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Allergome microarray for detection of total repertoire of allergen-specific IgE in human serum.

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Abstract

Allergy, or type 1 hypersensitivity, is defined as a malfunction of immune system where a person's body is hypersensitized to react immunologically to non-immunogenic substances. These substances are usually innocuous environmental antigens such as pollen, house dust mites, animal epithelia, moulds, etc and collectively are known as allergens. Type I hypersensitivity is characterised by excessive activation of mast cells by IgE resulting in a systemic inflammatory response. Therefore, elevated levels of allergen specific IgEs in the serum is the key in the pathogenesis of allergic diseases. The worldwide prevalence of allergy is dramatically increasing and it is of particular concern in industrialized countries. Fifty million Americans suffer from allergic diseases including asthma, rhinitis, sinusitis, dermatitis and food allergy and in the UK it affects one in four people at some point in their lives. Therefore, there is an increasing need for a sensitive *in vitro* test capable of rapidly and accurately quantify allergen-specific IgE from patient sera. The aim of this project is to assess the potential of high throughput technology for simultaneous *in vitro* measurement of allergen-specific IgEs to a variety of well-known environmental and food allergens and the development of a novel diagnostic biochip for clinical use. For this purpose, a protein microarray platform based on non purified allergens has been developed, evaluated, and validated to generate proof-of-principle and to demonstrate the specificity and sensitivity of the array

design. The protein microarray method developed for detection of specific IgE antibodies in human sera consist of four defined steps:

1. Printing: microspots containing total allergen extract and, antibodies are covalently immobilized onto activated glass microscope slides by means of high speed robotics. Minute volumes reagents are arrayed in 7 X 7 matrices, each spot diameter being 400 μm.
2. Processing: slides are initially developed with small volumes (100 μL) of serum samples. A sequential series of reagents are applied to the slide including detecting antibody and amplification reagents conjugated to a fluourophore. The total assay time required is less than 3 hours.
3. Scanning slides are processed by means of a high sensitivity fluorescence-detecting scanner that is able to gather the fluorescence signal emitted by each array component.
4. Quantification and analysis: computational analysis of the image data.

The studies carried out to validate the assay have shown that by using the tyramide amplification system it is possible to achieve an excellent degree of analytical sensitivity and analytical specificity. In addition, no cross-reactivity to other immunoglobulins at normal serum levels was detected. Reproducibility and repeatability are also within the standard requirements for this kind of immunoassay. Short-term stability studies

have been performed to assess the stability of the assay over-time and the results are satisfactory over 90 days. Finally, the clinical validation of the microarray assay showed that the data generated with this system is substantially better than the reference method in terms of sensitivity, correlation between class score and fluorescent signal. The microarray technology has several advantages over existing diagnostic methods such as ELISA. This system offers high throughput and true parallelism; it is highly economical in the use of specimens and reagents and also provides a rapid and accurate detection method for the quantification of specific IgEs in human serum. The knowledge and experience gained in this project will be used in the evaluation of a pre-market microarray-based in vitro diagnostic device.

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ABBREVIATIONS

APC	Antigen Presenting Cells
BSA	Bovine Serum Albumin
CAP	Cellulose Acetate membrane Precipitin
cm	Centimeter
CV	Coefficient of variation
DNA	Deoxyribonucleic Acid
DTT	Dithiotreitol
ELISA	Enzyme-Linked Immunosorbent Assay
FDC	Follicular Dendritic Cells
FcγR	Receptor for the Fc of immunoglobulins' γ heavy chain
FcγRI	Receptor I for the Fc of immunoglobulins' γ heavy chain
FcγRII	Receptor II for the Fc of immunoglobulins' γ heavy chain
FcγRIII	Receptor III for the Fc of immunoglobulins' γ heavy chain
FcεRI	Receptor I for the Fc of immunoglobulins' ε heavy chain
FcεRII	Receptor II for the Fc of immunoglobulins' ε heavy chain
fg	Femtogram
g	Gram
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GST	Glutathione-S-Transferase
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HRP	Horseradish Peroxidase

IDC	Interdigitating Cells
IFN-α	Interferon Alpha
IFN-β	Interferon Beta
IFN-γ	Interferon Gamma
IgA	Immunoglobulin A
IgA1	Immunoglobulin A1
IgA2	Immunoglobulin A2
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG2	Immunoglobulin G2
IgG3	Immunoglobulin G3
IgG4	Immunoglobulin G4
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-9	Interleukin 9
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13

IL-18	Interleukin 18
IL-22	Interleukin 22
IU/mL	International Units per millilitre
KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium phosphate monobasic
kIU/mL	KiloInternational Units per millilitre
L	Litre
LTC_4	Leukotriene C_4
M	Molar
MALT	Mucosa-Associated Lymphoid Tissue
MAST	Multi Allergy Screening Test
M-CSF	Macrophage Colony Stimulating Factor
mg	Milligram
µg	Microgram
µL	Microlitre
µm	Micron
MHC-I	Major Histocompatibility Complex Class I
MHC-II	Major Histocompatibility Complex Class II
mL	Millilitre
mm	Millimeter
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
NA	Not available

NaCl	Sodium chloride
Na_2CO_3	Disodium carbonate
Na_2HPO_4	Di-sodium hydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxyde
NCCLS	National Committee for Clinical Laboratory Standards
ng	Nanogram
NK	Natural Killer Cells
nL	Nanolitre
O.D.	Optical Density
PBS	Phosphate-buffered saline
pg	Picogram
pmc	Photomultiplier counts
PVP	Polyvinylpirrolydone
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RAST	Radioallergosorbent Test
RCA	Rolling Circle Amplification
RNA	Ribonucleic Acid
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SPT	Skin Prick Test
STAT4	Signal Transducer and Activator of Transcription 4

STAT6	Signal Transducer and Activator of Transcription 6
Tc	T cytotoxic lymphocytes
TCR	T Cell antigen Receptor
TGF-β	Transforming Growth Factor Beta
Th1	Type 1 T helper lymphocytes
Th2	Type 2 T helper lymphocytes
TNF-α	Tumor Necrosis Factor Alpha
TNF-β	Tumor Necrosis Factor Beta
ToRCH	Toxoplasma gondii, others, Rubella virus, Cytomegalovirus, Herpesvirus
TSA	Tyramide signal amplification
WHO	World Health Organization

ALLERGENS ABBREVIATIONS

D1	*Dermatophagoides pteronyssinus* (House dust mite)
D71	*Lepidoglyphus destructor* (Storage mite)
E1	Cat epithelium
E2	Dog epithelium
E3	Horse epithelium
F1	Egg white
F2	Cow's milk
F13	Peanuts
F14	Soybean
F49	Apple
G6	Timothy grass pollen
G12	Rye cultivated pollen
I1	Honeybee venom
I3	Wasp venom
K82	Latex
M1	*Penicillium notatum*
M2	*Cladosporium herbarum*
M3	*Aspergillus fumigatus*
M6	*Alternaria tenuis*
S901	Celery
T3	Birch pollen
W6	Mugwort pollen

W9	English plantain pollen
X901	Bet v 1
X903	Phl p 5
X904	Der p 1
X905	Der p 2

1.0 Introduction

1.1 The immune system

In our environment we are surrounded by a great variety of infectious agents: viruses, bacteria, fungi, protozoa and multicellular parasites. Some of these can cause disease and if they multiply unchecked they will eventually kill their host. However most infections in normal individuals are short-lived and leave little permanent damage because of the capacity of the immune system to fight the harmful infectious agents.

The immune responses involve firstly the recognition of the pathogen or other foreign material and secondly a reaction to eliminate it. There are two ways through which the immune system achieves this goal, i.e. by setting up innate (or non-adaptive) immune responses or adaptive immune responses. The innate immune responses are a first line of defense against infections and they are mediated primarily by phagocytic cells, such as monocytes, macrophages and neutrophils. These cells bind to microorganisms, internalise and kill them. In doing this, they use primitive non-specific recognition systems which allow them to bind to a variety of microbial products. On the contrary, an adaptive immune response displays a high degree of specificity for a specific pathogen. Differently from the innate immune responses, which do not alter on repeated exposure to a given infectious agent, the performance of adaptive responses improves with each successive encounter with the

same pathogen. This is achieved through mechanisms that allows the immune system to "remember" the infectious agent and to prevent it from causing disease later. The adaptive immune responses are primarily mediated by lymphocytes. These cells can specifically recognize individual pathogens, whether they are inside host cells or outside in the tissue fluids or blood and "remember" them upon a successive encounter.

1.2 The cells of the immune system

Immune responses are mediated by a variety of cells and by the soluble molecules which they secrete. The leucocytes are the cells primarily involved in all immune responses; however other cells in the tissues can participate to the immune responses, by signalling to the lymphocytes and responding to the cytokines released by T lymphocytes and macrophages. The cells of the immune system can be grouped into two main cathegories: cells involved in the innate immune responses (monocyte/macrophages, neutrophils, eosinophils, basophils, mast cells and platelets) and cells involved in the adaptive immune responses (antigen presenting cells, B and T lymphocytes and natural killer cells).

1.2.1 Phagocytes

The phagocytes belong to two major lineages: mononuclear phagocytes and polymorphonuclear granulocytes.

The group of **mononuclear phagocytes** consists of circulating cells (monocytes) and of cells that dwell within the interstitial spaces of

various organs (e.g. spleen, liver, lungs) where they display distinctive morphological features and perform different functions. The two main functions of this group of cells result from the activities of two different types of bone marrow-derived cells: "professional" phagocytic macrophages primarily involved in the innate immune responses (like the Kupffer cells in the liver, the synovial A cells in the synovial cavity and the microglial cells in the central nervous system), whose main role is to remove particulate antigens, and the antigen presenting cells, committed to take up, process and present antigenic peptides to T cells in the adaptive immune responses.

Myeloid progenitors in bone marrow differentiate into promonocytes and then into circulating **monocytes** which migrate through the blood vessel walls into the various organs to become macrophages. The monocytes are large cells (10-18 μm in diameter), with a horseshoe shaped nucleus and containing azurophilic granules. Ultrastructurally they are characterized by ruffled membranes, a well developed Golgi complex and many intracytoplasmic lysosomes which contain peroxidase and several acid hydrolases which are important for intracellular killing of microorganisms. At times, the monocytes can migrate from the blood into tissues where they develop into **tissue macrophages**. Monocytes/macrophages can actively phagocytose organisms or even tumour cells *in vitro*. Microbial adherence, followed by ingestion, occurs through specialized receptors (like scavenger receptors, toll receptors and mannose receptors) that recognize sugars or lipids on

the microbial surface. Monocyte/macrophages also have receptors for IgG antibodies and complement with which the microorganism may become coated.

The **antigen-presenting cells (APC)** are a heterogeneous population of leucocytes with very efficient immunostimulatory capacity. Some of them have a pivotal role in the induction of functional activity of T-helper cells, and in this regard they are seen as an interface between the innate and adaptive immune system. The APC are found primarily in in the skin, lymph nodes, spleen within or underneath most mucosal epithelia and in the thymus. A group of APC, the skin's Langherhans' cells take up antigens and then migrate as "veiled cells", via the afferent lymphatics into the paracortex of the draining lymph nodes. Here they are termed interdigitating cells (IDC) and they interact with T cells: the APC/IDC are infact rich in class II MHC molecules, which are important for presentation of the antigen to the T lymphocytes. Other APC occurring in the thymus, especially in the medulla, are also indicated as IDC and their presence in the thymus is of crucial importance because they induce T lymphocytes development and maturation, or T lymphocytes suppression in case the latter react against self-antigens. Another group of APC, the follicular dendritic cells (FDC), are located in the primary and secondary follicles of the B-cell areas of the lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT) where they present antigen to B lymphocytes. Contrarily to the skin's Langerhans' cells the FDC do not migrate and form a stable network by establishing strong intercellular

connections via desmosomes. They lack class II MHC molecules but they can bind antigens via complement receptors (CD21 and CD35), which can bind the complement associated with immune complexes. They also express Fc receptors.

The group of **polymorphonuclear granulocytes** mainly consists of neutrophils. They are released from the bone marrow at a high rate of about 7 million per minute, but are short-lived cells (2-3 days) compared to the other phagocytes which may live for months or years. Like monocytes, they can adhere to endothelial cells lining the blood vessels and leave the circulation by diapedesis in response to chemotactic stimuli. The granulocytes do not show any inherent specificity for the antigens, but they play an important role in acute inflammation (usually synergizing with antibodies and complement) in protection against microorganisms. Their predominant role is phagocytosis and destruction of pathogens. Their importance is shown by the marked increase in susceptibility to infections in subjects with a reduced white cells number or with genetic defects that prevent polymorphs diapedesis in response to chemotactic stimuli.

Up to 95% of circulating granulocytes are **neutrophils**. These cells display a characteristic multilobed nucleus (hence the term "polymorphonuclear") and are 10-20 μm in diameter. They can respond to stimulation by chemotactic agents, like protein fragments coming from the activation of the complement, factors derived from the fibrinolytic and kinin systems, products of other leucocytes and platelets and products of

certain bacteria. All these chemotactic stimuli ultimately result in neutrophil margination and diapedesis. Neutrophils have in their cytoplasm two kinds of granules with contain a large variety of antibiotic molecules. The primary or azurophilic granules are lysosomes and contain acid hydrolases, myeloperoxidase and muramidase (lysozyme). The secondary or specific granules contain lactoferrin, lysozime and antibiotic proteins (defensins, seprocidins, cathelicidins and bacterial permeability inducing protein). When microorganisms are ingested, they are contained within vacuoles termed phagosomes which in turn fuse with the lysosomes to form phagolysosomes, where the bacteria are killed. In addition to intracellular killing, the content of granules can be released extracellularly when the neutrophils are activated by immune complexes through their Fcγ receptors.

1.2.2 Eosinophils

These cells account for 2-5% of blood leucocytes in healthy non-allergic individuals. They are characterized by a bilobed nucleus and by many cytoplasmic granules that stain with acidic dyes (hence their name). Eosinophils are capable of phagocytosing and killing ingested microorganisms even if phagocytosis is not the primary function of these cells: rather than phagocyte, certain stimuli will cause the eosinophils to degranulate and release the granule contents into the extracellular environment. Eosinophils are thought to play a specialized role in immunity to parasitic worms using this mechanism: this type of reaction is

the only way these cells can use their granule armament against large pathogens (like parasites) which, given their dimensions, cannot be phagocytosed. Eosinophils also release histaminase and aryl sulphatase, which inactivate the mast cell products, histamine and some of the leukotrienes. The final effect of eosinophils is thus to dampen down the inflammatory responses and reduce granulocyte migration into the site of invasion.

1.2.3 Basophils and mast cells

Basophils are found in very small numbers in the circulation, accounting for less than 0.2% of leucocytes. The mast cells are located only in the mucosae and in the connective tissues and never in the circulation. They are indistinguishable from basophils for a number of properties, although they display distinctive morphological features. Both basophils and mast cells contain many cytoplasmic granules that stain with basic dyes. In both cell types the granules contain heparin, leukotrienes, histamine and the eosinophil chemotactic factor of anaphylaxis. The main role of both basophils and mast cells is played in allergic disease and will be discussed in detail in section 1.5.

1.2.4 Platelets

The platelets are not cells (since they lack the nucleus) but they derive from the fragmentation of megakaryocytes cytoplasm in the bone marrow. Their main role is played in blood clotting. However they play an

additional role in immune responses and especially in inflammation. The platelets express on their surface MHC class I products, receptors for IgG (FcγRII) and low-affinity receptors for IgE (FcεRII). Following injury to endothelial cells, platelets adhere to, and aggregate at, the endothelial surface of the damaged vascular tissue. Here they release substances contained within two types of granules, which include serotonin and fibrinogen. This results in an increased capillary permeability, activation of complement and hence attraction of leucocytes.

1.2.5 Lymphocytes

Lymphocytes are the main responsible for the specific immune recognition of pathogens, so they initiate adaptive immune responses. All lymphocytes are derived from bone-marrow stem cells, but T lymphocytes then develop in the thymus and are the main responsible for cell-mediated immune responses, whereas B lymphocytes develop in the bone marrow (in adult mammals) and become the main elicitors of the humoral immune responses.

1.2.5.1 T lymphocytes

The definitive T-cell lineage marker is the T cell antigen receptor (TCR). There are two defined types of TCR: one is a heterodimer of two disulphide-linked polypeptides (α and β); the other is structurally similar but consists of γ and δ polypeptides. Both receptors are associated with a set of five polypeptides, the CD3 complex, and together they form the T-

cell receptor complex. Approximately 90-95% of blood T cells are αβ T cells and the remaining 5-10% are γδ T cells. The αβ T cells are further subdivided into two distinct non-overlapping populations: a subset which carries the CD4 marker and mainly "helps" or "induces" immune responses (T-helper cells or Th cells), and a subset which carries the CD8 marker and is predominantly cytotoxic (T cytotoxic cells or Tc cells). $CD4^+$ T cells recognize their specific antigens in association with the MHC class II molecules, whereas the $CD8^+$ cells recognize their antigens in combination with MHC class I molecules. The T helper $CD4^+$ cells can be further subdivided into two types, each with their own functions. One group (type 1 T-helper cells or Th1 cells) interacts with the mononuclear phagocytes and helps them in destroying intracellular pathogens. Another group (type 2 T-helper cells or Th2 cells) interacts with B cells and helps them to divide, differentiate and make antibodies. The T cytotoxic $CD8^+$ cells are responsible for the destruction of host cells which have become infected by viruses or other intracellular pathogens: this kind of action is indicated as cytotoxicity, hence the name attributed to this T lymphocytes subgroup. In every case the T cells recognize antigens, but only when they are presented on the surface of the other cell by the so-called major histocompatibility complex (MHC) molecules. In doing this they use their TCR. This receptor is related, both in function and in structure, to the surface antibody which B cells use as their antigen receptors. T cells generate their effects, either by releasing soluble proteins, called cytokines, which signal to other cells, or by direct cell-cell interactions.

1.2.5.2 B lymphocytes

The B cells are a group of lymphocytes genetically programmed to encode surface receptors, each of them specific for a particular antigen. Upon recognition of their specific antigen, the B cells multiply and differentiate into antibody forming cells or **plasma cells**, which produce large amounts of the receptor molecule in a soluble form that can be secreted, the antibody. The antibody molecules, or immunoglobulins, are large glycoproteins found in the blood and tissue fluids and since they are virtually identical to the original receptor molecule, they bind to the antigen that initially activated the B cells. The majority of human B cells in the peripheral blood expresses two immunoglobulin isotypes on their surface, IgM and IgD. Less than 10% of the B cells in the circulation express IgG, IgA or IgE, although these are present in larger numbers in specific locations of the body, for example IgA-bearing cells in the intestinal mucosa. Plasma cells are not frequent in the blood, amounting to less than 0.1% of circulating lymphocytes: they are normally restricted to the secondary lymphoid organs and tissues but they are also abundant in the bone marrow. Antibodies produced by a single plasma cell are of one specificity and immunoglobulin class.

1.2.5.3 Natural killer cells

Natural Killer (NK) cells account for up to 15% of blood lymphocytes and express neither T-cell nor B-cell antigen receptors. A marker for the recognition of NK cells from a purified lymphocyte population is the

presence of FcγRIII receptors and/or CD56, in parallel with the absence of CD3. The function of the NK cells is to recognize and kill virus-infected cells and certain tumour cells. Cells of the body expressing MHC molecules are normally protected against the NK cells-mediated cytotoxicity; on the contrary, down regulation or modification of MHC molecules in virus-infected cells and in some tumour cells make them susceptible to NK cell-mediated killing. Another way for the NK cells to exert their cytotoxicity is the binding to IgG-coated targets via their FcγRIII receptor, a phenomenon indicated as antibody-dependent cellular cytotoxicity. NK cells can also release cytokines (IFN-γ, IL-1, GM-CSF) which may be important in the regulation of hemopoiesis and immune responses.

