

False-positive results of serological tests for allergy in alcoholics

RUNNING TITLE: Allergy tests in alcoholics

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Abstract

Background: Alcohol consumption is associated with enhanced Th2 immune responses.

Aim: To investigate the frequency of false-positive serological tests of allergy in alcoholics.

Methods: A total of 138 alcoholic patients consecutively admitted to hospital underwent a panel of allergy tests that included serum total IgE, a multiallergen IgE test (UniCAP Phadiatop), and skin prick tests to relevant aeroallergens in the area, which were considered the standard reference for atopy. In selected cases with positive specific IgE (sIgE) to cross-reactive carbohydrate determinants (CCDs) on ImmunoCAP, we determined sIgE to Hymenoptera venom components (ADVIA Centaur) and a microarray of 103 allergen components (ISAC).

Results: Increased (>170 IU/mL) serum total IgE was observed in 59/110 (54%) of non-atopic (skin prick test-negative) alcoholics. Forty-six non-atopic alcoholics (42%) had a positive multiallergen IgE test. This finding was closely associated with high serum concentrations of total IgE and sIgE to CCDs. The vast majority of alcoholics with positive CCD-sIgE showed positivity to glycosylated plant and Hymenoptera allergen components on ISAC and ADVIA Centaur. Only one out of 26 patients with positive sIgE to CCD and Hymenoptera venoms developed honeybee venom allergy after a median follow-up of 166 months. Measurements of sIgE to CCD markers on ImmunoCAP, ADVIA Centaur, and ISAC showed imperfect correlation.

Conclusions: Serological tests for allergy should be interpreted with caution in alcoholics, who frequently show increased levels of total IgE, CCD-sIgE and subsequent positivity of sIgE to glycosylated allergen components, irrespective of the method used.

Keywords: Alcohol, Cross-reactive carbohydrate determinants, Allergy, Glycans, Specific IgE, Total IgE

Resumen

Antecedentes: El consumo de alcohol se asocia con respuestas inmunes aumentadas de tipo Th2.

Objetivo: Investigar la frecuencia de falsos positivos en los tests serológicos de alergia en alcohólicos.

Métodos: En un total de 138 pacientes alcohólicos ingresados en el hospital de forma consecutiva se realizó un panel de pruebas de alergia que incluyó la determinación de IgE sérica total, un test de IgE específica multialérgico (UniCAP Phadiatop) y pruebas cutáneas en *prick* a una batería de aeroalérgenos relevantes que el área, cuya positividad se consideró la referencia para clasificar a los pacientes como atópicos. En casos seleccionados con positividad de IgE específica (sIgE) frente a carbohidratos con reactividad (CCDs) en el ImmunoCAP, se determinó la sIgE a componentes del veneno de Himenópteros (ADVIA Centaur) y a un microarray de 103 componentes alérgicos (ISAC).

Resultados: Se observó un aumento de las concentraciones de IgE sérica total (>170 IU/mL) en 59/110 (54%) de los alcohólicos no atópicos (*prick* test-negativos). Cuarenta y seis alcohólicos no atópicos (42%) presentaban un test de IgE específica multialérgico positivo. Este hallazgo estuvo estrechamente asociado con la presencia de concentraciones elevadas de IgE total y de sIgE a CCDs. La gran mayoría de los alcohólicos con positividad de sIgE a CCDs mostraron positividad a componentes moleculares glicosilados de plantas e himenópteros en el ISAC y el ADVIA Centaur. Sólo uno de los 26 pacientes con positividad de sIgE a CCDs e himenópteros desarrolló alergia clínica a picadura de abeja tras un seguimiento mediano de 166 meses. La correlación de las determinaciones de sIgE a marcadores de CCD en ImmunoCAP, ADVIA Centaur e ISAC fue imperfecta.

Conclusiones: Los tests serológicos de alergia se deben interpretar con precaución en pacientes alcohólicos, que frecuentemente muestran elevación de IgE total, positividad de sIgE a CCDs y, consecuentemente, positividad de sIgE a componentes alérgicos glicosilados, independientemente del método utilizado.

Palabras clave: Alcohol; Carbohidratos con reactividad cruzada; Alergia; Glicanos; IgE específica; IgE total.

Accepted Article

Introduction

Alcohol is a psychoactive substance with dependence-producing properties which has been widely used in many societies for centuries. The systemic effects of alcohol consumption include changes in innate and adaptative immune responses [1]. These alterations are directly related to the effects of ethanol itself on immune cells, and to enhanced intestinal absorption of microbial products, specifically lipopolysaccharide, a component of gram-negative bacteria [2]. Previous studies have shown that ethanol can induce profound changes on both Th1 and Th2 cytokine production, with predominant Th2-driven phenomena [3,4]. Accordingly, alcohol administration to rodents is followed shortly after by a significant increase in serum IgE concentrations [5]. In humans, total serum IgE is increased in most alcoholics [3,4,6-8] and is independent of liver disease [7]. The effect of alcohol on total IgE can also be observed in moderate alcohol consumers from general populations [9-11], and is independent of the effect of smoking [9,10]. Moreover, total serum IgE tends to decrease after alcohol abstinence [7]. Lastly, an increase in cord blood IgE is observed in children born to mothers who consume alcohol during pregnancy [12].

