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Development and Analytical Evaluation of a Microarray Assay for Quantitative Determination of Human Blood IgG to Food Antigens in the Italian Population

Running title: IgG to Food Antigens by Microarray

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SUMMARY

Research background. Food intolerance mediated by IgG antibodies is an adverse reaction depending on the difficulty to digest or metabolize a food or food component(s) and is manifested by numerous nonspecific symptoms, potentially also promoting systemic inflammation and allergy symptoms. Consequently, the detection of circulating food-specific IgG has a diagnostic value with possible clinical applications.

Experimental approach. We produced microarray chips able to analyse 16 blood samples at the same time. After validation of the characteristics and performance by stringent quality control criteria, we investigate their diagnostic validity for IgG-mediated intolerance on 6,575 subjects of the Italian population to 92 purified food proteins. Moreover, this assay was performed on capillary blood samples collected via fingertip, permitting a minimally invasive and practical collection of blood.

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Results and conclusions. 16 antigens showed an IgG response greater than 10 % and 8 aliments of these had a reactivity greater than 15 %. Wheat (20.4 %), cow's milk (30.8 %), beer's yeast (23.4 %) and mozzarella cheese (22.4 %) produced a very high IgG response greater than 20 % probably due to their large use in the Italian cuisine. Goat's milk, various milk derivatives (like gorgonzola, Grana Padano and parmesan cheese), durum wheat, kamut, egg and gluten had a reactivity greater than 15 %. 4 food antigens (emmer, pecorino cheese, ricotta and rye) caused a moderate IgG-reactivity ranging between 10 and 15 %. The other food extracts showed a mean low IgG reactivity lower than 10 %. No significant differences were observed between female and male subjects as well as in the various Italian regions analysed. Contrary, the highly reactive food antigens showed a decrease of the IgG response depending on age.

Novelty and scientific contribution. After their validation, our microarray chips were found to be a robust method with good reproducibility of results and low variation. Even if the primary aim of this study is the evaluation of the incidence of IgG-mediated reactivity in the Italian population by using a novel microarray technology and to compare results with a previous study conducted by using ELISA technology, this analytical approach can help reveal what triggers the symptoms of intolerance and help the doctor or nutritionist to choose the best treatment for the patients also with the possible aim to shed a light on the controversy about IgG testing. Finally, our microarray technology enables high processivity (high throughput), ensuring that a large number of samples can be analyzed with significant time, reagent and cost savings and minimally invasive for patients.

Keywords: microarray; IgG evaluation; food antigens; food intolerance; food hypersensitivity

INTRODUCTION

Food allergy is a reaction of the immune system to a food or its component(s) producing higher levels of IgE reactive versus that food or its protein(s) (*1*). The symptoms of allergy arise shortly after taking the food in question, in general 2–4 h, and are all the more severe the earlier they are. An estimated 6-8 % of children (*1*), almost 6 % of adults in Europe (*2*) and the United States (*3*), respectively, are suffering from food allergies, including multiple food allergies such as milk, eggs and peanuts at the same time (*1-3*). Food allergy is a disease having a high impact on the quality of life of patients and their families, with significant health care costs for the individual and the related National Health Systems. Consequently, since 2014 the European Regulation 1169/2011 has been applied in Italy (*4*) which makes allergen reporting mandatory even in non-prepacked foods. In fact, food allergy is defined a specific and reproducible immunological reaction related to food ingestion.

Contrary to food allergy, food intolerance is an adverse reaction that depends on the body's difficulty in digesting or metabolizing a food or food component (*5*) and is manifested by numerous nonspecific symptoms. The most commonly reported food intolerances leading to gastrointestinal symptoms (pain, abdominal distension, flatulence, and diarrhea) are wheat bread, milk and related products and spiced aliments (*6*). Food intolerance, mediated by IgG antibodies (*6*), can promote systemic inflammation and has been associated with allergy symptoms such as rashes, urticaria and asthma, but the pattern of the most common food specific IgG frequently observed may vary in different populations.

The detection of circulating food-specific IgG has a diagnostic value as it have been performed and associated with several pathological and clinical conditions such as subjects with small bowel stomas (*7*), affected by migraine and its comorbidities (*8*), autism spectrum disorder (*9*), inflammatory bowel disease (*10*), ulcerative colitis (*11*), childhood eczema (*12*), Crohn's disease (*13*), asthma (*14*) and in general with allergic symptoms (*5*,*6*).

Apart from some techniques and procedures without any scientific basis such as cytotoxic food testing, the ALCAT test, bioresonance, electrodermal testing (electroacupuncture), reflexology and applied kinesiology (*15,16*), diagnosis of IgG-mediated food intolerances are based on scientific sound tests named ELISA (Enzyme-Linked ImmunoSorbent Assay) used for the evaluation of serum immunoglobulins responses in normal and pathological subjects performed on 96-well plastic supports or on chips belonging to the last investigation technique known as microarray technology (*17*). Microarrays originated as useful tools for genotyping and gene sequencing with applications in the clinical field such as both diagnostic and prognostic tools (*17*). In addition to these uses, microarrays are also used in nutrigenomics to study individual responses based on one's genetic makeup (*18*). In the recent past, microarrays have also been used to detect IgE in rapid serological tests (*18*) and applied for the evaluation of possible allergens, food and environmental allergies (*19*).

In our previous study (*20*), we applied the ELISA test to quantify the IgG in the serum of 6,879 subjects belonging to the Italian population reactive versus 160 different food extracts. In the present study, we produced microarray chips and, after validation of their characteristics and performance by stringent quality control criteria, we investigated their diagnostic validity for intolerance of 6,575 subjects of the Italian population to 92 purified food proteins. Moreover, we performed this assay on capillary blood samples collected via fingertip on an appropriate swab according to our patent (*21*), permitting a minimal invasive and practical collection of blood. Finally, this study was conducted blindly with the primary aim of evaluating the incidence of IgG-mediated reactivity in the Italian population by using our microarray technology illustrated in the present study and by comparing the

results with the previous study conducted using ELISA technology (*20*). As such, the condition of the enrolled subjects, their possible pathologies or symptoms related to clinically relevant food intolerances, was unknown. A second study is currently underway with the aim of correlating possible pathologies with IgG-mediated reactivity to food-derived proteins.

