The deceptive effect of CCDs (short for “cross-reactive carbohydrate determinants”) in allergy diagnosis

THE DECEPTIVE EFFECT OF CCD’S IN ALLERGY DIAGNOSIS

The principle of blood-based allergy diagnosis is that presence of a ponderable amount of IgE that binds to an allergen indicates an allergic status towards this allergen (see also chapters 3 and 4). This amazingly simple view has been subject to numerous refinements, modifications and additions over the last decades. All too often, a positive laboratory result contradicted a patient´s experience. Contact with a given “allergen” did not cause any adverse effect despite considerable IgE levels towards this allergen (Altmann 2016; Homann, Schramm, and Jappe 2017; Platts-Mills et al. 2021).

Such “mimickers of allergy” (Ebo et al. 2004), may occasionally be proteins, but the major cause of “false-positive” and hence deceptive results are protein-linked N-glycans as they occur on all plant **glycoprotein**, on insect glycoproteins and some other invertebrates (Paschinger et al. 2005; Paschinger and Wilson 2019; Malandain 2005). A very different type of cross-reactive carbohydrate structure is found on proteins of mammals. This carbohydrate determinant works according to other principles and may in some cases lead to relevant symptoms as is discussed elsewhere (Platts-Mills et al. 2021; Roman-Carrasco et al. 2021; Hils et al. 2020).

Glycoproteins? Proteins can be divided into two large groups: Those consisting of amino acids only and the others, which are decorated with sugars - unusual and complex sugars, however. These complex structures have little resemblance to the sugar in your coffee or tea and therefore we call them “glycans” rather than sugars – hence, the term “glycoprotein”. (Fun fact: the Greek syllable “glyc” stands for sweet, but certainly no one has ever tasted such glycans.)

Depending on the origin of the protein, glycans exhibit different structures. The one type of glycans that is of interest here is that linked to an asparagine side chain via a nitrogen (atom symbol: N), hence they are called **N-glycans**. Mammals and thus us humans produce these so-called N-glycans in a way that differs significantly from that practiced

by **plants and insects**. From the various differences, the most significant one is the site to which a fucose is linked to the first building block of an N-glycan (see figures 1 and 2). The occurrence of the "**core alfa-1,3-linked fucose**" on proteins from plants as well insects is the one big difference (red arrows in figure below, (Bencurova et al. 2004; Tretter et al. 1993; Kurosaka et al. 1991; Fotisch and Vieths 2001) and this fucose positioning calls our immune system into action. In other words, the N-glycans on plant and insect glycoproteins are immunogenic. An antiserum raised against any such glycoprotein will thus react with extracts from virtually any plant material, with insect venoms and even smashed beetles or lobsters. Thus, such an antiserum is cross-reactive even though it binds to a well-defined conserved structure in all cases (Bencurova et al. 2004).

What makes this fact critical for the field of allergy diagnosis is that about a quarter of all allergic persons develops IgE directed against these cross-reactive carbohydrate determinants (CCDs), often at sizeable levels (Mari 2002). In a survey of 6000 serum samples, 22 % were CCD-positive, whereby the rate reached 35 % among teenage patients (Holzweber et al. 2013). An even higher percentage of 46 % was found in a cohort of 170 individuals preselected for polysensitization (Luo et al. 2021). These authors observed RAST classes 4 or higher in one third of the CCD-positive patients, in simpler words: rather high levels of anti-CCD IgE.