1.3 Antibodies

The recognition of a foreign antigen is the hallmark of the specific adaptive immune response. Two distinct types of molecules are involved in this process: the immunoglobulins and the T cell receptors. The immunoglobulins are a group of glycoproteins normally present in the serum and tissue fluid of all mammals. Some are carried on the surface of B cells, where they act as receptors for specific antigens. Others (antibodies) are free in the blood or lymph. Five distinct classes of immunoglobulin molecules are recognized in humans, namely IgG, IgA, IgM, IgD and IgE. They differ in size, charge, aminoacid composition and carbohydrate content. In addition to the differences between classes,

heterogeneity within each class has been described. The basic structure of all immunoglobulin molecules is a unit consisting of two identical light polypeptide chains and two identical heavy polypeptide chains. The latter are linked together by disulphide bonds. The light chains can be of two isotypes called κ and λ. Either of these isotypes can combine with any of the heavy chain types, but in one immunoglobulin molecule, both light chains and both heavy chains are of the same type. The C-terminal half of the light chains is constant in its aminoacidic sequence, whereas the N-terminal half of the chain shows much more sequence variability. The variable N-terminal halves of the light chains, in combination with the N-terminal parts of the heavy chains (which show sequence variability as well) form the antigen binding site. This antigen-binding site is very specific and unique for the antibodies produced by a given plasma cell.

The class and subclass of an immunoglobulin molecule are determined by its heavy chain type. Thus, the four human IgG subclasses (IgG1, IgG2, IgG3 and IgG4) have heavy chains called γ1, γ2, γ3 and γ4 respectively. These chains are slightly different although they are all recognizably as γ heavy chains. Apart from the four subclasses of human IgG there are also subclasses for human IgA (IgA1 and IgA2, bearing α1 and α2 heavy chains respectively), but none have been described, to date, for IgD, IgM and IgE (only one isotype of δ, μ and ε heavy chain is known). All immunoglobulins are glycoproteins, but the carbohydrate content ranges from 2-3% for IgG to 12-14% for IgM, IgD and IgE.

The **IgG** is the most represented antibody class in normal human serum, accounting for 70-75% of the total immunoglobulin pool. It is the most important class of immunoglobulins involved in the secondary immune responses and they are evenly distributed between the intravascular and extravascular pools.

The **IgM** accounts for approximately 10% of the immunoglobulin pool. Each IgM molecule is a pentamer of the basic four-chain structure. The IgM antibodies predominate in the primary immune responses. They are mainly confined to the intravascular pool and they are frequently associated with the immune responses to antigenically complex, blood-borne infectious organisms.

The **IgA** represents 15-20% of the human serum immunoglobulin pool. In humans more than 80% of IgA antibodies occur in a monomeric form of the four-chain unit. IgA is the predominant immunoglobulin in seromucous secretions such as saliva, colostrum, milk and tracheobronchial and genitourinary secretions.

The **IgD** accounts for less than 1% of the total plasma immunoglobulin but is a major component of the surface membrane of many B cells. The precise biological function of this class of immunoglobulins is still to be elucidated, although it may play a role in antigen-triggered lymphocyte differentiation.

The **IgE** antibodies, though scarce in serum, are found mainly on the surface membrane of basophils and mast cells in all individuals, where

they are bound through their FcεRI high affinity receptor. The role of IgE antibodies will be discussed in more details in section 1.5.

1.4 Cytokines and other molecules of the immune system

With the term cytokines it is indicated a large group of molecules involved in signalling between cells during immune responses. Each set of immune cells releases a particular blend of cytokines, depending on the type of cell and whether it has been activated. Equally important is the expression of cytokine receptors: only a cell which has the appropriate receptors can respond to a particular cytokine. All cytokines are proteins or glycoproteins and they can be grouped into a number of categories.

The **interferons** are a group of cytokines particularly important in limiting the spread of certain viral infections. One group of interferons (IFNα and IFNβ) is produced by cells which have become virally infected; another type, IFNγ, is released by certain activated T cells. Interferons induce a state of antiviral resistance in uninfected tissue cells. They are produced very early in infection and are the first line of resistance to a great many viruses.

The **interleukins** are a large group of cytokines (IL-1 to IL-22) produced mainly by T cells, but also by mononuclear phagocytes or by tissue cells. They have a variety of functions, but they are mostly involved in directing other cells to divide and/or differentiate.

The **colony-stimulating factors** are primarily involved in directing the division and differentiation of bone marrow stem cells and the precursors

of blood leukocytes. The balance of different colony-stimulating factors is partially responsible for the proportions of different cell types that will be produced. Some of these cytokines can also promote further differentiation of cells outside the bone marrow, like the M-CSF which promotes the development of monocytes in bone marrow and macrophages in tissues.

The **chemokines** are a large group of chemotactic cytokines which direct movement of cells around the body, from the blood stream into tissues and to the appropriate location within each tissue. Some of the chemokines also activate cells to carry out particular functions.

Other cytokines, like the TNFα, TNFβ and TGFβ, have a variety of functions and they are particularly important in mediating inflammation and cytotoxic reactions.

1.5 Biology of the allergic disease

When immune responses, normally beneficial, occur in an exaggerated or inappropriate form, causing inflammatory reactions and tissue damage, the term hypersensitivity is applied (Male, 1998). Particularly the allergies (type 1 hypersensitivity) are caused by innocuous environmental antigens (such as pollen, animal dander, foods, and dust mites) collectively called allergens.

The allergens usually enter the body via the mucosal surfaces or the skin, where they are taken up and processed by antigen presenting cells (APCs).

The latter present the processed allergens on their surface in form of short peptides associated with molecules of the major histocompatibility complex class II (MHC-II) to a subpopulation of T-helper $CD4^+$ lymphocytes, the Th0 naïve cells.

The developing, mainteinance, and ending of an allergic response relies then on two different subclasses of T-helper $CD4^+$ lymphocytes originating from the differentiation of the naïve Th0 cells in response to the antigenic stimulus: the Th1 cells that play a major role in the immune response to infectious diseases, and the Th2 cells that direct the immune response towards the allergy. The balance between these two cellular types will ultimately determine which way the immune system will follow in the response to an antigen.

The cytokine environment and the role of the antigen presenting cells are both essential in determining which T-cell subset will differentiate from the naïve Th0 cells (Lambrecht, 2001). Th1 responses to typical microbial antigens are favoured by the production of a pattern of cytokines of which the most relevant ones are IL-12 and IFNα. These cytokines direct the lymphocytes differentiation to the Th1 via the STAT4-mediated signalling pathway. Other cytokines, such as IL-18, IFNγ and IL-2, can contribute to the Th1 differentiation as well, especially in combination with IL-12. Th1 responses promote in turn the production of IgG by B cells, and directs the immune response towards the T-cell immunity. IFNγ and IL-2 play an additional role in suppressing Th2 cell development. (Lund et al., 2003; Male, 1998).

On the other side, a cytokine environment dominated by IL-4 and IL-13 favours the T-helper differentiation towards the Th2 development. This results in an inhibition of Th1 responses and in a parallel stimulation of the B cells to switch the antibody secretion to IgE rather than IgG,, directing thus the immune reactions towards the allergy.

The IgE antibodies produced in response to an allergen stimulation have a very short half-life in serum (few hours or a few days) before being degraded by serum proteases.

However connective tissues mast cells and blood basophils possess high-affinity receptors for IgE on their surface, the FcεRI. When the IgEs bind via their high affinity receptors to the surface of basophils and mast-cells they induce a sensitization of these two cellular types ("sensitization phase") (Matsson et al., 1997). The complex between the IgEs and the FcεRI receptor is characterized by an association constant ($K_a = 10^{10}\ M^{-1}$) much higher than the one occurring between IgG1 antibodies and their specific receptors (Maenaka et al., 2001; Ravetch and Kinet, 1991). This exceptionally high affinity is accompanied by a very slow dissociation rate (McDonnell et al., 2001), coupled with a restricted diffusion and rebinding to cells receptors (Ishizaka and Ishizaka, 1971; Geha et al., 1985). In addition the persistent local synthesis and secretion of IgE by B cells keeps on replacing the antibodies that are lost by degradation or by dissociation and that diffuse into the circulation or secretions (Gould et al., 2003). All these events account for the maintainance of mast-cells

sensitization and for the extension of IgEs half-life from few hours to about 14 days.

When the same allergen that had elicited the sensitization phase enters the body a second time and reaches the sensitized mast-cells, it binds to the specific IgEs raised against it on their surface. The receptor cross-linking of the membrane-bound IgEs sets off mechanisms of signal transduction (mainly via an increment in the levels of mast-cells intracellular Ca^{2+}) in the so-called "provocation phase" or "challenge phase" (Matsson et al., 1997; Ono, 2000). This event triggers the release of mediators, some of which are preformed, stored in cytoplasmic granules and awaiting release upon allergen provocation, while others result from the activation of enzymes. The products of activated mast cells include histamine, serotonin, lipid mediators (prostaglandins, leukotrienes), proteases (tryptase, chymase), chemokines (eotaxin, RANTES), and cytokines. Among the latter are TNF-α, GM-CSF, macrophage inflammatory protein-1α, and a number of "Th2-cell type" cytokines, such as interleukins IL-3, IL-4, IL-5, IL-9 and IL-13 (Gould et al., 2003).

Allergen activation of mucosal mast cells will also contribute to the enhancement of local IgE synthesis: the above-mentioned cytokines released during their degranulation increase the IgE production by B cells and augment the allergic response, like in a positive feedback.

The clinical symptoms that characterize the allergic outbreak result thus from the activities of the allergen-activated mast cells (Pawankar et al.,

1997). The local effects (e.g. in skin in atopic dermatitis or eczema) include (a) enhanced local vascular permeability, leading to leakage of plasma protein, such as fibrinogen (resulting in local deposition of crosslinked fibrin and tissue swelling); (b) increased cutaneous blood flow, with intravascular trapping of red cells, owing to arteriolar dilation; (c) increased loss of intravascular fluid from postcapillary venules to produce erythema (since the name of Immunoglobulin "E"); and other effects such as itching, owing to stimulation of cutaneous sensory nerves by histamine (Wedermeyer and Galli, 2000; Wedermeyer et al., 2000). Hayfever, asthma, reactions to food, and eczema are the most common clinical symptoms, caused by mast cell activation in mucosal tissues of, respectively, the nose, lung, gut and skin. Blood basophils are the IgE effector cells in the rare, but more dangerous, manifestation of systemic anaphylaxis (Gould et al., 2003) that can lead to the death of the patient.

1.6 Factors involved in the development of allergy

Over the last century the worldwide prevalence of allergy has increased dramatically. Allergic disease is a growing problem in industrialized countries (especially Western Europe, Australia, U.S.A.) where it affects approximately 25% of the population (ISAAC, 1998).

The occurrence of an allergic reaction is very individual, depending especially on genetic predisposition and environmental exposure to pollutants, but also on nutritional status, presence of chronic underlying infections or acute viral illnesses; these and other factors play an

important role in the expression of clinical symptoms mostly via an increment of the IgE production levels.

1.6.1 Genetical background of the allergic disease

A genetical predisposition to allergy is well proven: very early studies (Coca and Cooke, 1923) showed that allergic parents tended to have a higher proportion of allergic children compared to non allergic parents. Today it is well known that a clear difference in developing allergies exists between individuals having or lacking a genetic predisposition: the latter require higher and/or longer antigen exposure than the former (Aalberse, 2000). The tissue-specificity of an allergic reaction too appears to be inheritable: children from asthmatic parents are more likely to develop asthma than children from parents with atopic dermatitis and viceversa.

Despite all this clinical evidence it is very difficult to establish the precise mode of inheritance for atopy, since it is a multifactorial disease and greatly influenced by the interaction with environmental factors and can be transmitted from one generation to the next regardless of geographical or racial differences (Ono, 2000). No less than half of the susceptibility to asthma is determined by genetic predisposition (Duffy et al., 1990), and various genes or groups of genes have been proposed as candidates for the inheritance of atopic disease. However attempts to identify the responsible genes have often led to conflicting answers, owing to their multiplicity (Ono, 2000; Patiño and Martinez, 2001; Rosenwasser, 1997).

It has been suggested that a role may be played by the genes codifying for the molecules of the major histocompatibility complex MHC II on chromosome 6: the locuses HLA-DR4, -DR7 have been associated with susceptibility for the disease (Aron et al., 1996), whereas the locus HLA-DQ might confer protection against asthma (Ono, 2000). Some experimental evidence hypothesizes the role of these genes in the immune response to specific allergens: the house dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* (Lara-Marquez et al., 1999). However the genes of the major histocompatibility complex are not the only ones that appear to be associated with atopy: a mutation in chromosome 11q, that accounts for an Ile181Leu substitution in the β subunit of the FcεRI IgE high-affinity receptor, has been shown to be significantly linked to IgE levels, even if with some geographical, sexual and racial constraints (Cookson et al., 1992; Shirakawa et al., 1994). An important role in the genetic background that leads to the development of allergic disease may be also played by the genes coding for IL-4 and its receptor. A locus in human chromosome 5q31-q33 (the so-called "IL-4 cytokine cluster") has been associated with high total and specific IgE levels and asthma (Marsh et al., 1994; Xu et al., 1995); a linkage between this locus and specific IgE responses to some allergens (dog epithelium and american cockroach) in a group of African-American subjects was also observed (Hizawa et al., 1998). Another locus that has been indicated as a potential marker of atopy is the gene encoding the IL-4 receptor α-chain: this chain is common to the receptor for the cytokines

IL-4 and IL-13 both of which induce IgE synthesis in B cells and promote Th2 differentiation. It is hypothesized that an augmented signal transduction from the receptors for these cytokines might account for the atopic condition. Alternatively, signal transduction molecules linked to these receptors, such as STAT6, could also account for the elevated IgE synthesis and Th2-biased profiles in atopic individuals (Hershey et al., 1997; Ono, 2000).

1.6.2 Influence of environmental factors on the allergic disease

In addition and/or in combination with the genetic background, the influence of environmental factors is of great interest too in the pathogenesis of allergic disease. Individuals without a genetic predisposition can develop allergic disease only following strong antigenic stimuli (Aalberse, 2000). The quick rising of allergy in advanced countries or communities (in Africa asthma is an urban rather than a rural disease), shows the existence of a linkage between this disease and socio-economic advance (Riedler et al. 2001). A change in the pattern of early life microbial exposure may be a fundamental underlying factor in the rise of allergy (Hopkin, 2002).

Since the fetus is exposed to a Th2 environment in the uterus, the immune system is Th2-polarized at birth. It is believed that the immune deviation normally occurs by the induction of Th1 responses and the resulting suppression of Th2 responses following bacterial infections in the neonate. This may be averted (and the effect could be worst in

genetically predisposed individuals) by the high standards of hygiene in industrialized countries (the "hygiene hypothesis") and overexposure to allergenic substances such as the faecal antigens of the house dust mites or fungal spores in the atmosphere. These conditions are thought to have contributed to the dramatic rise in the prevalence of allergic disease in recent decades (Romagnani, 2000; Ono, 2000).

A further support for this theory comes from the demonstration that the presence of a cat or other pet in the domestic environment is associated with decreased incidence and prevalence of asthma amongst the preteens. This effect could be mediated by the induction of high titres of allergen-specific IgG4 antibodies that ultimately down-regulate the allergic response, even in presence of high titres of allergen-specific IgEs (Platts-Mills, 2002).

A theory alternative to the hygiene hypothesis has been recently proposed by observing that parasitic and helmintic infections, normally associated to a high level of IgE production, are accompanied by poor antigen-specific T cell-proliferative responses as well as reduced cytokine production (Mahanty and Nutman, 1995). Down regulatory molecules such as IL-10, TGF-β and Nitric Oxide have been implicated in this immunosuppression (Akdis et al., 1998; Doetze et al., 2000; King et al., 1996; Dai and Gottstein, 1999). These molecules, produced by antigen presenting cells and/or from the recently discovered regulatory T cells (Maloy and Powry, 2001) in response to a high pathogen burden (such as in chronic parasitic infections) can inhibit mast cell degranulation

providing a mechanism for the observed negative association between them and atopy. This "hypothesis of the anti-inflammatory network" suggests that instead of Th1 versus Th2 it may be the pro- versus anti-inflammatory axis with robust regulatory T cell network that has to be considered central to the balance and to the prevention of either Th1 and/or Th2 diseases (Yazdanbakhsh et al., 2002; Rook et al., 2000).

1.6.3 Influence of nutritional status on the development of allergy

The nutritional status of the individual may play an important role in the development of allergic diseases. A positive association between asthma and obesity in adults and children has been recently pointed out (Gennuso et al., 1998; Figueroa-Munoz et al., 2001). Particular attention has been recently focused on the proteohormone leptin. Since the serum levels of this hormone show a positive correlation with the body mass index (Considine et al., 1996), it has been recently suggested that it could also play a role in the occurrence of atopic asthma in children (Guler et al., 2004). Higher serum levels of leptin were found in asthmatic children (especially asthmatic males) compared to healthy children, despite no significant differences in body mass index. In addition a positive correlation between the levels of leptin and IgEs in serum has been observed, especially in obese children. This is probably due to the fact that more leptin is produced when body weight increases, given its role in decreasing appetite and increasing energy expenditure. Notwithstanding this primary effect in regulating fat mass, leptin plays also a

proinflammatory role inducing and/or enhancing the concentration in serum of TNF-α, IL-6 and IL-12, three cytokines whose levels are always increased (and which play a significant role) in allergic asthma. This observation, coupled to the report of the existence of receptors for leptin in human lungs, suggests that a role (possibly "involuntary") may be played by leptin in the development of allergic disease.

1.6.4 Biology of allergens: do they have any common feature capable to trigger the allergic response?

In physiological conditions the immune system triggers a reaction against harmful antigens, such as viruses and bacteria and/or their products. In the allergic disease the body reacts against harmless environmental antigens. In addition to a well-proven individual genetic predisposition and environmental influences, it has been proposed that there may be some common feature to the allergens themselves that allows them to divert the immune response to allergy. Aalberse (2000) reviewed several studies on this topic in order to try and establish what makes of an antigen an allergen. A common feature to all the allergens is their allergenicity. This characteristic can be defined in two ways. According to the clinical definition the allergenicity is the capacity of a molecule to elicit an allergic reaction, i.e. to induce clinical symptoms or a skin reaction in a patient. As stated by the immunological definition, the allergenicity indicates the ability of a molecule to sensitize, i.e. to induce the synthesis and secretion of high affinity antibodies (especially of the

IgE class) and/or to bind to IgE antibodies. Proteins and glycoproteins that possess allergenicity and that are capable to both sensitize and elicit an allergic reaction are considered as complete allergens. Molecules that can only elicit allergic symptoms but cannot sensitize are considered incomplete allergens. A well-known example of such a nonsensitizing elicitor, and thus of an incomplete allergen, is the major apple allergen Mal d 1.

An allergenic source or extract normally contains a mixture of molecules, some of which are capable to elicit the allergic response in a relevant percentage of allergic patients. These molecules are thus indicated as major allergens. However even when a molecule is a major allergen this does not mean that it is cause of major allergenic risk. The latter depends on the sensitizing potential of the allergen itself. So that the major allergens of dust mites represent a major allergenic risk than the major dog allergen (Can f 1) since the latter has a lower sensitizing power. Therefore, notwithstanding the fact that allergenicity is common to all the allergens, this characteristic is not expressed homogeneously.