The possible association between alcohol consumption and allergic sensitisation or allergic disease is controversial [6,9-11]. Alcohol consumption (particularly alcohol abuse) is consistently associated with the presence of serum-specific IgE (sIgE) to N-glycans from plant and invertebrate allergens, the so-called cross-reactive carbohydrate determinants (CCDs) [13-16]. CCDs are oligosaccharide side chains N-linked to asparagine residues in glycoproteins which are gaining attention as IgE-binding structures [17,18]. These CCDs are widespread in nature, particularly in pollens, foods, insect proteins, and parasites [17,18]. The currently held view is that IgE antibodies to CCDs display poor biological activity because they are associated with neither skin prick test (SPT) positivity nor clinical symptoms [17,18].

However, CCD-sIgE can interfere with *in vitro* determinations, by inducing extensive reactivity to plant, insect and helminth allergens [17,18].

Serological tools for allergy diagnosis include determination of total IgE, sIgE to whole allergen extracts, multiallergen sIgE tests that are intended for allergy screening, and determination of sIgE to allergen components, the so-called molecular diagnosis of allergy [19]. The last-mentioned includes determination of sIgE to individual molecular components and the use of microarrays. Microarray-based testing uses a small amount of serum samples and enables clinicians to determine sIgE antibodies against multiple recombinant or purified natural allergen components in a single assay [19,20]. These tests should take CCD interference into account, when using naturally glycosylated molecules or molecules obtained by recombination in microorganisms that imply glycosylation [17]. Interference might be different depending on the number of epitopes (mixture of allergens, single allergens or allergen components) and commercial method used [18]. This study sought to evaluate total IgE, sIgE to allergen extracts, multiallergen sIgE tests, and sIgE to single and microarray-based allergen components in alcoholics.

Methods

Study design and participants

This observational study took advantage of a previous survey on heavy drinkers consecutively admitted to the Internal Medicine Department of the University Teaching Hospital of Santiago de Compostela with a catchment area of approximately 500,000 people in NW Spain [21]. Previous reports based on this cohort include the study of atopy traits in relation to CD14 gene polymorphisms [8] and the study of CCD-sIgE sensitisation in combination with a survey of the general adult population [13]. Briefly, 138 out of 145 patients with a history of current chronic alcohol abuse admitted over a 2½-year period agreed to participate in the study. The median age of participants was

47 years (range, 27-78 years). One hundred-and-three patients (74.6%) were males. Median average alcohol intake was 120 g (12 units)/day (range, 50-300 g/day). The main reasons for hospital admission were alcohol withdrawal syndrome (67 patients), complications of advanced liver disease (35 patients), alcoholic hepatitis (20 patients) and miscellaneous causes in the remaining 16 patients. After a week of hospital admission, the participants: (a) signed an informed consent; (b) completed an interviewer-administered structured questionnaire; (c) underwent a panel of SPTs to aeroallergens; and, (d) had a blood sample taken for allergy studies in serum frozen until tested. These tests included sIgE to Hymenoptera venom (whole venom and components), sIgE to aeroallergens, sIgE to CCD markers, and sIgE to a microarray of allergen components (see below). No patient in the series reported systemic reactions to Hymenoptera stings at baseline in 2002-2004. The clinical records were reviewed in 2018 and the incidence of systemic reactions to Hymenoptera stings was registered. The study was reviewed and approved by the Institutional Review Board and conformed to the Helsinki Declaration.

Skin prick tests

In all cases, a panel of SPTs to respiratory allergens of local relevance was performed following standard procedures [8]. The panel included mites (*Dermatophagoides pteronyssinus*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*), pollens (*Lolium perenne*, *Plantago lanceolata*, *Betula alba*, *Parietaria judaica*), moulds (*Alternaria alternata*), and pets (dog and cat). Wheals ≥ 4 mm after 15 minutes were considered indicative of a positive reaction. Patients with at least one positive SPT (n=28, 20%) were considered atopic.

Serum total IgE

Total IgE was determined through a chemiluminiscent enzyme immunoassay (Immulite-2000, Siemens Medical Solutions, Llanberis, Gwynedd, UK). Serum IgE levels >170 IU/mL were deemed to be abnormally elevated [8].

Serum-specific IgE to mixtures of allergens (multiallergen screening tests)

Specific serum IgE to common aeroallergens was determined by means of a fluorimetric immunoassay (UniCAP-Phadiatop^R-ImmunoCAPTM, Phadia [currently Thermo Fisher Scientific], Sweden) (hereafter, Phadiatop) [8]. The Phadiatop test is useful in mass-screening programmes because it is a commercially available variant of serum-specific IgE assays based on the simultaneous testing for specific IgE to a mixture of relevant allergens causing common inhalant allergies. The test is qualitative, either positive or negative when fluorescence response is respectively higher or lower than that of the reference serum. A positive result thus indicates the presence of specific serum IgE against common inhalant allergens.