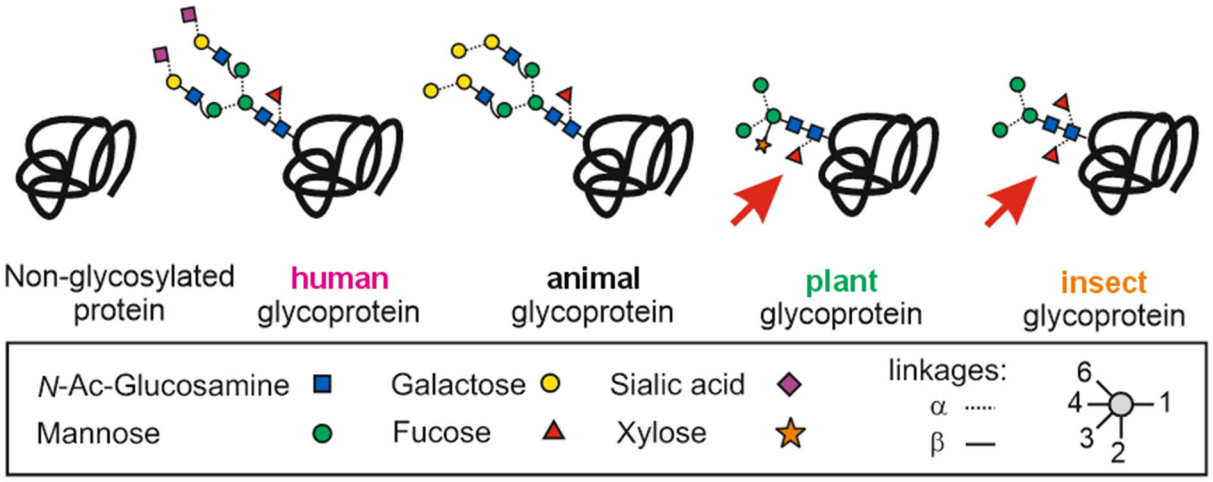

Figure 1. Modes of glycosylation of proteins with emphasis on cross-reactive glycan structures. The red arrows point at the critical "core alfa1,3-fucose" in plant/insect CCD structures.

The plural CCDs is used as we are confronted with a variety of similar structures all containing the **core-alfa1,3-fucose** as the pivotal element. The xylose residue, which does not occur in our own glycoproteins may - in very, very rare cases - also contribute to antibody binding. Terminal substitutions such as in $MGnXF^3$ probably play an inferior role. Noteworthy, presence or absence of the 3-linked mannose has at best a marginal

influence on binding affinity due to the spatial structure of the glycan (Kurosaka et al. 1991).

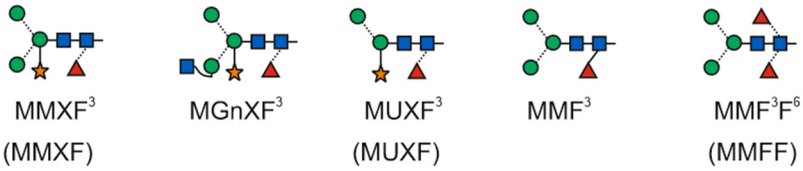

Figure 2. Examples from the variety of structures acting as cross-reactive carbohydrate determinants (CCDs) in proteins from plants and insects.

Following the structural definition of plant/insect CCDs, researchers tried to pinpoint the clinical relevance of anti-CCD IgE. *In vitro* studies at first appeared to substantiate the ability of CCDs to trigger histamine-release and thus allergic symptoms (Foetisch et al. 2003). A broad study with 420 patients with anti-CCD IgE, however, demonstrated that CCD-bearing proteins led to – at worst – very mild symptoms in only a few study individuals (Mari 2002). In the two decades to follow, no opposing view has ever been brought forward.

> **Simply speaking: anti-CCD IgE does not cause allergic symptoms. Conversely, if an allergen reacts with IgE via CCDs only, this will go unnoticed by the patient.**
>
> **However, the same reaction does give a signal when this patient´s sample is tested in the laboratory. Obviously, this is a deceptive, "false-positive" result, even though the measurement did proceed technically correct.**
>
> **Diagnostic results are the basis for preventive measures or therapeutic interventions. Obscuring the true culprit allergen by a number of such "false-positive" results may all too easily lead to ill-targeted therapies. In the worst case, a life-threatening allergy is overlooked and remains untreated.**

Understandably did it take a while to accept and disseminate the concept of "harmless CCDs". Likewise understandable is that the problem of way too many positive results for some patients became obvious only with the introduction of multi-allergen test systems about a decade ago. The cognitive dissonance caused by CCDs does not strike hard when only a small number of single allergens are tested. The doctor´s assumptions are confirmed, the patient is impressed and it takes a while until doubts about the results arise. One may nevertheless counter that this shrugging acceptance of **a large percentage of "false-positive" results** cannot be seen as a tolerable state of the art of single allergen-specific IgE determination.