To try and find a common ground to explain all these features, several studies of the protein structure have been conducted in order to assess their relevance as determinants of allergenicity. Protein structure has received much attention because of the hypothesis that common structural features could establish a base for the triggering of an allergic response by the immune system. Protein structure can be described at different levels: primary structure (given by the simple aminoacid

sequence), protein folding, domain structure and surface structure; the latter is particularly important being the most relevant for the antigen binding. It has been reported that stability, size and compactness of the overall folded allergenic protein are important features for its transport over mucosal barriers and its susceptibility to degradation by proteases. However, up to now very few structural characteristics are known to be common to allergens in general; some allergens have been clustered in classes that have been suggested to be more intrinsically allergenic, like 2S albumins from seeds (Teuber et al., 1998) and lipocalins (Virtanen et al., 1999). Given the importance of protein folding (whose structural basis is already contained in its peptide backbone) studies by using proximity plots to predict the tridimensional structure of a protein have been conducted on a number of major allergens and this lead to a clustering of allergenic molecules in a few groups depending on their folding (Holm and Sander, 1993; Fedorov et al., 1997; McTigue et al., 1995). Proximity plots are used to visually compare the foldings of allergens which are represented in the format of proximity matrices. By using these plots as fingerprints it emerged that the more different are the plots and the more different are the folds. This approach has been used also to try and explain the often occurring phenomenon of cross-reactivity between allergens. By comparing different allergens it emerged that sharing a similar plot is not enough to account for a similar folding; however different plots will definitely bring to different folds. According to these studies the allergens investigated were clustered in 5 groups according to

their protein folds: 1) antiparallel β-strands, 2) antiparallel β-sheets intimately associated with one or more α-helices, 3) (α+β) structures in which the α and β structural elements are not intimately associated, 4) α-helical and 5) other structures. The overall conclusion of these studies however is that to date there is no structural evidence that brings an antigen to behave like an allergen: the only characteristic shared by all allergens, as far as it is known, is their ability to reach and stimulate immune cells and mast cells and the capacity to avoid activation of Th2 suppressive mechanisms. Features other than structure can be relevant for allergenicity as well like the knowledge of the sensitization pathways that can be different for different allergens.

All of these data point out that a number of factors must contribute to allergy. The term "allergic breakthrough" is commonly used to indicate all these conditions that, alone or in combination, can cause an exceeding of immunological activity that at the end leads to the atopy.

1.7 Clinical diagnosis of allergic disease

It can be difficult to make a precise diagnosis of an allergic status since several other disease aetiologies are present with similar symptom profiles, and there is no single gold standard for achieving a reliable diagnosis (Ahlstedt, 2002). The latter can only be made by the expert physician when all clinical and laboratory findings have been evaluated. The clinical diagnosis should be based on a careful evaluation of patient's history, symptoms, findings at physical examination, laboratory test results, and any other investigations considered necessary. A correct and early diagnosis of an allergic status is of primary importance in facilitating the selection of appropriate management strategies such as immunotherapy or allergen avoidance (Holgate and Lack, 2005). As with most medical conditions, the starting point for diagnosing allergy is a careful clinical history and physical examination. The clinical history in particular should assess if the patient is likely to be allergic and, if allergy is suspected, which allergens are involved (Eriksson, 1994). However, although valuable, a good clinical history is not always definitive. In these circumstances, given the central role played by the reaction of IgEs with their receptors in the phenomenon of allergy (Gould et al., 2003), the detection of IgE antibodies either via cutaneous reactions or laboratory tests is the chosen strategy. Even if a normal level of serum IgE cannot exclude the atopy and the threshold of serum IgE necessary to trigger a clinical reaction can differ from allergen to allergen (Sampson and Ho,

1997; Sampson, 2001; Soderstrom et al., 2003), IgE levels are usually high when an allergic disease occurs (Male, 1998). It follows that during the clinical evaluation of a person suspected of having an allergic disease, total serum IgEs and allergen-specific IgEs are the primary analytes that are commonly monitored (Matsson et al. 1997). The most common monitoring systems currently used can be divided in two groups: the cutaneous reactions and the laboratory tests.

1.7.1 Cutaneous reactions

(I) The skin prick test

It is a very simple diagnostic test and it provides a rapid means of assessing allergies in a clinical context. It is performed with an appropriate skin testing device (e.g., lancet) which punctures the skin through a drop of glycerinated extract and appropriate positive (histamine) and negative controls (saline). The immediate wheal-and-flare reaction results by the release of preformed mediators triggered by the allergen introduced into the skin. This ultimately leads to increased vascular permeability, local oedema and itching. Skin prick tests are judged positive if there is a mean wheal diameter of 3 mm or greater than those induced by the negative control (Crespo et al. 2004). This wheal reflects the presence of mast cell-bound IgE specific to the tested allergen (Li, 2002).

A positive skin test usually correlates with a positive radioallergosorbent test (RAST) or other laboratory tests for allergen-specific IgE in serum,

and a positive provocation test of the relevant area (e.g. challenge of the nasal or bronchial mucosa with the allergen). The fact that patients with a variety of atopic disorders show the classical immediate wheal-and-flare response following skin prick tests demonstrates that IgE is bound to skin mast cells, even though their allergic symptoms may occur in the nose or bronchi. This test is still considered as the gold standard for IgE antibody detection to date, mainly because of its unrivalled clinical sensitivity.

(II) The skin patch test

It is very similar to the skin prick test, but it involves the application of the allergen to a lightly abraded skin area that is not affected by the allergic lesions (for example in atopic eczema) (Male, 1998).

(III) Intradermal testing

This test is performed by injecting the allergen extracts within the dermal layer and it is usually reserved for venom and drug allergy testing when percutaneous tests (i.e. skin prick and skin patch) are negative but there is high suspicion of allergy. The test allergen is 10- to 100-fold more diluted and although this approach may increase the clinical sensitivity of skin testing, it does so at the cost of a loss in clinical specificity (Plebani, 2003).

1.7.2 Laboratory tests

Traditionally, laboratory procedures have employed antibody-based methods for the detection of serum IgE. A variety of detection end points (radioactive, colorimetric, fluorescence, etc.) as well as several solid-phase chemistries have been developed. Particularly enzyme immunoassays, at present widely employed in diagnostics, are based on two important biological phenomena: (i) the extraordinary discriminatory power of antibodies, based on the ability of the immune system of vertebrates to produce a virtually unlimited variety of glycoproteins (antibodies), each with an affinity for a specific foreign compound (antigen or hapten); and (ii) the extremely high catalytic power and specificity of enzymes, which may quite often be detectable with great ease. Enzyme immunoassays consist thus of a two-pronged strategy: the reaction between the immunoreactants (antibody with the correspondent antigen) and the detection of that reaction using enzymes, labeled to the reactants, as indicators (Tijssen, 1985).

Here it follows a brief description of the most commonly employed laboratory tests in allergology to date.

(I) Radioallergosorbent Test (RAST)

This test, originally described by Wide and coworkers (1967), is based on the covalent coupling of an allergen to a paper disc solid phase. Given the capability retained by the coupled allergen to bind its specific antibody (IgE, IgG and IgA isotypes), the complex can be incubated with

the serum of a patient whose allergic status is to be investigated. If antibodies against this allergen occur in the serum, they will react with the conjugated allergen. Then, after removal of all the unbound serum components, radioactive-labelled (^{125}I) antibodies anti-IgE are added, and they will likely bind the serum IgEs. The uptake of labelled antibodies, measured in terms of radioactivity, will result proportional to the amount of IgE antibodies occuring in the serum. In its original form the results of the test were reported in classes or in arbitrary units by interpolation from a heterologous IgE anti-birch pollen reference curve.

This test has been made commercially available in 1974. Since then, there have been several attempts to improve it. The clinical sensitivity of the RAST infact was lower than the skin prick test's. In addition the occurrence of competitive inhibition of IgE antibody binding to limited insolubilized allergen by IgG antibodies in serum from individuals with high-dose natural exposure or allergen immunotherapy was of concern. As a consequence of these drawbacks, Nalebuff and coworkers (1979) used longer incubation times and a different scoring system to achieve a clinical sensitivity similar to that of skin testing, but they ended up in a loss of clinical specificity. Other strategies focused on the replacement of the original solid phase with other support media, such as cellulose in the form of filter paper discs (Ceska et al., 1972), mycrocristalline particles (Gleich and Jones, 1975), thread (Miller et al., 1984), minicolumns (Bousquet et al., 1990), paramagnetic particles (Kleine-Tebbe et al.,

1992), nitrocellulose (Derer et al., 1984), polystyrene tubes (Zeiss et al., 1973) and microtiter plates (Olivieri et al., 1993).

More recently immunoassays have undergone several refinements, including substitution of the original radiolabeled polyclonal antibodies with enzyme-tagged monoclonal detection antibodies, the development of solid-phase support systems with higher binding capacities, and the introduction of semi or totally automated systems.

(II) Multi Allergy Screening Test (MAST)

A method whose chemistry is similar to the RAST (since it employs a radioactive-labelled secondary antibody for the detection of allergen-specific IgEs) but with differences and advantages, is the MAST system described by Miller and coworkers in 1984.

The main difference consists in the solid phase to which the allergens are adsorbed: this is not a paper disk but a nitrocellulose thread containing one or more allergen specificities to be measured. Up to 38 threads carrying allergen specificities, positive and negative controls and calibration controls are all packed into a unique analytical test chamber. In this way no volume-dependent pipetting steps or separate standards are required: quality controls are infact internal to the chamber itself. The detection system involves also immunography onto high-speed Polaroid® instant film; an inexpensive microprocessor-controlled automatic recording infrared transmittance densitometer quantifies results from the

immunographic film strip; and a patient-result form allows easy interpretation of the test results.

(III) Enzyme-Linked Immunosorbent Assay (ELISA)

Originally described by Engvall and Perlmann (1971), it is very similar to the RAST and MAST tests, above described, consisting of an antigen adsorbed to a solid phase and incubated with serum of a patient suspected of allergy, in order to reveal the presence of IgE against one or more allergens. The main difference consists in the way the signal is detected: instead of incubating the IgE bound to the antigen adsorbed onto the solid phase with a radioactive anti IgE, an enzyme-linked anti-IgE with its substrate is added. Since the action of the enzyme on its substrate gives rise to a coloured product, the measurement of the colour intensity allows to quantify the serum IgE. The advantage of the enzyme-based immunoassays over RAST and RAST-analogues is the employment of an enzymatic amplification that compensates for the low abundance of circulating IgEs, providing thus greater clinical sensitivity (Lebrun et al., 2005).

(IV) Cellulose Acetate membrane Precipitin (CAP)

This is another in vitro test system, very similar to ELISA, for the determination of circulating specific IgE antibodies. The main difference is given by the solid phase: the allergen of interest is covalently coupled to a flexible hydrophilic carrier polymer encased in a capsule (ImmunoCAP).

The carrier consists of a CNBr-activated cellulose derivative with a very high protein binding capacity compared to other substrates like traditional coated tubes or paper (Axen et al., 1988; Yman, 1991). The ImmunoCAP can react with specific IgEs of the patient's serum. After washing away unbound IgE, enzyme-labelled antibodies against IgE are added to form a complex. After incubation, unbound enzyme-labelled anti-IgE is washed away and the bound complex is then incubated with a fluorogenic substrate. The latter forms a fluorescent product upon reaction with the anti-IgE conjugated enzyme. As well as in ELISA the colour intensity will provide indication of the levels of allergen-specific IgEs in the patient's serum, via interpolation to a heterologous total serum-IgE dose-response curve used as a calibration system. In the clinical practice CAP results must be interpreted with care. The diagnostic performance of this test varies in an allergen-specific manner, and CAP scores do not always correlate with clinical severity (Kelso et al., 1991; Kong-Ling and Kue-Hsiung, 1994). It has been reported that CAP clinical sensitivity, clinical specificity and positive predictive values agree well with skin prick test for house dust mite and grasses, but poorly with tests for cat dander and peanuts (Thompson et al., 1999).

1.7.3 Characterization of immunoassays for allergen-specific IgEs

According to the NCCLS guidelines (Matsson et al., 1997) the IgE antibody immunoassays can be generally grouped into one of three

cathegories depending on the kind of results that they provide: qualitative, semiquantitative and quantitative.

(I) Qualitative assays

A qualitative assay produces one of two possible results: either the analyte is non detectable/negative/non reactive or detectable/positive/reactive/present. Qualitative assays do not provide any precise estimate of the analyte concentration. A positive test result only implies that the assay signal exceeds the analytical threshold or positive cut-off point that has been set to obtain an arbitrary combination of diagnostic sensitivity and specificity. Qualitative assays maintain their value in clinical practice, particularly when used as a first step in the diagnostic algorithm and/or as an objective tool in epidemiologic studies. The *in vivo* tests (skin prick and the cutaneous reactions) are examples of qualitative assays.

(II) Semiquantitative assays

These assays report not only the positivity or negativity of a sample for one or more allergens, but they also provide information on the differences between positive signals (i.e. the magnitude of the response). These differences are usually given in a series of increasing grades or class scores (e.g. 0 to 6; low to high). These classes are, in turn, defined by arbitrarily defined International Units per milliliter (IU/mL), or per liter (kIU/L) determined relative to a supplier-specific, heterologous

dose-response curve; an endpoint dilution to which the signal becomes negative (e.g. titer); or in comparison to a qualitative grading scheme (e.g. color chart). Ideally, but not exclusively, response signal units generated by semiquantitative assays correlate with the level of IgE antibodies in the blood and the patient's sensitivity to the test allergen specificity. The results produced however are not traceable to any common reference material and the assays do not always (consistently) meet the criteria of linearity, dilution recovery and parallelism that are typically achieved by quantitative assays.

Examples of IgE immunoassays belonging to this category are the RAST and most "RAST-analogues".

(III) Quantitative assays

This kind of assays provides an accurate and reproducible estimate of the concentration of an analyte, such as the allergen-specific IgE antibodies. In addition these assays fulfill the analytical criteria for quantification, including parallelism, recovery, precision and linearity across the whole assay's working range. The quantitative assays employ a multipoint standard or calibration curve from which unknown data are interpolated. The reference curve can be constructed by using heterologous or homologous interpolation methods. The calibrated titration assay that uses heterologous interpolation is unanimously considered an acceptable alternative because IgE antibody for each allergen is interpolated from a heterologous or different IgE reference

curve, such as a total serum IgE reference curve. Therefore the assay has to be traceable to the WHO 75/502 internationally recognized total serum IgE standard. The results can be reported in gravimetric (mg/L) and/or International Units (kIU/L).

It has been demonstrated that threshold levels for specific IgE are likely to be useful in clinical practice to distinguish symptomatic from asymptomatic allergy in patients with positive skin test results (Pastorello et al., 1995). Therefore quantitative results might really have an added value in clinical practice, allowing physicians not only to detect the presence of sensitization to a given allergen, but also to relate IgE concentrations to symptoms and risk of diseases.

1.7.4 Allergy diagnosis: the way forward

The skin prick test (and the cutaneous reactions in general) has two main advantages over laboratory *in vitro* testings, i.e. its inexpensiveness and its immediate accessibility. The results of the skin prick are immediately available to the physician (in about 15 minutes) and visible to a doubting patient. In addition the clinical reasoning is facilitated, the physician being able to provide the patient with an overall evaluation of clinical history, symptoms and test results together, in the face of the patient (Plebani, 2003). This test, however quick and simple, suffers from several drawbacks. Patients (particularly children) will only tolerate a limited number of tests, and people who are receiving antihistamine or anti-depressants treatment cannot be tested unless the

drug treatment is interrupted several days in advance. A variability in the results has been reported due to season, time of day and area of the body tested. Patients have to be kept under observation for some time in order to avoid a possible anaphylactic reaction caused by the allergen extract applied on the skin. Finally, most of the allergen extracts (especially food) are not standardized, therefore different product batches may give different results even if they come from the same manufacturer (Vanto et al., 1980; Holgate and Lack, 2005). In addition, the skin prick test gives only a "yes-or-no" answer for the allergenicity to a given extract, but no correlation with allergen-specific IgE levels in serum is provided.

In vitro tests for IgE antibodies have several advantages over skin testing, including a reliable definition of the performance parameters (analytical sensitivity, analytical specificity, precision and stability). The reagents employed for the *in vitro* testing have normally long shelf-lives and are amenable to ongoing quality control for stability and reproducibility. In addition they are not influenced by pharmacotherapy so that medications do not have to be withdrawn before testing. The risk of systemic reactions can also be avoided (Dolen, 2001). For these reasons, *in vitro* testing is the preferred method for IgE measurement in the following circumstances: a) extreme sensitivity to allergens, as demonstrated by near-fatal reactions that may occur; b) abnormal skin conditions, such as severe dermatographism and active dermatitis; c) during the refractory period of mast cell mediator depletion immediately after a severe allergic

reaction; d) use of medications that can interfere with skin reactivity; e) uncooperative patients and/or patients who eventually refuse skin testing (Bernstein and Storms, 1995). In the clinical practice results of skin testing and modern immunoassays are concordant more often than not (Dolen, 2000).

A clinical pathway has been proposed in a recent review (Plebani, 2003) for the diagnosis and treatment of allergy considering both *in vivo* and *in vitro* testing. In cases where the patient presents with unequivocal symptoms and clinical history, and just one or few allergens are supposed to be the causative agent(s), a selected measurement of specific IgE in blood is suggested. In cases where diagnosis of allergy remains doubtful, or many allergens are suspected to play a causative role, skin testing or a multiallergen screening test in blood should be performed. If positive, a selected measurement of *in vitro* specific IgE with quantitative assays is then requested to properly diagnose and treat the patient.

1.7.5 Alternative *in vitro* methods for the detection of allergen-specific IgE antibodies: cell-based technologies

Since the late sixties, attempts have been made to use the secretory response of human basophils, in presence of IgE cross-linking doses of allergen, as an alternative way to measure allergen-specific IgE antibodies (Lichtenstein et al., 1967; Crockard and Ennis, 2001). Assays were set up based either on the release of specific mediators by

basophils (histamine and cysteinyl leukotrien C_4 – LTC_4) or on the expression of specific molecules on the basophils surface (especially CD63 and CD203c) following the cross-linking between allergen and the surface-bound IgE antibodies. However, a number of drawbacks have emerged that did not allow them to be used in the clinical diagnostics. These cell-based technologies involve whole blood, which need to be processed within 24 hours, they are time consuming and expensive and they require special technical skills. In addition not all sensitized individuals release mediators from their basophils on allergen challenge. Finally both reagents and assay methods have not been standardized yet and their clinical sensitivity and specificity vary in accordance to the allergen used for the challenge (Nolte, 1993; Ebo et al., 2002). This is the reason why these tests are used only for research purposes to date and their applicability to a clinical diagnostic routine is not seen as feasible in the near future (Hamilton and Adkinson, 2004).

1.8 Microarray technology

It was widely believed, in the early 1990s, that to achieve high analytical sensitivity in detecting an analyte it was necessary to bind the majority of the analyte itself present in a test sample (Hay et al., 1991). The key concept of the microarray stems from the idea that using far smaller amounts of binding agent (located at high surface density onto a solid support) very high sensitivities are achievable, higher than those that for decades have been regarded as obligatory. By using high-specific-activity labels (like fluorescent dyes), a sufficient mass of capture agent could be accommodated onto a microspot a few μm in diameter and ultrasensitive detection of the target analyte under investigation can be achieved. This ultimately permitted the creation of microarrays, each microspot therein recognizing a different analyte (Ekins and Chu, 1999).
A microarray is thus made by ordered rows and columns (arrays) of capture molecules (genetic elements, proteins, or antibodies) laid down in well-defined positions onto a solid substrate. The microarrays are exposed to samples containing appropriate analytes which bind to their immobilized counterparts. The concentration of the analyte in the sample can be qualitatively and/or quantitatively determined by detecting the resulting ligand-analyte complex in the deposited microspots.

1.8.1 DNA Microarrays

The microarray technology has been initially employed in genetic studies where high density DNA microarrays have been shown to be the ideal assay format to study the expression profiles of thousands of genes simultaneously in tissues and organs under different experimental conditions (DeRisi et al., 1997; Duggan et al., 1999; Liotta and Petricoin, 2000). The basic principle of DNA microarray consists in the hybridization of an unknown mRNA (purified and collected from the sample to be analyzed) to an ordered array of DNA sequences corresponding to known genes or expressed sequence tags. Every mRNA, or transcript, is labelled with a marker, e.g. a fluorescent dye. The location of the signal gives an indication of which transcripts occur and the signal intensity gives a quantitative estimate of each transcript in the sample. A DNA microarray can contain DNA sequences corresponding to more than 30,000 human genes, i.e. the genome, ordered on a miniature glass slide. It is thus possible to measure all transcripts in the sample, i.e. the transcriptome. It follows that the gene expression profiling is one of the main achievements of this technology (Benson et al., 2004).

