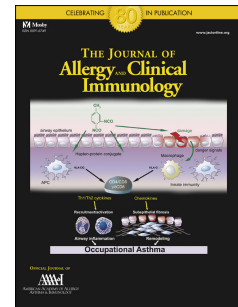


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Immunoglobulin G and G4 to 91 allergenic molecules in early childhood by route of exposure, current and future IgE-sensitization: a MAS birth cohort study

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Abstract

Background: Studies of a limited number of allergens suggested that non-sensitized children produce immunoglobulin G (IgG) responses mainly to foodborne allergens, while IgE-sensitized children also produce strong IgG responses to the respective airborne molecules.

Objective: To systematically test the hypothesis that both, the route of exposure and IgE-sensitization, affect the IgG responses to a broad array of allergenic molecules in early childhood.

Methods: We examined sera of 148 children participating in the Multicentre Allergy Study, a birth cohort born in 1990. IgG to 91 molecules of 42 sources were tested with ImmunoCAP[®]ISAC (TFS, Sweden). IgE-sensitization at age 2y and 7y was defined by $\text{IgE} \geq 0.35 \text{ kU}_A/\text{L}$ to ≥ 1 of 8 or 9 extracts from common allergenic sources, respectively.

Results: The prevalence and geometric mean levels of IgG to allergenic molecules in non-sensitized children were at age 2y lower than in IgE-sensitized children and they were extremely heterogeneous: highest for animal food ($87 \pm 13\%$; 61 ISU/L, [95%CI 52.5-71.5]), intermediate for vegetable food ($48 \pm 27\%$; 13 ISU/L, [95%CI 11,2-16,1]) and lowest for airborne allergens ($24 \pm 20\%$; 3 ISU/L, [CI95% 2.4-3.4])(p for trend < 0.001 [%], p for trend < 0.001 [levels]). IgG4 antibodies were infrequent (<5%) and contributed poorly (<3%) to overall IgG antibody levels. IgG responses at age 2y were slightly more frequent and stronger among children with than in those without IgE sensitization at age 7y.

Conclusion: The children's repertoire of IgG antibodies at 2y of age to a broad array of animal foodborne, vegetable foodborne and airborne allergenic molecules is profoundly dependent on the route of allergen exposure and the child's IgE-sensitization status and only marginally involves the IgG4 isotype.

Key messages: A) The frequency and intensity of the IgG response induced by natural exposure to allergenic molecules is, at 2y of age, highly dependent on the route of allergen exposure, being extremely frequent for animal food allergens, very frequent for vegetable food and moderately frequent for airborne allergens; B) These IgG responses are more frequent and stronger in children with current IgE-sensitization; C) The contribution of IgG4 antibodies to this IgG response, which is induced by natural exposure, is marginal.

Capsule summary: IgG responses to an array of 91 allergenic molecules were significantly more frequent in IgE-sensitized than in non-sensitized 2-year-old probands of the MAS birth cohort study, and they followed in both population subsets the same order (animal foodborne>vegetable foodborne>airborne).

Author Keywords

Allergenic molecules, IgE sensitization, birth cohort, childhood, immunoglobulin E, immunoglobulin G, immunoglobulin G4, microarray, prediction

Abbreviations

CI Confidence interval

D. pt. *Dermatophagoides pteronyssinus*

IgE Immunoglobulin E

IgG Immunoglobulin G

IgG4 Immunoglobulin G4

ISAC ImmunoCAP solid phase allergen chip

ISU ISAC Units

kU Kilounits

MAS Multicentre Allergy Study

PR.10 Pathogenesis-related proteins 10

SD Standard deviation

y Years

Conflict of interest

Johannes Forster's institution received support for travel to meetings and fees for participation in review activities for the study by the German Ministry of Health. Outside the submitted work he received payment for the development of educational presentations from BVKJ (German Association of Pediatricians). Travel expenses were paid for Fred Zepp as an expert for the European Union IMI-Program in March 2015.

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Introduction

The role played by immunoglobulin G (IgG) antibodies in allergic reactions, unlike that of immunoglobulin E (IgE),^{1,2} is still debated. IgG antibodies are considered to be nonpathogenic³ or even protective⁴, and since they occur within the course of as well as after immunotherapy⁵ they are labeled as “blocking antibodies”⁶. However, allergen-specific IgG may also play a role in anaphylactic events⁷ and may develop in parallel with or even preceding IgE antibodies.⁸ IgG has been seen generally as a marker of natural exposure.⁹ Thereby, the IgG response against allergens starts in general as an IgG1-dominated response and, upon continued exposure, may gradually shift towards an IgG4-dominated response¹⁰, a phenomenon also characterizing allergen-specific immunotherapy.¹¹

The IgG responses to animal food are frequent in childhood,¹² whereas little epidemiological information is available about IgG responses to inhalant allergens in early childhood. The impact of allergic predisposition on allergen-specific IgG responses has been investigated only in individual studies, each one focusing on one or a limited variety of allergenic sources, such as mites¹³, pollen¹⁴, pets¹⁵, and a few foods.^{16,17,18} Overall, these studies suggested that the allergen-specific IgG responses are stronger or more frequent in IgE-sensitized than in non-sensitized subjects.¹³⁻¹⁹ It has been also suggested that IgG responses in early childhood to certain allergenic molecules may even predict IgE-sensitization later in life.^{20,21} Altogether, information on the impact of the route of exposure and the allergic predisposition on allergen-specific IgG responses is sparse and no study in early childhood has simultaneously investigated the IgG repertoire to a wide range of molecules.