Serum-specific IgE to Hymenoptera venom components

Serum sIgE to whole *Apis mellifera* (honey bee) venom and *Vespula spp* (wasp) venom was measured by the Immuno-CAP-250 system (Thermo Fisher Scientific), and expressed in kU_A/L [13]. Determination of sIgE to Hymenoptera venom components was performed in a subsample of 26 alcoholics with positive sIgE to CCDs. Molecular components of Hymenoptera venoms included phospholipases from honeybee and wasp (Api m 1 and Ves v 1 respectively), hyaluronidases from honeybee and wasp (Api m 2, Ves v 2 respectively), and antigen 5 from wasp (Ves v 5). Natural honeybee phospholipase A2 (Api m 1) was obtained from Sigma-Aldrich (Madrid, Spain). Recombinant honeybee venom hyaluronidase (Api m 2) was expressed in baculovirus-infected insect cells, purified and characterized as previously reported [22,23], and kindly provided by the authors for studies by the ADVIA-Centaur. Wasp venom

allergens were purified from lyophilised venom (ALK-Abelló Source Material, Spring Mills, Pennsylvania, USA), as indicated [24,25]. Natural venom components are glycosylated proteins, with the exception of wasp antigen 5 (Ves v 5) and wasp phospholipase (Ves v 1) [26]. Recombinant proteins obtained in the baculovirus-infected cell model are also glycosylated [27]. Purified venom components were biotin labelled, and the levels of specific IgE to these allergens were tested using the ADVIA-Centaur platform (Siemens Medical Solutions Diagnostics, Munich, Germany) and expressed in kU/L [25].

Serum-specific IgE to cross-reactive carbohydrate determinants (CCDs)

Serum sIgE to the glycan MUXF (o214) in the 138 alcoholic patients was determined by ImmunoCAP (Thermo Fisher Scientific) and results were reported elsewhere [13]. The MUXF notation refers to the substitutions (Mannose, Unsubstituted, Xylose, Fucose) to the backbone of two N-acetyl-glucosamines and a mannose that is common to all N-glycans [17,18]. The MUXF glycan is widespread in nature and is the only glycan in the pineapple protein bromelain [17,18]. Natural bromelain is included in the ISAC (Thermo Fisher Scientific). Sensitisation to the bromelain protein is very rare, and thus the presence of bromelain-sIgE is considered indicative of CCD sensitisation [17,18]. We also determined sIgE to horseradish peroxidase (HRP) in cases studied on the ADVIA platform. Horseradish peroxidase mainly contains MMXF-type (Mannose, Mannose, Xylose, Fucose) N-glycans. Sensitisation to the HRP protein is also very rare, and thus the presence of HRP-sIgE is considered indicative of CCD sensitisation [17,18]. Natural Hymenoptera venom components commonly bear MMF3F6 (Mannose, Mannose, not xylosylated and doubly fucosylated) N-glycans [17,18].

Serum-specific IgE to an array of allergen components

Determination of sIgE in the ISAC microarray (Thermo Fisher Scientific) was available in a subsample of 18 alcoholics with positive sIgE to CCDs by ImmunoCAP. In order to

further define the profile of multiple positive sIgE results in CCD-positive alcoholics, their results were compared to those of 36 patients (12 males, median age 34 years, range 20-79 years) with respiratory allergy, polysensitization to aeroallergens, and no history of alcohol abuse who were attended in an Allergy clinic from the same area during 2007-2009. The allergen microarray chip immunoassay was the same in all individuals and contained 103 allergen components from 43 allergen sources, including a marker of CCD sensitisation (natural bromelain, nAna c 2). The commercial array of allergens in the ISAC is ever changing and the composition of the version used was only available up to 2011. Following the manufacturer's instructions, 20 microlitres of patient serum were pipetted onto the chip, and specific antibodies for the attached allergens to the chip surface were left to bind during a 2-hour incubation period. Following a buffer wash, bound IgE antibodies were detected with a fluorescence-labelled anti-IgE antibody. The chip was read in a fluorometer, and fluorescent signal units were interpolated into ISAC Standardised Units (ISU), which correspond to IgE antibody levels in the ng/mL range. This technique is a semi-quantitative test. Values of 0.3 ISU or higher were regarded as positive.

Statistical analyses

The Mann-Whitney U-test was used to compare numerical variables between groups, the Chi-square test and Fisher exact test were used to compare proportions, and the Spearman's rank test was used to assess correlations. For incidence rate, person-years were calculated from the date of the hospital admission until death or until the last reliable contact with the health care system, to account for potential misclassification owing to change of residence. Two-tailed P-values lower than 0.05 were considered statistically significant.

Results

Total serum IgE

High serum total IgE concentrations (>170 IU/mL) were observed in 78/138 (57%) alcoholics, with 21 (15%) of these registering concentrations >1000 IU/mL. Among the 110 SPT-negative alcoholics, 59 (54%) displayed IgE concentrations >170 IU/mL and 14 (13%) displayed IgE concentrations >1000 IU/mL; among the 28 SPT-positive alcoholics, 19 (68%) displayed IgE concentrations >170 IU/mL and 7 (25%) displayed IgE concentrations >1000 IU/mL. These differences between SPT-positive and SPT-negative alcoholics in the proportions of increased IgE were not statistically significant ($P>0.05$).

Multiallergen IgE screening tests

A total of 70 out of 138 alcoholics (51%) showed a positive multiallergen screening test (Phadiatop). While most of the SPT-positive alcoholics (24/28 individuals, 86%) were Phadiatop-positive, a sizeable proportion of SPT-negative alcoholics (46/110 individuals, 42%) were likewise Phadiatop-positive. These apparently false-positive Phadiatop results were closely linked to higher serum concentrations of total IgE and sIgE to CCDs (Figure 1). There was also a significant correlation between total IgE and sIgE to MUXF (coefficient 0.639, $P<0.001$). A total of 47 out of 138 alcoholics (34%) showed sIgE to MUXF ≥ 0.1 kU_A/L and 26 (19%) showed sIgE to MUXF ≥ 0.35 kU_A/L.