The widespread use of this technology for monitoring gene expression has generated valuable insight into various disease states, particularly in cancer research. Some kinds of cancers are often characterized by similar histopathological appearance notwithstanding their differences in clinical course and response to chemotherapy. By using a DNA microarray platform it was possible for example to identify differential

gene expression in leukaemias with the same histomorphological features. The information obtained, allowed to set up a basis not only to better recognize different kinds of leukaemia, but also to introduce new classes and subclasses of tumours. The advantages of this approach would result in better targeted therapies providing a maximized efficacy and a minimized toxicity (Golub et al., 1999). In another work the same technique has been used to better characterize the gene expression patterns of breast carcinomas not only to have a better characterization and classification of the carcinomas but also to spot the genes whose expression is associated to a better prognosis (Sorlie et al., 2001). By using the opposite strategy, the DNA microarray platform can be useful in targeting and better characterizing genes or groups of genes commonly expressed by different kind of tumours in order to unravel fundamental mechanisms that cut across distinct types of cancers.

DNA microarrays have proven to be particularly valuable in analyzing clustered gene expression, revealing co-regulated gene networks. They have been used for example to analyze group of genes in the allergic disease: microarray analyses of Th2 cells from allergic patients showed up-regulation of gene families such as chemokines, chemokine receptors and metallo proteinases (Wohlfahrt et al., 2003). Microarray analysis has been used to elucidate the effects of specific cytokines relevant to allergy. As an example, a better characterization of the way IL-12 and IL-4 induce the differentiation of Th0 cells into the Th1 or Th2 subtypes respectively was achieved by establishing which genes were more expressed after

stimulation by either of these two cytokines (Lund et al., 2003). In another study the Th2 cytokine IL-13 was shown to induce distinct transcriptional programs in human airway epithelial cells, smooth muscle cells and fibroblasts, highlighting thus the way this cytokine affects the clinical manifestations of asthma (Lee at al., 2001). All these studies indicate a unique feature of DNA microarrays, i.e. the possibility to identify whole groups of functionally related genes, rather than individual genes.

1.8.2 Protein microarrays

Gene expression analysis does not readily predict protein abundance nor does it provide any information about protein function: it is not possible to predict the protein expression level of a cell by simply quantitatively correlating the mRNA and protein levels (Gygi et al., 1999). Given the central role played by proteins in biology and physiology, better methods were needed in order to study protein abundance and activity in high throughput. Protein-based microarrays offered such approach. They have been introduced in order to unravel protein-protein and protein-nucleic acid interactions and for high-throughput screening of recombinant antibodies (Cahill, 2001; Emili and Cagney, 2000; deWildt et al., 2000). Arrays of proteins and/or antibodies, when covalently bound to glass slides by means of pin-based or micro-dispensing liquid handling robots, have been shown to retain their ability to interact specifically with other proteins or with small molecules in solution (MacBeath and Schreiber, 2000) similarly to their DNA/RNA counterparts. However

proteins have several properties that render their microarraying a more challenging issue than microarraying their DNA counterparts. Unlike the simple hybridization chemistry of nucleic acids, proteins demonstrate a staggering variety of chemistries, affinities and specificities. Moreover proteins may require multimerization, partnership with other proteins or post-translational modification to demonstrate activity or binding. In addition, there is no equivalent amplification process like PCR that can generate large quantities of proteins. Expression and purification of proteins is a tedious task and does not guarantee the functional integrity of the protein at the end of the process. Finally, many proteins are notoriously unstable, which raises concerns about microarray shelf-life. Despite these challenges, the development of protein microarrays has begun to achieve some recent success (LaBaer and Ramachandran, 2005).

The development of protein microarrays has followed two different ways, namely abundance-based microarrays and function-based microarrays.

The abundance-based microarrays aim to measure the abundance of specific biomolecules using analyte-specific reagents such as proteins and/or antibodies. Antibody arrays have the potentiality to detect the presence or absence of specific proteins, and to quantify their level of expression in normal versus pathological tissue or body fluid (Cahill, 2001). Protein arrays were used to develop comparative fluorescence assays to measure the concentrations of several specific proteins and antibodies in serum and other complex solutions (Wiltshire et al., 2000;

Haab et al., 2001; Huang et al., 2001; Wiese et al., 2001). Protein and antibody arrays have been recently described to work as a useful diagnostic tool in the identification of chemical compounds in blood, not necessarily of proteic nature, such as drugs of abuse (Du et al., 2005). One of the main potential application of protein microarray is their use as a diagnostic tool for infectious and autoimmune and allergic diseases and can be easily optimized for clinical use as an alternative to ELISA. This technique has potentially important advantages in throughput, convenience and cost because it can provide a practical and convenient method to quantify thousands of different protein species in clinical or research applications, using minimal amounts of reagents (especially biological samples) and increasing the cost effectiveness of the analysis. This is ultimately due to the miniaturization that comes with this method: microarrays use smaller volumes than conventional testing systems and therefore they have proved to be highly economical in the use of specimens and reagents. Finally, the use of the microarray in contrast to traditional immunoassay systems, provides a remarkable saving in time required to process samples. In studies conducted in this laboratory, it was shown that the ToRCH assay performed equivalently to a commercial ELISA (Mezzasoma et al., 2002; Bacarese-Hamilton et al., 2004a; Bacarese-Hamilton et al., 2004b).

Microarray-based systems for the parallel detection of autoantibodies in human sera have been described as well. In one of them, known autoantigens were arrayed in known concentrations within one array. This

format allowed the determination of 18 different autoantibodies' titer by using a minimal amount of serum in a single immunoassay experiment (Joos et al., 2000).

The second strategy, function-based microarrays, is meant to examine protein function in high throughput by printing a collection of target proteins on the array surface and assessing their interaction and biochemical activities (LaBaer and Ramachandran, 2005).

As an example, protein microarrays have been used to analyse specific properties of membrane proteins. Membrane protein arrays were generated to study the ligand-binding properties of membrane receptors. Because important members of signalling pathways, such as receptors and their associated signalling molecules, are membrane located, systematic analysis of membrane proteins provides a valuable task for proteomics (Fang et al., 2002).

Another application of function-based protein microarrays is the monitoring of enzymatic reactions on a chip. Several studies are reported concerning the use of function-based protein microarrays for the quantitative measurement of kinase activity. By applying Surface Plasmon Resonance, fluorescence and phosphorimaging for the detection of phosphorylation a quantitative evaluation of three inhibitors was achieved (Houseman et al., 2002). Microwells containing arrayed proteins were used to analyse nearly all the protein kinases from the yeast *Saccharomyces cerevisiae*. Many novel activities were described, including 27 protein kinases with an unexpected tyrosine kinase activity

(Zhu et al., 2000). Protein-protein interactions were investigated on a large scale through the production of the first proteome chip. For the generation of the chip, 5800 open reading frames of yeast were cloned and the corresponding proteins were overexpressed in *E. coli*, purified and spotted. The resulting protein microarray was used to screen for interactions with calmodulin and phospholipids and allowed the identification of binding motifs (Zhu et al., 2001).

All these studies point out at the big potential of the microarray as a tool to be used both in research and in diagnostics. The advantages offered by miniaturization, high-throughput and parallelism will allow this technique to keep on developing and improving and to widen its range of potential applications.

1.9 The protein microarray as a novel diagnostic tool for the determination of allergen-specific IgE in human serum

The avoidance of an allergenic source is the primary strategy when allergy occurs. For this reason a wide range of allergens to which patients can be potentially exposed, from food to environmental sources, have to be tested for both diagnosis and prevention. However, such wide spectrum of allergens has not been tested yet because with the currently available detection methods the volumes of serum samples and the costs would be prohibitive. Microarray technology offers advantages in diagnostic applications because the amounts of reagents required, and thus the cost per assay, are greatly reduced. In this miniaturized platform crude or purified native and recombinant allergens can be spotted in microdot arrays on chips to permit extensive panels of specific IgE measurements to be performed with small quantities of serum. This is particularly attractive for allergy testing where high volumes of serum samples are requested to date to screen for allergen-specific IgE. For this reason the latest technology trend in allergy diagnostics looks at microarrays (Hamilton and Adkinson, 2004).

Some basic general steps are applied in the production of allergen microarrays. The first consists in the deposition of the purified or recombinant allergen components onto chemically preactivated glass slide in the form of a circular shaped spots by way of an automated process termed spotting. Delivery of nanolitre volumes of the protein

solution can be achieved in a contact or non-contact manner. If the coating chemistry of the glass slide and the spotting conditions are optimised for the immobilization of proteins, a sufficient proportion of the protein retains its biologically active form and can be consequently recognized by affinity interaction partners, e.g. antibodies. In total not more than a few hundred picograms of protein become covalently attached to the surface. As a consequence, with only a few micrograms of allergenic protein, a vast number of allergen microarrays can be produced. Basically, about 10,000 protein spots can be deposited on a standard microscope glass slide (75 X 25 mm), that by far exceeds the number of allergens known to date. This will allow, in turn, to accommodate a number of replicas of the array on the same slide surface and, separating them with a Teflon mask or other similar device, to test either different samples, or the same sample for other antibody reactivities, such as IgG.

During the first processing step the immobilized allergens on the arrays are exposed to the patient's serum. Consequently, allergen-specific IgEs bind to the individual allergen spots on the array. In the successive steps, bound IgE can be detected by using fluorescently-labelled secondary antibodies or various other available chemistries and signal amplification techniques.

Allergen arrays can be scanned with a conventional microarray laser scanner and the resulting images can be quantified with any available microarray analysis software (Harwanegg et al., 2003).

One of the first allergen microarrays to be described (Kim et al., 2002) showed a poor analytical sensitivity (1 IU/mL). This value was higher than the 0.35 IU/mL used by CAP and most ELISA systems as a cut-off to discriminate a positive from a negative sample and thus to detect concentrations of allergen-specific IgEs that are already indicative of an allergic status. Using a test with such analytical sensitivity means that all the reactivities falling into class score (or grade) 1 and part of the reactivities falling into class score (or grade) 2 (according to the classification adopted today by most of the allergen-specific IgE in vitro tests) are missed out. In addition the heterologous calibration system used in this study, based on a serial dilution of human IgE spotted on the slide, was not sensitive and reproducible either. Finally in order to reduce the volume of serum required for the incubation of the arrays, the allergens had to be spotted at high concentrations (1 mg/mL or above) in order to "capture" the most of the very scarce allergen-specific IgE antibodies occurring in the diluted serum. A positive feature of this work was the choice of spotting heterogeneous protein mixtures from crude extracts rather than purified allergens. Many protein components in the extracts can potentially play a role as allergens and it is only for very few extracts that a clear characterization in terms of major allergenic molecules has been achieved. It follows that the use of extracts is more appropriate for diagnostic purposes and their use in a miniaturized platform is one of the main advantages offered by the protein chip technology.

However the use of crude allergen extracts is not considered as a breakthrough by everybody. Other groups chose to spot on the slides the specific molecules responsible to elicit the allergic reaction either obtained by purification procedures from the crude extracts, or as recombinant proteins (Hiller et al., 2002). In this way the mass of allergen spotted onto the slide surface could be reduced and the volume of serum sample employed could be kept at low levels (i.e. 40 μL per test). The clinical sensitivity to the different allergen sources was well reflected by the IgE reactivity profile to the microarrayed allergen components from the respective sources and the level of reproducibility was high as well. However the clinical sensitivity of the assay remained low, and very similar to the RAST's. In addition no strict association between the magnitude of the wheal reactions in the skin prick test and the fluorescence intensity was observed. Though this kind of strategy is still far from replacing the use of the whole extracts and to be fully applied in the clinical diagnostics, the characterization and production/purification of specific allergenic molecules could be useful in improving the current diagnostic strategy: it has been suggested that low-abundant allergens could be spiked, for example, in crude allergenic extracts (Lundberg et al., 2001).

An attempt to test the performance of an allergen biochip made up with recombinant allergens from timothy grass pollen and birch pollen was made (Jahn-Schmid et al., 2003). Notwithstanding the advantages of a further reduction in the serum volume (down to 20 μL), the analytical

parameters did not significantly improve compared to the allergen arrays previously described: the analytical sensitivity was claimed better than CAP/RAST system, but comparable to the ELISA; the values of coefficient of variation indicating the intra-assay and inter-assay precision were higher than the specifications suggested in the NCCLS guideline for IgE immunoassays (Matsson et al., 1997); the clinical sensitivity and specificity, as well as the other parameters, varied according to the allergen considered.

The problem of the inadequate sensitivity (both analytical and clinical) is of particular concern especially in children and patients who are developing an allergy and in subjects under immunotherapy, where it is extremely important the evaluation and monitoring of low levels of allergen-specific IgE. As a consequence of the small feature size (about 200 μm in diameter) and the low amount of immobilized allergen, microarray assays fall into the category of the so-called "ambient analyte assays". These assays do not measure the total concentration of the analyte present in the sample, but only a small concentration of it: if the fluorescence read-out device is sensitive enough and the surface coating chemistry provides an optimal signal-to-noise ratio, detection limits and assay linearity as for conventional immunoassays can be achieved with microarray-based assays (Ekins, 1998).

Other studies have thus focused on strategies and chemistries that could further increment the analytical sensitivity of the system. One strategy consisted in the application of the Rolling Circle Amplification (RCA)

technique to the immunoassay for the detection of allergen-specific IgE antibodies in serum (Wiltshire et al., 2000). This method initially described to detect point mutations in human genomic DNA and to analyze rare somatic mutations (Lizardi et al., 1998), was subsequently adapted for the detection of protein antigens and in this form it is better known as immunoRCA (Schweitzer et al., 2000). In the immunoRCA an oligonucleotide primer is covalently attached to an antibody; thus, in the presence of circular DNA, DNA polymerase and nucleotides, the DNA molecule is amplified to contain hundreds of copies of the circular DNA sequence that remain attached to the antibody and that can be detected in a variety of ways. The use of this method in immunoassays resulted in a significant increment in the analytical sensitivity, enabling thus to carry out immunoassays in microspot and microarray formats. However when applied to the allergy diagnostics, the clinical sensitivity varied according to the allergen tested: only for some allergens the clinical sensitivity was higher than the reference CAP and, amongst them, the clinical sensitivity was more pronounced for some of them and not for others. The clinical specificity, clinical accuracy and correlation with the results of the skin prick test were however matching the specifications.

Another strategy consisted of a more "traditional" microarray assay format coupled with a non-linear signal amplification technique based on the tyramide system (Molecular Probes - Invitrogen Detection Technologies, 2005 – www.invitrogen.com). This is an enzyme-mediated detection method that employs the catalytic activity of horseradish

peroxidase (HRP) to generate high-density labelling of a target protein or nucleic acid sequence in situ (Bobrow et al., 1989; Chao, 1996; Van Gijlswijk et al., 1997; Speel et al., 1999). The tyramide labelling is the combination of four elementary processes: binding of a probe (allergen-specific IgE) to the target (allergen) via immunoaffinity, followed by secondary detection of the probe with an HRP-labelled secondary antibody or streptavidin conjugate; this is followed by the activation of multiple copies of a fluorophore-labelled tyramide derivative by HRP; finally the covalent coupling of the resulting highly reactive, short-lived tyramide radicals to nucleophilic residues in the vicinity of the HRP-target interaction site occurs, resulting in minimal diffusion-related loss of signal localization. The signal amplification conferred by the turnover of multiple tyramide substrates per peroxidase label translates to ultrasensitive detection of low-abundance targets (such as the allergen-specific IgE antibodies) and the use of smaller amounts of tracer antibodies and hybridization probes (Figure 1.1). This strategy has been already successfully employed in our laboratory and allowed to get an analytical sensitivity for the system lower than 1 fg of bound IgE, regardless the allergen considered (Bacarese-Hamilton et al., 2002; Bacarese-Hamilton et al., 2004a) corresponding to a concentration comparable to the 0.35 IU/mL widely accepted as cut-off value.

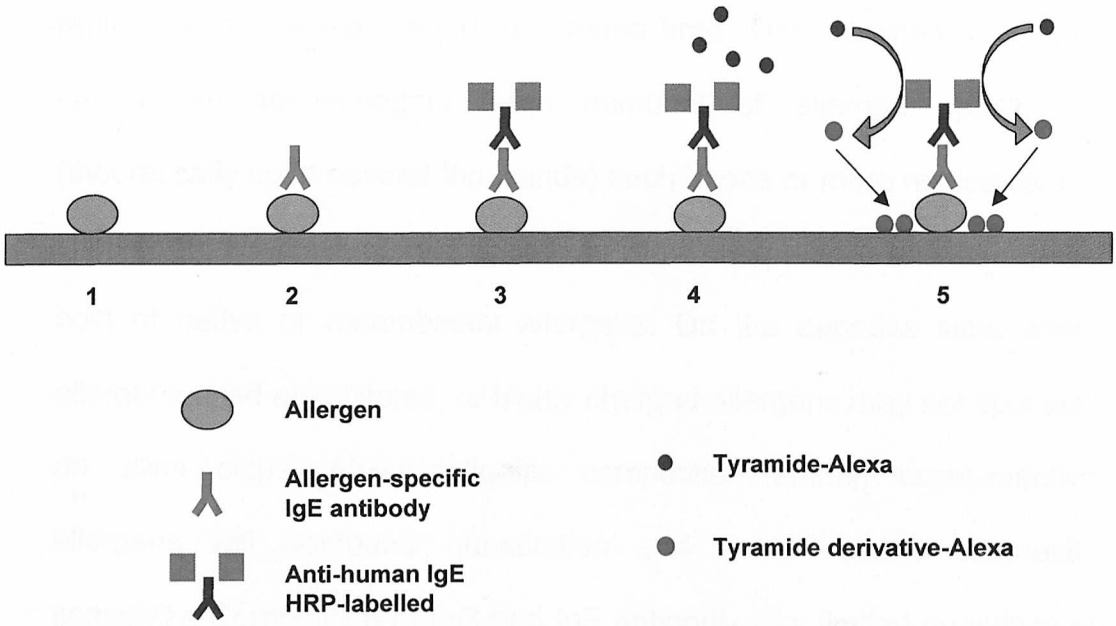


Figure 1.1. Tyramide amplification system applied to the allergen microarray format.
1. Allergens are spotted onto a glass slide (only one allergen is depicted here for graphic convenience). **2.** The array is then incubated with a serum sample containing allergen-specific IgE antibodies that will bind to their specific allergens. **3.** The binding between allergen and allergen-specific IgE can be revealed by using an HRP-labelled secondary antibody raised against human IgEs. **4.** The next step consists of the incubation of the array with fluorophore-labelled tyramide. **5.** In presence of H_2O_2 the HRP acts on the tyramide molecules transforming them in tyramide derivatives. The latter are extremely unstable and are characterized by a very short half-life; therefore they fall on the slide surface where they bind to nucleophilic residues in close proximity to the spot where the HRP-target interaction occurs. The fluorophore coupled to the tyramide enables to detect the spot where all the reactions have taken place. The figure shows the basic steps of the tyramide amplification system that make use of a HRP-labelled secondary antibody; however the intermediate steps of the protocol can vary according to the degree of signal amplification that is required.

Miniaturization with chip technology and purified allergens has distinct advantages and disadvantages. The main advantage is the smaller serum requirement for the chip assay that could allow broad-spectrum allergy evaluations with finger-stick blood samples, a particularly attractive feature, especially for pediatric patients. The chip-based immunoassay has the potential for complete automation, with the benefit of reduced assay variation, more precise quantitation by using multiple

replicates, and a more rapid turnaround time. The activated silica chip can easily accommodate large numbers of allergen specificities (theoretically up to several thousands) each in one or more replicates, but such comprehensive testing is likely to remain expensive because of the cost of native or recombinant allergens. On the negative side, some allergens used as mixtures, or highly charged allergens may not spot well on silica chips. Also, antigenic competition among cross-reactive allergens will confound quantitation and could reduce diagnostic sensitivity. Competition of IgG and IgE antibodies for limited quantities of spotted allergens will also be a concern, especially for foods and in patients with current or previous immunotherapy where high titres of allergen-specific IgGs are produced. Lastly, any fully automated large-capacity IgE-detection system will likely lead to the inclusion of unvalidated specificities, as it has been already happened, especially in food and drug allergy testing (Hamilton and Adkinson, 2004).