Before 2000, most studies of allergen-specific IgG responses were performed with assays based on allergenic extracts. These tests were hampered by a high non-specific background signal, caused by extracts' contaminants recognized by the 'normal' human IgG repertoire. The availability of purified native or recombinant allergenic molecules, printed on microarrays, has recently allowed systematic studies of the serum antibody repertoire with a few microliters of serum only.^{8,15} By using this microarray approach, we have recently compared the IgG and IgE responses to a panel of seven foodborne and three airborne pathogenesis-related proteins 10 (PR.10) molecules in birch-sensitized and non-sensitized children of the Multicentre Allergy Study (MAS) cohort.⁸ This study allowed the discrimination of a "default" weak IgG response, driven by *foodborne* PR.10 molecules and detectable in both healthy and birch-sensitized children, from a "(pre-)atopic" strong IgG response, driven by *airborne* PR.10 molecules and detectable in birch-sensitized children only.⁸

The present study, therefore, aimed to test the working hypothesis that IgG responses to environmental non-infectious antigens in infancy are influenced by two major determinants: the route of exposure in early childhood to that antigen and the individual predisposition of IgE-sensitization. To this end, we examined the IgG responses at 2 years (y) of age against 91 allergenic molecules in a subset of 148 children of the MAS cohort, and we related these responses to the presence of IgE-sensitization at 2y and 7y of age.

Methods

Study design and setting

The Multicentre Allergy Study (MAS), a prospective observational birth cohort study, recruited 1314 of 7609 infants born in 1990 on 6 delivery wards in 5 German cities (Berlin, Düsseldorf, Mainz, Freiburg, and Munich). A detailed description of the stratified sampling scheme and study subjects is given elsewhere.^{22,23} The study was approved by the local

ethics committee. Each parent gave written informed consent at the time of enrolment. For the present study only children whose serum data on IgE-sensitization were available at both 2y and 7y of age and whose serum was still available at 2y of age were included.

Definition of IgE-sensitization

IgE-sensitization at 2y or 7y of age was defined as the presence of specific IgE antibodies in the serum (cut-off ≥ 0.35 kU_A/L) (UniCAP FEIA; TFS; Lund, Sweden) to at least one of the common allergenic extracts tested in the MAS birth cohort study at age 2y (milk, egg, soy, wheat, house dust mite, cat, birch and grass) or age 7y (milk, egg, soy, wheat, house dust mite, cat, dog, birch and grass), respectively.²⁴

Assessment of IgG and IgG4 antibodies against allergenic molecules

IgG antibodies to native and recombinant allergenic molecules were tested in sera diluted 1:50 with a multiplex microarray approach (ImmunoCAP ISAC™103 and 112, TFS, Sweden), according to the manufacturer's instructions. The 91 molecules that were available and comparable in both multiplex assays were taken into account for the present analysis. Results were expressed in ISAC units (ISU) per liter, with a cut-off point for positivity of 0.1 ISU/L.¹⁵ IgG4 antibodies against the same molecules have been tested with ImmunoCAP ISAC™ 112, TFS, Sweden and the results have been expressed in ISU/L (cut-off for positivity ≥ 0.1 ISU/L).¹⁵

Assessment of IgG antibodies against allergenic sources

Results obtained at molecular level were also used to analyse outcomes at source level. Molecules belonging to sources with irrelevant exposure in the first 2y of life in Germany (e.g. Brazil nut, pellitory, olive pollen) were excluded from analyses at source level. When two highly cross-reacting molecules belonging to the same source were tested (e.g. Phl p 5 and

Phl p 6), one of them was excluded (e.g. Phl p 6). Highly cross-reacting molecules, belonging to different categories with different routes of exposure (e.g. PR.10 molecules, Profilin, Serum Albumin) have also been excluded, with only the exception of Bet v 1, which has been used as a marker of sensitization to birch pollen. Overall, the analyses at source level included 35 molecules and 16 sources. **[Figure e1]** The presence of serum IgG to a given molecule was defined by a level equal or above 0.1 ISU/L, as previously described.^{8,15} IgG responses to a given allergenic source were defined by the presence of IgG antibodies against at least one of its examined species-specific molecules. The IgG concentration to each allergenic source was calculated by summing-up the positive levels of IgG antibodies to each of the non-cross-reacting molecules belonging to that allergenic source.

Statistical Methods

Comparison of prevalence values - Comparisons of the frequency of general characteristics between participants included and excluded from the present analysis were examined by Chi square test. Comparisons of the general characteristics as well as the prevalence of IgG responses to allergenic molecules and sources between IgE-sensitized and non-sensitized participants at 2y or 7y of age were examined by Chi square test or by the exact Fisher test, when required.

Comparison of levels of IgG to allergenic sources - Geometric mean levels of positive IgG responses to allergenic sources were calculated within the subpopulations of IgE-sensitized and non-sensitized subjects and expressed in ISU/L. A t test was used to compare the logarithmic average values of IgG levels to allergenic sources of sensitized versus non-sensitized children at 2y and 7y of age.

Comparison of prevalence of IgG responses to the three allergenic categories - The mean prevalence (and its standard deviation, SD) of IgG responses to the allergenic

molecules within the three categories of animal foodborne, vegetable foodborne and airborne allergens were calculated in IgE-sensitized and non-sensitized children at 2y of age. These mean values were then compared and a p value was estimated using a mixed logistic model, where subjects accounted for the random effects. A mixed logistic regression was also used to evaluate the trend of IgG prevalence across the three categories of exposure. In this last analysis, the three categories were hierarchically ordered according to the proportion and dose of ingested allergen: animal foodborne (ingested early in infancy and at high frequency and dose), vegetable foodborne (ingested later in infancy or at a lower frequency and dose), and airborne (inhaled at minimal doses).

Comparison of the levels of IgG responses to the three allergenic categories - The overall concentration of IgG antibodies to animal foodborne, vegetable foodborne and airborne allergens were calculated in each participant by summing-up the positive levels of IgG antibodies against each of the molecules belonging to the respective category. The geometric mean (95% Confidence interval, CI) of these values was obtained in the groups of IgE-sensitized and non-sensitized children at 2y of age, respectively. Then a t test on logarithmic values was used to compare the category level of IgE-sensitized versus non-sensitized children at 2y of age.

Software and p values - Statistical analyses were performed using Stata 13.1 (StataCorp, College Station, Tex). A p value <0.05 was considered as statistically significant.