Serum IgE specific to allergen components in CCD-positive alcoholics

The results of sIgE to whole Hymenoptera venom and Hymenoptera venom components in the 26 CCD-positive alcoholics are shown in Table 1. Nearly all CCD-positive alcoholics displayed dual ImmunoCAP-IgE positivity to honeybee and wasp whole venom extracts. None of these patients referred a history of Hymenoptera venom allergy at baseline. After a median of 166 months of follow-up (range, 5-193 months), only one patient (a 50 year-old male with baseline honeybee venom-sIgE of 15.7 kU_A/L and MUXF-sIgE of 2.0 kU_A/L) developed honeybee venom allergy and underwent specific immunotherapy (incidence rate, 0.35 cases per 100 person-years).

As regards to venom components, a high proportion of patients showed positivity to both recombinant (glycosylated) honeybee hyaluronidase and natural (also glycosylated) wasp hyaluronidase. Concentrations of sIgE to these glycoproteins were particularly high, namely >50 kU/L in 7/25 individuals (28%) and 3/26 individuals (11%) for honeybee and wasp hyaluronidase respectively. Detectable concentrations of sIgE to non-glycosylated allergens such as wasp antigen 5 (Ves v 5) and wasp phospholipase (Ves v 1) were observed in nearly half of alcoholics (Table 1).

The results of sIgE to an array of 103 allergenic components in 18 CCD-positive alcoholics are depicted in Figure 2. Among the 12 individuals with negative SPT to aeroallergens, the overall number of positive results on ISAC was 89/1236 (7%). Most of these positive results corresponded to natural, glycosylated plant allergens, particularly from *Phleum pratense* (nPhl p 4 [berberine bridge enzyme]; 11/12 cases, 92%), *Cynodon dactylon* (nCyn d 1 [grass group 1]; 11/12 cases, 92%), *Platanus acerifolia* (nPla a 2 [polygalacturonase]; 9/12 cases, 75%), *Cryptomeria japonica* (nCry j 1 [pectate lyase]; 7/12 cases, 58%), *Cupressus arizonica* (nCup a 1 [pectate lyase]; 7/12 cases, 58%), *Olea europaea* (nOle e 1 [common olive group 5]; 5/12 cases, 42%), and *Salsola kali* (nSal k 1 [pectin methylesterase]; 4/12 cases, 33%), frequently at low-to-moderate titres (Figure 2). Most SPT-negative alcoholics were also positive to natural, glycosylated Hymenoptera allergen from *Apis mellifera* (nApi m 1 [phospholipase A2]; 10/12 cases, 83%). However, the same patients were negative to some natural plant allergen components from *Actinidia deliciosa*, *Prunus persica*, *Arachis hypoagea*, *Olea europaea*, *Corylus avellana*, *Triticum aestivum*, *Sesamum indicum*, *Glycine max*, *Artemisia vulgaris*, and *Ambrosia artemisiifolia* (Figure 2). Most SPT-negative alcoholics were uniformly negative to recombinant proteins related to plants, with the exception of rPru p1 (*Prunus persica* PR-10 protein), which was positive in 4/12 cases (33%). The same SPT-negative alcoholics tended to be uniformly negative to proteins of mite, mould, helminth, crustacean, mammal, avian,

fish or arthropod (*Blatella germanica*) origin, whether natural or recombinant. Among the 6 CCD-positive alcoholics with positive SPT to aeroallergens, all patients were sensitised to mites, and patients number 16 and 17 were also sensitised to *Plantago lanceolata*. The overall number of positive results on ISAC in SPT-positive patients was 75/618 (12%). The pattern of positive results was similar to that in SPT-negative individuals, with the addition of some specific sensitisations, particularly to mites, the most common aeroallergens in the area (Figure 2).

The profile of positive sIgE ISAC results in CCD-positive alcoholics was different from that of non-alcoholic patients with respiratory allergy and multiple IgE sensitizations. A comparison of representative allergens is summarized in Table 2. Thus, patients with respiratory allergy showed the most common sensitization profile in our region and whenever sIgE to profilin (represented by rBet v 2), nCyn d 1 and/or nPhl p 4 was positive, it was accompanied by positive results to other recombinant allergens from the same source (Table 2), suggesting a genuine sensitization. However, the sensitization profile detected in alcoholics did not follow the same pattern and only sIgE natural allergens were positive, with the exception of rPru p 1. Moreover, alcoholics also showed a uniform sIgE positivity to *Apis mellifera* (nApi m 1), which was absent in polysensitized allergic patients from the area (Table 2). These results suggest a role of CCD-bearing proteins in these false positive results.

