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IgE to cross-reactive carbohydrate determinants (CCD) in childhood: Prevalence, risk factors, putative origins

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Abstract

Background: IgE antibodies to cross-reactive carbohydrate determinants (CCD) are usually clinically irrelevant but they can be a cause of false positive outcomes of allergen-specific IgE tests in vitro. Their prevalence and levels have been so far cross-sectionally examined among adult allergic patients and much less is known about their origins and relevance in childhood.

Methods: We examined CCD with a cross-sectional approach in 1263 Italian pollen allergic children (Panallergen in Paediatrics, PAN-PED), as well as with a longitudinal approach in 612 German children (Multicenter Allergy Study, MAS), whose cutaneous and IgE sensitization profile to a broad panel of allergen extracts and molecules was already known. The presence and levels of IgE to CCD were examined in the sera of both cohorts using bromelain (MUXF3) as reagent and a novel chemiluminescence detection system, operating in a solid phase of fluorescently labelled and streptavidin-coated paramagnetic microparticles (NOVEOS, HYCOR, USA).

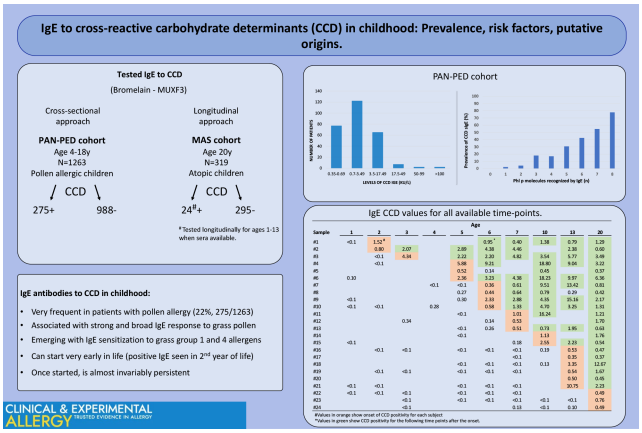
Results: IgE to CCD was found in 22% of the Italian pollen allergic children, mainly in association with an IgE response to grass pollen. Children with IgE to CCD had higher total IgE levels and were sensitized to more allergenic molecules of Phleum pratense

For Affiliation refer page on 205

than those with no IgE to CCD. Among participants of the German MAS birth cohort study, IgE to CCD emerged early in life (even at pre-school age), with IgE sensitization to group 1 and 4 allergen molecules of grasses, and almost invariably persisted over the full observation period.

Conclusions: Our results contribute to dissect the immunological origins, onset, evolution and risk factors of CCD-sIgE response in childhood, and raise the hypothesis that group 1 and/or 4 allergen molecules of grass pollen are major inducers of these antibodies through an antigen-specific, T-B cell cognate interaction.

KEYWORDS
allergens and epitopes, allergic rhinitis, CCD, grass pollen, IgE, pediatrics



GRAPHICAL ABSTRACT:
IgE antibodies to CCD in childhood, tested in the PAN-PED (cross-sectional approach) and MAS (longitudinal approach) cohorts, are as follows: (1) very frequent in patients with pollen allergy (22%, 275/1263), (2) associated with strong and broad IgE response to grass pollen, (3) emerging with IgE sensitization to grass group 1 and 4 allergens, (4) can start very early in life (positive IgE seen in second year of life) and (5) once started, are almost invariably persistent.

1 | INTRODUCTION

Patients with IgE-mediated allergic diseases develop specific IgE (sIgE) antibodies against protein epitopes of allergenic molecules from natural sources, which translate into positive sIgE results in vitro and skin prick test (SPT) in vivo.[1,2] In addition, many patients present IgE reactivity to a few glycans attached to a wide variety of carrier proteins and thus, as defined since their discovery, IgE antibodies to "cross-reactive carbohydrate determinants" (CCD).[3]

CCDs consist of asparagine (Asn)-linked oligosaccharides on glycoproteins, and are believed to be the most frequent individual IgE epitope structures.[4] These N-glycans—MUXF, MMXF—are found in all main plant allergen sources and rubber latex. Classical CCD epitopes display core α1,3-linked fucose and/or β1,2-linked xylose—common posttranslational modifications of plant (both) and insect glycoproteins (fucose) that are absent in humans—considered to be the cause of their immunogenicity.[5]

Key Messages

- Cross-reactive carbohydrate determinants (CCD) investigated in a cross-sectional and a longitudinal cohort of children.
- IgE response to CCD is very frequent among children with pollen allergy in these cohorts.
- Group 1 and/or 4 allergen molecules of grass pollen may be major inducers of CCD.