Results

Participants - Overall, 148 children (75 males) of the 1314 children recruited in the MAS birth cohort were included in the present study. The included children did not differ significantly from those not included regarding sex, parental education and parental history of allergy, whereas they had more frequently German nationality, older siblings, and they had

been more frequently breastfed. Among the 148 participants, 25 (17%) children were IgE-sensitized at 2y of age and 51 (34%) at 7y of age. **[Table e1]** At 2y of age most of the IgE-sensitized children (52%) were sensitized to milk (foodborne), compared to only a quarter (25%) at 7y of age. On the other hand, at 7y of age more than half of the children (65%) were sensitized to grass (airborne), compared to only 24% at 2y of age. **[Table e2]** The children with IgE-sensitization at 2y of age did not significantly differ from the non-sensitized ones in regard to sex, nationality, parental education, older siblings, breast-feeding and parental history of allergy. 64% of the IgE-sensitized children at 2y were still sensitized at 7y of age, whereas only 28% of the children non-sensitized at 2y of age became sensitized at 7y of age. **[Table 1]** Interestingly, 97% of those seroconverted children became sensitized to at least one airborne source at 7y of age, whereas just 37% became sensitized to a foodborne source. Only 4 of the 9 children losing their IgE-sensitization at age 7y were sensitized to foodborne sources, while 5 were sensitized to airborne sources at age 2y. **[Table e3]**

Prevalence of IgG responses in non-sensitized children - The frequency of the IgG responses to the 91 molecules was extremely heterogeneous in non-sensitized children at 2y of age and ranged from 0% (Art v1, mugwort) to 98% (Bos d 5, milk). **[Figure 1]** IgG responses to foodborne and airborne molecules tended to rank in the upper and lower half of the prevalence spectrum, respectively. Among IgG responses to food proteins, the five to cow milk proteins ranked all in the first five positions, while IgG responses to Gal d 2 and Gal d 1 ranked 6th and 9th in the spectrum. The majority of the IgG responses to vegetable food proteins appeared in the first half of the spectrum. **[Figure 1]** A few extreme exceptions to these trends can be observed: for example, IgG to nGal d 5 (egg) ranked 90th, IgG to Phl p 1 and Phl p 4 (timothy grass) 14th and 12th, respectively, while IgG to Der f 1 (*Dermatophagoides farinae*) and Bla g 1 (*Blattella germanica*), could be observed in the 11th and 8th position. **[Figure 1]**

Prevalence of IgG responses in IgE-sensitized children - A higher percentage of IgG responses to allergenic molecules was detected among IgE-sensitized vs non-sensitized children (45% vs 36%, $p < 0.001$). A statistical significance was reached in the case of 19 molecules, including: Gal d 5 (egg), Act d 1 (kiwi), Ana o 2 (cashewnut), Ara h 1, Ara h 2, Ara h 3 (peanut), Cor a 1.0401 (hazelnut), Gly m 5, Gly m 6 (soy), Can f 3 (dog), Fel d 4 (cat), Der p 1, Der p 2 (*Dermatophagoides pteronyssinus*, *D. pt.*), Alt a 6 (*Alternaria*), Art v 3 (mugwort), Cup a 1 (cypress), Phl p 4 (timothy grass) Phl p 5 (timothy grass), Pla a 2 (plane tree). By contrast, IgG responses to only 2 molecules (Hev b 8 (latex), Act d 5 (kiwi)) were significantly less frequent in the sensitized than in the non-sensitized group. **[Figure 1]**

Prevalence and levels of IgG4 responses – We detected IgG4 responses to only 38 of the 91 examined molecules. The prevalence of the observed responses ranged from 1% to less than 20% in most cases (33/38) and overcame 20% for five molecules only (Bos d 4, Bos d 5, Bos d 8, Gal d 2 and Der p 2). **[Figure e2]** In a subset analysis of 21 participants, IgG4 was positive only in <5% of all tests performed and represented less than 3% of the IgG level in sera positive for both, IgG and IgG4, tested on the same laboratory session **[Table 2, Figure e3]**. IgG4 responses to most of the 38 molecules were more frequent or exclusively seen in participants with IgE-sensitization **[Figure e4]**. Nevertheless, the propensity of IgE-sensitized children to produce a more frequent and stronger IgG response **[Figure 1]** persisted when the sera with IgG4 responses to the examined molecule were excluded from the comparison **[Figure e5]**.

Prevalence and concentration of IgG responses to allergenic sources - To test our working hypothesis, we analysed the frequency and concentration of detectable IgG responses in IgE-sensitized and non-sensitized children at 2y of age at source level. **[Figure 2]** Reflecting the trends at molecular level, the IgG responses to (A) animal foodborne sources (milk and egg) were detected in over 90%. To (B) vegetable foodborne sources they ranged between 47% (sesame) and 86% (hazelnut), and to (C) airborne sources between 16% (birch) and 79% (timothy grass). In each source the prevalence of IgG responses

tended to be higher in IgE-sensitized than in non-sensitized children and this difference was significant for cashew nut ($p<0.05$), peanut ($p<0.01$) and *D. pt.* ($p<0.01$). The levels of IgG to milk ($p<0.01$), egg ($p<0.01$), kiwi ($p<0.05$), soy ($p<0.05$) and peanut ($p<0.05$) were significantly higher in IgE-sensitized versus non-sensitized children. **[Figure 2]**

Prevalence and concentration of IgG responses by allergen exposure - Within both groups, non-sensitized and IgE-sensitized children, the overall prevalence of IgG responses was highest against animal-foods, intermediate to vegetable-foods, and lowest to airborne allergens (p for trend <0.001). These associations were also replicated when the geometric mean levels of IgG antibodies against the three categories of molecules were analysed. Last, IgE-sensitized children showed significantly higher prevalences of IgG and higher levels of the sum of detectable IgG (with the exception of the airborne group) than their non-sensitized peers in each of the three categories. **[Figure 3]**