There was a significant correlation between sIgE to MUXF (o214) on ImmunoCAP and sIgE to MUXF-bearing natural bromelain (nAna c 2 [*Ananas comosus*]), the CCD marker on ISAC (Figure 3). However, only 6 out of 18 patients (33%) with positive ($\geq 0.35 \text{ kU}_A/\text{L}$) sIgE to CCDs (MUXF) on ImmunoCAP presented a positive sIgE (≥ 0.3 ISU) to the CCD marker (bromelain) on ISAC. The correlation between sIgE to the CCD marker (horseradish peroxidase) on the ADVIA platform and sIgE to bromelain on ISAC followed a similar pattern (Figure 3). The correlation between sIgE to horseradish peroxidase (ADVIA) and sIgE to MUXF (ImmunoCAP)

was stronger and more consistent (Figure 3). The majority of patients (19/26, 73%) with positive (≥ 0.35 kU_A/L) sIgE to CCDs (MUXF) on ImmunoCAP presented a positive sIgE (≥ 0.35 kU_A/L) to the CCD marker (horseradish peroxidase) on ADVIA Centaur.

The concentrations of sIgE to MUXF on ImmunoCAP tended to be correlated with serum total IgE concentrations (correlation coefficient 0.411, $P=0.03$). The correlation between serum total IgE and sIgE to bromelain on ISAC (coefficient 0.302, $P=0.23$) and sIgE to horseradish peroxidase on ADVIA (coefficient 0.130, $P=0.23$) was weaker. There was a significant correlation between sIgE to CCD markers (as measured by any method) and sIgE to glycosylated allergen components, particularly from Hymenoptera venoms (Api m 1, Api m 2, Ves v 2) and plants (nPhl p 4, nCyn d 1, nPla a 2, nCry j 1, nCup a 1, nOle e 1, and nSal k 1) (data not shown).

Discussion

Increased total serum IgE, increased serum CCD-sIgE, and subsequent positive sIgE results to glycosylated plant and insect allergens in alcoholic patients were already known from previous reports, including the present study population [3,4,6-8,13,16]. To the best of our knowledge, this is the first study showing the prevalence of increased total serum IgE and positive multi-allergen sIgE tests after systematically categorizing alcoholics as atopic or non-atopic using a standard panel of SPT. Among non-atopic (SPT-negative) alcoholics, 54% showed increased (>170 IU/mL) and 13% showed very high (>1000 IU/mL) serum total IgE concentrations. Furthermore, 42% of non-atopic alcoholics had a positive (i.e., presumably false-positive) multi-allergen IgE test in close relationship with the presence of CCD-sIgE. As expected, alcoholics with positive CCD-sIgE on ImmunoCAP showed positivity to multiple naturally glycosylated allergens on ISAC multiarray and, positivity to glycosylated Hymenoptera venom components on ADVIA Centaur, although significant discrepancies in CCD-sIgE positivity were observed depending on the method used. Taken together, these results demonstrate that serological tests for allergy should be interpreted with caution in alcoholics.

Multiallergen sIgE tests are rarely used in allergy clinics but are intended for screening for allergic sensitisation in primary care. In general populations, the test has significant specificity and the false-positive rate (i.e., a positive multiallergen test in SPT-negative individuals) is lower than 10% [28]. In contrast, a third of all alcoholic admitted to the hospital in our series showed false-positivity of the multiallergen sIgE. Like general populations [14,15,28], such false-positivity of multiallergen tests in alcoholics is closely associated with the presence of CCD-sIgE. Indeed, tests that mix several allergens are particularly prone to CCD interference [18]. These findings indicate that multiallergen sIgE tests have lower specificity among alcoholics.

Alcoholics with CCD-sIgE had dual positivity of sIgE to honeybee and wasp venoms, as previously reported in this series [13]. Moreover, they displayed sIgE positivity to glycosylated phospholipases and in particular, to hyaluronidases from both venoms. These findings may be of importance, since SPTs to Hymenoptera venoms are frequently negative in cases of true allergy, and diagnosis of Hymenoptera venom allergy often depends on intradermal tests, serum sIgE, and clinical history, which may be less reliable in alcoholics [29]. The mechanisms for CCD sensitisation in alcohol drinkers are not known. CCDs are probably the most common epitopes to which humans are exposed [17]. Exposure could derive from pollen inhalation, vegetable consumption or Hymenoptera stings. Some circumstantial evidence supports the contention that the latter could be of particular importance. Lifetime history of Hymenoptera stings is associated with CCD sensitisation in patients without Hymenoptera venom allergy [30]. CCD-sIgE concentrations increase after Hymenoptera stings [31]. In the present series, a sizeable proportion (nearly 50%) of alcoholics with CCD-sIgE showed positive sIgE to wasp antigen 5 (Ves v 5) and wasp phospholipase (Ves v 1), that are not glycosylated [26], suggesting that Hymenoptera stings were probably common in this population. The fostering effect of alcohol consumption on IgE synthesis [3-11] could explain the high levels of sIgE to venom

components in those exposed. Unfortunately, the history of previous Hymenoptera stings was not registered in the presented cases. None of 138 alcoholic patients reported Hymenoptera venom allergy at baseline, and only one patient developed Hymenoptera venom allergy during follow-up. The positivity of sIgE to Hymenoptera venom components further exemplifies two facts of CCD interference on sIgE determinations. Firstly, CCD sensitisation can interfere, not only with determination of sIgE to naturally glycosylated molecular components, but also with determination of sIgE recombinant molecular components that are obtained in baculovirus-infected cells (such as honeybee venom hyaluronidase in the present study) that are can be glycosylated [17]. In contrast, recombinant molecules that are obtained in procaryote organisms (bacteria) are not glycosylated [17]. Secondly, aside from the ImmunoCAP, other devices supposedly less prone to CCD interference, e.g., the ADVIA Centaur [18], could also be affected.