MATERIALS AND METHODS

Materials

For the analytical procedure porous nitrocellulose film Microarray slides (Grace Bio-Labs, Bend, OR, USA) were used. Technical grade TRIS(Hydroxymethyl)-Aminomethane (TRIS), sodium chloride (NaCl), Proclin 300, Tween® 20 and 3,30,5,50-tetramethylbenzidine (TMB) were from Merck Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Protease Inhibitor cocktail tablets were from Roche Diagnostics GmbH (Mannheim, Germany). Human IgG from serum and goat-produced Anti-human IgG (Fab specific)-peroxidae (HRP) conjugated were from Invitrogen (Waltham, Massachusetts, USA). Bovine Serum Albumin (BSA) was from NZYtech (Lisbon, Portugal). Elisa 96-well plates from Greiner Bio-One (Cassina de Pecchi, Milano, Italy) were also used to sample preparation.

Production of food antigens

Food antigens were produced from 92 aliments (Table S1) by a proprietary extraction procedure. Briefly, all aliments were diluted in pH=7.5 buffer containing 50 mM TRIS, 120 mM NaCl, 0.1 % Tween 20, 0.1 % BSA and protease inhibitor cocktail. The solutions were minced by an ultra-turrax and the homogeneous food mixtures were transferred in pre-signed tubes, vortexed, incubated for 10 minutes at -20 °C and subjected to cold agitation for 30 min. After the extraction procedure of approximately 2 h, the food mixtures were centrifuged at 5,000×*g* for 15 min and the supernatants were then collected into a pre-labelled tubes and incubated overnight at 4 °C. Subsequently, the food protein extracts were centrifuged at 20,000 g for 15 minutes at 10 °C and the supernatants diluted in 50 % glycerol and frozen at -20 °C.

The extraction of food proteins was quantitatively evaluated by a Bradford spectrophotometric assay (*20*) and qualitatively by SDS-PAGE procedure (*20*).

Human subjects

For this study, 6,575 subjects aged between approx. 1 and 93 years were selected from the Italian population: 1,258 males, 3,106 females and 2,211 whose sex was unknown. The mean age of males was 41±16 years, while for the female group was 42±17 years.

Biological samples were provided by a private laboratory (Unifarco SpA, Belluno, Italy). Samples were anonymized and sent to the second laboratory (Diagnostica Spire, Reggio Emilia, Italy), which carried out the IgG-mediated reactivity analyses. After the analyses, the non-identifiable aggregated data were collected, which were used in this research.

IgG microarray

Food antigen extracts were diluted in pH=7.5 buffer containing 50 mM TRIS, 120 mM NaCl, 0.1 % Tween 20, and 0.1 % BSA. Diluted food antigen extracts were spotted using the Spotter iONE M24YOU (Berlin, Germany) on porous nitrocellulose film microarray slides, which were then stored at 4 °C overnight.

Microarray slides were subsequently used to analyze the capillary blood samples by a colorimetric assay based on ELISA (Enzyme-Linked ImmunoSorbent Assay) technology for the determination of IgG immunoglobulins reactive towards food antigens. Briefly, microarray slides were immersed in a blocking solution capable of covering non-specific sites for 30 min. After a blocking phase, the diluted capillary blood samples were added to each microarray slide pad and incubated for 30 min. After washing with an appropriate buffer, anti-human IgG conjugated with horseradish peroxidase was added. The unbound conjugate was then eliminated by washing with an appropriate buffer. Finally, a solution containing the substrate of the peroxidase enzyme (TMB) was added for appropriate minutes to allow the correct colorimetric enzymatic reaction, which was stopped with a final washing in water.

As it is well known that food allergy is not uniquely accounted by a single subclass of IgG, the reactivity of food antigens was tested against the entire class of IgG (see Results and Discussion section).

Statistical analysis

Data are expressed as mean values±standard deviation percentage (coefficient of variation %=CV %). Statistical analysis was performed by analysis of variance (ANOVA) and Student-Newman-Keuls test when appropriate by means of SPSS Statistics software v. 19.0 for Windows (*22*). The eventual statistical significance of differences was set at $p<0.05$.

RESULTS AND DISCUSSION

Food antigens

According to the previous study (*20*), food antigens were produced from different groups of aliments, such as meats, fishes, crustaceans, milk products and eggs, vegetables, seeds, fruits and dried fruits, legumes and cereals, spices, yeast and mushrooms (Table S1). Total proteins from each aliment were extracted by using an owner procedure in bulk as previously illustrated. Final proteins were dissolved in a solution composed of a cocktail of protein inhibitors and stabilizers. Protein concentration expressed in mg/mL for each aliment permits us to have a marker of reproducibility of extraction and purification protocol adopted as well as the concentration useful for further analysis and microarray spotting. SDS-PAGE and densitometric scanning were performed for each protein extract to determine the relative percentage of each electrophoretic band and proteins molecular mass (see Fig. 1 of Reference *20*). These analytical approaches along with their IgG response were useful to evaluate the general protein composition of the extracts and the quality control of the production process as well as to guarantee the reproducibility of the microarray chips.

Analytical procedure for microarray chips

The present microarray platform is a rapid and high throughput colorimetric test based on ELISA method to quantitatively measure IgG antibodies to different food antigens, in human fresh serum or plasma or dried blood. This is not a test for IgE-mediated allergy. IgG antibodies present in dried blood samples are extracted by an appropriate buffer (see above section) and the produced solutions tested for reactivity to food antigens. After incubating the microarray slide with the blocking solution, diluted fresh plasma or serum or extracted dried blood samples are incubated with food antigens (see above section) immobilized on pads. After washing away unbound serum or plasma components, anti-human IgG conjugated to horseradish peroxidase is added, and this binds to surface-bound antibodies in the second incubation. A step of washing is necessary to remove unbound conjugate and a solution containing enzyme substrate is added to trace specific antibody binding and color development. After removing excessing substrate solution, slides are dried by centrifugation for 30 sec. The optical density of the standards, positive controls and samples are measured using a high-resolution scanner and its dedicated software. Results are indicated as picograms of IgG, calculated by using a standard curve of human IgG at different concentration, from 0 to 24 picograms.