The clinical significance of CCDs remains a debated topic. Although several studies observed that they do not usually induce degranulation after interaction with IgE on mast-cells or basophils, and therefore do not elicit in vivo a positive reaction to SPT with the allergen extract, nor allergic symptoms upon allergen exposure,[3,6–8] others have shown in vitro activation of degranulating

cells mediated by IgE to CCD.[9,10] Their role in causing false positive outcomes in in vitro singleplex or multiplex IgE tests has been confirmed throughout populations,[3] making them a causal agent for cross-reaction that hinders correct allergy diagnosis.[11] The debated lack of degranulating capacity of IgE to CCD remains unexplained. A proposed explanation for this has been the very low affinity of IgE to CCD, compared to IgE to protein epitopes,[12] but this hypothesis has been confuted.[13] Another possible cause might be the interference of CCD-specific IgG antibodies, induced by a diet rich in vegetable food containing plant N-glycans.[14]

Notwithstanding their apparent lack of function, IgE to CCD are quite frequent in humans. IgE antibodies to CCD were found in 22%[11] and 23%[7] of European allergic patients. This prevalence increased to 31% among pollen-allergic patients, and to 71%[7] among patients with multiple pollen sensitizations.[7] A significant correlation was also observed between total IgE (tIgE) levels and CCD positivity.[7]

The evolutionistic meaning and the inducing factors of the IgE response to CCD are still hotly debated.[15] Within a Ugandan population endemically infected by *Schistosoma mansoni*, the prevalence of IgE to CCD reached 40% and was inversely associated with asthma,[16] suggesting their protective role in the context of the immune response against helminths. In a European, westernized population, the prevalence of IgE to CCD increased with age and an onset in childhood was hypothesized.[11] Allergen molecules of pollen, insect venom and mites express CCDs that are recognized in vitro by IgE antibodies of allergic patients,[3] but it is still unclear which molecule of these allergen sources is responsible for the induction of these antibodies and what is the mechanism of induction.[15]

Studies of the IgE response to CCD in childhood that may address some of the above listed questions are lacking. To fill this knowledge gap, we examined the IgE response to CCD, both with a cross-sectional approach in a large cohort of Italian children affected by seasonal allergic rhinitis (SAR), and with a longitudinal approach in a birth cohort of German children.

2 | MATERIALS AND METHODS

2.1 | Study populations

We examined the biodata banks of two large populations with a cross-sectional (PAN-PED cohort, Italy) and a longitudinal approach (MAS cohort, Germany). The population sample and study design of the PAN-PED[17,18] and the MAS[19] cohorts have been described elsewhere and are briefly summarized below.

2.1.1 | PAN-PED cohort

- Study population—The "Panallergen in Pediatrics" (PAN-PED) enrolled 1360 patients during routine appointments in 16 paediatric outpatient clinics in 14 Italian cities between 2009 and 2011. Criteria for eligibility were: (i) age 4–18 years, (ii) history of pollen-induced allergic rhino-conjunctivitis (AR) and/or asthma in one of the two last pollen seasons, and (iii) positive SPT for the relevant pollen extracts. Exclusion criteria were: (i) previous specific immunotherapy (SIT) for any pollen allergen, and (ii) any other severe chronic disease. Recruited children's parents answered a standardized questionnaire, and patients underwent SPT and blood draw. IgE to extracts, molecules and tIgE were performed (description in Appendix S1).[20]

2.2 | MAS birth cohort

- Study design—The Multicenter Allergy Study (MAS), a prospective birth cohort study, enrolled 1314 infants born in 1990 throughout 5 German cities. It was approved by the local ethics committees. Each parent provided written informed consent at the time of enrolment. All children were asked to undergo a blood draw during follow up visits at the age 1, 2, 3, 4, 5, 6, 7, 10, 13 and 20 years. Sera of consenting children were tested for tIgE and sIgE antibodies (ImmunoCAP, TFS, Uppsala, Sweden) to five airborne (mites, cat, dog, birch, grass) and four food (milk, egg, soy, wheat) allergen extracts.
- Examined population subset—In the present study, we first addressed participants examined in 2010 around their 20th birthday, then we focused on the patients with a positive response to CCD and examined backwards all their available sera collected at the time points 1, 2, 3, 4, 5, 6, 7, 10 and 13 years of age.

2.2.1 | IgE to CCD

The presence and levels of IgE to CCD were examined in sera of both cohorts using bromelain (MUXF3) (NOVEOS, HYCOR, USA) (description in Appendix S1).[21] We have previously validated the IgE concentration to CCD obtained with the NOVEOS by matching it against that obtained with the ImmunoCAP (MUXF3) (Thermofisher, Uppsala, Sweden) (data not shown).

2.2.2 | Statistics

Quantitative variables were summarized as mean and standard deviation (sd), categorical variables were summarized as number and percentage (%). The Chi-square test was used for categorical variables, to test differences between groups and T-test was used to compare quantitative variables between the two groups (positive or negative patients tested for CCD).

A logistic regression was used to evaluate the relationship between CCD positivity and the number of Phl p molecules. The odd ratio (OR), the relative confidence interval at 95% (CI 95%) and the *p* value were reported.

TABLE 1 Characteristics of the "Panallergen in Pediatrics" (PAN-PED) study population.