Association of IgG to airborne sources at age 2y with IgE to the same sources at age 7y - Overall, IgG responses at 2y of age to the 91 molecules were only slightly more frequent among children with than in those without IgE-sensitization at 7y (40% vs 36%, $p>0.05$). IgG responses to nine molecules only (Gal d 1, Pru p 1, Ara h 3, Act d 1, Act d 8, Der p 2, Art v 3, Can f 1, and Der p 1) were significantly more frequent among children with than in those without IgE-sensitization at 7y of age. **[Figure e6]** Interestingly, 7y old children with an IgE-sensitization to airborne allergenic sources showed a higher prevalence of IgG responses to the same allergen category at 2y of age than their non-sensitized peers, while this association was not found in the case of food allergens. **[Figure e7]**

Discussion

Major outcomes and study novelty - In a subset of 148 children of the MAS birth cohort, we found that – with a few exceptions only – IgG responses in infancy to environmental non-

infectious antigens are (a) associated to the oral route of exposure and (b) to the subject's capacity to produce IgE to common allergens and (c) receive only infrequent and minimal contribution from the IgG4 isotype. To our knowledge, this is the first study describing the IgG repertoire to a broad range of allergenic molecules and their major determinants in a birth cohort.

The impact of exposure on IgG responses - The association between the route of allergen exposure and IgG responses clearly emerged, in our study, at molecular- and source level. For example by the quite impressive difference in the prevalence of IgG production between Cor a 1.0401 and Cor a 1.0101. Cor a 1.0401 (33th position) is a foodborne PR.10, contained in the hazelnut, while Cor a 1.0101 (73th position) is an airborne PR-10, contained in the hazel pollen. **[Figure 1]** A few other studies examined this issue in the previous decades. In an American study IgG antibodies to milk and egg proteins were found in over 95% 2y-old children at risk of allergic diseases.¹² By contrast, only 41 (54%) out of 76 American children, highly exposed to Fel d 1, developed an IgG response to Fel d 1.⁴ Among 31 non-allergic American children, only 30% had an IgG response to Der p 1.¹³ Finally, only one of 14 non-allergic Swedish children had detectable IgG1 to Bet v 1.²⁵ These pieces of information from different populations, each focusing on a quite limited set of molecules or sources,^{4,12,13,25} anticipated the comprehensive scenario shown by our study and suggested that IgG antibodies can be seen as a marker of allergen exposure.⁹ The route of exposure could as well help to explain the opposite trend, when non-sensitized children show more often IgG production than IgE-sensitized children, in some molecules. Because, other than for milk, egg and pollen the exposure to molecules of some allergenic sources (e.g. *Anisakis simplex*, Insect venom, Cod fish, etc.) can be extremely dependent on casual events (ingestion of contaminated fish, insect sting, family dietary habits, etc.). This heterogeneity may therefore explain the trends of IgG responses to Gad c 1, Ani s 1, Act d 5, Api m 4. However, Hev b 8 (profilin) represents an exception to this explanation. Additionally, not just the route but as well the amount of allergen exposure plays a role for the IgE-sensitization. Indeed, a 2-year-

old child who drinks one glass of milk (about 100 grams) a day, annually ingests about 80 grams (i.e. 80 millions of micrograms) of β -lactoglobulin.²⁶ The annual intake of vegetable food protein in European infants is in general lower than that of animal food protein.²⁷ Last, a single grain of grass pollen contains on average only 2.3 *picograms* (one millionth of a microgram) of Phl p 5 and in Germany approximately 2000 grass pollen grains are cumulatively dispersed per m³ per year.²⁸ This suggests that a German child inhales no more than a few *micrograms* of Phl p 5 per year. Our results, therefore, also suggest that not only the route (ingestion versus inhalation) but also the overall amount of allergen exposure determines whether or not an IgG response to allergenic molecules will occur in early childhood. A further determinant could be the antigenic potency of the molecule. IgG responses to Phl p 1, the most potent allergen of *Phleum pratense* and the most frequent initiator of an IgE response to this pollen²², were indeed observed in over 70% of the non-sensitized children. Therefore, one could assume that Phl p 1 is potent in inducing IgG antibodies as well as IgE antibodies.

The impact of IgE-sensitization on IgG responses - Our working hypothesis was that an IgG production in early childhood is a marker for a higher propensity of producing antibodies in general (including IgE). Hence, we examined the association of IgG production with IgE-sensitization to common allergens, rather than to each individual molecule. Our results show, that the higher IgG prevalence in IgE-sensitized children is - at 2y of age - spread all over the 91 molecules and is not strictly limited to the antigen inducing the IgE-sensitization.

Our data clearly show that IgE-sensitized children produce more and stronger IgG responses to allergenic molecules. Our systematic observations have been anticipated by studies limited to a few allergenic molecules.^{13,14,18,20,29,30} Among Swedish children, allergic sensitization or symptoms correlated with high levels of IgG responses to ovalbumin, birch and cat²⁹ and IgG levels to β -Lactoglobulin were higher in patients with mild-moderate atopic dermatitis than in controls.³⁰ In Finnish children, IgG responses to ovalbumin were associated

with allergic disorders.¹⁸ Moreover, serum IgG to rye grass pollen was related to total IgE in American children.¹⁴

Last, the contribution of IgG4 to the observed IgG responses was infrequent (<8% of the IgG positive responses) and – when detected – quantitatively marginal (<3% in the values positive for both, IgG and IgG4). This result is consistent with data showing that IgG4 responses are mostly induced by continuous administration of high levels of allergen (such as in the case of allergen immunotherapy)¹¹ and are negligible in early childhood.⁸ Accordingly, the highest prevalences of IgG4 responses were found in animal food allergenic molecules.