According to our analytical procedure, fresh whole or dried blood samples were found stable for 72 h at 4 °C. Fresh blood is centrifuged at 2,500×*g* (4,000 rpm) to obtain separation of plasma or serum that are then stable for 30 days if stored at -20 °C. Similarly, the dried blood extracts were observed to be stable at -20 °C for 30 days. Before using them, all tested samples are allowed to reach room temperature and mixed in the diluent solution before use. Results can be impaired if

plasma/serum/dried blood extracts samples are old or not properly stored. Finally, in general, the repeated freezing and thawing of samples should be avoided.

Performance and quality control of microarray chips

An example of microarray formed of 16 nitrocellulose pads is illustrated in Fig. S1. Each pad of microarray slide includes 4 positive controls, 1 negative control and increasing concentration of IgG standard spots. An in-house made and patented specific software integrated with artificial intelligence operates the quality control test for each pad, identifying at least 3 out 4 positive controls, negative control, and the linearity of the standard curve (*21*). The linear regression acceptability limit (R^2) calculated on the values of the calibration curve of the IgG standards for each pad must be greater than 0.900. It is necessary for each pad to comply with the requirements to pass the test and obtain the final report.

Repeatability was assessed with three serum samples under constant parameters (same operator, measurement system, day and kit lot) obtaining values of 12.7 %, 11.3 % and 13.1 % calculated as CV %. The reproducibility was assessed with three serum samples under varying parameters (different operator, measurement systems, days and kit lots) having values of 12.7, 11.8 and 12.6 of CV %. Values of repeatability and reproducibility of CV % below ~15 % were calculated when dried blood samples were tested instead of fresh serum both collected from the same human subjects,

The analytical sensitivity of 3.6 picograms for plasma/serum/dried blood extracts has been estimated based on the increasing concentration of the IgG standards used to obtain the standard curve. The linearity of the system stated as the ability of a method to provide results proportional to the concentration of analyte in the test sample within a given range was assessed with a serial dilution of two different serum/dried blood extracts samples with known activity. Mean values of 100.6 % for the first sample and of 95.4 % for the second one were obtained as percentage of recovery. Finally, the accuracy was assessed by means of the dilution of a serum/dried blood extract with known activity with a serum/dried blood extract with unknown activity by considering the dilution factor used. A recovery of 105.0–103.7 % was calculated for the first sample and a recovery of 109.6–106.5 % was obtained for the second one.

According to stability tests, microarray slides were found stable for 12 months when correctly stored in their vacuum-sealed package at 2–8 °C. Once opened, they are stable for 2 months if stored at room temperature, in a dry place protected from light.

IgG response to food antigens

By considering the analytical sensitivity of our chip microarray, we fixed a limit of 5 % (=4.60 picograms) for very low IgG reaction, 10 % (=5.6 picograms) for medium reactivity, 15 % (=6.60 picograms) for medium and >20 % (=7.60 picograms) for very high reactivity antigens.

Fig. 1 shows the values of IgG reactivity of the antigens measured on 6,575 subjects of the Italian population. As evident, 16 antigens show an IgG response greater than 10 % and 12 aliments of these have a reactivity greater than 15 %. Four food extracts were observed to produce a very high IgG response greater than 20 %, in particular wheat (20.4 %), cow's milk (30.8 %), beer's yeast (23.4 %) and mozzarella cheese (22.4 %) probably for their large use in the Italian cuisine. The other food extracts show a mean low IgG reactivity lower than 10 %. It is worth mentioning that these are mean values determined over a very large representative sample of Italian population, 6,575 subjects, and IgG response was found to range between 0 and 100 % for each of the food antigens studied depending on the human subject. Milk (from cow and goat), various milk derivatives (like gorgonzola, grana Padano, Parmesan cheese and mozzarella cheese), soft and durum wheat and kamut, were found to produce a high IgG-response along with egg, yeasts and gluten (Fig. 1). Moreover, 4 food antigens (pecorino cheese, ricotta, emmer and rye) were found to give a moderate-high IgG-reactivity ranging between 10 and 15 %.

Fig. 1

No significant differences were observed between female and male subjects both for low (Fig. 2) and highly reactive (Fig. 3) 92 food antigens studied.

Fig. 2

Fig. 3

We also analysed the IgG response to the reactive food antigens greater than 20 % depending on the age of the cohort of 6,575 subjects (Fig. 4). Seven subgroups of subjects were formed, the first one having the age lower than 18 years, five groups with a time span of about 10 years and the last one with very old group greater than 70 years. All the 14 highly reactive food antigens showed a decrease of the IgG response depending on age. By considering the mean values derived from the sum of the IgG-reactivity percentages related to the 14 highly reactive foods reported in Fig. 4 (Table S2), a linear decrease in total reactivity was observed (Fig. S2). Finally, significant differences with $p<0.05$ were measured for the groups of age greater than 30 years old compared to the group of 0-18 years (Table S2).

Fig. 4

Finally, we evaluated the trend of the highly reactive food antigens in the different Italian regions (Fig. 5). In general, apart from small variations, no significant differences were observed and the food reactivity was found independent from the different regions and their peculiar eating habits.

Fig. 5

ELISA, due to its accuracy, specificity and sensitivity, is a versatile immunological analysis method belonging to the category of immunoenzymatic tests (*19,20*). In this type of analytical assay, a substance to be measured, defined as an analyte, binds to another, generally represented by an antibody, which detects its presence. The ELISA assay aims to detect and identify, both qualitatively and quantitatively, a specific substance within a sample, in general biological matrices. For these reasons, ELISA is the unique scientifically proven test for the determination of plasma/serum IgG (and immunoglobulins belonging to other classes such as IgE) titer in healthy and pathological subjects. ELISA tests are generally performed on plastic supports known as 96-well plates which however require the use of large quantities of samples and reagents, a long time for analysis and the possibility to analyse only one sample at a time. Contrary to this, we develop a microarray system formed of chips able to analyse 16 samples at the same time and to quantitatively determine the IgG reactivity to 92 food antigens for sample. Moreover, thanks to the nanotechnology characteristics of microarrays (*17-19*), the present system permits us to manage a high number of samples by a single operator with the use of small quantities of blood sample with considerable benefit for the patient with its limited invasiveness, high repeatability of the analysis compared to the classic 96-well plates, reduced consumption of material (such as plates, plastic material, tips, *etc*.), minimum use of space (as warehouse, fridge, workstation, *etc*.). Finally, it shows a remarkable sensitivity and precision based on the use of automation for both chip preparation and its analysis. This is possible because the automation is compact and small in size given that it has to work on very small supports.