	CCD negative[a]		CCD positive[a]		
	n = 988		*n* = 275		
	n	%	*n*	%	*p*[b]
Male	664	67.27	194	70.55	.304
Age (y) (mean, SD)	40.48	3.34	10.39	3.58	.652
Parental allergy	653	66.09	186	67.6	.632
Parental smoking	481	48.68	125	45.45	.343
Age at SAR onset (y) (mean, SD)	5.27	2.84	5.13	2.74	.455
Duration SAR (y) (mean, SD)	5.22	3.33	5.25	4.86	.864
SAR severity (ARIA)					.939
Mild intermittent	240	24.3	68	24.7	
Mild persistent	236	23.89	63	22.91	
Moderate/severe intermittent	93	9.41	29	10.55	
Moderate/severe persistent	419	42.41	115	41.82	
RC VAS (index) (mean, SD)	58.76	21.34	58.89	22.36	.928
Asthma	391	39.57	113	41.09	.650
Asthma VAS (index) (mean, SD)	46.79	19.96	48.28	21.59	.493
OAS	230	23.28	81	29.45	.036
Anaphylaxis	66	6.68	7	2.55	.009
Urticaria/Angioedema	210	21.26	54	19.64	.559
Atopic dermatitis	351	35.53	102	37.09	.632
GI allergic symptoms	59	5.97	26	9.45	.041
Other allergies	88	8.91	28	10.18	.517

Note: Data were summarized as numbers (*n*) and frequencies (%) if they were categorical, or mean and standard deviation (SD) if quantitative.

[a]Numbers refer to negative and positive patients tested for cross-reactive carbohydrate determinants (CCD) with cut-off >0.35 kU/L.

[b]Chi-square test was used to compare categorical data between groups; T-test was used to compare continuous variables between groups.

Pearson correlation was used to evaluate the relationship between tIge and CCD IgE in CCD-positive patients.

Univariable and multivariable logistic regression was used to investigate the relationship between CCD positive and Phl p molecules. OR, CI95% and p-value have been reported. Two multivariable regressions were implemented using recombinant Phl p 4 or native Phl p 4.

A *p*-value of <.05 was considered statistically significant. The statistical analysis was performed with stata software (stata 16.2, College Station TC77845).

3 | RESULTS

3.1 | Characteristics and sensitization pattern of patients with IgE to CCD

Overall, 1263 Italian children with seasonal allergic rhinoconjunctivitis were included in the cross-sectional study. Among them, 275/1263 (22%) had IgE to CCD (cut-off >0.35 kU/L). Their clinical condition (Table 1), and sensitization to allergen extracts (Table S1) and molecules (Table 2) were compared to those of the 988 patients with no IgE to CCD:

- Clinical characteristics—Patients with IgE to CCD were not different from those without IgE to CCD in terms of age, parental allergy and parental smoking. They were also not different in terms of age in relation to SAR onset, duration and severity. However, they had more oral allergy syndrome (OAS) (29.5% vs. 23.3%, $p = .036$) and gastrointestinal symptoms (9.5% vs. 6.0%, $p < .041$), but less anaphylaxis (2.6% vs. 6.7%, $p < .009$) (Table 1).
- Sensitization to allergen extracts—When compared with the 988 patients without IgE to CCD, the CCD-positive ($n = 275$) had more frequently a positive SPT to allergen extracts of pollen and vegetable food (Table S1). This difference was selective and stronger for some allergens, with Timothy (97%) and Bermuda (89%) grass showing very high correlation, followed by Farinaccio (78%), Plantago lanceolate (73%), Hazelnut (55%), Birch (50%), Plane tree (48%), Russian thistle (36%) and Mugwort

TABLE 2 Sensitization to pollen, vegetable food, mite, cat and alternaria, by CCD-sIgE seropositivity in the "Panallergen in Pediatrics" (PAN-PED) study population.

		CCD-sIgE negative[a]		CCD-sIgE positive[a]		
		n = 988		*n* = 275		
Extract	Molecule	*n*	%	*n*	%	*p*[b]
Timothy grass	rPhl p 1	748	85.29	256	97.34	<.001
	rPhl p 2	354	40.41	187	71.10	<.001
	rPhl p 4	284	34.13	158	63.71	<.001
	rPhl p 5	448	51.32	226	85.93	<.001
	rPhl p 6	387	44.33	217	82.51	<.001
	rPhl p 7	19	1.94	21	7.75	<.001
	rPhl p 11	164	18.83	127	48.29	<.001
	rPhl p 12	177	18.12	110	40.59	<.001
Birch	rBet v 1	215	22.01	80	29.63	.009
Olive	nOle e 1	520	53.22	202	74.54	<.001
Cypress	nCup a 1	303	79.53	104	90.43	.008
Mugwort	rArt v 1	83	29.23	35	29.66	.930
Pellitory	rPar j 2	247	59.23	63	53.39	.256

[a]Numbers refer to negative and positive patients tested for cross-reactive carbohydrate determinants (CCD) with cut-off >0.35 kU/L.

[b]Chi-square test was used to compare data between groups.