Limitations of the study - We have to acknowledge some limitations of our study. First, the included participants do not represent the whole MAS cohort or the German general population. Therefore, although general conclusions are robust enough, associations concerning individual molecules may be context dependent and less reproducible in different settings. Second, the ISAC test for IgG antibodies may not be sensitive enough to detect low IgG antibody levels. However, this method was validated in previous studies in our⁸ as well as other labs.^{15,31} Third, we did not test IgG1 antibodies, as the ISAC kit is not considering this possibility. Nevertheless, IgG2 and IgG3 responses to allergenic molecules, to our knowledge, are regarded as being irrelevant. Therefore, considering the very low frequency and intensity of IgG4 responses, we speculate that the vast majority of the allergen-specific IgG responses observed in our study population are of the IgG1 isotype. Fourth, as we used the IgE results already produced in 1992 in the MAS,²⁴ relevant allergens, such as peanut and tree nut, are missing in the panel of extracts for defining IgE-sensitization in this study. However, these molecules play a much smaller role in the life of a child as the ones used for the definition of IgE-sensitization. Fifth, as two different ImmunoCAP ISAC™ kits (103 and 112) were used for the assessment of IgG antibodies, the results could be effected. Nonetheless, we have tested the reproducibility to validate comparability of the results across

the two generations and concluded that the “inter-kit” variability among the ISAC versions was similar to the “intra-kit” variability. [not shown, results available on request]

Biological implications – At the moment, a routine determination of IgG to potential allergens in the clinical setting cannot be recommended. However, our study has biological implications. A recent study⁸ of antibody responses to seven foodborne and three airborne PR.10 molecules introduced the concept of (A) a “default” (i.e. physiologic) IgG response, directed mainly at ingested, non-infectious antigens as opposed to (B) a “pre-atopic” IgG response, directed mainly at airborne allergens in children developing also IgE-sensitization. The present study supports and further expands both concepts by systematically analysing a very broad range of molecules from ingested and inhaled allergenic sources, and by showing a greater frequency and concentration of IgG responses to allergens in children with IgE-sensitization at 2y of age.

The functional role of a “default” IgG response is unknown. It may be linked to the development of tolerance, especially to food allergens, or it may be just an epiphenomenon with no functional relevance. We also do not know whether IgG default responses play any role (preventing, facilitating) in the onset or evolution of an “atopic” response. Weak and transient IgE responses may be also a “normal” epiphenomenon accompanying IgG default responses in children with a stronger propensity to produce antibodies in general. Accordingly, we³² and others³³ have shown that weak IgE responses (i.e. $<1 \text{ kU}_A/\text{L}$) in infancy are often transient and a high proportion of them disappear after a few years. Altogether, these responses might be an expression of the fluctuation of the phenotype of T-cell responses to environmental antigens in early life, first described two decades ago in longitudinal studies.³⁴⁻³⁷ On the other hand, this and our previous study of the MAS cohort suggest that IgG to some airborne allergenic proteins (e.g. PR.10)⁸ mark true “pre-atopic” responses, i.e. a response preceding and somehow predicting an IgE response to the same allergens. Accordingly, it has been previously proposed that high levels of IgG to some allergens early in life might predict the development of IgE-sensitization later in life.^{20,21,38} This

area of investigation is therefore very complex and further studies are now needed to define how “default” and “pre-atopic” IgG responses can be better discriminated.

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Legends to Figures

Figure 1 – Prevalence of IgG responses to 91 allergenic molecules in 148 children of the MAS cohort at 2 years of age, by IgE-sensitization at 2 years of age

Red and blue bars are the prevalence of detectable IgG responses (≥ 0.1 ISU/L) at 2 years of age in children with or without IgE-sensitization at 2 years of age. P values were calculated with a chi-square test or exact Fisher test, when appropriate. Significant differences were highlighted (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The column on the right represents the exposure category (animal foods [red], vegetable foods [orange], inhaled [yellow], others [grey]) to which the allergenic sources of individual molecules belong. The marked boxes refer to molecules used for analyses at source level.

Figure 2 – Prevalence and levels of IgG responses to allergenic sources in 148 children of the MAS cohort at 2 years of age, by IgE-sensitization at 2 years of age and main exposure route

Circles show the prevalence (y axis) and the levels (diameter) of detectable IgG responses (≥ 0.1 ISU/L) to the allergenic sources (see methods for definition) at 2 years of age in children with (red) or without (blue) IgE-sensitization at 2 years of age. P values refer to comparisons of the prevalences and levels and were calculated with a chi-square test and a t test, respectively. Statistically significant differences were highlighted (* $p < 0.05$ for prevalence, $p < 0.05$ for level).

Figure 3 -- Average prevalences and levels of IgG responses to allergenic molecules, by exposure route, in 123 non-sensitized and 25 IgE-sensitized 2y-old children

Table 1 - Characteristics of the sensitized and non-sensitized children at 2 years of age

	IgE sensitized at 2y (n=25)*		non-sensitized at 2y (n=123)*		p°
	n	%	n	%	
Males	16	64	59	48	0,144
Nationality					
German	23	100	121	99	0,663
Other origin	0	0	1	1	
Parental education ($\geq 12y$)	16	64	55	45	0,091
Older siblings	13	52	64	52	0,998
Breast-feeding (>1 month)	21	84	96	78	0,569
Parental history of allergy (at least one parent)	14	56	70	57	0,963
IgE sensitized at 7 years of age	16	64	35	28	0,001

* sporadic missing values for a few parameters

°Chi square test is used for comparing frequencies

Table 2 - IgG vs IgG4 to 91 molecules in 21 MAS participants age 2y*

IgG	IgG4	n	%	IgG level°	IgG4 level°	%'
POS	POS	79	4,1	18,7	0,5	2,8
neg	POS	0	0,0	n.a.	n.a.	n.a.
POS	neg	1010	52,9	0,6	n.a.	n.a.
neg	neg	822	43,0	n.a.	n.a.	n.a.