According to the previous study (*20*), the quality of food antigen extracts is essential for an accurate IgG reactivity evaluation (*23*) as they have several limitations mainly related to the extraction processes potentially producing loss of some allergenic proteins, protein acquisition from unknown sources, different protein concentration and composition batch-to-batch production. This can cause the absence or low concentration of proteins in the extracts producing false negative results during diagnosis and ineffectiveness of the following therapy. By having this in mind, food antigens used to produce the present microarrays are tested for protein content and quality by using mono-dimensional SDS-PAGE electrophoresis (*20*) to assure an accurate quality control of the entire purification process and batch-to-batch reproducibility. Furthermore, each food production was tested for biological activity and IgG reactivity by using specific serum samples reactive towards that specific antigenic extract.

By comparing the IgG reactivity in 6,880 subjects of the Italian population tested by classic ELISA test on 96-well support determined in the previous study (*20*) and in this last research by means of microarray, we observed a quit similar behaviour regarding the highly reactive food antigens. In fact, milk and several milk derivatives, egg, wheat and yeast produced a IgG response greater than 15-20 % by using both technologies. Wheat produced a reactivity between 15 and 20 % and rye a IgG response between 10 and *15 %* by ELISA assay on 96-well support (*20*) while kamut and gluten were not tested in the previous study. In general, by using microarray technology applied to immune-enzymatic test for serum IgG quantification we observed a strong reactivity to proteins belonging to well-known allergen-food antigens (*24,25*). It is worth of mention the high reactivity of gluten extract, 18.7 % in the total population with 18.1 % in male and 19.3 % in female, showing a large part of Italian population potentially suffering of non-celiac gluten sensitivity that is a disorder that identifies all those cases in which a patient manifests characteristic symptoms of celiac disease, and benefits from a gluten-free diet, although medical tests can exclude the presence of celiac disease or wheat allergy (*26*). This high reactivity to gluten was also reported by Ryu *et al.* (*25*) on 30 Korean subjects. According to the previous study (*20*), pineapple, banana, peanut and mushrooms were found moderately reactive with a % between 5 and 10.

We also confirm that no significant differences were observed between male and female groups but contrary to the previous study (*20*), we measured a general decrease of the IgG response to the highly reactive food antigens in relationship with the age. This discrepancy to the previous study can be due to the different selection of groups. In fact, in the first study only two groups with a time span of 40 years were selected while in this latest research we selected 7 groups with a smaller age range and therefore more reliable. It is possible to hypothesize that subjects who present high levels of plasma IgG also show symptoms of discomfort and various types of disorders and therefore tend, also on the advice of professionals in the sector, to reduce those foods that cause the onset of these same disorders (*10,27*). The plasmatic IgG content accordingly decreases.

More frequently consumed foods have been found to produce an increase of IgG compared with less frequently eaten foods and serum IgG levels for immunogenic food groups are higher than non-immunogenic food groups (*24*). IgG-mediated reactivity against food antigens can arise due to their repeated exposure which may increase the probability of food-specific antigens coming in contact with their antigen-specific T and/or B cell in an inflammatory environment (*27*). This process subsequently activates and induces proliferation of cells similar to those that are implicated in the generation of autoantibodies against self-tissue and immune diseases (*28-30*). It is now clear that the increase in specific plasmatic IgG is a defense mechanism against antigens (non-self molecules)

which can cause inflammatory pathologies and in particular allergies (*31-33*). In fact, antigens can be ingested due to damage of the intestinal mucosa or due to binding at the level of the intestinal cells between antigens and mast cells and basophilic granulocytes present in the lymphatic system or with receptors exposed on the cells. It is estimated that approximately 15 % of ingested proteins are not fully digested (*32*). The increase in IgG allows to bind the antigen and this complex in turn binds with mast cells and basophilic granulocytes on a receptor antagonistic to the receptor that binds IgE, stimulating the allergic response (*31-33*). This receptor is known as FCγRIIb and its binding with antigens reduces the response of both mast cells and basophilic granulocytes by reducing the activation of TH_2 leukocytes with lower production of inflammatory cytokines, histamine, heparin, etc. (*32*). This is a defense mechanism against particularly dangerous molecules known as tolerance. This mechanism prevents the development of allergies but also produces stress in the organism with excess production of IgG and formation of cell-IgG-antigen complexes which can deposit in tissues and organs producing inflammation and tissue damage (*27*). In fact, there is a balance between IgG and IgE levels than can be altered with the increase of the antigen intake and production of IgG immunoglobulins. We should also consider that the blood amount of IgG to neutralize the antigen able to develop allergies is 100 times greater in the blood (10 mg/mL of IgG vs 0.1 mg/mL of plasma IgE) potentially producing a huge accumulation of this class of immunoglobulin (*32*). With the increase of antigens intake and with their continuous exposure, in particular for highly allergenic foods, activation and proliferation of all cells (mast cells, basophilic granulocytes and T and B lymphocytes) occurs with loss of tolerance with a mechanism similar to the production of autoantibodies and autoimmune diseases and inflammation (*27*). These autoantibodies can also be directed towards components of the junctions of the intestinal epithelium such as occludin with increased intestinal permeability (*27*) and also development of food allergies with production of IgE which has been shown to be produced more in situations of intestinal permeability (*27,34*). Several findings suggest that the presence of IgG to food antigens, rather than the presence of symptoms, is perhaps more indicative of a simultaneous increase in intestinal permeability and that in some cases these IgG-mediated reactions to food antigens reach clinical significance producing symptoms and in other cases no (*27,35*). Consequently, an individual gastrointestinal symptom status, *e.g*. symptomatic vs. asymptomatic, should not exclude the possibility of increased permeability as, due to growing evidence highlighting the connection between increased intestinal permeability and many chronic diseases, this may have prevention-oriented implications in clinical practice. We should also keep in mind that clinicians suggesting food IgG assays should also consider the increased permeability that may be present in patients resulted

positive to the food IgG test and sensitivity to food antigens should be considered during clinical evaluation as a potential cause but also as a consequence of increased intestinal permeability (*27*).