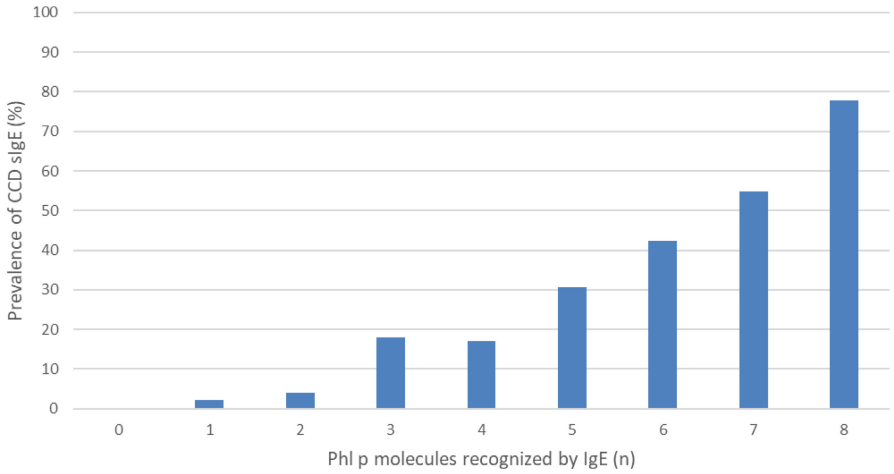


FIGURE 1 Prevalence of IgE to CCD by number of Timothy grass molecules recognized by IgE. Percentage of IgE to bromelain MUXF3 is shown according to the amount of timothy grass molecules recognized by the grass pollen allergic Italian children (*n* = 1131) affected by seasonal allergic rhino-conjunctivitis.

(34%) ($p < .001$); while no association was found between IgE to CCD and skin sensitization to pellitory, cypress and ragweed. Moreover, no association was found between CCD sIgE and IgE sensitization to allergen extracts of *Dermatophagoides pteronyssinus*, cat and Alternaria. Interestingly, SPT reactivity to the extract of palm tree (whose main or only allergen is profilin) was almost double as frequent among patients with IgE to CCD (Table S1).

- IgE sensitization to allergen molecules—Among patients with IgE to CCD, 256 (97%) had IgE antibodies to rPhl p 1, while 158 (63.7%) had IgE to recombinant Phl p 4. IgE sensitization to Phl p molecules and Ole e 1 were those most strongly associated with IgE to CCD ($p < .001$) (Table 2). The prevalence of IgE to CCD in grass pollen allergic patients was also highly related (OR = 1.84 (CI 95% 1.67–2.03), $p < .001$) to the number of allergen molecules of timothy grass recognized, ranging from 0% in patients with no apparent response to almost 80% in those with sIgE to eight *Phleum pratense* allergen molecules (Figure 1).

3.2 | Relationship between IgE levels to CCD, allergen extracts, molecules and total IgE

Among the 275 patients with IgE to CCD, we examined the distribution of the levels of these antibodies (Figure 2), the relationship between the levels of IgE to CCD and those of IgE antibodies to allergen extracts (Figure 3A), their initiator molecules (major allergens that start sensitization cascades) (Figure 3B) and all the molecules of timothy grass (Figure 3C).

- Distribution of CCD-sIgE levels—In 103 (37%) patients, the CCD-sIgE antibody levels ranged between 0.35 and 1 kU/L, in 143 (52%) between 1 and 10 kU/L and only in 29 cases (11%) above 10 kU/L. (Figure 2).
- Relationship of CCD-sIgE levels with those of IgE to other allergens—IgE levels to CCD were more related to those of IgE to the extracts of Timothy and Bermuda grasses (Figure 3A) and their initiator molecules Phl p 1 and Cyn d 1 (Figure 3B) than to the levels of IgE to other pollen, such as Pellitory, and Olive (Figure 3A) and their initiator molecules Par j 2 and Ole e 1 (Figure 3B). No correlation was observed between the levels of IgE to CCD and those of IgE to *Dermatophagoides pteronyssinus*, cat and Alternaria (Figure 3A). Lastly, the analysis within Phleum pratense showed a hierarchy of correlation between the levels of IgE to CCD and those of IgE to each of the eight molecules examined (Spearmann correlation coefficient), with rPhl p 1 and rPhl p 7 showing the best and the worst correlation, respectively (Figure 3C).
- Relationship of CCD-sIgE levels with those of total IgE—IgE levels to CCD were related to tIgE levels (Figure S1). However, the average ratio between CCD-sIgE and tIgE was as low as <1%.

3.3 | IgE to CCD in the MAS birth cohort

Within the 1314 participants in the follow-up at 20y of the MAS birth cohort, 612 were examined. Of these, 330 (54%) were atopic and 319 had available aliquotes, and therefore had CCD-sIgE determination done in their serum. Only 24 subjects were positive, with a prevalence of 3.9% (24/612) in the whole population sample and of 7.5% (24/319) in the examined atopic fraction (Figure S2). Major characteristics of the population sample, by serological condition, are shown in the e-repository (Table S2). From these 24 subjects, a number of 7, 9, 8, 2, 17, 18, 20, 17, 19 and 24 sera were collected at 1, 2, 3, 4, 5, 6, 7, 10, 13 and 20 years of age, respectively, for a total of 141 serum samples (Table 3). Age at first detection of CCD-sIgE was widely distributed from 2 years to 20 years of age, with 3 children showing a first response already within the first 3 years of life, 10 other children between the 5th and the 7th years, and two, six and three children at 10, 13 and 20 years of age, respectively (Table 3). Interestingly, once started, the IgE response to CCD persisted in 23/24 (95%) subjects and was transiently remittent in one case only (Table 3). A deeper analysis showed that at the time of first detection of their IgE sensitization to CCD: (A) almost all the 24 children were already sensitized to more than one airborne extract among grass and birch pollen, mite, cat and dog; (B) all but two (22/24, 90%) were already sensitized to grass pollen, in most cases at high or very high intensity; (C) many of the grass pollen-sensitized children already presented IgE to many different allergen molecules of Phleum pratense; and (D) children sensitized to grass pollen were already sensitized to Phl p 1, while three were not sensitized to Phl p 5, and two were not sensitized to Phl p 4 (Table 4).