REMEDIES FOR THE CCD PROBLEM

Three options for answering the CCD problem in blood-based allergy diagnosis are available:

a.) switching to alternative diagnostic methods
b.) use of CCD-free allergen preparations
c.) preventing the binding of anti-CCD IgE

2a) Alternative diagnostic methods

The classical methods skin-prick test and oral-challenge pose the advantage of directly observing what is to be observed at the cost of a high time requirement for the patient and the therapeutic personal. In addition, the number of allergens that can be screened in one session is limited.

An alternative where the cellular processes occurring in the patient´s body upon contact with an allergen are mimicked in a test-tube is the basophil-activation test (Ebo et al. 2021).

A third option shall – if only for the sake of comprehensiveness and entertainment value – not be concealed, and that is bioresonance (Hammond and Lieberman 2018; Wuthrich 2005), which certainly is as good as the anamnestic intuition of the conduction medic.

2b) CCD-free allergen preparations = component-resolved allergy diagnosis

An allergen, be it a pollen, a food, pet hair or an insect venom consists itself of several soluble components, which are the actual causative agents of an allergic episode. The symptom-triggering effect of the different components differs widely. To date, the overwhelming majority of allergen components, almost exclusively proteins, has been identified and their relative importance has been assessed. These proteins can be recombinantly produced in the laboratory.

Therefore, it is possible to use the most relevant allergic components for targeted sIgE determinations either as single components or as multi-component arrays. The perspectives of component-resolved allergy diagnosis are amply discussed elsewhere (Barber et al. 2021; Jakob et al. 2017; Sato and Ebisawa 2024).

In the context of this discourse, two aspects where CCDs play a role even if allergen components are used shall be shown.

First, some single-component tests use (or have used) carrier matrices made of cellulose isolated from cotton. While cellulose per se is a polysaccharide, it does contain proteins which in part carry CCD structures (Hemmer et al. 2018).

Second, a handful of allergen components have so far resisted recombinant expression in bacteria and need to be isolated from their natural source. Unfortunately, these

components (Cry j 1, Cup a 1, Cyn d 1, Ole e 1 and Phl p 4) are glycoproteins carrying CCDs (www.allergome.org).

To conclude, as of now, component resolved allergy diagnosis has not grown out of the CCD problem zone.

2c) Preventing the binding of anti-CCD IgE = use of a CCD-blocker

All of the above-mentioned solutions of the CCD-problem entail resorting to concepts other than the classical ELISA-like formats, often at considerably greater costs. However, there is a genuinely simple and cost-effective solution and that is preventing (blocking) CCD-specific IgE from binding to the immobilized allergen by the use of a CCD blocker.