* in all 91X21=1911 tests performed

° ISU/L, geometric mean levels, cut-off ≥ 0.1 ISU/L

n.a. not applicable

%' of the IgG level in subjects positive to both, IgG and IgG4

Table 2 - Average prevalences and levels of IgG responses to allergenic molecules, by exposure route, in 123 non-sensitized and 25 IgE-sensitized 2y-old children

Table 2 - Average prevalences and levels of IgG responses to allergenic molecules, by exposure route, in 225 non-sensitized and 25 IgE-sensitized 2-6-year-old children															
		oral vs inhaled route	amount	n of molecules	sources	Prevalence of IgG (≥ 0.1 ISU/l)*					Levels of IgG†				
						non-sensitized children		IgE sensitized children		p value^	non-sensitized children		IgE sensitized children		p value§
						%	SD	%	SD		mean (ISU/l)	CI 95%	mean (ISU/l)	CI 95%	
1	animal foodborne	oral	high	7	Bos d 4, Bos d 5, Bos d 8, Bos d lactoferrin, Gal d 1, Gal d 2, Gal d 3	87.0	12.6	96.6	7.5	0.002	61.0	52.5-71.5	105.6	75.9-146.9	0.003
2	vegetable foodborne	oral	intermediate	13	Cor a 9, Glym 5, Glym 6, Tri a aA_II, Tri a 19.0101, Ana o 2, Act d 1, Act d 2, Act d 5, Ara h 1, Ara h 2, Ara h 3, Ses i 1	48.5	# 27.1	64.6	# 29.7	<0.001	13.5	11.2-16.1	26.8	19.5-37.0	0.001
3	airborne	inhalative	low	15	Der p 1, Der p 2, Fel d 1, Fel d 4, Can f 1, Can f 2, Phl p 1, Phl p 2, Phl p 5, Phl p 11, Pla a 1, Pla a 2, Alt a 1, Alta 6, Bet v 1	24.0	19.8	35.7	22.5	<0.001	2.9	2.4-3.4	3.9	2.4-6.4	0.134

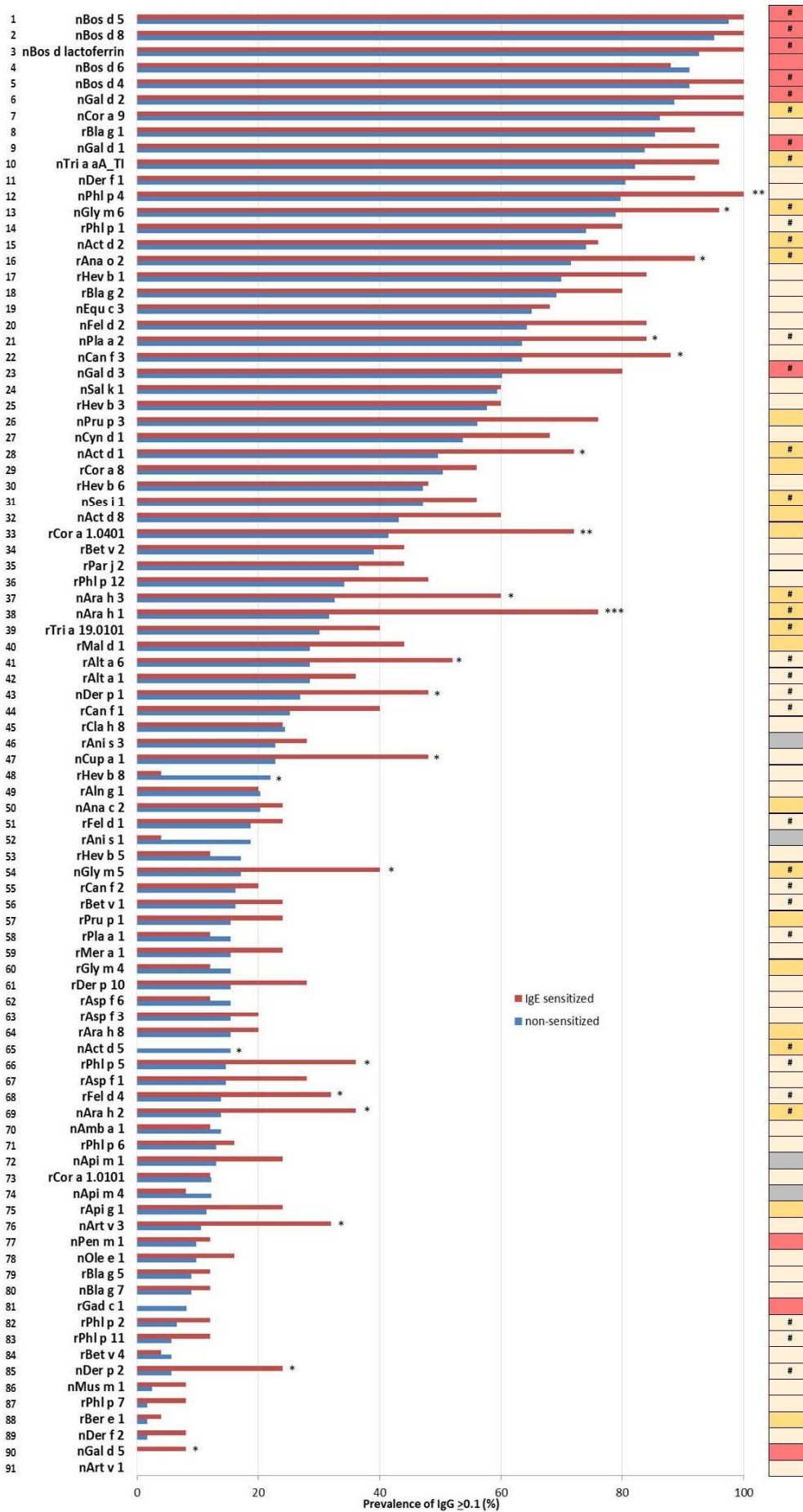
* average ($\pm$ SD) prevalence of detected IgG (≥ 0.1 ISU/L) to each of the molecules in the category