Determination of sIgE to microarrays of allergen components is a promising tool for molecular diagnosis of allergy [19,20]. Our results confirm that the test instrument can also be affected by CCD interference [32-34]. In a subsample of alcoholics with CCD sensitisation, a uniform pattern of sIgE positivities was observed including -as expected- naturally glycosylated allergen components of plant and insect origin. Microarrays have a changing allergen composition; in fact, the device that was used for the present study changed its composition in recent versions. However, the composition of arrays commonly includes natural (n) plant and insect allergens that can be prone to CCD interference. Of note, non-atopic alcoholics with CCD sensitization frequently showed IgE positivity to nApi m 1 from *Apis mellifera*, nPhl p 4 from *Phleum pratense*, nCyn d 1 from *Cynodon dactylon*, nPla a 2 from *Platanus acerifolia*, nCry j 1 from *Cryptomeria japonica*, nCup a 1 from *Cupressus arizonica*, and nOle e 1 from *Olea europaea*. A similar pattern was observed in atopic alcoholics, even in cases negative to the same allergens on SPT. However, the same patients were more rarely

positive to nAct d 1 from kiwi and nSal k 1 from *Salsola kali*, and were uniformly negative to additional natural plant allergens, supporting the notion that the behaviour of these allergens as “CCD-bearing proteins” is not uniform. Of note, this pattern of apparent IgE polysensitisation among alcoholics was entirely different from that of non-alcoholic polysensitised patients with respiratory allergy from the same area.

Unexpected positivity to rPru p 1 (PR-10 from *Prunus persica*) was observed in a sizeable number of alcoholics with CCD sensitization. The same patients were negative to analogous PR-10 from Mal d 1 and Bet v 1, suggesting that positivity to rPru p 1 does not represent genuine sensitization in these cases. The exact method for obtaining rPru p 1 was not available from the manufacturer and the reason for such sIgE positivity in alcoholics is unknown. Microarrays provide a large data load, which means that misinterpretation of data becomes a real risk when clinical information is unreliable. It should be noted that sIgE to the CCD marker used in the array presented (bromelain) proved negative in two-thirds of alcoholics who showed sIgE to MUXF on ImmunoCAP. Bromelain from pineapple (nAna c 2) is a well-recognised CCD marker, which contains a single MUXF epitope [17,18]. For unknown reasons, which may include microarray methodology or allergen processing for the array, sIgE to the marker was not detected in the majority of cases, which could further favour misinterpretation of microarray results. More recent versions of the ISAC include the MUXF molecule instead of bromelain, which could increase diagnostic accuracy. The peculiar profile of positive sIgE results to glycosylated natural allergens may be strongly indicative of CCD sensitization. Additionally, inhibition studies are a well-known strategy to confirm CCD interference [13,15,34]. Specifically, Holsweber et al. demonstrated the neutralizing effect of a CCD blocker against a group of well-known CCD-bearing proteins (nCyn d 1, nJug r 2, nCry j 1, nCup a 1, nOle e 1, nPla a 1, and nPhl p 4) [34], most of them involved in false-positive results in our series of alcoholics.

The study has some limitations that warrant mention. Determination of sIgE to molecular components was only performed on a small subset of alcoholics with positive CCD-sIgE. As previously mentioned, the molecular components in the array are ever changing, but CCD interference will remain a problem whenever natural plant or insect allergens are included. We did not perform inhibition experiments, which would have demonstrated that sIgE reactivity to glycosylated allergens was due to CCD interference [13,15,34]. This study included very heavy drinkers, with a median daily alcohol consumption of 120 g (12 standard drinking units). The results cannot be fully extrapolated to all alcohol drinkers, though previous studies have shown that less heavy and even moderate alcohol drinkers display higher IgE concentrations and higher rate of CCD-sIgE positivity than does the non-drinking population [13-15].

Harmful alcohol use remains unrecognised by many clinicians, and specifically by allergologists for at least two reasons. Firstly, estimation of alcohol consumption is not easy and is time-consuming. Clinical assessment of alcohol intake is often reduced to a simple question, such as "*Do you drink?*", and answers are often reduced to a simple statement such as "*Average*". Denial and minimisation are hallmarks of alcohol abuse, with many individuals underreporting their use of alcohol [35]. A positive response including "*in the last 24 hours*" to additional questions such as "*When was your last drink?*" has a positive predictive value of 68% and negation has a negative predictive value of 98% for detection of alcohol abuse [36]. Although imperfect, there are effective strategies that clinicians can use, as well as short questionnaires [36]. A precise estimation of the average amount of alcohol consumption requires reviewing a standard day and summing up the number of units of alcohol (glasses of wine, beer and spirits, each approximately equivalent to one standard unit or 10 g of pure ethanol). Secondly, the potential effect of alcohol consumption on immune responses is often underestimated. During history-taking related to lifestyle factors, clinicians tend to focus on smoking, particularly when facing a patient with allergic respiratory disease.

Yet, part of the effects on IgE responses which were previously attributed to smoking, could actually be due to the confounding effect of alcohol [6,9,13]. In summary, the prudent allergologist should include a questionnaire on alcohol consumption as a routine part of standard clinical practice. Results of serological tests for allergy should be interpreted carefully in alcoholic patients, who frequently display increased levels of total IgE and sIgE to CCDs, with subsequent widespread positivity of sIgE to glycosylated allergens and their components, irrespective of the method used.