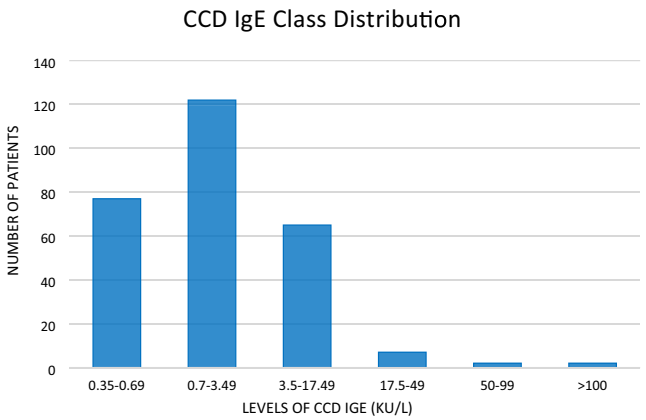


FIGURE 2 Frequency distribution of CCD IgE levels in the PanPed population. Distribution of IgE antibody levels to bromelain MUXF3 (>0.35 kU/L) among 275 CCD-sIgE positive Italian children with seasonal allergic rhino-conjunctivitis.

4 | DISCUSSION

In our Italian and German paediatric populations, we found that IgE response to CCD: (1) is very frequent among children with pollen allergy; (2) is associated, in both populations, with an IgE response to grass pollen; (3) emerges with IgE sensitization to group 1 and 4 allergen molecules of grasses; (4) can start early in life (even at preschool age); and (5) once started, is almost invariably persistent. To our knowledge, this is the first study evaluating the IgE to CCD in paediatric patients and including a longitudinal design.

4.1 | CCD prevalence in our study populations

The prevalence rate of CCD-sIgE was very high in our Italian population, composed mostly of grass pollen allergic children. However, this prevalence is consistently lower than those reported 20 years ago among Italian pollen allergic adults (31%) or their polysensitized subset (71%).[7] Although from a relatively similar setting, the mean age of our population was much lower, suggesting that IgE sensitization to CCD may also start after the first two decades of

FIGURE 3 Correlation between CCD-sIgE levels and allergen-sIgE levels. The relationship between the serum levels of IgE to bromelain MUXF3 and the serum levels of IgE antibodies to (A) Allergen extracts—Bermuda grass, Timothy grass, Birch, Olive, Cypress, English Plantain, Mugwort, Ragweed, Alternaria, Pellitory, HDM and Cat, (B) Initiator molecules—Cyn d 1, Phl p 1, Bet v 1, Ole e 1, Cup a 1, Par j 2, Art v 1 and Pru p 3, and (C) Molecules of Phleum pratense—Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 7, Phl p 11, Phl p 12. Units are shown as kU/L.