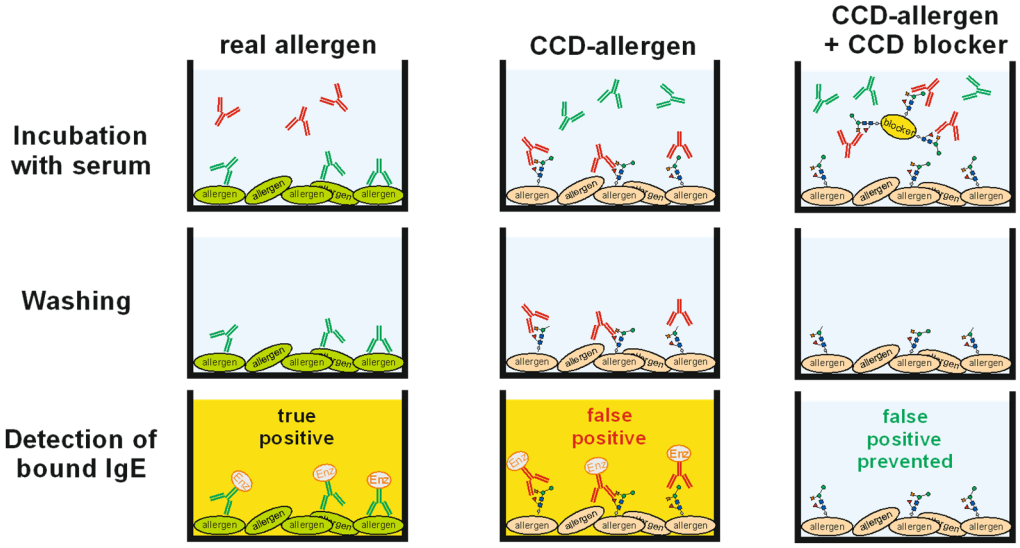

Figure 3. Blocking of anti-CCD IgE with a soluble glycoprotein prevents

For this purpose, Proglycan has developed a polyvalent semi-synthetic glycoprotein that combines high inhibitory efficacy with a conceivably lowest risk of unintended reduction of signals for other components. The volume of **Proglycan CCD-Blocker** added to a serum (or plasma) sample amounts to only 2 % of the sample volume. The effect is obvious as can be seen in Fig. 4.

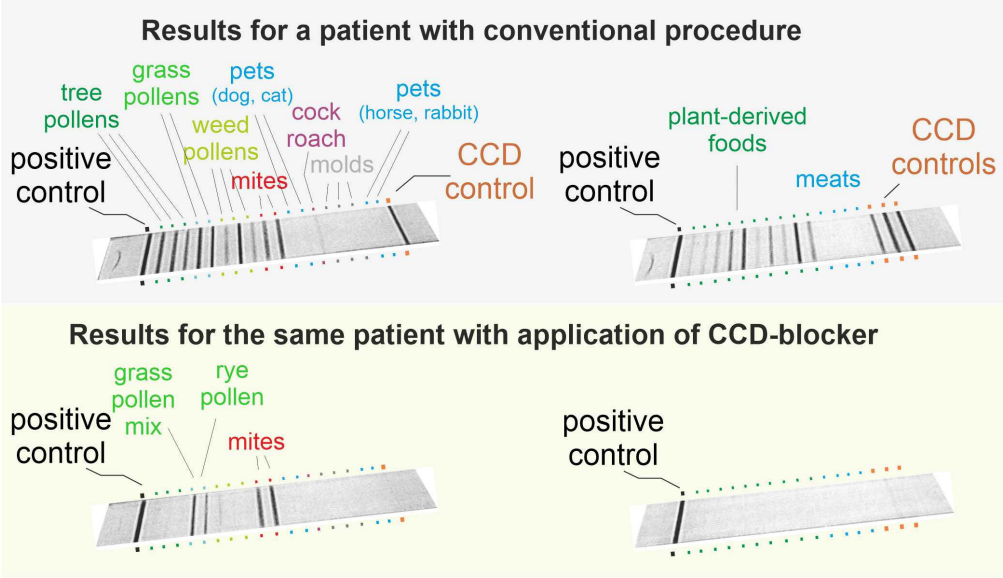

Figure 4. Effect of the addition of CCD-blocker on the number of positive results. Note, that most pollens, cock roach and all plant-foods have turned negative.

The major benefit of the application of the CCD-Blocker is a drastically reduced number of positive results, more exactly of results indicating an sIgE content of < 0.35 U/mL (or kU/L; or < 0.84 ng/mL). Examples of this outcome are shown in Fig. 5 and 6.