1.10 Aim of the project

This project aims to employ the allergen microarray as a novel and powerful tool to screen the antibody reactivity against the whole range of allergenic molecules capable to elicit an immune response in sensitized and/or atopic people, i.e. the allergome. Such a tool will have potential applications for both research and diagnostic purposes.

The tests used to date for allergy diagnostics, like ELISA, RAST, CAP and others (previously described in chapter 1.3.2), provide enough clinical sensitivity and specificity to detect IgE in human serum; however they are time-consuming and require large quantities of serum samples to screen against a limited number of allergen specificities. In addition they make use of poorly characterised antigen preparations and, as a consequence, for most of them the clinical sensitivity differs from allergen to allergen and from batch to batch of the same allergenic extract and the detection capability never reaches the values provided by the skin prick test. The latter, on the other side, requires specifically trained physicians and needs to be carried out in a clinical environment where adrenaline-based drug treatments are readily available in the event of anaphylactic shock.

Furthermore none of the assays currently used in the diagnosis of allergies has the throughput to screen against the repertoire of the most common allergens (that is estimated to exceed 300 antigens with this number increasing every day) with true parallelism.

The microarray technology offers the possibility to circumvent most of the current limitations in the allergy diagnostics by allowing the simultaneous and multiparametric analysis of serum reactivity against a variety of allergens.

Indirect fluorescence has previously been used to determine the concentration of antibodies to various antigens in human serum. However to increase the analytical sensitivity for the determination of sub-nanomolar concentrations of analytes such as allergen-specific IgE a signal amplification is required.

The aim of this project is thus the application of the microarray technology, combined with signal amplification techniques, to set up an immunoassay capable to achieve the quantitative and simultaneous determination of the allergen-specific IgE antibodies to a wide variety of well-known environmental and food allergens within human sera and to build a panel for the diagnosis of allergy to the widest number of allergens contemporarily.

The assembling of such solid-phase immunoassay starts with the assessment of the right support in terms of material and surface chemistry. The following steps involve first the assessment of the right spotting conditions for the allergenic extracts (in terms of spotting diluents and spotting concentration of each allergenic extract) and finally the evaluation of the right processing protocol for the detection of serum samples reactivity.

Once these basic prerequisites are accomplished, and the feasibility of such a system proves to be convincing, the next step is the validation of the immunoassay. It follows that these parameters have to be monitored:

- the analytical sensitivity, or detection limit of the system;
- the analytical specificity against other analytes occurring in the samples (like other classes of antibodies);
- the intra-assay (reproducibility) and inter-assay (repeatability) precision;
- the stability of the assay over time after the printing and after the processing;
- the clinical sensitivity and specificity of the assay evaluated with a number of clinical samples from allergic and non-allergic patients.

The validation of the above-mentioned parameters will utimately enable to have a system with all the potentialities for routinely laboratory practice. The ultimate goal of this project would be to get an overall throughput for this system that would render it more reliable and feasible compared to the current diagnostic methods and that overcomes the drawbacks of both skin prick tests and currently employed in vitro tests.

To achieve this goal the microarray immunoassay should own some extra features compared to the present detection systems. Notably other than being sensitive, specific, precise and stable over time, it should also allow to be carried out by people who have received a minimal training, it should save time in the processing routine and it would ideally employ lower quantities of reagents. These last two features would ultimately

translate in a remarkable saving in money too. Amongst the reagents employed to process an arrayed slide the most critical one is the serum. At present the in vitro tests can screen for specific IgE only when a venous blood sample is taken. This is due to the lower concentration of IgE in blood, compared to other classes of antibodies: IgE is the least abundant antibody class in serum with a concentration of ~ 100 ng/mL, compared with 10 mg/mL of IgG in the circulation of normal ("nonatopic") individuals (Gould et al., 2003). The use of microarray technology in the diagnosis of allergy, via the determination of the amount of specific IgE in blood, would mean the possibility to potentially test for IgE against a wide range of allergens with just a small amount of blood that can be ideally provided by the same patient via a pin prick without the need of medical intervention.

2.0 Materials and Methods

Materials and methods for the following processes are described below: (1) the preparation of proteins and antibodies for printing, (2) the printing process, i.e. the immobilisation of allergens and antibodies onto the solid substrate, (3) the processing protocol of the arrayed slides for determination of allergen-specific IgE, (4) the scanning of processed slides and the quantitative analysis of the image data, (5) the optimization studies conducted in order to assess the right solid surface, the right spotting conditions for antibodies and allergens and the right processing protocol, and (6) the methodology used for the characterization and validation of the microarray assay.

2.1 Preparation of proteins and antibodies to be arrayed

The arrays were printed onto aldehyde-activated glass microscope slides (VALS-25, Cel Associates) which were identified as the substrate of choice (see Results, section 3.3.1 and Discussion). Most of the arrays employed in this study were designed as 7 X 7 or 8 X 8 matrices. This format incorporated anti-human IgE antibodies, allergens and controls.

2.1.1 Preparation of anti-human IgE antibodies

A combination of two monoclonal anti-human IgE antibodies was printed. These antibodies (Mouse anti-human IgE - 4.HIgE, 7G6E4C9,

Rei 12/157, 22.07.99, "capture" antibody and Mouse anti-human IgE - 2.HIgE, 8B3C10B8, Wei 40/81, 13.03.03, "tracer" or "detection" antibody) were both supplied in a lyophilised form by Allergopharma (Joachim Ganzer KG, Hermann-Körner-Str. 52, D-21465 Reinbek, Germany). Both antibodies were reconstituted to a concentration of about 4 mg/mL in 1X phosphate-buffered saline (PBS: 0.2 g/L KCl (P-9333, Sigma), 1.44 g/L Na_2HPO_4 (444425M, BDH), 0.24 g/L KH_2PO_4 (102034B, BDH), 8 g/L NaCl (10241AP, BDH)) and stored at –20°C. In order to avoid freeze-thaw cycles the resulting solutions were aliquoted prior to storage. The combination of the two mouse monoclonal Anti-IgE antibodies (capture and tracer) was diluted in PBS (x1) containing 2 g/L sodium dodecyl sulphate (SDS L4390, Sigma) and printed on slides at the following concentrations: 31.75 ng/mL, 62.5 ng/mL, 125 ng/mL, 250 ng/mL and 500 ng/ml. This dilution curve served as a signal control for the myeloma IgE spiked in the blocking solution (see section 2.3). A monoclonal antibody, by definition, only recognizes and bind to one given epitope on a target molecule. Being this dilution curve made up of a mixture of two different monoclonal antibodies raised against two different epitopes on human IgE was meant to maximize the capture of the myeloma IgE, increasing the signal intensity of the dilution curve and providing thus a better signal control. However since one of these same monoclonal antibodies was used as tracer antibody in the processing protocol, no significant increment in signal from the anti-IgE dilution curve so designed could be expected.

2.1.2 Preparation of allergen extracts

The allergen extracts used for the assay belong to a wide range of categories of clinical relevance: mite allergens (*Dermatophagoides pteronyssinus D1, Lepidoglyphus destructor D71, Der p 1 X904, Der p 2 X905*), epidermal allergens (*Cat hair E1, Dog hair E2, Horse hair E3*), food allergens (*Egg white F1, Cow's milk F2, Celery S901, Peanuts F13, Soybean F14, Apple F49*), grass pollens (*Timothy grass G6, Rye cultivated G12, Phl p 5 X903*), venom allergens (*Bee venom I1, Wasp venom I3*), occupational allergens (*Latex K82*), mould allergens (*Penicillium notatum M1, Cladosporium herbarum M2, Aspergillus fumigatus M3, Alternaria tenuis M6*), tree pollens (*Birch pollen T3, Bet v 1 X901*), weed pollens (*Mugwort W6, English plantain W9*).

The allergens, provided by Allergopharma as lyophilised extracts, were reconstituted in bidistilled water to a theoretical protein concentration of 2 mg/mL. The protein content of each extract was indicated as mg of protein per mg of lyophilised material. The actual protein concentration of the reconstituted material was determined by the Bradford method (Bradford, 1976) before dilution with individual spotting diluents.

2.1.3 Preparation of controls

The arrays were generally designed to incorporate positive controls (goat anti-rabbit IgG-Alexa 546 dye - A11010, Invitrogen - in 1X PBS) and negative controls (10 g/L Bovine Serum Albumin - A7906, Sigma - in 2X PBS containing 0.1 mL/L Tween 20 - 663684B, BDH). The

positive control serves as a means to assess the deposition process. Since the anti-rabbit IgG is conjugated with a fluorophore, the slide can be read prior to processing. If, upon reading, a clear and saturated signal is detected from the spot containing the positive control and if the spot itself displays a regular circled shape, then the deposition has occurred correctly. The choice of the fluorophore Alexa 546 in the positive control was based on the fact that the signal amplification technique employed in the processing of the arrays made use of this same fluorophore coupled to the tyramide.

Print solutions containing antibodies, allergens and controls were finally transferred into a 384-well microtiter plate (221006, Thermo Life Sciences). Depending on the number of replicate arrays required, a corresponding number of pins were employed and the appropriate number of wells was filled with each printing solution. At least 50 µL of solution were added to each well.

All the solutions were prepared and transferred into the source plate just before the start of the print run.

2.2 Printing

Antibodies, allergens and controls were contact-printed on the slides by means of computer-controlled high-speed robotics (Total Array Systems; Biorobotics/Genomic Solutions). The samples were transferred from the 384-well microtiter plate to glass slides by use of spring-loaded

stainless steel solid pins of 200 μm diameter (supplied by Genetix – K2900). Each pin is estimated to transfer ~ 1 nL of sample to the slide and to produce spots with a diameter of about 0.3-0.4 mm and a pitch of 0.6 mm. After deposition of each print solution, pins were washed first with distilled water for 4 seconds, then with 70% ethanol for 2 seconds and finally dried for 4 seconds. This cycle was repeated three times.

On completion of the printing process, slides were kept inside the robot printer cabinet for at least 12 hours. This stable and controlled environment (40 to 55% humidity and 20 to 25°C) is conducive to the binding of the antibodies and the allergens to the slide surface.

On removal of slides from the robot, printed slides were stored in the dark in boxes containing desiccant silica gel bags (S8394-50EA, Sigma). It is critical that the slides are kept dry: slides that have been exposed to moisture on storage give imprecise and varying signal.

Various assay formats were used for different experiments, depending on the particular aim.

Each biochip was designed to contain 2 arrays per slide; for the evaluation of intra-slide precision, allergens were printed as 4 replicates; for evaluation of inter-slide allergen precision data from 3 slides were analysed, i.e. a total of 12 replicates for each allergen.

2.3 Processing of the microarrayed slides for the determination of IgE in human serum

Pooled sera positive (NH1/28) and negative (M11/9) for the allergens of the panel and 46 clinical samples were supplied by Allergopharma. Prior to being used in the microarray, each serum sample had been previously tested by Allergopharma by using the ELISA RV 5 immunoassay (Specific IgE ELISA RV 5 kit - Enzyme immunoassay for quantitative determination of allergen-specific IgE in serum or plasma, Allergopharma – Cat. No. 36020). Therefore for each serum sample the allergen-specific IgE reactivity against all the allergens spotted was given in terms of ELISA class scores from 0 to 5. The ELISA method used for this assessment is described in detail in section 2.6.5.

The first step in the processing of slides was the sticking of an adhesive frame (Gene-Frame 65 µl - 1.5 X 1.6 cm - AB-0577, Abgene Limited) around the printed array. This frame served two purposes: first, samples and reagents were contained within the array area; secondly, the frame limited the developing area to where the array was printed thereby minimising reagent use.

In order to block non-specific binding to the activated slide surface, the printed slides were incubated for 60 minutes at room temperature (RT) in the slide blocking solution (20 g/L bovine serum albumin (BSA - A-7906, Sigma) in 1X PBS containing 1µg/ml of human myeloma IgE (supplied by Allergopharma). The latter was added in order to generate a signal

control in the anti-human IgE dilution curve. Staining jars with at least 50 mL of blocking solution were used for this purpose. The BSA used for the blocking step was in great excess respect to the myeloma IgE in the final solution (1 g of BSA vs. 50 μg of IgE from myeloma in 50 mL: the mass of IgE in the blocking solution was thus one million times less than the mass of BSA). Therefore no background interference from the human IgE from myeloma was expected nor it could be detected, upon processing. After being blocked and rinsed, the slides were incubated with the serum sample. In order to avoid a further dilution of an analyte already occurring in very small amounts in human serum (i.e. the allergen-specific IgE antibodies) the serum was always used neat for processing the arrays. The serum was followed by incubation with a monoclonal mouse IgG raised against human IgE (2.HIgE, 8B3C10B8, Wei 40/81, 13.03.03, "tracer" or "detection" antibody, supplied by Allergopharma) at a working strength concentration of 0.14 μg/mL, followed by a peroxidase-conjugated AffiniPure goat anti-mouse-IgG (Horseradish peroxidase-anti IgG conjugate, Fcγ fragment specific, minimal cross-reaction to Human, Bovine and Horse Serum Proteins - 115-035-071, Jackson Immunolabs) at a working strength concentration of 1.6 μg/ml. The anti-human IgE and anti-mouse IgG-HRP were dissolved in a conjugate diluent consisting of 2X PBS containing 0.1 mL/L Tween 20 and 10 g/L BSA.

The final incubation employed a Tyramide-Alexa 546 conjugate diluted 200 times in a solution of Amplification Buffer containing 0.015 mL/L

H_2O_2. Tyramide-Alexa 546 conjugate, Amplification Buffer and H_2O_2 were all supplied by Invitrogen in the TSA kit #23 (Catalogue number T-20933). A wash step was introduced after every incubation. The washing solution employed consisted of 1X PBS with 0.1 mL/L Tween 20.

Upon completion of the final processing step, the gene-frames were removed and the slides were air-dried at +37°C for 10-15 minutes before being scanned.

2.4 Scanning and quantification of processed slides

Scanning was performed by using the S5000 scanner (Perkin Elmer Life Sciences), according to the manufacturer's instructions. All the slides were scanned under constant instrument settings, i.e. 90% of laser power and 60% of photomultiplier gain. The images were generated with the ScanArray Express™ software provided by Perkin Elmer Life Sciences Inc. Acquired images were stored as Tiff files.

Quantification was achieved by using the same ScanArray Express™ software according to the manufacturer's instructions. Background signal was subtracted from each individual slide. Data were stored as Excel files.

2.5 Optimization studies

2.5.1 Choice of the optimal solid surface for the allergen microarray

Before selecting the aldehyde glass slides (VALS-25, Cel Associates) as the solid surface of choice for the allergen microarray, various surface chemistries were tested. Specifically the performances of amino-silane coated glass slides (CSA-100, Cel Associates), aldehyde glass slides (VALS-25, Cel Associates), silylated glass slides (CSS-100, Cel Associates) and Black Polymer MaxiSorp™ plastic slides (230305, Nunc) were compared.

2.5.2 Optimization of spotting diluents and spotting concentrations for allergen extracts

All allergens were spotted at a range of concentrations and by using a broad range of buffers.

Initially all the allergens were spotted in bidistilled water at 500 μg/mL of protein concentration. Since these conditions did not allow the detection of any signal in more than 50% of the allergen extracts, the spotting concentration was raised to 1 mg/mL of protein and a number of diluents, other than bidistilled water, were used. The choice of diluents was primarily made according to their pH values (see Discussion).

1) Acidic buffers:

   a) Glycine buffer pH 1.4: 26.4 mL of (0.1M glycine (10119CU, Sigma) + 0.1M NaCl (10241AP, BDH)) and 73.6 mL of 0.1M HCl (101254I, BDH).

   b) Glycine buffer pH 2.4: 62.3 mL of (0.1M glycine + 0.1M NaCl) and 37.7 mL of 0.1M HCl.

   c) Glycine buffer pH 2.4 containing 0.1 mL/L Tween 20.

   d) Glycine buffer pH 2.4 containing 2 g/L SDS.

   e) Citrate buffer pH 2.6: 90 mL of 0.1M citric acid (277814N, BDH) and 10 mL of 0.2M Na_2HPO_4.

   f) Citrate buffer pH 2.6 containing 2 g/L SDS.

   g) Citrate buffer pH 5.4: 44.7 mL of 0.1M citric acid and 55.3 mL of 0.2M Na_2HPO_4.

   h) Citrate buffer pH 5.4 containing 2 g/L SDS.

   i) 1X PBS pH 6.5 (see section 2.1.1; pH adjusted at 6.5)

   j) 1X PBS pH 6.5 containing 2 g/L SDS.

2) Buffers with a neutral pH:

   a) 1X PBS pH 7.4 (see section 2.1.1; pH adjusted at 7.4).

   b) 1X PBS pH 7.4 containing 0.1 mL/L Tween 20.

   c) 1X PBS pH 7.4 containing 10 g/L sucrose (S-0389, Sigma).

   d) 1X PBS pH 7.4 containing 10 g/L Polyvinylpirrolydone (PVP, P-5288, Sigma).

   e) 1X PBS pH 7.4 containing 2 g/L SDS.

f) 1X PBS pH 7.4 containing 2 g/L SDS and 10 g/L sucrose.

3) Alkaline buffers:

a) 1X PBS pH 8.0 (see section 2.1.1; pH adjusted at 8.0).

b) 1X PBS pH 8.0 containing 2 g/L SDS.

c) Carbonate/Bicarbonate buffer pH 9.2: 10 mL of 0.1M Na_2CO_3 (102404H, BDH) and 90 mL of 0.1M $NaHCO_3$ (102475W, BDH).

d) Carbonate/Bicarbonate buffer pH 9.2 containing 0.1 mL/L Tween 20.

e) Carbonate/Bicarbonate buffer pH 9.2 containing 2 g/L SDS.

f) Borate buffer pH 9.4: 87 mL of (12.37 g/L boric acid (100583R, BDH) + 100 mL/L NaOH (102524X, BDH)) and 13 mL of 0.1M NaOH.

4) Other diluents:

a) 1 mM Dithiotreitol (DTT, D-9779, Sigma) in bidistilled water.

b) 5 mM DTT in bidistilled water.

c) 200 mL/L Propan-1-ol (103454V, BDH) and 1 mL/L Tween 20 in bidistilled water.

After establishing the optimal spotting diluent, the optimal spotting concentration of each extract was assessed too. This was made by printing each allergen at various concentrations, ranging from 2 mg/mL to 8 μg/mL. Different spotting concentrations were chosen for every extract after checking the response of single extracts, printed at 1 mg/mL, to clinical samples of known reactivity. Spotting diluents and spotting concentrations were both identified by using the panel of allergen-specific serum samples (see Materials and Methods, section 2.3).

2.5.3 Choice of the method employed to detect the signal from allergen-specific IgE in human serum

Previous studies carried out in this laboratory (Bacarese-Hamilton et al., 2002; Bacarese-Hamilton et al., 2004a) made use of a mouse anti-human IgE (2.HigE, 8B3C10B8, Wei 40/81, 13.03.03, Allergopharma) conjugated with Sulfo-NHS-LC-Biotin (as supplied in the EZ-Link™ Sulfo-NHS-LC-Biotinylation kit - 21430, Pierce) followed by an incubation with a Streptavidin-Horseradish Peroxidase conjugate (TSA kit #23, Invitrogen) and by the Tyramide amplification system. However because of a high incidence of false negatives and the inconsistencies of the biotinylation process (in terms of unpredictable levels of biotin incorporation per antibody molecule in every biotinylation process) this detection protocol was rejected in favour of a method that does not make use of the biotin-avidin system (see Results, section 3.5, and Discussion).

A range of working strength dilutions, incubation times and temperatures were evaluated for all steps of the test protocol.

The **blocking step** was conducted overnight at +4°C or at room temperature either for 2 hours or for 1 hour. Since no difference was observed between these three conditions, the 1 hour incubation at room temperature was chosen because it allowed saving time in the overall processing.