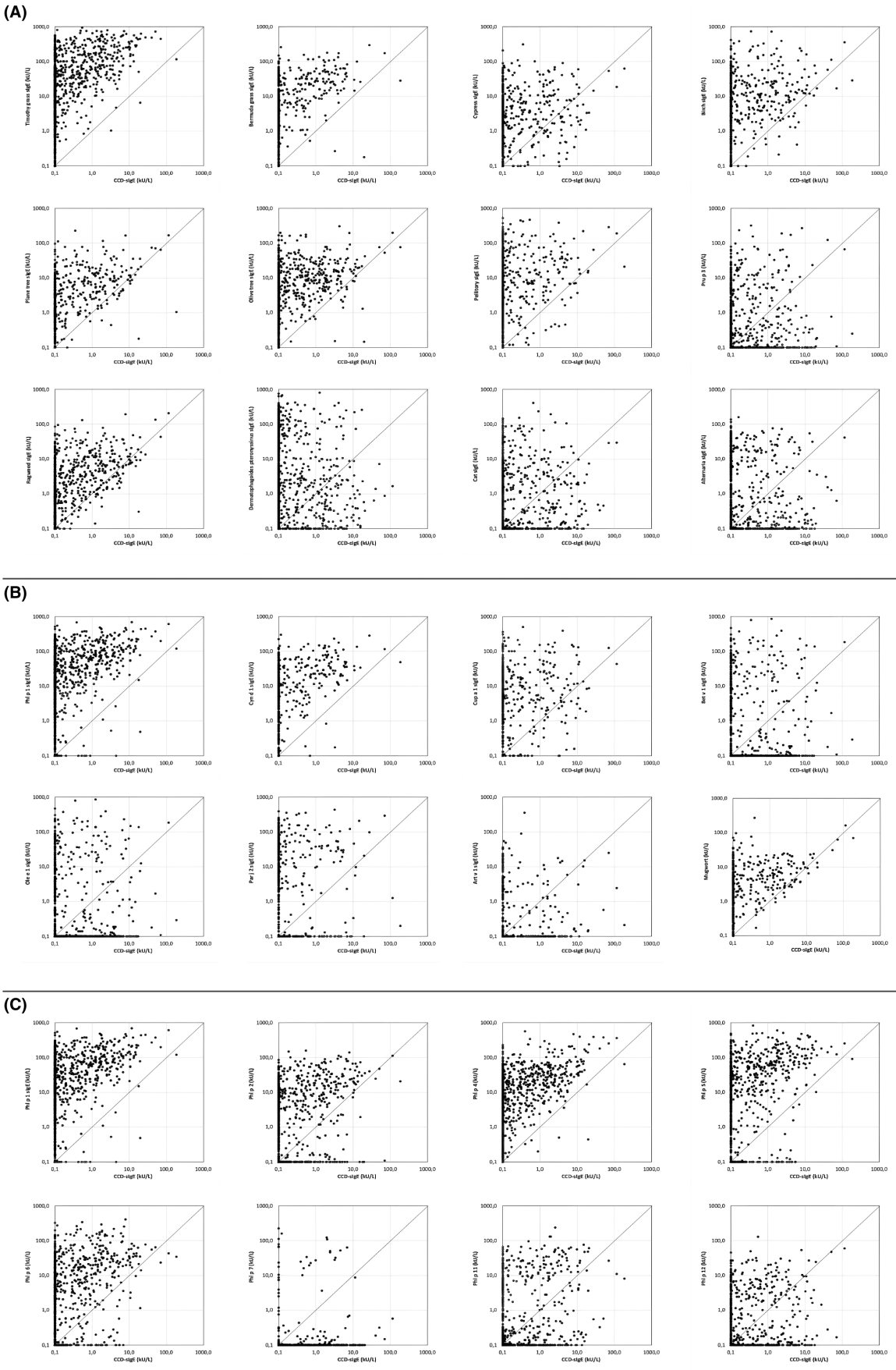


TABLE 3 IgE values to CCD at all tested time-points for the 24 CCD-positive subjects of MAS at 20 years of age.

	Age									
Sample	1	2	3	4	5	6	7	10	13	20
#1	<0.1	1.52[a]				0.95[b]	0.40	1.38	0.79	1.29
#2		0.80	2.07		2.89	4.38	4.46		2.38	0.60
#3		<0.1	4.34		2.22	2.20	4.82	3.54	5.77	3.49
#4		<0.1			5.88	9.21		18.80	9.04	3.22
#5					0.52	0.14		0.45		0.37
#6	0.10				2.36	3.23	4.38	18.23	9.97	6.36
#7				<0.1	<0.1	0.36	0.61	9.51	13.42	0.81
#8					0.27	0.44	0.64	0.79	0.29	0.42
#9	<0.1				0.30	2.33	2.88	4.35	15.16	2.17
#10	<0.1	<0.1		0.28		0.58	1.33	4.70	3.25	1.31
#11					<0.1		1.01	16.24		1.21
#12			0.34			0.14	0.53			1.70
#13					<0.1	0.26	0.51	0.73	1.95	0.63
#14					<0.1			1.13		1.76
#15	<0.1						0.18	2.55	2.23	0.54
#16		<0.1	<0.1		<0.1	<0.1	<0.1	0.19	0.53	0.47
#17							<0.1		0.35	0.37
#18					<0.1	<0.1	<0.1	0.13	3.35	12.67
#19		<0.1	<0.1		<0.1	<0.1	<0.1		0.54	1.67
#20									0.50	0.45
#21	<0.1	<0.1			<0.1	<0.1	<0.1		10.75	2.23
#22	<0.1	<0.1	<0.1		<0.1	<0.1	<0.1			0.49
#23			<0.1		<0.1	<0.1	<0.1	<0.1	<0.1	0.76
#24			<0.1				0.13	<0.1	0.10	0.49

[a]Values in orange show onset of CCD positivity for each subject.

[b]Values in green show CCD positivity for the following time points after the onset.

life. The steady incidence observed in our MAS study population, as discussed below, further supports this interpretation. On the other hand, the low and higher frequency of IgE to CCD in the MAS and PAN-PED cohorts, respectively, is reflecting the different target population, coming from the general or selective pollen allergic population. The prevalence rate to CCD may also change geographically, reflecting the different sensitization profiles and patterns that exist throughout the world, and therefore systematic studies on this topic are required. The methodology used in our study (Hycor platform) differs from the one used in the majority of other studies (ImmunoCAP). Nevertheless, this should not originate discrepancies in CCD positivity, as the systems have been compared previously by our group and showed good correlation (data not shown).