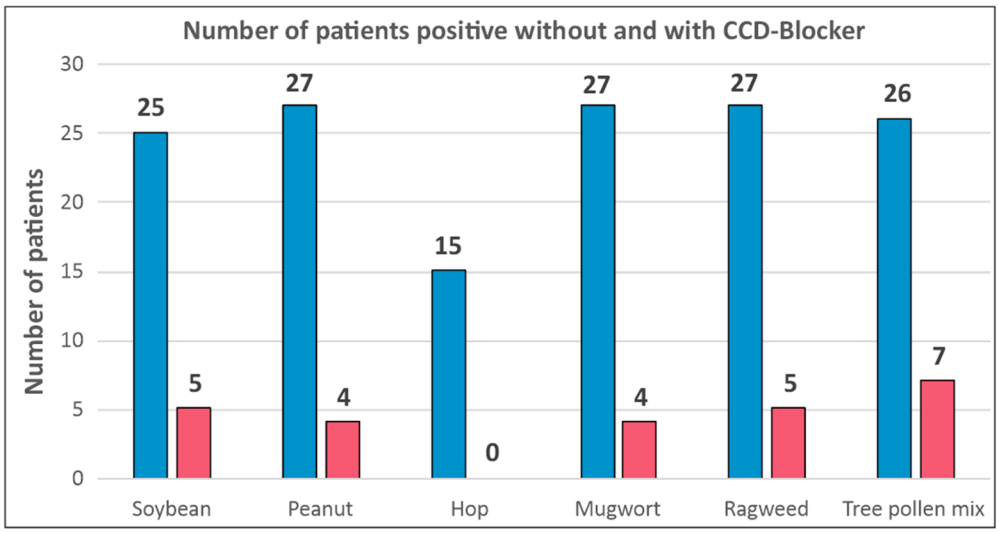

Figure 5. Numbers of sera positive to the given allergen in a cohort of 170 patients without and with use of CCD-Blocker.