Plasma IgG testing is clearly not indicated for the diagnosis of food allergies (*30*) and although its clinical implications remain under discussion (*36*), according to the previous considerations, IgG determination against food antigens has several scientifically proven benefits, in particular 1) to evaluate a potentially harmful state for the organism at a pre-clinical level and without obvious symptoms, 2) highlight a highly probable potential inflammatory state of the intestinal mucosa (also with implications of alteration of the microbiota and therefore dysbiosis), 3) prevent and limit the state from evolving towards a highly inflammatory and allergic condition with IgE production and serious symptoms and 4) to reduce a pre-clinical and sub-chronic inflammatory state. This is confirmed by several studies showing that elimination of foods based on IgG food sensitivity test results also in reduced gastrointestinal symptoms associated with various diseases (*17,37-39*). Finally, it is now well known that food allergy is not uniquely accounted by the single subclass of IgG4, but it is also exerted by other IgG subclasses, all of which are known to have measurable affinity for FcgRIIb and, consequently, only the assay of all IgG classes can give correct information (*32*).

In a recent paper (*25*), a microarray was fabricated by immobilizing 66 food antigens on activated glass slides and serum IgG of 30 Korean subjects was analyzed also in relation to subjects' dietary patterns. According to the authors, the developed microarray showed very good performances and potential to be used as an automated assay system. Moreover, the authors observed that immunogenic and most consumed foods, such as egg, milk and gluten, induced relatively high immune responses. These results obtained in Korean subjects with food antigens typical of that population agree with our results even if obtained on antigens typical of Italian nation. Finally, in the same study (*25*), the authors demonstrated that the use of serum or directly tested whole blood produced comparable results. This also agrees with our procedures in which we used dried blood devices instead of serum or plasma in our microarray technology by obtaining similar results (see also reference *21*).

CONCLUSIONS

The microarray test used in this study is a tool used to identify and measure levels of specific IgG antibodies in the blood. It can help reveal what triggers the symptoms of intolerance and help the doctor or nutritionist choose the best treatment for the patient. Microarray is a robust method with good reproducibility of results and low variation, as demonstrated in this validation study. Moreover,

microarray technology enables high processivity (high throughput), ensuring that a large number of samples can be analyzed with significant time, reagent and cost savings.

FUNDING

This study received no funding.

ETHICAL APPROVAL

The authors have informed the University of Modena and Reggio Emilia about their research. The collection of blood samples was done by a third party, and therefore does not fall into the competence of the Unimore University Ethics Committee for Research. Laboratories that have conducted sampling and IgG-mediated reactivity analyses are certified laboratories that comply with the national ethical regulations and standards, and GDPR regulation of the European Union.

CONFLICT OF INTEREST

V. Mantovani and V. Ghirelli are employees of Humanbiocare. However, all the authors declare that they have no known competing financial interests that could have appeared to influence the work reported in this paper.

SUPPLEMENTARY MATERIAL

Supplementary material is available at www.ftb.com.hr.

AUTHORS' CONTRIBUTION

V. Mantovani, F. Mantovani, F. Maccari and F. Galeotti performed the analytical procedures. N. Volpi and V. Mantovani participated in the research design. N. Volpi wrote the manuscript.

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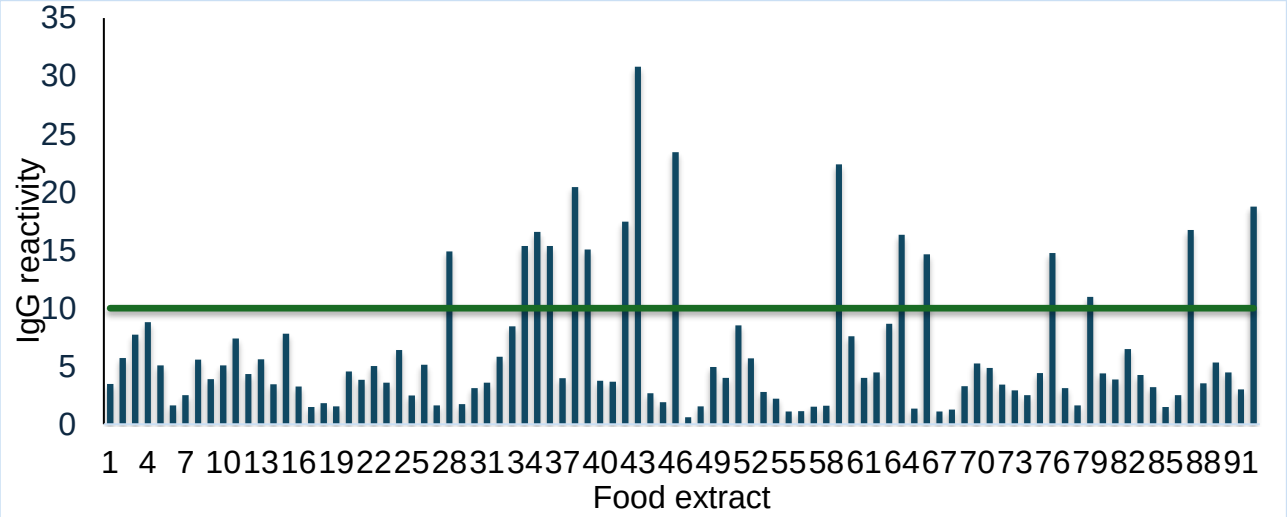

Fig. 1. Percentages of IgG reactivity of the 92 food antigens evaluated by the validated microarray test measured on 6,575 subjects of the Italian population. The 92 food extract types are illustrated in Table S1