The **serum incubation** temperatures and times tested were: room temperature (1 hour); 37°C (1 hour); 4°C (1 hour); 4°C (5 hours); 4°C (overnight). The best performance resulted from 1 hour incubation conducted at 37°C.

Secondary and tertiary antibodies were tested in terms of incubation time and temperature. Different combinations of incubation times (15', 30', 45') were assessed as well as different working strength concentrations: 27 μg/mL, 2.7 μg/mL and 0.27 μg/mL for the anti-human IgE and 1.6 μg/mL, 0.8 μg/mL and 0.4 μg/mL for the anti-mouse IgG-HRP conjugated. Once determined, the best combination in terms of time and working strength dilution for both the antibodies, the incubations were carried out at room temperature and at 37°C and the latter provided the best performance.

The **Tyramide-Alexa 546** was similarly tested in terms of incubation times (5', 10', 15', 20', 30'), concentration (1 in 100, 1 in 150, 1 in 200, 1 in 300, 1 in 400) and temperature (incubation conducted at room temperature or at +37°C). An incubation lasting 15' and conducted at +37°C at a working strength dilution of 1 in 200 was established as the optimal condition.

The **optimized protocol** for the determination of serum IgE is detailed in the Results, section 3.4.

The **calibration system** initially employed for the allergen microarray immunoassay was based on the spotting of a dilution curve of human IgE from myeloma (supplied by Allergopharma). A polyclonal biotinylated anti-human IgE (16-10-04 – KPL) was used as a control secondary antibody to compare with the monoclonal anti-human IgE (Mouse anti-human IgE - 2.HIgE, 8B3C10B8, Wei 40/81, 13.03.03, "tracer" or "detection" antibody - Allergopharma) used in the ultimate protocol.

2.6 Methodology used for the characterization and validation of the microarray assay

Analytical and clinical parameters for the characterization and validation of the microarray assay have been assessed for each allergen by using the negative and positive serum pools and a panel of 46 serum samples. The positive control pool was used to set precision ranges

(intra-slide estimations were based on 4 replicates, inter-slide estimations were based on 12 replicates generated over three slides). Clinical sensitivity was estimated by using the panel of clinical samples provided by Allergopharma: it was based on the percentage of sera classified as ≥ Class Score 1 in both ELISA and microarray tests.

2.6.1 Analytical sensitivity

The analytical sensitivity indicates the smallest quantity of an analyte that can be reproducibly and statistically distinguished from the variance of the background or a zero calibrator in a given assay system. It is also indicated as the lower detection limit or the positive threshold of the assay. The analytical sensitivity is of primary importance in a validation study, especially when the analyte to be detected (i.e. the IgE antibodies) is normally occurring in very small amounts in the sample.

This parameter was determined by printing slides with 10 μg/mL of a goat anti-human IgE as "capture antibody" (Bethyl Laboratories, catalogue no. A80-108A) in phosphate buffered saline containing 2 g/L Sodium Dodecyl Sulphate and 21 replicates of a non-specific protein (2 g/L Bovine serum Albumin, BSA). Each slide was then incubated with one of the 5 Reference Standards (provided in the Allergopharma ELISA RV 5 kit) containing the following concentrations of human IgE: 0.35 IU/mL (Standard 1), 1.0 IU/mL (Standard 2), 3.5 IU/mL (Standard 3), 10 IU/mL (Standard 4) and 50 IU/mL (Standard 5). The sensitivity was calculated, on three different occasions, as the concentration of IgE corresponding to

the mean + 2 standard deviation of the signal generated by the non-specific protein (BSA), and interpolated from the curve generated with the IgE standards.

The maximum theoretical sensitivity was determined by maximising the amount of capture antibody printed on the slide surface to 250 μg/mL and diluting the lowest reference standard to a concentration of 0.00175 IU/mL.

2.6.2 Analytical Specificity

This parameter indicates the ability of an analytical method to determine only the component it purports to measure and thus the extent to which the assay responds only to a specified analyte and not to other substances present in the sample (Matsson et al., 1997). Particularly, in the context of the present work the specificity refers to an assay which is specific for IgE and shows no cross-reactivity of the anti-human IgE reagent with other classes of human antibodies or other substances present in the sample.

Specificity was determined by spotting slides with two dilutions (5 μg/mL and 10 μg/mL) of the following antibodies: anti-human IgE (Bethyl Laboratories, catalogue no. A80-108A), human IgG (Reagent grade, Sigma, Cat no I-4506) and human IgM (reagent grade, Sigma, Cat no I-8260). After being blocked, the arrays were first incubated with Human myeloma IgE (Allergopharma) at a concentration of 1 μg/mL for 60 minutes at 37°C then with mouse anti-human IgE (Allergopharma 2.HIgE,

8B3C10B8, Wei 40/81, 13.03.03) followed by anti-mouse IgG-horseradish peroxidase conjugate and Tyramide-Alexa 546 conjugate (as per optimized protocol). The analytical specificity was calculated by assessing the reactivity obtained with the 3 antibodies spotted on the slide surface and expressing it in terms of signal. In particular for each of the two concentrations of the spotted antibodies, the signal gathered from the IgG and IgM (cross-reactivity) was expressed as a percentage of the signal collected from the anti-IgE (specific signal). The specificity was then calculated as the difference between the specific signal and the cross-reactivity signal.

2.6.3 Precision

The precision indicates the agreement between replicate measurements of the same analyte. Particularly intra-assay precision, or reproducibility, refers to agreement within an individual assay run. The interassay precision, or repeatability, refers to results obtained from different sets of assays. In the present study one assay is one printed slide. Thus the precision is assessed by measuring the agreement between replicate measurements of the same allergen within the same slide (intra assay) or between different slides (inter assay) belonging to the same batch.

The intra-slide allergen precision (or reproducibility) was determined by printing four replicates of each allergen on the same slide and processing the slide with the positive serum pool. For each allergen the average and

standard deviation of the signal were calculated and the precision was finally expressed as coefficient of variation, i.e. the percentage of the ratio between standard deviation and average signal.

The inter-slide allergen precision (or repeatability) was determined by using data from three different slides each printed with four replicates of every allergen. The inter-slide signal precision was then obtained by calculating average and standard deviation of the signal from the 12 replicates of each allergen and expressing it as coefficient of variation, i.e. percentage of the ratio between standard deviation and mean signal.

2.6.4 Stability

A study was carried out to assess the stability of immobilized allergens on the glass surface after being spotted. This study was performed by printing 6 allergens, each representative of one of the categories listed in section 2.1.2: *Dermatophagoides pteronyssinus* D1, Cat epithelium E1, Peanuts F13, Timothy grass G6, Honeybee venom I1 and Birch pollen T3. The printed slides were processed using the pool positive serum at 1, 28, 60 and 90 days after the printing. The stability of the signal over time was then assessed for each allergen by evaluating the percentage of change in the signal over time expressed as coefficient of variation (standard deviation/mean signal*100).

In order to evaluate the stability of the signal on processed arrays, some slides were re-read 138 days after being processed. The second reading

was carried out by maintaining the same settings (i.e. 90 of laser power and 60 of photomultiplier gain) used during the first reading.

2.6.5 Specific IgE ELISA RV 5

The reference method provided by Allergopharma (Specific IgE ELISA RV 5) was employed when a direct comparison with the microarray immunoassay was necessary. The assay was performed following the Manufacturer's Instructions for Use provided in the kit.

This test is a classical non-competitive enzyme immunoassay for the determination of circulating specific IgE in serum and/or plasma. In this assay the solid phase is given by chemically activated paper discs with covalently bound anti-IgE antibodies (reference discs) or allergens (allergen discs). By using sterile forceps, reference and allergen discs were added to the bottom of the wells of a 96-well microtiter plate with flat bottom (supplied by Greiner). The reference discs were then incubated with one of the 5 Standards provided, each with a known concentration of human IgE (reported in section 2.6.1); the allergen discs were incubated with the patient's serum. Each paper disc containing well was filled up with 50 μL of either standard or serum sample. The plate was then covered with cling film (to avoid evaporation) and allowed to incubate at 37°C for 1 hour. During this time the IgE of the standards could bind to the anti-IgE absorbed on the reference discs and the allergen-specific IgEs of the serum could bind to the allergens absorbed on the allergen discs. Surplus standard/serum were removed by a washing step. The

washing solution was provided in the kit as a concentrate and it was diluted 1:20 with bidistilled water prior to use. The washing (4 cycles) occurred in a washer for microtiter plates (TECAN Columbus-Washer, Allergopharma). The second step consisted of an incubation with an alkaline phosphatase-labelled anti-human IgE (conjugate) that recognizes (and binds to) both the solid phase bound IgE from the standards and solid phase bound allergen-specific IgE from serum samples. 50 μL of the conjugate were added to each well. The plate, once again, was covered with cling film and allowed to react for 90 minutes at 37°C. Unbound anti-human IgE was then removed by a further washing step, performed as described above. 15-20 minutes before the end of the conjugate incubation the substrate solution of p-nitrophenyl-phosphate was prepared by dissolving a substrate tablet per 5 mL of substrate diluent (both provided in the kit). Therefore in the next step 200 μL per well of substrate solution were added. The plate was then covered with cling film and the enzyme was allowed to react with its substrate at 37°C for 1 hour in the dark. By the activity of the alkaline phosphatase a coloured solution emanates. The enzymatic reaction was finally stopped by adding 100 μL per well of a stop solution (1M sodium hydroxide, provided in the kit). The optical density of the coloured solution was then measured in a photometer within 2 hours from the addition of the stop solution. The calculation of results was achieved by interpolating the values of optical density read off the allergen discs to the standard curve generated through the optical density values read off the reference discs.

2.6.6 Clinical Trial

To classify the IgE reactivity, 46 serum samples and the positive and negative pool sera (previously tested with the reference system Allergopharma ELISA RV 5 on all the 27 allergens of the panel) were assessed with the microarray immunoassay and the response from individual samples was classified by signal intensity.

The raw signal data obtained with the microarray test were plotted against the class score of reactivity provided by Allergopharma ELISA RV 5.

The reactivity of the clinical samples to each allergen was regarded as negative when falling into class score 0 and positive for class scores 1 to 5, according to the ELISA reference system. The signal obtained in the microarray from the negative samples was averaged and this value, separately calculated for each allergen, was used as a cut-off. The reactivity of the clinical samples to each allergen in the microarray was then analysed and the signals obtained were compared to the cut-off value.

All the samples with ELISA scores 1 to 5 whose signal in the microarray was higher than the cut-off were considered reactive (i.e. true positives), whereas those whose signal was below the cut-off were regarded as non-reactive, and thus as false negatives. Clinical sensitivity was then calculated as the percentage ratio between the true positives and the ELISA positives.

Accordingly, the samples with ELISA score 0 whose signal in the microarray was below the cut-off were considered true negatives and those whose microarray signal fell above the cut-off were regarded as false positives. Clinical specificity was thus calculated as the percentage ratio between the true negatives and the ELISA negatives.

This analysis was performed on each single allergen by testing the reactivity of all the 48 serum samples provided.

Class scores are defined in the Allergopharma ELISA RV 5 by the following IgE concentrations in human serum: class 0: <0.35 IU/mL; class 1: 0.35 to 0.7 IU/mL; class 2: 0.7 to 3.5 IU/mL; class 3: 3.5 to 17.5 IU/mL; class 4: 17.5 to 50 IU/mL; class 5: >50 IU/mL.

Microarray class-scores were defined by interpolating the photomultiplier counts (pmc) signals from the allergens to a dose-response curve generated with the ELISA standards in the microarray system by using the same experimental format employed for the analytical sensitivity. In this way class score 0 in microarray is defined by a pmc interval 0 to cut-off; a signal falling between the cut-off and 13178 pmc is considered class 1; a signal between 13179 and 30426 pmc is regarded as class 2; signals between 30427 and 47674 pmc fall into class 3; signals from 47675 to 58925 pmc fall into class 4; finally class 5 gathers all the signals above 58926 pmc.

3.0 Results

3.1 Allergens abbreviations:

A list of the allergens used in the microarray immunoassay (with their respective abbreviations) is repeated here for the convenience of the reader, since these same allergens' codes rather than the complete names are used in Figure 3.1.

D1	*Dermatophagoides pteronyssinus* (House dust mite)
D71	*Lepidoglyphus destructor* (Storage mite)
E1	Cat epithelium
E2	Dog epithelium
E3	Horse epithelium
F1	Egg white
F2	Cow's milk
F13	Peanuts
F14	Soybean
F49	Apple
G6	Timothy grass pollen
G12	Rye cultivated pollen
I1	Honeybee venom
I3	Wasp venom
K82	Latex
M1	*Penicillium notatum*

M2	*Cladosporium herbarum*
M3	*Aspergillus fumigatus*
M6	*Alternaria tenuis*
S901	Celery
T3	Birch pollen
W6	Mugwort pollen
W9	English plantain pollen
X901	Bet v 1
X903	Phl p 5
X904	Der p 1
X905	Der p 2

3.2 Array design

The allergen arrays were produced by printing all the allergens in 3 replicates within a 7 x 7 matrix. The replicates of the allergens were randomly distributed within the arrays in order to better evaluate the reproducibility in their performance. The arrays were designed to contain two copies of a dilution curve of anti-human IgE ("detection" and "capture" antibodies – see Materials and Methods, section 2.1.1), a blank (phosphate buffered solution) and the fluorophore Alexa 546 as a source of reference signal. The fluorescent signal was visualized in a pseudo-color scale (dark blue to white) corresponding to increasing fluorescence (Figure 3.1).

Anti-IgE D+C 500 ng/mL	Positive Control	E1	I1	X901	E1	Blank
Anti-IgE D+C 250 ng/mL	M2	T3	M1	X904	X905	T3
Anti-IgE D+C 125 ng/mL	M1	G12	E3	M6	E2	I3
Anti-IgE D+C 62 ng/mL	Blank	X904	M2	I1	M1	G12
Anti-IgE D+C 31 ng/mL	M6	E2	I3	Blank	X905	M2
E3	T3	Blank	G12	M6	X901	X904
X901	E1	X905	E2	I1	E3	I3

Anti-IgE D+C 500 ng/mL	Blank	F2	W9	D1	F2	Blank
Anti-IgE D+C 250 ng/mL	F13	W6	K82	D71	G6	W6
Anti-IgE D+C 125 ng/mL	K82	X903	F14	S901	F14	F49
Anti-IgE D+C 62 ng/mL	M3	D71	F13	W9	K82	X903
Anti-IgE D+C 31 ng/mL	S901	F1	F49	M3	G6	F13
F14	W6	M3	X903	S901	D1	D71
D1	F2	G6	F1	W9	F14	F49

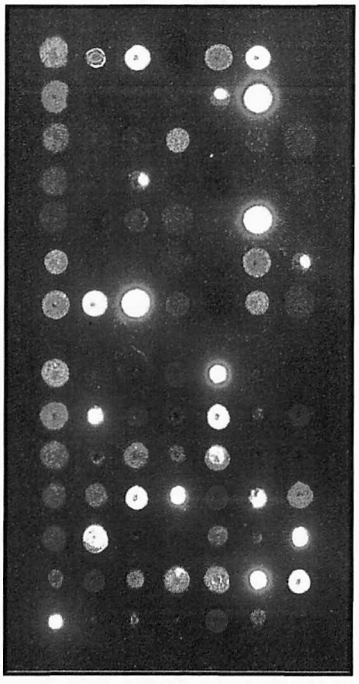


Figure 3.1. Array design.
On the left the schematic of how the three replicates of each allergen extract, the anti-IgE dilution curves and the controls were positioned on the array. Anti-IgE D+C indicates a solution containing detection (D) and capture (C) antibody. On the right an example of how the allergen microarray looks like once processed with a clinical sample (serum 5492) with a broad spectrum of reactivity.

3.3 Evaluation of print conditions

3.3.1 Choice of the optimal solid surface to accommodate the allergen microarray

Allergens were immobilized on glass surfaces using different chemistries (silanated, aldehyde and silylated) and on a plastic surface (Black Polymer MaxiSorp™). Amino-silane coated glass slides (CSA-100, Cel Associates) were initially employed for the allergen microarray. These slides had been chosen on the basis of earlier experiments conducted in our laboratory, indicating that proteins such as antibodies and albumin bound with high efficiency and reproducibility to this surface (Mezzasoma et al., 2002; Bacarese-Hamilton et al., 2002; Bacarese-Hamilton et al., 2004a; Bacarese-Hamilton et al., 2004b). However, for this specific project the amino-silane slides provided a higher degree of non-specific signal and the signal-to-background ratio observed with the silylated slides was unacceptably low. In contrast, the aldehyde slides showed the best performance in terms of high signal-to-background ratio and a good spot morphology (Figure 3.2). Consequently this surface was chosen as the solid support for the allergen microarray.

The Black Polymer MaxiSorp™ plastic slides showed more specific signal than amino-silane glass slides and a signal-to-background ratio comparable to the aldehyde glass slides; however the spot morphology was of poor quality (Figure 3.3). Because of this feature this surface too was considered unsuitable for the allergen microarray and thus discarded.

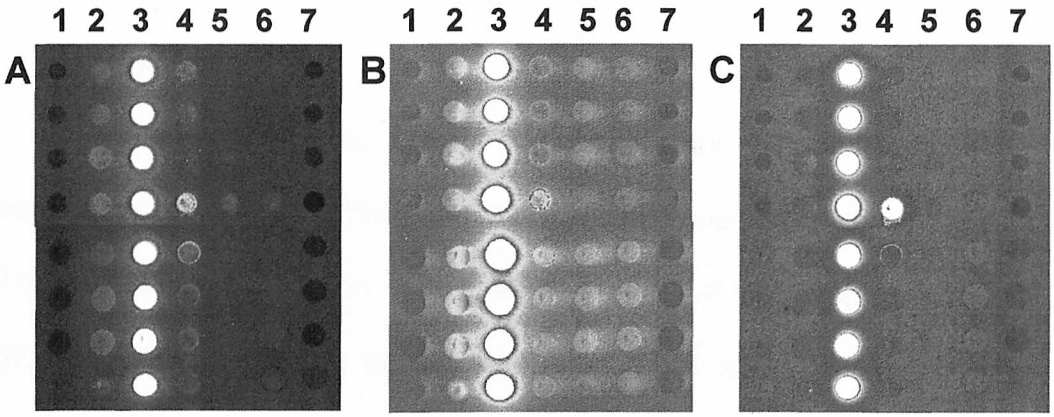


Figure 3.2. Evaluation of the performance of allergen extracts printed on different glass slides.
A: Aldehyde glass slides; **B:** Silylated glass slides; **C:** Amino-silane glass slides. All the slides were printed and processed at the same time. Lane 1: English plantain pollen; Lane 2: Honeybee venom; Lane 3: Der p 2; Lane 4: Bet v 1; Lane 5: Soybean; Lane 6: Celery; Lane 7: Mugwort pollen. Every allergen was spotted by using 8 different spotting diluents. From top to bottom: PBS pH 6.5 + 2 g/L SDS; PBS pH 7.4 + 2 g/L SDS; PBS pH 8.0 + 2 g/L SDS; 200 mL/L Propan-1-ol + 1 mL/L Tween 20; PBS pH 6.5; PBS pH 7.4; PBS pH 8.0; Carbonate/Bicarbonate pH 9.2. The slides were processed with serum NH1/28.