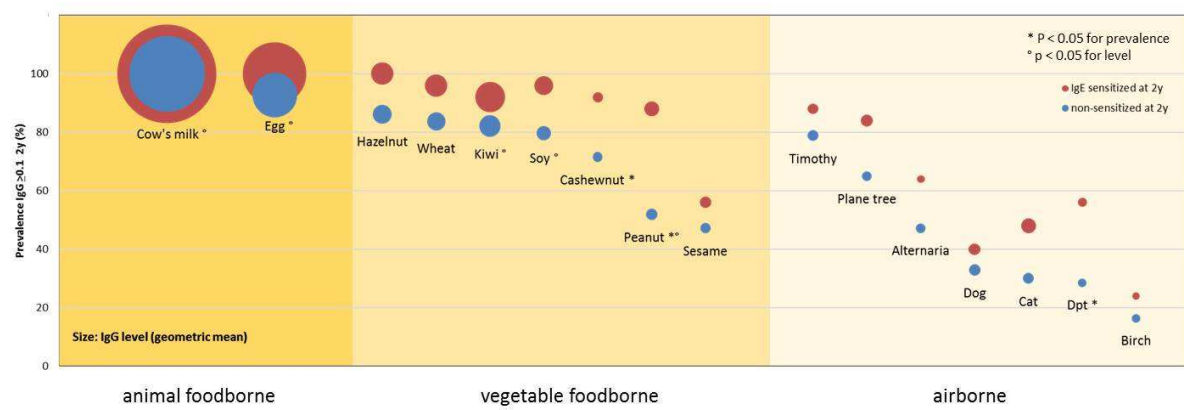
[†] geometric means (CI 95%) of the sum of detectable IgG levels to the molecules in the category (mean level in the category)

[^] calculated by two level mixed logistic model

[§] t student test

p value for trend calculated by mixed logistic regression, $p < 0.001$





ELECTRONIC REPOSITORY

Immunoglobulin G and G4 to 91 allergenic molecules in early childhood by route of exposure, current and future IgE-sensitization: a MAS birth cohort study

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Tables: 5

Figures: 6

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Results

Predictive factor of IgG at 2 years of age

Looking at the difference between weak and strong IgG producers, a tendency can be seen. Just 5% of the children belonging to the quartile of weak IgG producers at 2y were IgE-sensitized at 2y, whereas in the quartile of strong IgG production a higher percentage (22%) was IgE-sensitized. At age 7y that difference was less significant. [Table e4]

IgG prevalences to many foodborne molecules (27 out of 41, 66%) at 2y of age were higher in children IgE-sensitized at 7y than in their non-sensitized peers. Additionally, for more than half of the molecules (28 out of 41, 68%) as well the titers were higher in children sensitized at 7y. However, only in four out of these 28 cases (14%) (Ara h 1, Ara h 2, Gly m 5, Gly m 6) these titers were significantly higher. Thus, justifying that global IgG responses to foodborne molecules at 2y of age do not predict IgE-sensitization at 7y. [Figure e6]

Legends to figures

Figure e1 -- Selection of the molecules at source and category level

Figure e2 – Prevalence of IgG4 responses (cut-off 0.1 ISU/L) to allergenic molecules at 2 years of age in 148 children of the MAS cohort.

Figure e3 – Relationship between the levels of IgG (y-axis) and IgG4 (x-axis) to 91 allergenic molecules in a subset of 21 children of the MAS cohort. Each serum has been tested against the 91 molecules in parallel for IgG and IgG4 antibodies on the same day by the same operator. Overall 1911 comparisons have been performed.

Figure e4 – Prevalence of IgG4 responses to allergenic molecules in 148 children of the MAS cohort at 2 years of age, by IgE-sensitization at 2 years of age. Only the molecules with at least one positive value (≥ 0.1 ISU/L) in either group are shown. P values were calculated with a Chi-square test or exact Fisher test, when appropriate. Significant differences were highlighted (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure e5 – Prevalence of IgG responses to allergenic molecules in 148 children of the MAS cohort at 2 years of age, by IgE-sensitization at 2 years of age. Only the results obtained in sera with no IgG4 response to the examined molecule have been taken into account. IgG4 antibodies, therefore do not contribute to the observed prevalence values. P values were calculated with a Chi-square test or exact Fisher test, when appropriate. Significant differences were highlighted (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure e6 – Prevalence of IgG responses to 91 allergenic molecules at 2 years of age in 148 children of the MAS cohort, by IgE-sensitization at 7 years of age

Red and blue bars are the prevalence of detectable IgG responses (≥ 0.1 ISU/L) at 2 years of age in children with or without IgE-sensitization at 7 years of age. P values were calculated with a chi-square test or exact Fisher test, when appropriate. Statistically significant differences were highlighted (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure e7 – Prevalence and levels of IgG responses to nine allergenic sources at 2 years of age in 148 children of the MAS cohort, by IgE-sensitization to the same sources at 7 years of age.

Red and blue bars represent the prevalence (left panels) and the levels (right panels) at 2 years of age of detectable IgG responses ($\text{IgG} \geq 0.1$ ISU/L) to foodborne (upper panels) and airborne (lower panels) allergenic sources in children with (red) or without (blue) IgE-sensitization to the same source at 7 years of age. P values refer to comparison of the prevalences and were calculated with a chi-square test or Fisher exact test for the separate sources and with two level logistic mixed model to take into account multiple data per subjects for the grouped sources. P values for geometric means were calculated with t test for unpaired data on logarithmic values for separate sources and with two level linear mixed model on log IgG levels. Statistically significant differences were highlighted (* $p < 0.05$