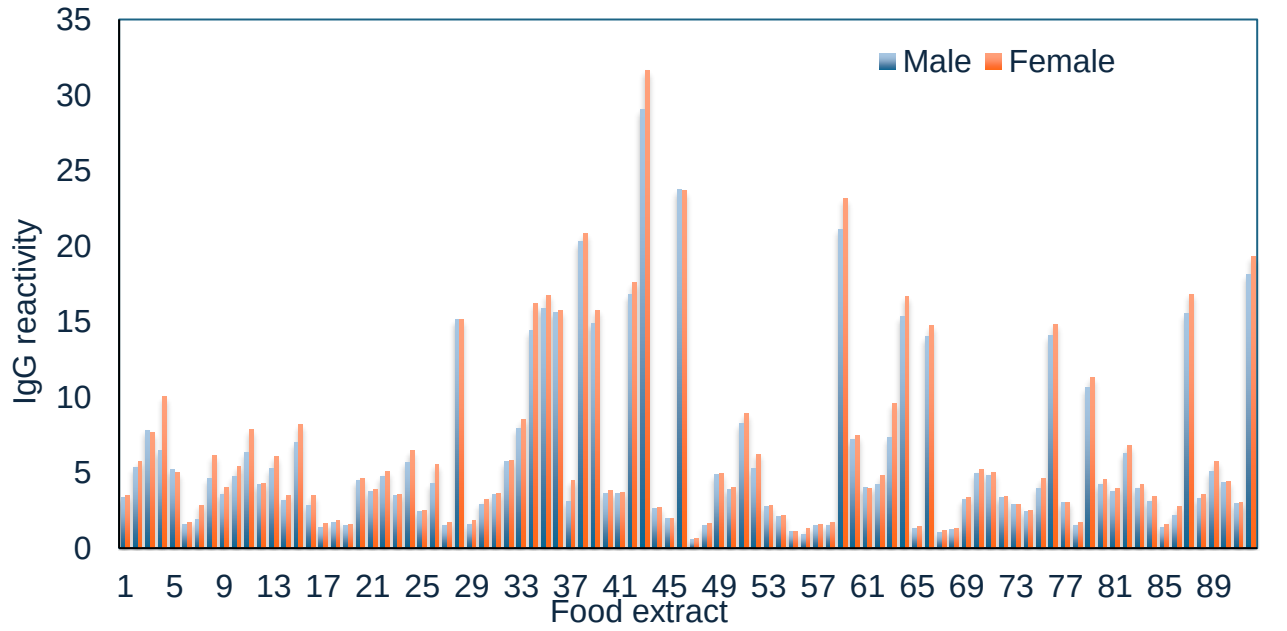

Fig. 2. Percentages of IgG reactivity of the 92 food antigens in females and males of Italian population. The 92 food extract types are illustrated in Table S1

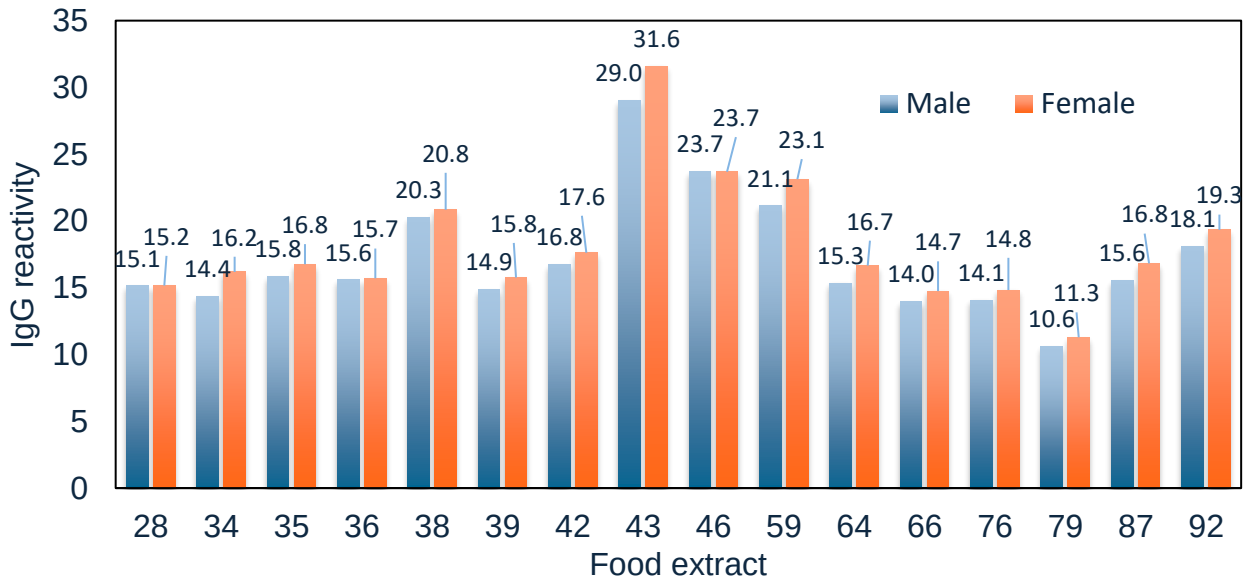

Fig. 3. Percentages of IgG reactivity of the 16 highly reactive (>15 %) 92 food antigens in females and males of Italian population. 28=Emmer cheese, 34=gorgonzola cheese, 35=Grana Padano cheese, 36=durum wheat, 38=wheat, 39=kamut, 42=goat milk, 43=cow milk, 46=beer's yeast, 59=mozzarella cheese, 64=Parmesan cheese, 66=Pecorino cheese, 76=ricotta cheese, 79=rye, 87=egg, 92=gluten