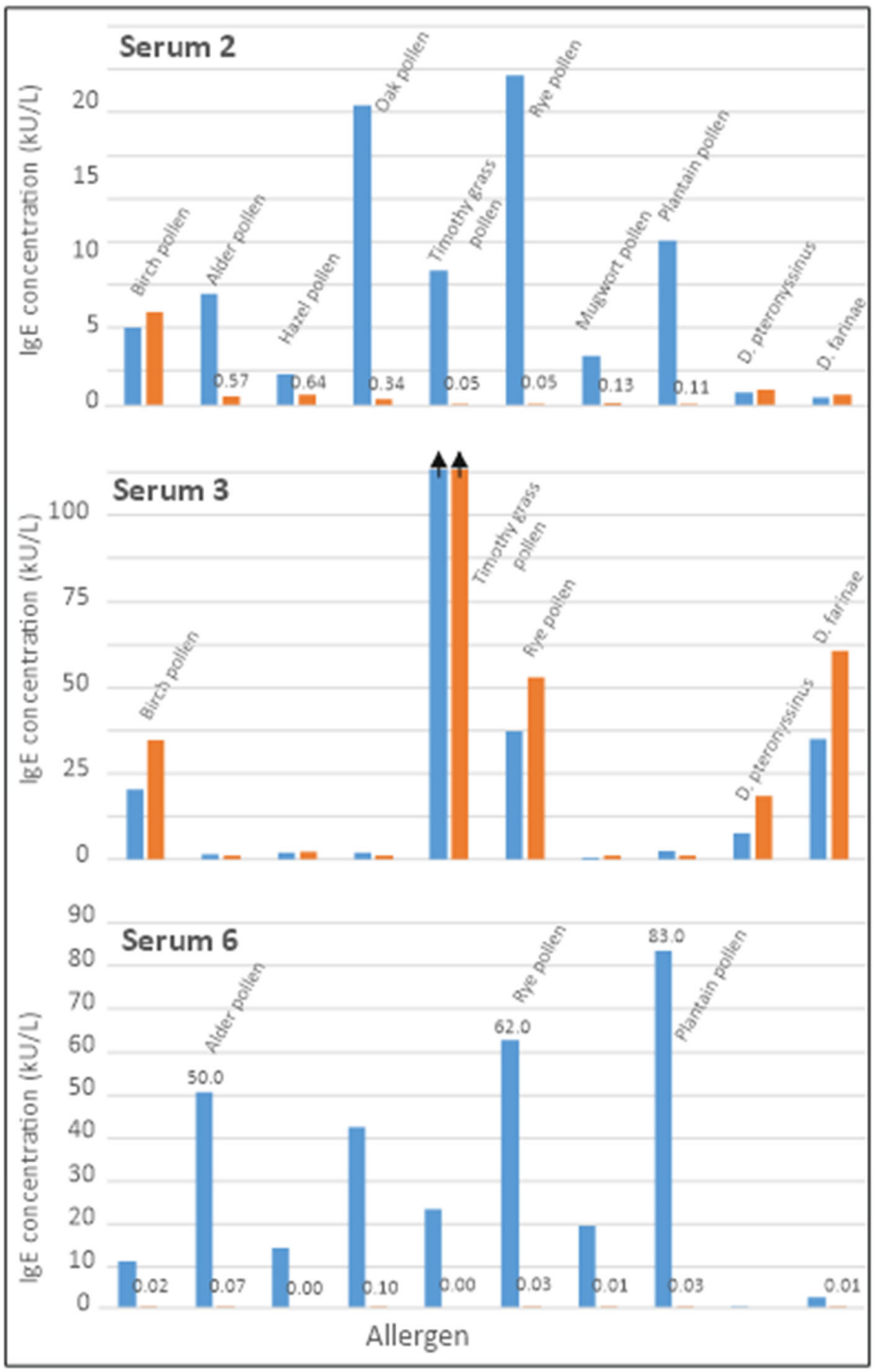

Figure 6.
Numbers of allergens positive in three different patients without and with use of CCD-Blocker.

Data provided by Biocheck GmbH, Münster, generated with the help of Polycheck® Allergy diagnostics.