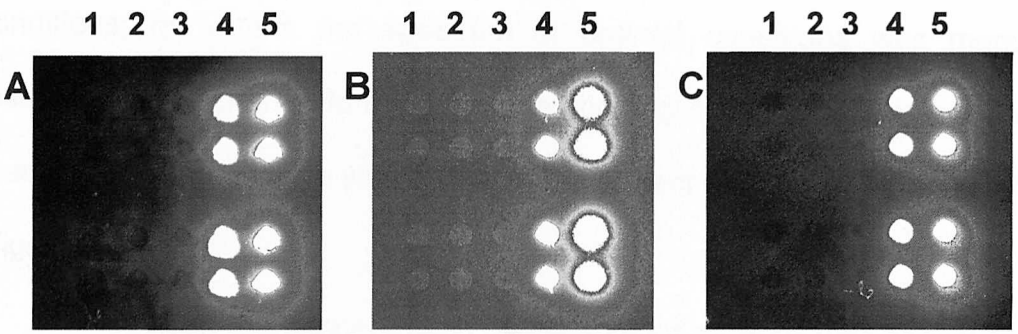


Figure 3.3. Evaluation of the performance of allergen extracts printed on different slide surfaces.
A: Black Polymer MaxiSorp™ plastic slides (Nunc); **B:** Amino-silane glass slides; **C:** Aldehyde glass slides. All the slides were printed and processed at the same time. The low-quality spot morphology of the Black Polymer MaxiSorp™ plastic slides rendered them unsuitable for use as the substrate for allergen chips. Lane 1: Apple; Lane 2: *Penicillium notatum*; Lane 3: *Alternaria tenuis*; Lane 4: Der p 2; Lane 5: Signal control (Goat anti-rabbit IgG Alexa 546 conjugated). The slides were processed with serum NH1/28.

3.3.2 Evaluation of the spotting conditions for the allergenic extracts

For every allergen extract different spotting diluents and spotting concentrations were tested (see Materials and methods, section 2.5.2). The protein concentration of the spotted material was approximately 1 mg/mL. Optimal binding conditions of individual allergens to the slide surface were chosen on the basis of their clinical response when tested with serum samples whose reactivity had been previously assessed with an ELISA reference method (Allergopharma ELISA RV 5). Specifically, the condition which led to the lower number of false positive and false negative results was considered the more suitable (Figure 3.4).

Some extracts showed a good response to a range of spotting conditions; for others the spectrum of optimal conditions was more restricted and a change in the pH of the spotting diluent or the presence or absence of an additive was critical to the correct response of the assay (Figure 3.5).

In addition the concentration of the spotted material was shown to significantly influence the results. Extracts spotted with a low concentration will result in a weak or absent signal. On the other side, when an excess of material is spotted either an increment in non-specific signal can occur or, alternatively, a weakening of the signal itself presumably due to the tight packing of allergenic and non-allergenic molecules and consequently to the non accessibility of the epitopes by the allergen-specific IgEs (Figure 3.6).

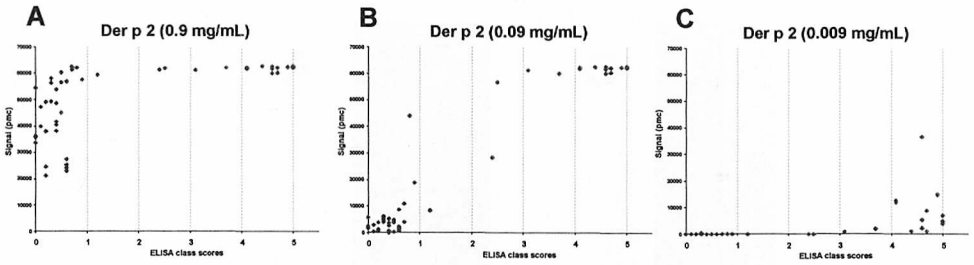


Figure 3.4. Evaluation of the optimal spotting concentration of the allergen Der p 2 (X905).
The allergen was spotted at three different concentrations and the reactivity with clinical samples was evaluated. **A:** At 0.9 mg/mL of spotted protein the signal in the microarray saturated very quickly leading to a high number of false positives (i.e. poor clinical specificity). **B:** At 0.09 mg/mL of spotted protein the microarray signal matched more closely the results of the ELISA reference test. **C:** At 0.009 mg/mL the protein concentration was too low to detect a signal from most positive samples leading thus to a high number of false negatives (i.e. poor clinical sensitivity). 0.09 mg/mL was thus chosen as the optimal spotting concentration for this allergen.

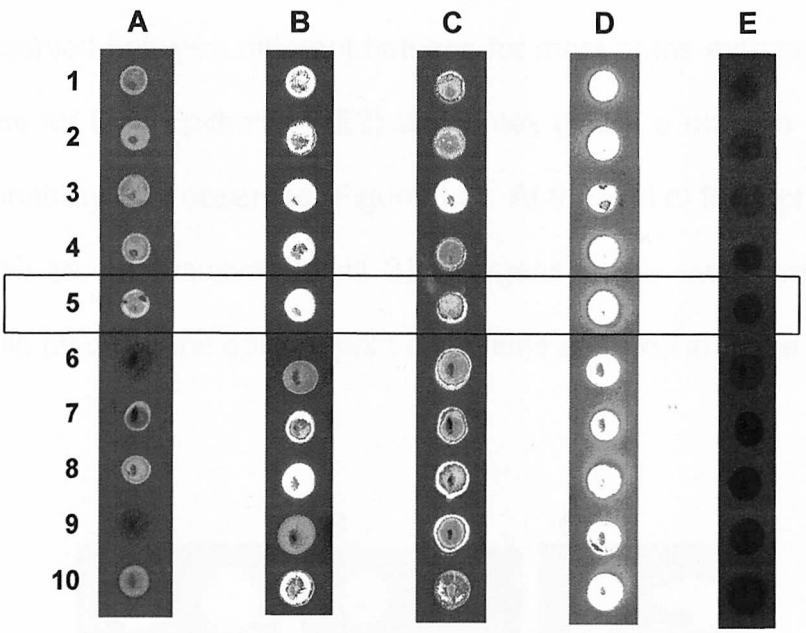


Figure 3.5. Evaluation of the optimal spotting diluent for Cat epithelium (E1).
The extract was diluted at 1 mg/mL of protein concentration and spotted using the following buffers: **1:** Glycine pH 2.4; **2:** Glycine pH 2.4 with 0.1 mL/L Tween 20; **3:** Glycine pH 2.4 with 2 g/L SDS; **4:** 1X PBS pH 7.4; **5:** 1X PBS pH 7.4 with 0.1 mL/L Tween 20; **6:** 1X PBS pH 7.4 with 2 g/L SDS; **7:** Carbonate/Bicarbonate pH 9.2; **8:** Carbonate/Bicarbonate pH 9.2 with 0.1 mL/L Tween 20; **9:** Carbonate/Bicarbonate pH 9.2 with 2 g/L SDS; **10:** 200 mL/L Propan-1-ol with 1 mL/L Tween 20. The arrays were processed with 4 positive (**A** to **D**) and one negative (**E**) serum samples. **A:** Serum 36; **B:** Serum 5266; **C:** Serum 5625; **D:** Serum 5478; **E:** Serum 96 (negative control). The best performance was observed when the allergen extract was diluted and spotted with buffer 5.

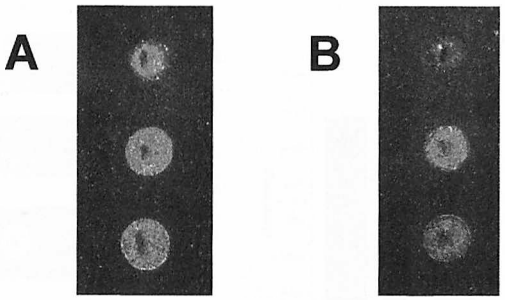


Figure 3.6. Optimization of spotting concentration for Celery (S901).
A: Array processed with serum 5658 (ELISA class score 2.9). **B:** Array processed with serum 35 (ELISA class score 2.5). The extract has been spotted at three different protein concentrations. From top to bottom: 1.6 mg/mL, 0.16 mg/mL, 0.08 mg/mL. The signal from the allergen gets weak when the protein concentration is higher or lower of the optimal value of 0.16 mg/mL.

In addition several batches of material were tested for the majority of allergens under evaluation; no significant differences in performance were observed between different batches for most of the extracts (Figure 3.7). Only for Dog Epithelium (E2) and Latex (K82), a marked batch-to-batch variability was observed (Figure 3.8). At the end of this optimization study, the serum reactivity to all 27 allergens under investigation was detectable by using the optimal print conditions as listed in Table 3.1.

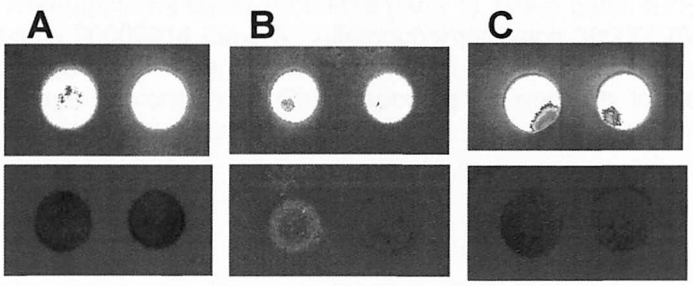


Figure 3.7. Comparison between different batches of three representative allergen extracts.
A: Mugwort pollen (W6): batches 30007391 (left) and 30009982 (right). **B:** Honeybee venom (I1): batches FIV-159 (left) and AM260206 (right); **C:** English plantain pollen (W9): batches 30008610 (left) and 30010200 (right). The three allergens were processed with positive (top panels) and negative (bottom panels) serum samples. All the batches of the extracts were provided by Allergopharma.

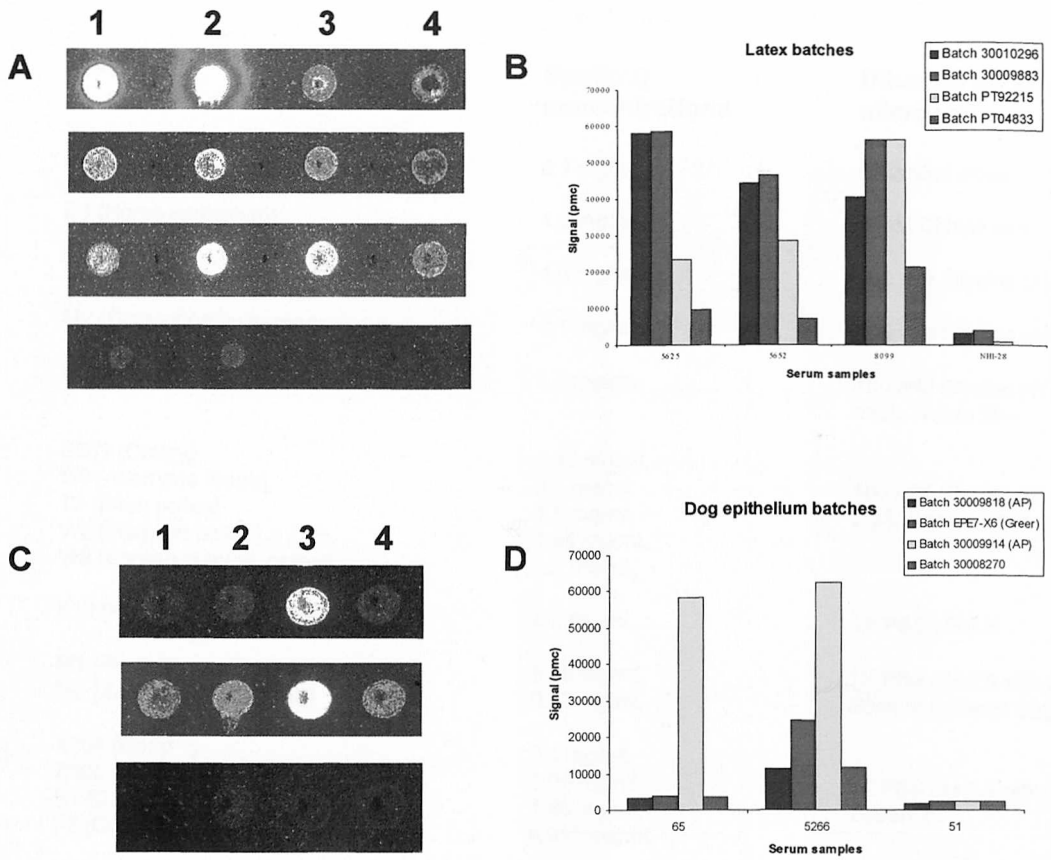


Figure 3.8. Batch-to-batch variation observed in Latex (K82) and Dog epithelium (E2) extracts.
A: Arrays were made by spotting 4 different batches of latex: batch 30010296 (lane 1), batch 30009883 (lane 2), batch PT92215 (lane 3), batch PT04833 (lane 4). All the batches of the extract were provided by Allergopharma. The arrays were processed with 4 serum samples. From top to bottom: serum 5625, serum 5652, serum 8099, serum NH1-28 (negative control). **B:** Chart reporting the reactivity to the different latex batches of the serum samples tested. **C:** Arrays were made by spotting 4 different batches of dog epithelium: Allergopharma batch 30009818 (lane 1), Greer batch EPE7-X6 (lane 2), Allergopharma batch 30009914 (lane 3), Allergopharma batch 30008270 (lane 4). The arrays were processed with 3 serum samples. From top to bottom: serum 65, serum 5266, serum 51 (negative control). **D:** Chart reporting the reactivity to the different dog epithelium batches of the serum samples tested.

Allergen	Spotting concentrations	Diluents for spotting of allergens
F1 (Egg white)	0.1 mg/mL	Bidistilled water
E3 (Horse epithelium)	1.2 mg/mL	5 mM Dithiotreitol
F49 (Apple)	1.0 mg/mL	100 mM Glycine pH 1.4
M2 (*Cladosporium herbarum*)	1.1 mg/mL	100 mM Glycine pH 2.4
I1 (Honeybee venom)	1.8 mg/mL	100 mM Glycine pH 2.4 with 0.1 mL/L Tween 20
S901 (Celery) M6 (*Alternaria tenuis*) T3 (Birch pollen) W6 (Mugwort pollen) W9 (English plantain pollen)	0.16 mg/mL 0.7 mg/mL 1.5 mg/mL 1.65 mg/mL 1.2 mg/mL	100 mM Glycine pH 2.4 with 2 g/L Sodium Dodecyl Sulphate
F13 (Peanuts)	0.2 mg/mL	1X PBS pH 6.5
M1 (*Penicillium notatum*) M3 (*Aspergillus fumigatus*)	0.08 mg/mL 0.13 mg/mL	1X PBS pH 6.5 with 2 g/L Sodium Dodecyl Sulphate
X904 (Der p 1) X905 (Der p 2) E1 (Cat epithelium) F2 (Cow's milk)	0.1 mg/mL 0.09 mg/mL 1.25 mg/mL 0.013 mg/mL	1X PBS pH 7.4 with 0.1 mL/L Tween 20
X903 (Phl p 5) G12 (Rye, cultivated) I3 (Wasp venom)	0.008 mg/mL 0.08 mg/mL 0.2 mg/mL	1X PBS pH 7.4 with 2 g/L Sodium Dodecyl Sulphate
D1 (*Dermatophagoides pteronyssinus*) K82 (Latex)	1.6 mg/mL 0.14 mg/mL	100 mM Carbonate/Bicarbonate pH 9.2
D71 (*Lepidoglyphus destructor*) F14 (Soybean) G6 (Timothy grass) X901 (Bet v 1)	0.7 mg/mL 1.6 mg/mL 0.1 mg/mL 0.6 mg/mL	100 mM Carbonate/Bicarbonate pH 9.2 with 0.1 mL/L Tween 20
E2 (Dog epithelium)	1.0 mg/mL	100 mM Borate pH 9.4

Table 3.1. Diluents and spotting concentrations optimized for each allergen.
All the lyophilized allergen extracts were initially reconstituted in bidistilled water at 2 mg/mL of protein concentration and subsequently diluted at a final spotting concentration and with the spotting diluents indicated.

3.4 Optimized test protocol to process the arrays

Previous investigations carried out in our laboratory have demonstrated that positive serum samples with IgE reactivity determined as having a class score less than 4 are not detectable unless a protocol incorporating a signal amplification step is employed (Bacarese-Hamilton et al., 2002). Using the Tyramide Amplification System (Invitrogen), the optimal protocol developed for IgE determination in serum was:

60' Block in 20 g/L BSA spiked with 1 μg/mL human IgE from myeloma (room temperature);

3' Rinse;

60' incubation with serum at 37°C;

3' Rinse;

45' incubation at 37°C with "detection" anti-human IgE at 0.14 μg/mL;

3' Rinse;

45' incubation at 37°C with HRP/anti-mouse IgG at 1.6 μg/mL ;

3' Rinse;

15' incubation at 37°C with Tyramide-Alexa 546 diluted 1:200 ;

3' Rinse;

Dry at 37°C for 20';

Reading/quantification of fluorescent signal.

All sample and reagent incubations were performed using 100 μL of solution per slide; wash cycles were performed in a staining jar containing 50 mL of wash solution.

3.5 Evaluation of processing conditions

Previous work conducted in this laboratory on the allergen assay employed the biotin-streptavidin system as an intermediate step in the non-linear amplification protocol (Bacarese-Hamilton et al., 2002; Bacarese-Hamilton et al., 2004a). After the serum incubation, the arrays were incubated with a biotinylated secondary antibody, followed by the conjugate streptavidin-HRP and finally by the Tyramide-Alexa 546. The biotin-avidin system makes use of the very high affinity between biotin and avidin (dissociation constant: 10^{-15} M) that is much higher than the affinity between antigen and antibody (Guesdon et al., 1979). However the amplification provided was insufficient to detect allergen-specific IgE in low-reactive serum samples: the latter were often ranked as negative or non-reactive, impairing thus the clinical sensitivity of the assay. The biotinylation procedure was shown to result in different biotinylation ratios of antibody which in turn resulted in the binding of differing amounts of streptavidin-HRP (Figure 3.9).

The clinical sensitivity was significantly improved when bound allergen-specific IgE was revealed using a native anti-IgE rather than the corresponding biotinylated secondary antibody as employed initially (Figures 3.10, 3.11).

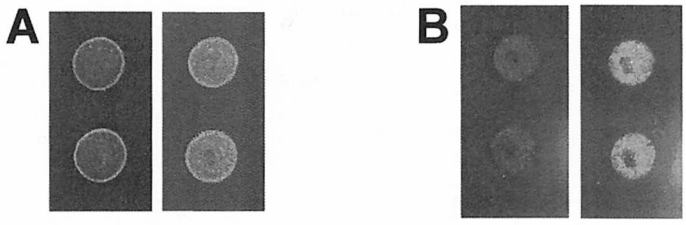


Figure 3.9. Evaluation of allergen reactivity in arrays processed with secondary antibodies having different biotin incorporation.
A: *Lepidoglyphus destructor* (D71) incubated with serum 5492 (ELISA class score 4.7) **B:** Apple (F49) incubated with serum 8046 (ELISA class score 2.1). The arrays were incubated with two biotinylated anti-human IgE antibodies. The assays on the left were processed with a secondary antibody containing an average of 0.5 molecules of biotin per molecule of antibody. The assays on the right were processed with a secondary antibody containing an average of 2.2 molecules of biotin per molecule of antibody.

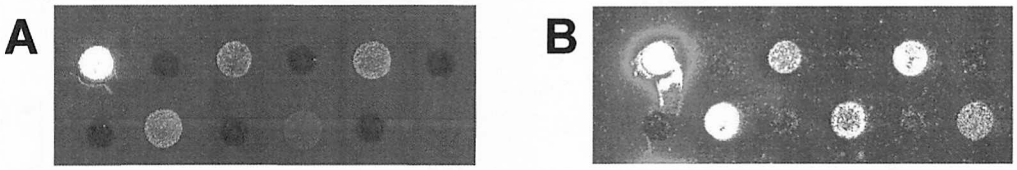


Figure 3.10. Evaluation of allergen reactivity in arrays processed with different detection protocols.
A: Protocol making use of biotin-avidin system **B:** Protocol making use of a native anti-human IgE followed by a tertiary antibody-HRP conjugated. The arrays contain the allergen Bet v 1 (X901 – top left corner) and replicates of Birch pollen extract (T3) spotted at serial dilutions (from top center to bottom right: 2.5 mg/mL, 2.0 mg/mL, 1.5 mg/mL, 1.0 mg/mL and 0.75 mg/mL). Both arrays were incubated with serum 36 (ELISA class score 4.9 for T3 and 5.0 for X901). The signal from T3 is barely detectable when using the protocol with the biotin-avidin system notwithstanding the high reactivity of the serum sample for this allergen.