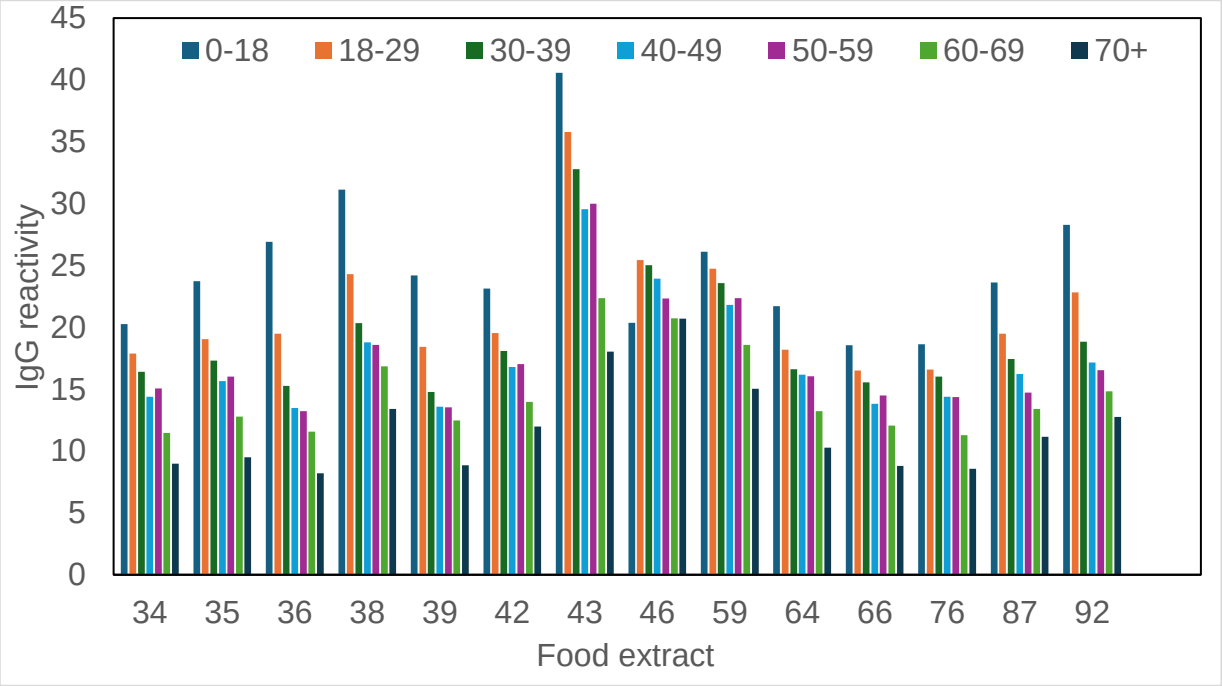

Fig. 4. Percentages of IgG reactivity of 14 highly reactive (>15 %) food antigens in relation to the age of Italian subjects grouped in 7 groups. 34=gorgonzola cheese, 35=Grana Padano cheese, 36=durum wheat, 38=wheat, 39=kamut, 42=goat milk, 43=cow milk, 46=beer's yeast, 59=mozzarella cheese, 64=Parmesan cheese, 66=Pecorino cheese, 76=ricotta cheese, 87=egg, 92=gluten