4.2 | CCD-sIgE is mainly associated with IgE to grass pollen extract and its molecules

CCD-sIgE was primarily associated in both population samples with IgE sensitization to pollen, and especially grass pollen. Albeit this association is expected among the Italian population of patients with history of pollen-induced allergic rhinitis, the fact that it was also observed in the MAS cohort is very interesting and rebuts possible bias of results arising from the characteristic of the cohort. Moreover, considering the much lower prevalence of venom allergy, compared to pollen allergy, in any general population of westernized countries, it can be inferred that pollen origin of CCD-sIgE is also more frequent in the general population. Actually, insect venom allergy is quite infrequent in childhood, and although it is well known that insect venom is also an inducer of IgE to CCD,[22] the epidemiological impact of this induction mechanism is probably marginal in the general population. This finding confirms previous observations that some pollen is more active than other in inducing CCD-sIgE. We did not find, for example, any birch pollen monosensitized patients with CCD-sIgE, which is in line with previous reports.[23,24] We cannot conclude, however, that grass pollen was the only inducer of CCD-sIgE in our Italian patients. In particular, IgE sensitization to cypress was also very frequent, and a few patients with CCD-sIgE were sensitized to cypress but not to grass pollen. Hence, we may suspect that in a small minority of patients, cypress extract and its major allergenic molecule (Cup a 1) may have contributed to generate a CCD-sIgE response. By expanding our analysis at molecular

TABLE 4 IgE sensitization profile of 24 MAS cohort participants at onset of their IgE response to CCD.

		Allergens															
Sample	Age Onset	CCD	Cat	Dog	HDM	Birch	Timothy Grass	Phl p 1	Phl p 2	Phl p 4	Phl p 5	Phl p 6	Phl p 7	Phl p 11	Phl p 12	Cyn d 1	Ole e 1
#1	2	0.95	<0.1	0.55	0.57	2.18	84.10	20.81	5.88	15.43	24.03	1.40	<0.1	5.49	<0.1	14.01	0.47
#2	2	0.80	<0.1		<0.1	<0.1	3.18	8.22	<0.1	0.34	<0.1	<0.1	<0.1	<0.1	<0.1	2.62	<0.1
#3	3	4.34	0.37	<0.1	<0.1	<0.1	51.10	22.76	0.38	5.79	47.55	6.75	<0.1	<0.1	<0.1	12.52	<0.1
#4	5	5.88	4.94	<0.1	<0.1	8.64	70.20	19.22	<0.1	18.58	75.60	8.86	<0.1	<0.1	5.90	19.58	<0.1
#5	5	0.52	<0.1	<0.1	79.50	<0.1	<0.1										
#6	5	2.36	82.90	6.07	6.76	10.60	99.00										
#7	6	0.36	<0.1	0.76	<0.1	2.23	93.00										
#8	6	0.27	0.46	<0.1	0.89	0.53	<0.1										
#9	6	0.30	<0.1	<0.1	0.65	99.00	82.10	22.06	28.58	13.57	58.30	15.96	<0.1	<0.1	<0.1	5.20	<0.1
#10	6	0.58	0.92	<0.1	<0.1	<0.1	7.14	7.37	<0.1	5.48	1.09	<0.1	<0.1	0.69	<0.1	0.80	<0.1
#11	7	1.01	<0.1	<0.1	<0.1	2.22	85.20	17.35	<0.1	23.73	84.35	17.44	<0.1	32.05	<0.1	16.00	<0.1
#12	7	0.14	<0.1	<0.1	<0.1	<0.1	3.98										
#13	7	0.26	<0.1	0.54	<0.1	23.10	12.10	6.28	<0.1	3.29	<0.1	<0.1	<0.1	<0.1	<0.1	4.73	<0.1
#14	10	1.13	<0.1	<0.1	<0.1	1.04	48.30	24.81	<0.1	24.59	70.12	13.36	<0.1	<0.1	<0.1	19.46	<0.1
#15	10	0.18	<0.1	<0.1	0.62	<0.1	25.70	7.87	<0.1	9.23	1.67	<0.1	<0.1	<0.1	<0.1	3.04	<0.1
#16	13	0.19	<0.1	<0.1	<0.1	<0.1	27.90	10.63	<0.1	11.69	30.83	2.88	<0.1	<0.1	<0.1	5.20	<0.1
#17	13	0.35	<0.1	0.50	0.73	0.42	13.10	4.36	<0.1	0.25	<0.1	<0.1	<0.1	<0.1	<0.1	0.76	<0.1
#18	13	0.13	<0.1	1.38	1.95	2.01	100.00	107.30	<0.1	5.84	120.04	32.17	<0.1	<0.1	<0.1	93.68	1.21
#19	13	0.54	0.82	0.51	1.43	8.99	100.00	48.76	54.62	2.44	27.12	0.78	<0.1	42.30	<0.1	37.65	<0.1
#20	13	0.50	<0.1	<0.1	<0.1	2.76	6.07										
#21	13	10.75	1.45	15.50	24.50	36.30	43.20										
#22	20	0.49	0.22	0.38	<0.1	6.09	76.80										
#23	20	0.76	0.16	0.33	0.19	1.81	2.20										
#24	20	0.13	<0.1	<0.1	<0.1	<0.1	58.70	14.86	8.22	19.70	23.88	4.58	<0.1	<0.1	<0.1	14.43	<0.1

level, we found that the association, both qualitative and quantitative, of CCD-sIgE with IgE to Phl p 1, was stronger than that to other "initiator" molecules, such as Bet v 1, Cup a 1, Ole e 1, Art v 1, Par j 2. However, a strong association was also found between the levels of CCD-sIgE and those of IgE to nPhl p 4. Taken together, this data confirm that grass pollen can be a major inducer of CCD-sIgE[3,7] and suggests that Phl p 1 and Phl p 4 may both contribute to induce this response.[15,25]

4.3 | Onset and persistence of CCD-sIgE antibodies

Our study highlights that the IgE response to CCD may start at any point in the first two decades of life, even at pre-school age. Moreover, once started, this response tends to be persistent over time, at least within the limit of the monitoring period of our birth cohort study. This finding suggests the existence of long-living plasma cells producing IgE to CCD, similar to those existing for any other plasma-cell producing IgE to protein derived B-cell epitopes. Stable incidence and persistence also explain why the prevalence of CCD-sIgE increases with age, and the above discussed remarkable difference in the prevalence of this response in populations polysensitized to pollen allergens and characterized with different average ages.

