched in amino acids was associated with a good response to omalizumab, while the uric acid metabolic pathway was involved in non-responders.

For the first time, this study paves the way to a possible a-priori identification of children who are most likely to benefit from omalizumab based on their metabolic arrangement. Further studies, also based on targeted approaches, may expand these results.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

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Functional *CTLA-4* variants associate to both allergic asthma and rhinitis potentially by modulating naïve regulatory T cells

To the Editor,

A genome-wide linkage study previously indicated the chromosome 2q region containing the cytotoxic T-lymphocyte protein 4 (*CTLA-4*) gene might be a candidate asthma locus.¹ However, prior reports of associations between *CTLA-4* genetic variants and asthma are conflicting and inconclusive.²⁻⁴ This study aimed to determine the role of *CTLA-4* single nucleotide polymorphisms (SNPs) on allergy risk by carrying out genetic association and functional analyses. Detailed information on the cohorts, sample processing and experimental methods have been provided in Appendix S2.

We identified 5 tag-SNPs (rs733618, rs4553808, rs16840252, rs231775, and rs3087243) in *CTLA-4* from the Hapmap Chinese Han population (CHB, Figure S1). These tag-SNPs were genotyped in a cohort of 1703 Singapore Chinese adults (age: 22.2 ± 5.6, 42% male, Table S1). Of these 5 *CTLA-4* tag-SNPs genotyped, rs3087243 has the highest significance level of associations with AR without asthma ($p = 4.69 \times 10^{-3}$, OR = 1.38), asthma without AR ($p = 3.49 \times 10^{-4}$, OR = 1.58), and AR with asthma ($p = 6.67 \times 10^{-4}$, OR = 1.52, Table 1).

Next, we determined if rs3087243 has a functional effect on *CTLA-4*. We extracted *CTLA-4* mRNA expression data from 31,300 whole blood samples constituting 36 different cohorts collected by the eQTLgen consortium. In these cohorts, meta-analysis showed a strong correlation between allele "A" of rs3087243 and increasing *CTLA-4* mRNA expression (meta p -value = 2.67E-69, combined Z-score = 17.60, Figure 1A). From the HapMap CHB data, tag-SNP rs3087243 is in a strong linkage disequilibrium ($r^2 = 1$) with rs11571316 that is located ~1.4 kb upstream of the *CTLA-4* gene (Figure S1). We cloned -1686bp to +110bp of the *CTLA-4* gene to conduct an in vitro luciferase assay and showed a significantly higher *CTLA-4* promoter activity associated with allele "A," as compared to allele "G" of rs3087243 ($p < .01$, Figure 1B,C). This suggests rs11571316 might represent a functional SNP that influences the *CTLA-4* promoter activity and causes the apparent genetic association between the tag-SNP rs3087243 and respiratory allergies.

Lastly, when analyzing the *CTLA-4* surface expression of T cells by flow cytometry, we demonstrated a functional effect of

TABLE 1 Genetic association of *CTLA-4* tag SNPs to allergic rhinitis and asthma in Singapore Chinese population

Reference/Phenotype		AR without asthma (N = 455) vs. Nonatopic Nonallergic controls (N = 525)		AR with asthma (N = 338) vs. Nonatopic Nonallergic controls (N = 525)		Asthma without AR (N = 379) vs. Nonatopic Nonallergic controls (N = 525)	
SNP	Allele	Logistic p	OR	Logistic p	OR	Logistic p	OR
rs733618	G/A	3.04E-03*	0.76 (0.63-0.91)	5.64E-02	0.82 (0.66-1.01)	4.27E-02*	0.81 (0.67-0.99)
rs4553808	G/A	9.08E-01	0.98 (0.74-1.31)	2.33E-01	0.82 (0.58-1.14)	1.92E-01	0.81 (0.60-1.11)
rs16840252	T/C	7.96E-01	0.96 (0.73-1.28)	2.34E-01	0.82 (0.59-1.14)	2.00E-01	0.82 (0.60-1.11)
rs231775	A/G	9.24E-03*	1.30 (1.07-0.157)	2.86E-02*	1.29 (1.03-1.61)	4.91E-02*	1.24 (1.00-1.54)
rs3087243	A/G	4.69E-03*	1.38 (1.10-1.73)	3.49E-04*	1.58 (1.23-2.02)	6.67E-04*	1.52 (1.19-1.93)

Note: Logistic regression test was performed adjusting for age and gender.

Abbreviations: CI, confidence interval; Logistic p , adjusted logistic regression p -value; OR, adjusted odds ratio, using minor allele as the reference category.

*Logistic $p < .05$ is considered significant association.