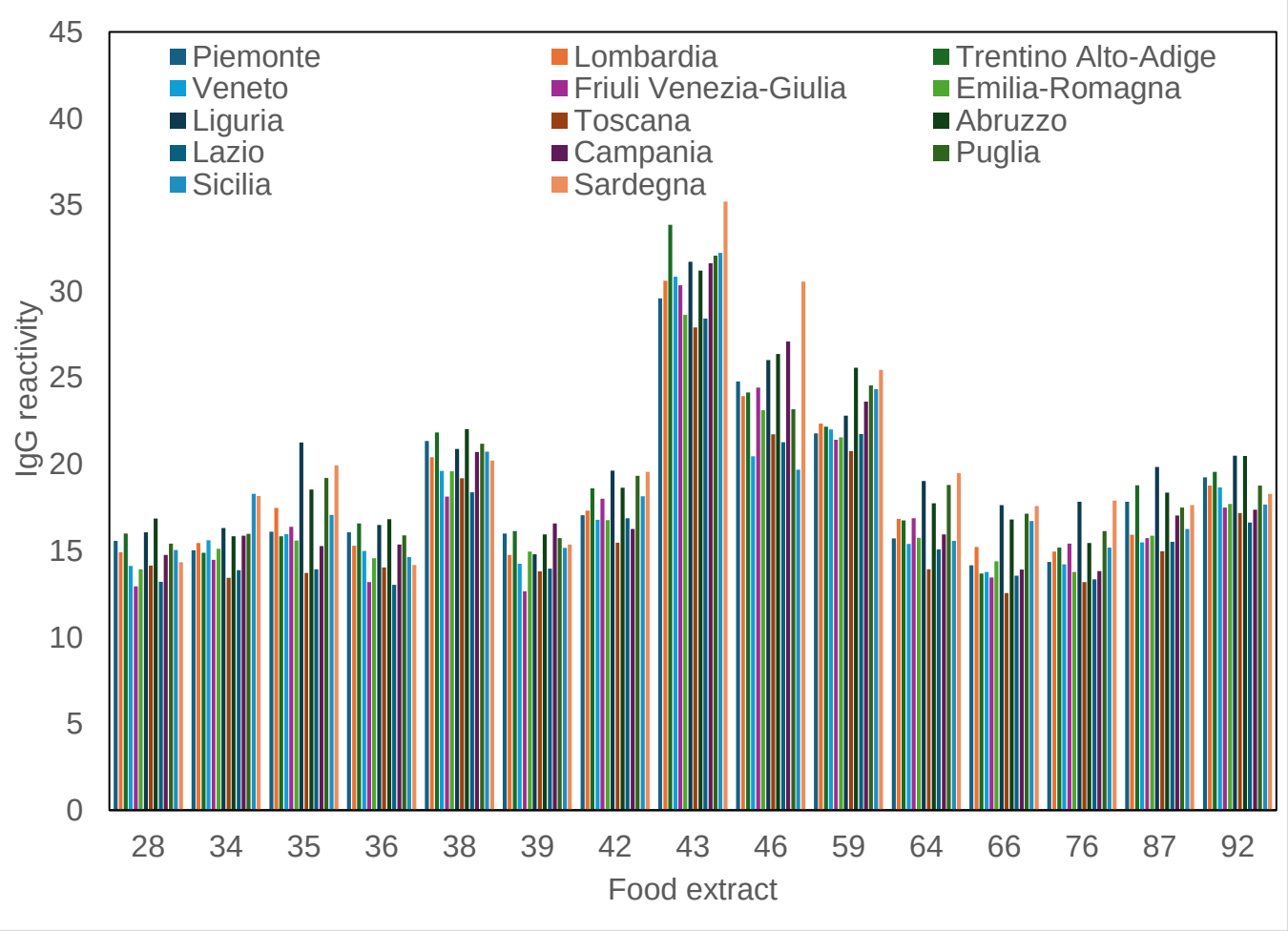

Fig. 5. Percentages of IgG reactivity of 15 highly reactive (>15 %) food antigens determined in 14 Italian regions. 28=Emmer cheese, 34=gorgonzola cheese, 35=Grana Padano cheese, 36=durum wheat, 38=wheat, 39=kamut, 42=goat milk, 43=cow milk, 46=beer's yeast, 59=mozzarella cheese, 64=Parmesan cheese, 66=Pecorino cheese, 76=ricotta cheese, 87=egg, 92=gluten

Supplementary material

Table S1. The list of 92 various aliments from which food protein antigens were prepared

Food extract	Label
Apricot	1
Amaranth	2
Cashew nut	3
Pineapple	4
Peanut	5
Orange	6
Asparagus	7
Oat	8
Avocado	9
Salted cod	10
Banana	11
Chard	12
European bass	13
Broccoli	14
Cocoa	15
Coffee	16
Artichoke	17
Carrot	18
Cauliflower	19
Chickpea	20
Cucumber	21
Chicory	22
Onion	23
Coconut	24
Rabbit	25
Green beans	26
Bean	27
Emmer	28
Broad bean	29
Fennel	30
Strawberry	31
Champignon mushroom	32
Shrimp	33
Gorgonzola	34
Grana Padano	35
Durum wheat	36
Buckwheat	37
Wheat	38
Kamut	39
Kiwi	40
Raspberry	41

Goat milk	42
Cow milk	43
Lettuce	44
Lentils	45
Beer's yeast	46
Lemon	47
Pig	48
Corn	49
Mandarin	50
Almond	51
Beef	52
Apple	53
Eggplant	54
Melon	55
Cod	56
Honey	57
Blueberry	58
Mozzarella cheese	59
Hazelnut	60
Walnut	61
Gilthead bream	62
Barley	63
Parmesan cheese	64
Potato	65
Pecorino cheese	66
Peppers	67
Pear	68
Fishing	69
Peas	70
Chicken	71
Tomato	72
Grapefruit	73
Prune	74
Quinoa	75
Ricotta	76
Rice	77
Salmon	78
Rye	79
Mackerel	80
Sole	81
Soy	82
Spinach	83
Turkey	84
Tuna	85
Trout	86
Egg	87
Cabbage	88
Veal	89

Pumpkin	90
Zuchini	91
Gluten	92

Table S2. The mean values derived from the sum of the IgG-reactivity percentages for the 14 highly reactive foods reported in Fig. 3 are reported depending on the group of age. The CV % and the significance of the differences versus the 0–18 group is also illustrated. The linear regression of the mean values of the IgG-reactivity for the 7 age groups (from 0–18 years=group 1 to >70 years=group 7) is also illustrated with the related equation and R^2 value

Parameter	Age group						
	0–18	18–29	30–39	40–49	50–59	60–69	>70
Mean	24.7	21.4	19.1	17.5	17.4	14.6	11.9
CV/%	23.6	24.4	26.8	26.9	26.4	24.6	32.2
p-value		0.80639	0.03660	0.00193	0.00164	0.00001	0.00000

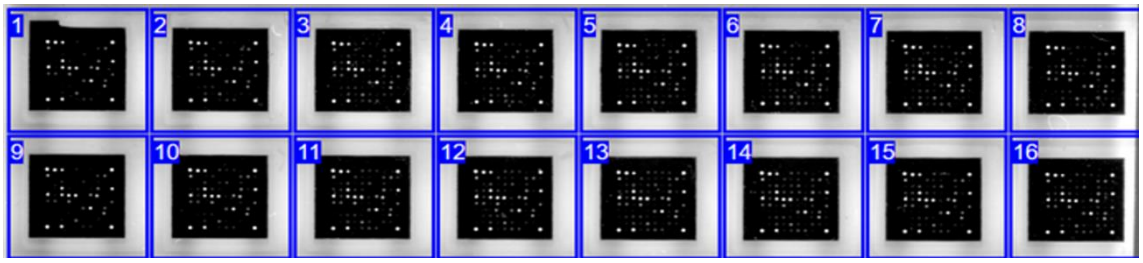

Fig. S1. An example of the produced microarray chip composed of 16 pads each bearing the 92 food antigens is reported. Four positive controls located at the four corners of each pad, 1 negative control and increasing concentration of IgG standard spots visible on the top left side of each pad, are illustrated

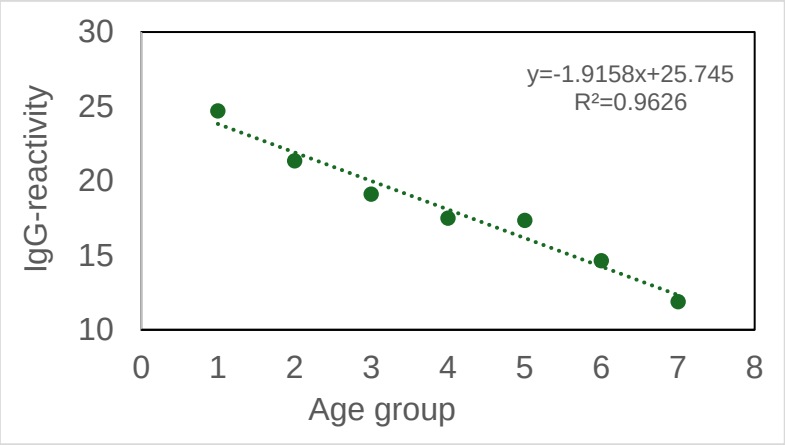

Fig. S2. The linear regression of the mean values of the IgG-reactivity for the 7 age groups (from 0–18 years=group 1 to >70 years=group 7) (see Table S2) is illustrated with the related equation and R^2 value