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Table 1. Serum levels of specific IgE to Hymenoptera venoms and their components in 26 heavy drinkers with cross-reactive carbohydrate sensitisation and without a history of systemic reaction to Hymenoptera stings.

	Hymenoptera venom						
	Whole venom		Phospholipases		Hyaluronidases		Antigen 5
	Honey bee	Wasp	Honey bee (Api m 1)	Wasp (Ves v 1)	Honey bee (Api m 2)	Wasp (Ves v 2)	Wasp (Ves v 5)
Method	ImmunoCAP	ImmunoCAP	ADVIA	ADVIA	ADVIA	ADVIA	ADVIA
Median (and range) of specific IgE (kU/L)	6.9 (1.0-74.3)	3.4 (0.21-56.9)	3.2 (0-115)	0.48 (0-119)	12.9 (0.45-348)	8.8 (0.12-112)	0.12 (0-88.7)
Percentage with specific IgE \geq0.35 kU/L (n/N)	100 (26/26)	96 (25/26)	77 (20/26)	50 (13/26)	100 (25/25)	92 (24/26)	46 (12/25)

ImmunoCAP, UniCAP-250 system (Thermo Fisher Scientific, Uppsala, Sweden) [13].

ADVIA, ADVIA-Centaur system (ADVIA-Centaur (Siemens Medical Solutions Diagnostics, Munich, Germany). Allergens were natural (purified) with the exception of honeybee venom hyaluronidase (Api m 2), which was obtained by recombination in baculovirus-infected cells and was therefore also glycosylated.

Table 2. Comparison of ISAC results of IgE specific to representative allergens in alcoholic patients with CCD sensitization on CAP and polysensitised patients with respiratory allergy from an allergy clinic.

Allergen-sIgE positivity (≥0.30 ISU)	Alcoholics with CCD-specific IgE (n=18)	Polysensitised patients with respiratory allergy from an allergy clinic (n=36)	P-value
nAna c 2 [n (%)]	6 (33.3)	0 (0.0)	<0.001
nApi m 1 [n (%)]	16 (88.8)	0 (0.0)	<0.001
rPru p 1 [n (%)]	7 (38.8)	1 (2.7)	0.001
nAct d 1 [n (%)]	3 (16.6)	0 (0.0)	0.032
nPla a 2 [n (%)]	14 (77.7)	0 (0.0)	<0.001
nCry j 1 [n (%)]	12 (66.6)	1 (2.7)	<0.001
rBet v 1 [n (%)]	0 (0.0)	6 (16.6)	0.162
rBet v 2 [n (%)]	1 (5.5)	9 (25.0)	0.137
nCup a 1 [n (%)]	10 (55.5)	2 (5.5)	<0.001
nOle e 1 [n (%)]	9 (50.0)	1 (2.7)	<0.001
nArt v 1 [n (%)]	1 (5.5)	1 (2.7)	0.999
nArt v 3 [n (%)]	1 (5.5)	1 (2.7)	0.999
nCyn d 1 [n (%)]	16 (88.8)	19 (52.7)	0.014
nSal k 1 [n (%)]	7 (38.8)	0 (0.0)	<0.001
nPhl p 4 [n (%)]	17 (94.4)	11 (30.5)	<0.001
Isolated nPhl p 4 positivity* [n (%)]	14 (77.7)	0 (0.0)	<0.001

*Positivity of specific IgE to nPhle p 4 with negativity to rPhle p 1, rPhle p 2, rPhle p 5, rPhle p 6, and rPhle p 12.

LEGEND OF FIGURES

Figure 1. Serum concentrations of IgE specific to cross-reactive carbohydrate determinants (MUXF, top) and total IgE (bottom) in a sample of 138 alcoholics, stratified according to the results of skin prick tests (SPTs) and multiallergen IgE (Phadiatop) results. (+ve), positive; (-ve), negative.