Table e1 - Selection of the molecules at source and category level

Category	Molecules	Inclusion	Reason for exclusion	Category	Molecules	Inclusion	Reason for exclusion
animal foodborne	Cod			airborne	Alder		
	rGad c 1	no	low exposure		rAln g 1	no	PR.10, CR
	Milk				Alternaria		
	nBos d 4	yes			rAlt a 1	yes	
	nBos d 5	yes			rAlt a 6	yes	
	nBos d 6	no	Serumalbumin, CR		Aspergillus		
	nBos d 8	yes			rAsp f 1	no	low exposure
	nBos d lactoferrin	yes			rAsp f 3	no	low exposure
	Egg				rAsp f 6	no	low exposure
	nGal d 1	yes			Bermuda		
vegetable foodborne	nGal d 2	yes			nCyn d 1	no	low exposure
	nGal d 3	yes			Birch		
	nGal d 5	no	Serumalbumin, CR		rBet v 2	no	Profilin, CR
	Shrimps				rBet v 1	yes	
	nPen m 1	no	Tropomiosin, CR		rBet v 4	no	Polcalcin, CR
	Ananas				Cat		
	nAna c 2	no	CCD, CR		rFel d 1	yes	
	Apple				nFel d 2	no	Serumalbumin, CR
	rMal d 1	no	PR.10, CR		rFel d 4	yes	
	Brazilnut				Cladosporium		
other	rBer e 1	no	low exposure		rCla h 8	no	low exposure
	Cashewnut				Cockroach		
	rAna o 2	yes			rBla g 1	no	low exposure
	Hazelnut				rBla g 2	no	low exposure
	nCor a 9	yes			rBla g 5	no	low exposure
	rCor a 8	no	LTP, CR		nBla g 7	no	low exposure, Tropomiosin, CR
	rCor a 1.0401	no	PR.10, CR		Cypress		
	Kiwi				nCup a 1	no	low exposure
	nAct d 1	yes			Dermatophagoides farinae		
	nAct d 2	yes			nDer f 1	no	IgE extract was available for
animal foodborne	nAct d 5	yes			nDer f 2	no	Dpt
	nAct d 8	no	PR.10, CR		Dermatophagoides pteronyssinus		
	Peach				nDer p 1	yes	
	rPru p 1	no	PR.10, CR		nDer p 2	yes	
	nPru p 3	no	LTP, CR		rDer p 10	no	Tropomiosin, CR
	Peanut				Dog		
	nAra h 1	yes			rCan f 1	yes	
	nAra h 2	yes			rCan f 2	yes	
	nAra h 3	yes			nCan f 3	no	Serumalbumin, CR
	rAra h 8	no	PR.10, CR		Hazelnut		
vegetable foodborne	Sellery				rCor a 1.0101	no	PR.10, CR
	rApi g 1	no	PR.10, CR		Horse		
	Sesame				nEqu c 3	no	Serumalbumin, CR
	nSes i 1	yes			Latex		
	Soy				rHev b 1	no	low exposure
	rGly m 4	no	PR.10, CR		rHev b 3	no	low exposure
	nGly m 5	yes			rHev b 5	no	low exposure
	nGly m 6	yes			rHev b 6	no	low exposure
	Wheat				rHev b 8	no	low exposure, Profilin, CR
	nTri a aA_TI	yes			Mercury		
other	rTri a 19.0101	yes			rMer a 1	no	Profilin, CR
	Anisakis				Mouse		
	rAni s 1	no	low exposure		nMus m 1	no	low exposure
	rAni s 3	no	low exposure, Tropomiosin, CR		Mugwort		
	Bee				nArt v 1	no	low exposure
	nApi m 1	no	low exposure		nArt v 3	no	low exposure, PR.10, CR
	nApi m 4	no	low exposure		Olive pollen		
					nOle e 1	no	low exposure
					Plane tree		
					nPla a 2	yes	
					rPla a 1	yes	
animal foodborne					Ragweed		
					nAmb a 1	no	low exposure
					Saltwort		
					nSal k 1	no	low exposure
					Timothy		
					rPhl p 1	yes	
					rPhl p 2	yes	
					nPhl p 4	no	CCD, CR
					rPhl p 5	yes	
					rPhl p 6	no	CR to Phl p 5
vegetable foodborne					rPhl p 7	no	Polcalcin, CR
					rPhl p 11	yes	
					rPhl p 12	no	Profilin, CR
					Wall pellitory		
					rPar j 2	no	LTP, CR

CCD - Cross-reactive carbohydrate determinants

CR - Crossreaction

LTP - Lipid transfer protein

PR.10 - Pathogenesis-related protein 10

Table e1 - Characteristics of the population sample

	included (n=148)*		not included (n=1166)*		p°
	n	%	n	%	
Males	75	51	609	52	0.721
Nationality					
German	144	99	1055	93	0.004
Other origin	1	1	75	7	
Parental education (≥ 12y)	71	49	592	53	0.369
Older siblings	77	52	463	40	0.004
Breast-feeding (>1 month)	117	79	776	68	0.004
Parental history of atopy (at least one parent)	84	57	596	52	0.054
IgE sensitized at 2 years of age	25	17	146	28	0.005
IgE sensitized at 7 years of age	51	34	231	42	0.082

* sporadic missing values for a few parameters

°Chi square test is used for comparing frequencies

Table e2 - Prevalences of IgE sensitized children at 2y and 7y

	IgE sensitized at 2y (n=25)		IgE sensitized at 7y (n=51)		p*
	n	%	n	%	
Milk	13	52	13	25	0,038
Egg	12	48	6	12	0,001
Soy	4	16	10	20	1,000
Wheat	5	20	13	25	0,776
Mite	3	12	19	37	0,031
Cat	7	28	12	24	0,779
Dog	°	°	14	27	-
Birch	4	16	18	35	0,109
Grass	6	24	33	65	0,001

° not tested

*Fisher's exact test was used to compare frequencies between groups.

Table e3 - Characteristics of the sensitized children at 2 and 7 years of age

	IgE sensitized at 2y (n=25)				IgE sensitized at 7y (n=51)			
	IgE sensitized at 7y (n=16)		non-sensitized at 7y (n=9)		IgE sensitized at 2y (n=16)		non-sensitized at 2y (n=35)	
	n	%	n	%	n	%	n	%
foodborne sources[°]	11	69	4	44	11	69	13	37
airborne sources[°]	14	88	5	56	14	88	34	97

[°] sensitized to at least one source

Table e4 - Association between IgG production and IgE sensitization

	lowest quartile of IgG producers at 2y (37)		highest quartile of IgG producers at 2y (37)		p value°
	n	%	n	%	
IgE sensitized at 2y (25)	2	5,4	8	21,6	0,085
IgE sensitized at 7y (51)	11	29,7	13	35,1	0,804

° Fisher's exact test was used to compare frequencies between groups