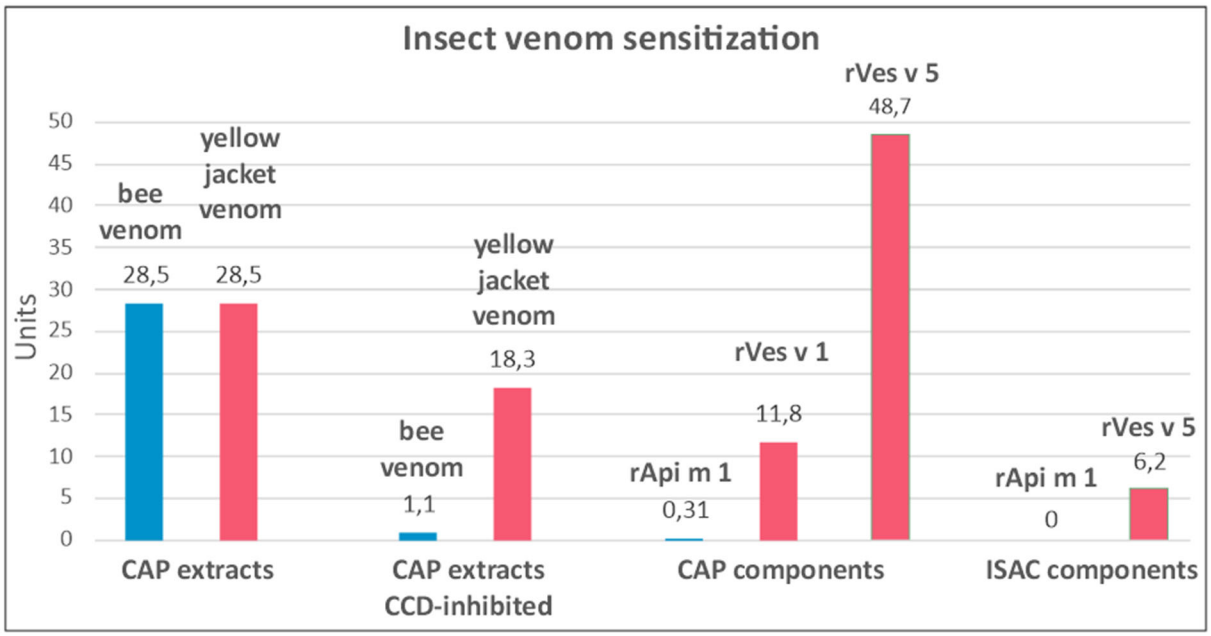

Figure 7. The case of an insect sting sensitized patient with concomitant CCD-reactivity. Classical CAP test with whole venoms indicated double-sensitization. CAP or ISAC with recombinant (CCD-free) components clearly identified yellow jacket venom as the culprit allergen.
The same conclusion could be drawn from CAP with venoms when the CCD-blocker was applied despite an "*only*" 96 % reduction of CCD-based signal.

Remark: Results for CAP are given in U/mL, those for ISAC in a test specific unit.

A POSSIBLE EXPLANATION FOR THE BENIGN NATURE OF CCD'S

Does the harmlessness of CCDs result from their nature as carbohydrates? Though at first sight intuitive, this notion would be an unscientific shortcut. One source for this superficial assumption is the indeed only weak binding of lectins to their carbohydrate binding partners. However, the well-known role of blood-group determinants (the ABO / abh system) and of bacterial surface polysaccharides as highly specific, firmly binding antigen reminded that this is not a general situation.

Figure 8. Illustration of weak versus strong binding of an antibody to its antigen.

To decide if binding strength could explain the performance of CCDs, the effort was undertaken to purify CCD-specific IgE from patients´ blood and determine its binding

affinity to the classical CCD structure MMXF[3]. The same laboratory had just determined the binding strengths of IgE and IgG for relevant protein allergens from grass and birch pollen. This allowed for a direct and thus rather dependable comparison of affinities. Interestingly, for all allergens the IgEs had very high affinities in the nanomolar to subnanomolar range (Hantusch et al. 2005; Jin et al. 2008).

	MMF	MMXF	Phl p 5	Bet v 1
	Dissociation constants (nM)			
IgE	0.6	0.07	0.3	0.02
IgG	21	11	1800	490
ratio	33	160	6400	2200

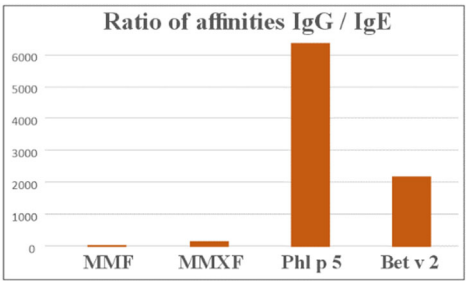

Table 1 / Figure 9. Dissociation constants for the binding of patients´ IgE and IgG to either glycans (bound to protein) or proteins. Note that lower numbers imply higher affinity!

So, while IgEs bound to glycan and peptide epitopes with comparable affinity, the binding strength of the IgGs differed substantially (Fig. 8). In other words: IgG vs. glycan determinants (CCDs) has a comparably much higher affinity than is observed with protein allergens. Now, the serum concentration of IgG is much higher[1] than that of IgE, but with such significantly lower affinities, that IgG cannot compete with IgE.

We assume that the comparably higher IgG affinities observed for MMF[3] and MMXF[3] may be just enough to compete for binding to these determinants. Hence, mast cell degranulation remains below the threshold of perceptibility. An increase of the IgG4 antibodies may contribute to this effect (Grilo et al. 2021).

How does the comparably higher affinity of anti-CCD IgG arise? We speculate that the inevitable frequent contact with plant food from earliest childhood on acts like a specific immunotherapy much the same way as it is performed later in life in the course of targeted desensitization.

[1] Typical concentrations of IgG and IgE in serum are 9000 and 1 µg/mL respectively.

CONCLUSION

Cross-reactive but harmless structures on plant and insect allergens are a frequent cause of false-positive diagnostic results. Professional ethics of those performing allergy diagnosis demands to resolve a problem affecting up to a quarter of the patient population. Application of the Proglycan CCD-blocker constitutes a cost-effective and simple strategy towards dependable diagnostic results for this group of patients.

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Proglycan GmbH, Vienna, November 2024

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