4.4 | Profile of molecular IgE sensitization at/before onset of CCD-sIgE

The analysis of the IgE molecular sensitization profile before and at the time of the first detection of the IgE response to CCD has been quite informative. Phl p 1, not Phl p 4 nor Phl p 5, is invariably observed at the very first detection of a CCD-sIgE response among participants in the MAS birth cohort. A sensitization to Cypress or other potential CCD-sIgE inducer (insect venom, mites) was not found in these sera (not shown), suggesting that Phl p 1, not only Phl p 4, may be an inducer of CCD-sIgE, as previously hypothesized.[15] Indeed, group 1 grass pollen molecules are not only extremely potent allergens[26] but are glycosylated and may express two different forms of CCD.[27] Nevertheless, our data are also consistent with the hypothesis that, in some sera, also Phl p 4, a molecule containing multiple CCDs,[28] has contributed in part or totally to the induction and expansion of the patient's CCD-sIgE response.

4.5 | Biological implications: origin and evolution of the CCD-sIgE response

Our results suggest that IgE to CCD emerge with time during the intra-molecular, B-cell-epitope spreading process of the IgE response. Namely, we hypothesise that the IgE response would start against dominant B-cell epitopes formed by the protein skeleton of the inducing molecule (e.g. Phl p 1, Phl p 4) and only at a later stage, especially in patients with a high atopic propensity, would spread also to the much weaker antigenic determinants, such as CCD. At this very advanced stage, the patient's IgE responses have already involved dominant B-cell epitopes of other allergenic molecules of the same allergen source (e.g. Phl p 5), through a process of intermolecular B-cell epitope spreading, also defined as "molecular spreading",[29] so that the CCD-sIgE positive patient is invariably also polymolecularly sensitized to the allergen source (e.g. grass pollen). The ability to produce IgE to a weak B-cell epitope such as CCD, also implies that the atopic/genetic propensity of the patient to produce IgE responses is strong enough to cause sensitization to other, weaker pollen, thus explaining the strong association between CCD-sIgE and pollen polysensitization. In this perspective, IgE production to CCD would be part of a cognate, antigen-specific T-B cell interaction,[30,31] a hypothesis to be further explored. Interestingly, the hypothesis that CCD-containing initiator molecules are essential to generate an IgE to CCD is further supported by the lack of any IgE to CCD in allergic patients monosensitized to birch pollen, whose initiator is Bet v 1, a non glycosylated allergen molecule.[32] The CCD positive patients within the PAN-PED population presented more frequently OAS and gastrointestinal symtoms, but less anaphylaxis. A possible protective role of CCD IgE, like in the helminth endemic population against asthma, leading to a decrease in anaphylaxis is an interesting hypothesis that should be studied in more depth.

4.6 | Diagnostic implications

The high frequency of CCD-sIgE and the evidence that high levels (i.e. >10 kU/L) of these antibodies can be found in allergic children may have an impact on the diagnostic precision in paediatric allergology. Inhibition tests confirm that these antibodies may generate false positive results not only against extracts containing CCDs[3,15] but also against any reagent, including for example Ara h 2, in diagnostic tests whose solid phase or cellulose matrix contains CCD epitopes.[5] Thus, our results highlight the need of raising awareness among allergists, paediatricians and laboratory doctors about the risk of these false positive results and subsequent unnecessary preventive/therapeutic measures in allergic children.[12,33] The impact of CCDs as culprits of false positive results within non-allergic children and adult populations also merits further investigation.

5 | CONCLUSIONS

In conclusion, our results contribute to dissect the immunological origins, onset, evolution and risk factors of CCD-sIgE response in childhood, and raise the hypothesis that group 1 and/or 4 allergen molecules of grass pollen are major inducers of these antibodies through an antigen-specific, T-B cell cognate interaction.

AUTHOR CONTRIBUTIONS

EP was involved in acquisition of data, laboratory analysis, interpretation of data, and drafted the manuscript. ST, RB, CC, AC, RC, LC,

PC, GdC, MMdG, IDI, ARB, MG, AG, VM, IS, EV, RA, AB, MC, TF, FM, NM, FP, UP, DP, GP, MT, AMZ, GR, LG, KI, AG, CM, FZ, AS, UE, SL and TK were involved in acquisition of data. VP was involved in statistical analysis. GR was involved in acquisition of data and laboratory analysis. PMM was involved in acquisition of data, interpretation of data and drafted the manuscript. All authors reviewed and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

Dr. Matricardi reports grants and personal fees from Euroimmun AG, grants and personal fees from Thermo Fisher Scientific and personal fees from Hycor Biomedical Inc, outside the submitted work. All other authors declared no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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