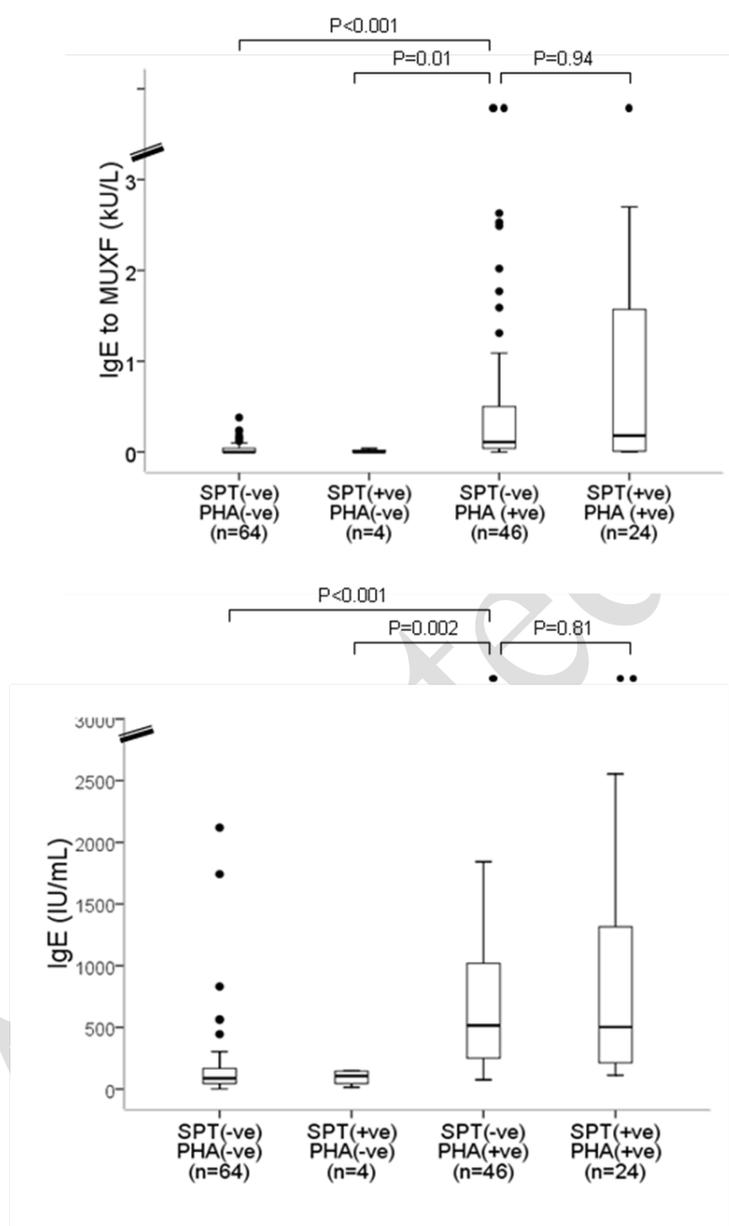


Figure 2. Representation of positive results of specific IgE (sIgE) to 103 allergens in the microarray ISAC system in 18 alcoholic patients with positive (≥ 0.35 kU_A/L) sIgE to cross-reactive carbohydrate determinants (CCDs) on ImmunoCAP. Serum sIgE to the remaining allergen components proved to be negative in all patients, including components from crustaceans (rPen a 1; nPen i 1; nPen m1), helminths (rAni s 1), animal danders (rFel d 1, 2, & 4; rCan f 1, 2, & 3), mammals (nEqu c 3; nMus m 1; nBos d 4, 5, 6, & 8), fish (rCyp c 1; rGad c 1), avian (nGal d 1, 2, 3, & 5), moulds (rAlt a 1 & 6; rCla h 8; rAsp f 3, 4 & 6), arthropods (rBla g 1, 2, 4, 5, & 7), latex (rHev b 1, 3, 5, & 6), vegetable foods (nAct d 2, 5, & 8; rMal d 1; rApi g 1; rDau c 1; rAna o 2; rBer e 1; nAra h 2, & 3; rAra h 8; rCor a 1.0101, 1.0401 & 8; nCor a 9), tree pollens (rAln g 1; rBet v 1; rBet v 4; rPla a 1; nOle e 2), weed pollen (nAmb a 1; rPar j 2), grass pollens (rPhl p 7 & 11), and seeds (nSes i 1; nTri a 18; nTri a gliadin; rTri a 19.0101; rGly m 4 & 6). Recombinant components are denoted by 'r' and natural purified proteins by 'n'. ISU, ISAC standardised units. SPT, skin prick test. On SPT, the 6 positive patients were sensitised to mites; patients number 16 and 17 were also sensitised to *Plantago lanceolata*.

		SPT-negative patients												SPT-positive patients					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
CCD marker	nAna c 2	■		■		■								■	■		■		
Mites	nDer p 1															■			
	nDer p 2													■	■	■		■	
	rDer p 10					■													
	rDer f 1															■			
	rDer f 2													■	■	■		■	
	rEur m 2													■	■	■			
Hemiths	rAni s 3					■													
Mammals	nBos lactoferrin													■					
Mould	rAsp f 1		■												■				
	rAsp f 2					■													
Insect	nApi m 1	■	■	■	■	■	■	■	■	■				■	■	■	■	■	■
	nApi m 4						■												
Latex	rHev b 8																		■
Vegetable foods	nAct d 1	■	■																■
	rPru p1	■		■	■			■						■	■	■			
	rPru p3													■					
	nAra h 1								■										
Tree pollens	rBet v 2																		■
	nPla a 2	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■
	nCry j 1	■		■	■			■		■		■		■	■	■	■	■	■
	nCup a 1	■		■	■			■		■		■		■	■	■	■	■	■
	nOle e 1					■											■		
Grass and weed pollens	nArt v 1																		■
	nArt v 3																		■
	nCyn d 1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	rMer a 1																		■
	rPhl p 1	■		■															■
	rPhl p 2																		■
	nPhl p 4	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	rPhl p 5																		■
	rPhl p 6																		■
	rPhl p 12																		■
Seeds	nSal k 1	■		■		■		■					■	■		■			
	nTri a α-amylase	■												■					
	nGly m 5	■												■					

■	Undetectable (<0.30 ISU)
■	Low (0.30-0.99 ISU)
■	Moderate-high (1-14.99 ISU)
■	Very high (≥15 ISU)

Figure 3. Correlation between markers of cross-reactive carbohydrate sensitisation in alcoholic patients with positive (≥ 0.35 kU_A/L) sIgE to cross-reactive carbohydrate determinants (CCDs) on ImmunoCAP.

Accepted Article