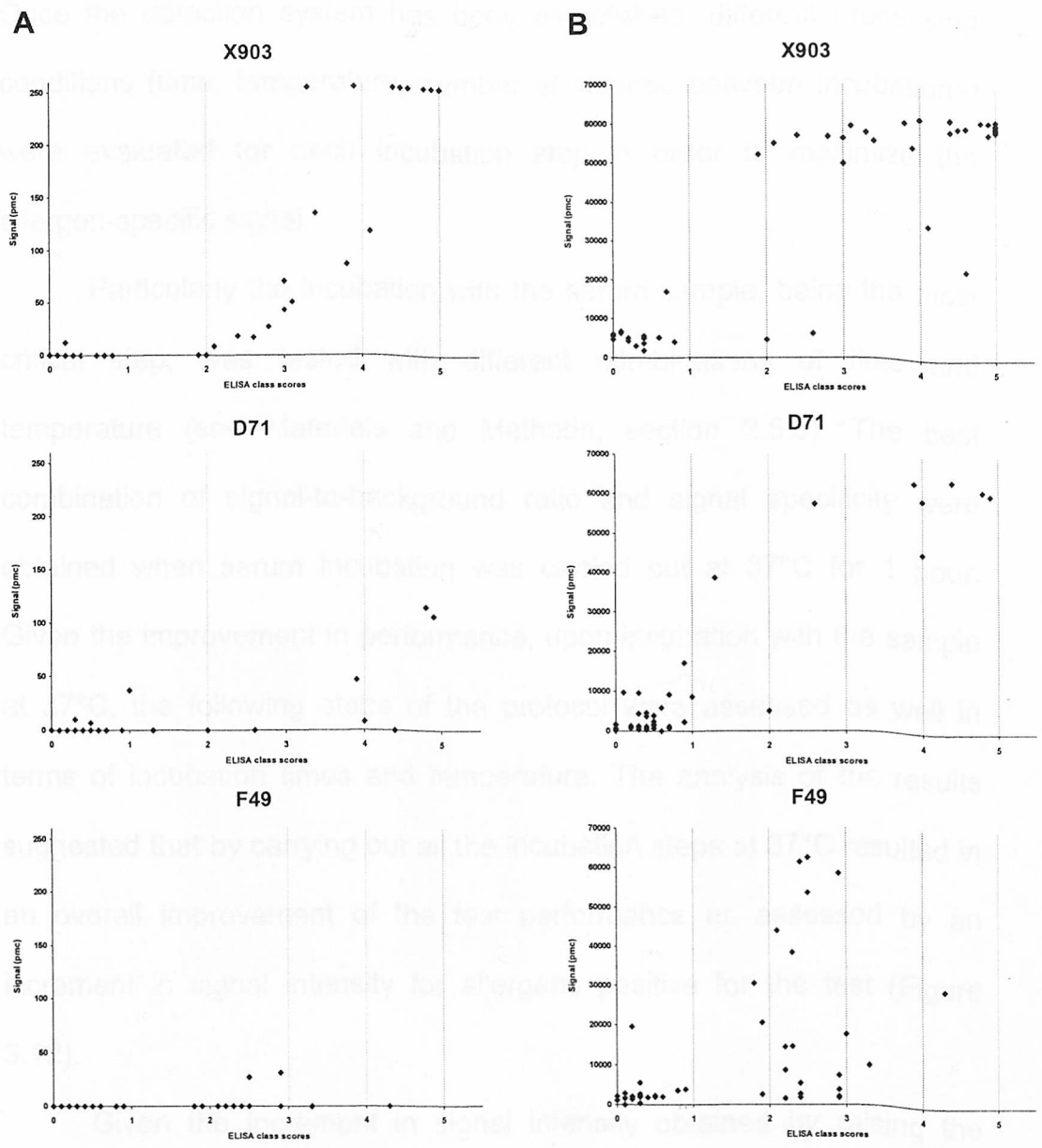


Figure 3.11. Pattern of reactivity of three representative allergens in arrays processed with different detection protocols.
The allergens Phl p 5 (X903), *Lepidoglyphus destructor* (D71) and Apple (F49) were processed with 48 serum samples and the microarray signal was plotted as a function of the ELISA class scores. **A:** The arrays were processed with the protocol making use of the biotin-avidin system **B:** The arrays were processed with the protocol making use of a native anti-human IgE followed by a tertiary antibody-HRP conjugated.

Once the detection system has been established, different processing conditions (time, temperature, number of washes between incubations) were evaluated for each incubation step in order to maximize the allergen-specific signal.

Particularly the incubation with the serum sample, being the most critical step, was tested with different combinations of time and temperature (see Materials and Methods, section 2.5.3). The best combination of signal-to-background ratio and signal specificity were obtained when serum incubation was carried out at 37°C for 1 hour. Given the improvement in performance, upon incubation with the sample at 37°C, the following steps of the protocol were assessed as well in terms of incubation times and temperature. The analysis of the results suggested that by carrying out all the incubation steps at 37°C resulted in an overall improvement of the test performance as assessed by an increment in signal intensity for allergens positive for the test (Figure 3.12).

Given the increment in signal intensity obtained by raising the incubation temperature in all the phases of the processing, attempts were made to evaluate if the incubation of the slides at higher temperature would have allowed to shorten one or more incubation steps (and thus the whole protocol). For this purpose the incubation times of both secondary and tertiary antibodies were tested in various combinations. At the end the best performance was achieved when each antibody was incubated for at least 45 minutes (Figure 3.13).

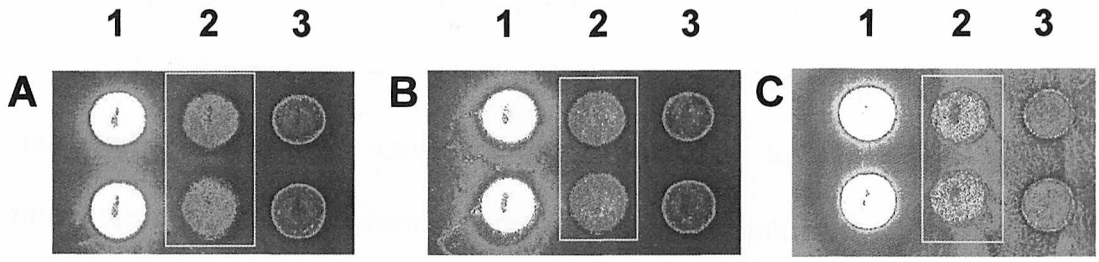


Figure 3.12. Incubation of slides carried out at different temperatures.
A: All incubation steps carried out at room temperature. **B:** Serum incubation carried out at 37°C, all other incubation steps carried out at room temperature. **C:** All incubation steps carried out at 37°C. The arrays were designed to contain 3 allergen extracts: X901 (Bet v 1 – Lane 1); T3 (Birch pollen – Lane 2); D71 (*Lepidoglyphus destructor* – Lane 3). The arrays were incubated with serum 19, positive for all the three allergen extracts.

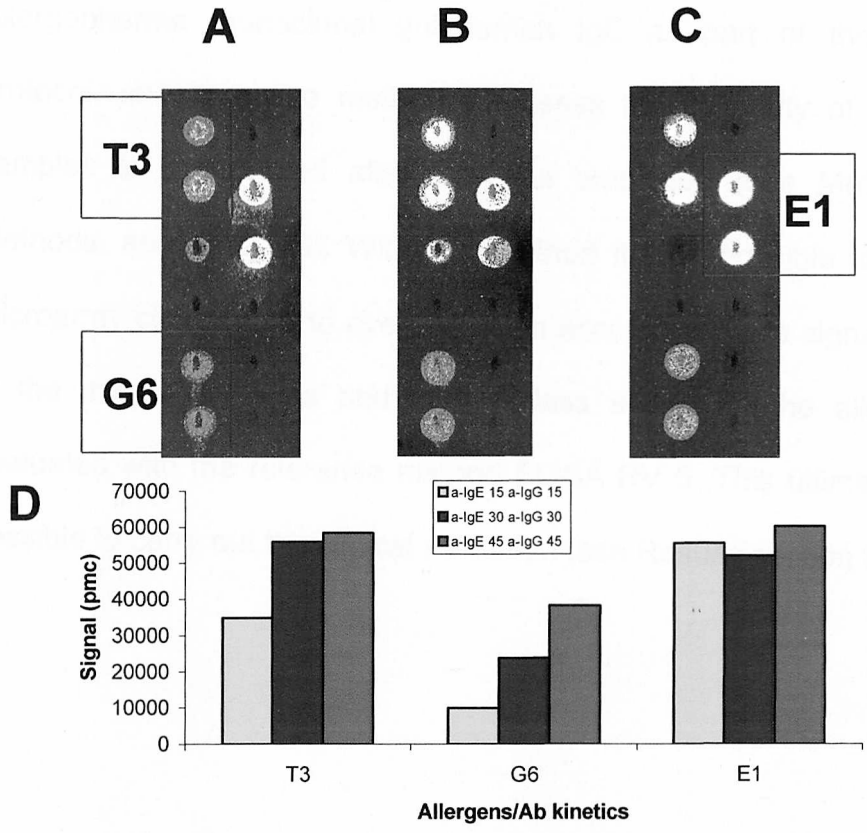


Figure 3.13. Secondary and tertiary antibodies kinetics.
A: Incubation with anti-human IgE and anti-human IgG-HRP conjugated carried out for 15 minutes each. **B:** Incubation with anti-human IgE and anti-human IgG-HRP conjugated carried out for 30 minutes each. **C:** Incubation with anti-human IgE and anti-human IgG-HRP conjugated carried out for 45 minutes each. **D:** Chart showing the different signals for the 3 allergens tested for the antibody kinetics. The arrays were incubated with serum 51, positive for the three allergens printed. All the incubation steps were carried out at 37°C. T3: Birch pollen; G6: Timothy grass pollen; E1: Cat epithelium.

3.6 Calibration of the IgE immunoassay

Initial attempts to print Allergopharma myeloma IgE to be used as internal calibration curve for the microarray immunoassay were unsuccessful. Some evidence suggests that the epitope recognized by the anti-human IgE secondary antibody is the same epitope that binds the myeloma IgE to the slide surface. However the myeloma IgE is recognized when it binds to the anti-human IgE immobilized on the slide surface (Figure 3.14). For this reason, and in order to maintain the Allergopharma monoclonal anti-human IgE as part of the detection protocol, an alternative method to assess the reactivity of the serum samples to the printed allergens was employed (see Materials and Methods, section 2.6.6). With this method it was possible to assign a microarray class score to every allergen according to the signal obtained in the microarray tests and to the class scores of the allergens as evaluated with the reference method ELISA RV 5. This ultimately made possible to carry out the clinical validation (see Results section 3.7.4).

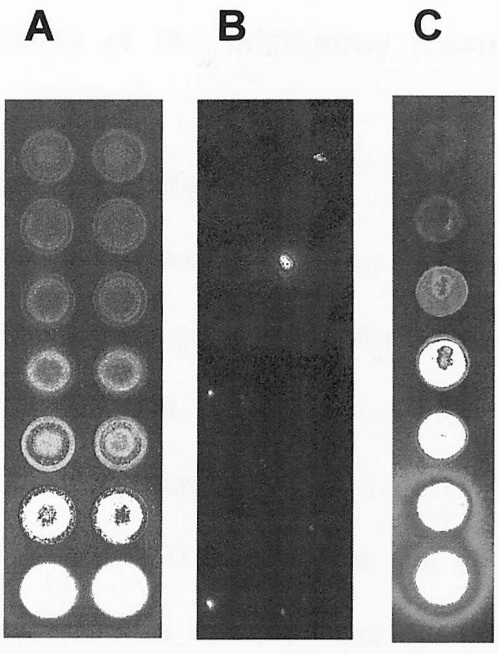


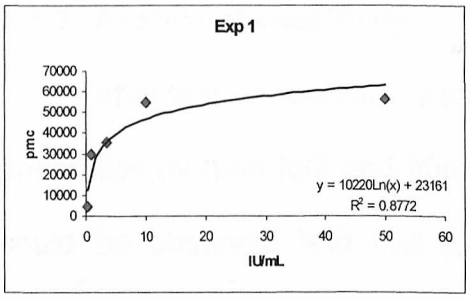
Figure 3.14. Attempts to calibrate the microarray IgE immunoassay.
A: Array produced by printing a dilution curve of human IgE from myeloma and processed with a polyclonal secondary antibody anti-human IgE. **B:** Array produced by printing the same dilution curve of human IgE from myeloma, as in A, and processed with monoclonal anti-human IgE. **C:** Array produced by printing a dilution curve of a monoclonal anti-human IgE antibody and processed with human IgE from myeloma followed by the same monoclonal anti-human IgE used for the experiment in B. Human IgE from myeloma and monoclonal antibodies anti-human IgE were sourced from Allergopharma; polyclonal anti-human IgE was purchased from KPL.

3.7 Performance of the microarray immunoassay: validation studies

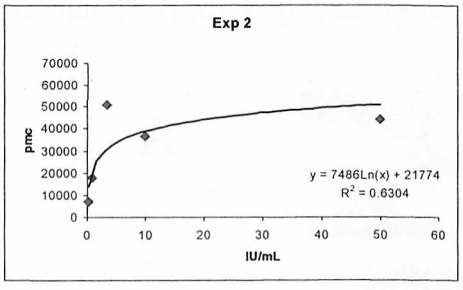
3.7.1 Analytical sensitivity

This parameter was assessed by running the experiment three times. Every experiment provided good curve fits and dose-response curves over the range 0 to 50 IU/mL (or 0 to 122 ng/mL) of IgE. The analytical sensitivity of the allergen microarray was determined to be typically less than 0.2 IU/ml (or less than 0.5 ng/mL) i.e. well below the value of 0.35 IU/mL normally accepted as detection limit for the IgE immunoassays (Figure 3.15).

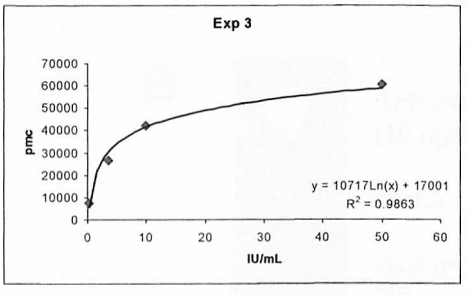
The theoretical maximum sensitivity of the allergen microarray was determined to be approximately 0.002 IU/ml (or 0.005 ng/mL) (Figure 3.16).



Average BSA signal	2364.52 pmc
SD BSA signal	775.516 pmc
Signal sensitivity (Average + 2*SD)	3915.552 pmc
Sensitivity	**0.15 IU/mL**

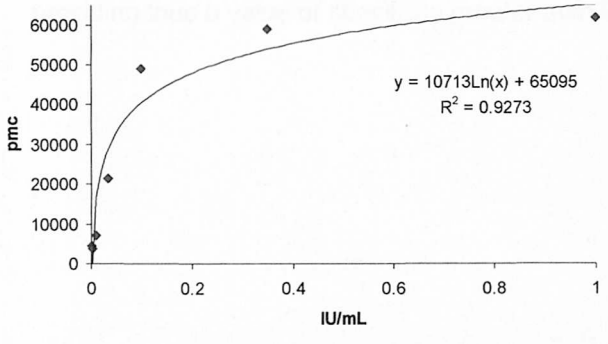


Average BSA signal	4191.214 pmc
SD BSA signal	642.3983 pmc
Signal sensitivity (Average + 2*SD)	5476.011 pmc
Sensitivity	**0.11 IU/mL**



Average BSA signal	255.5238 pmc
SD BSA signal	410.8336 pmc
Signal sensitivity (Average + 2*SD)	1077.191 pmc
Sensitivity	**0.23 IU/mL**

Figure 3.15. Analytical sensitivity.
The analytical sensitivity was carried out on three occasions. The detection limit was calculated by interpolating the signal sensitivity value (as given by the zero reference or negative control) to the curve fits obtained by processing IgE standards onto arrays printed with 10 pg of capture anti-human IgE antibody. In the charts the microarray signal (pmc) has been expressed as a function of the IgE concentration.



Signal sensitivity (Average + 2*SD)	1711.561 pmc
Sensitivity	**0.0026 IU/mL**

Figure 3.16. Maximum theoretical sensitivity.
This value was calculated by increasing the amount of capture anti-human IgE printed and diluting the lowest IgE reference standard. The procedure to calculate the sensitivity is the same as described for Figure 3.15.

3.7.2 Analytical specificity

Analytical specificity was evaluated against other classes of antibodies (human IgG and human IgM). No detectable reactivity to IgG could be observed and that to IgM ranged from 0 to 0.1% thereby meeting the target specifications of >95% specificity for IgE (Figure 3.17).

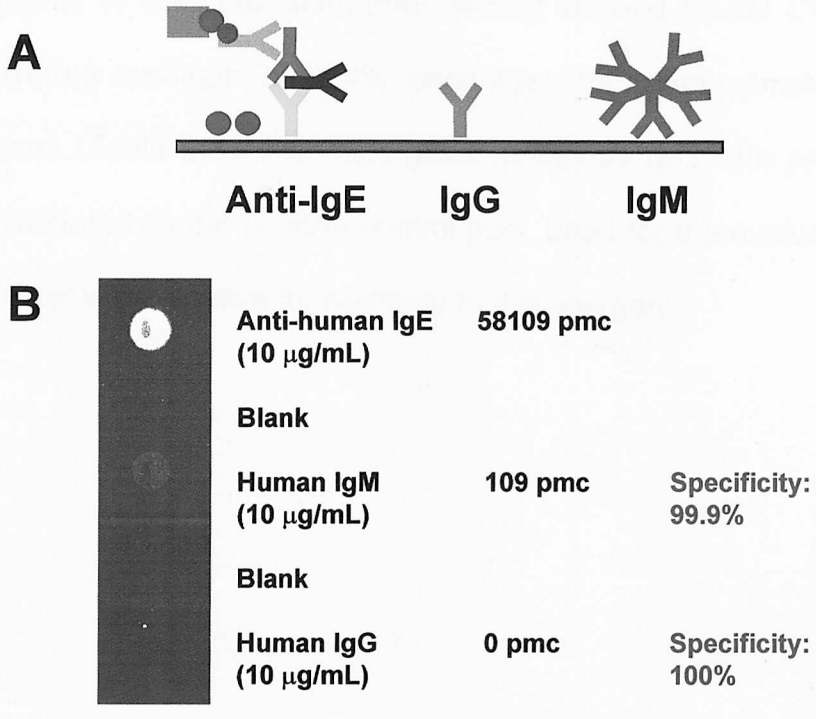


Figure 3.17. Analytical specificity.
A: Schematic of the experimental design: the slide was printed with anti-human IgE, human IgG and human IgM. Then the array was processed with the established protocol (see results section 3.3). **B:** Strong signal was detectable only by the anti-human IgE. Very weak signal or no signal at all was recorded from human IgG and human IgM, providing thus a value of specificity greater than 99% for both these antibodies.

3.7.3 Allergen intra- and inter-slide precision

For the allergen microarray the intra-slide allergen precision analysis revealed that the signal generated by the bound allergen-specific IgEs resulted in typical CVs less than 5% (with a maximum of 10.5% for Latex extract) for all the allergens tested (Table 3.2). An analysis of inter-slide variability (4 reps per slide, three slides) showed typical CVs less than 8% (with a maximum of 16.4%, once again for Latex extract) for all the allergens (Table 3.3). For *Aspergillus fumigatus* (M3) the precision was not evaluated as the positive control pool, used for the evaluation of this parameter was negative for reactivity to this allergen.

Allergen	Mean signal (pmc)	St. Dev.	C.V.%
Dermatophagoides pteronyssinus (D1)	60477.5	69.5	< 1
Lepidoglyphus destructor (D71)	60539.5	37.6	< 1
Cat epithelium (E1)	60100.8	356.8	< 1
Dog epithelium (E2)	60987.1	53.7	< 1
Horse epithelium (E3)	47468.7	4098.0	8.6
Egg white (F1)	59627.8	1615.7	2.7
Cow's milk (F2)	61045.8	1.9	< 1
Peanuts (F13)	54728.3	660.1	1.2
Soybean (F14)	37375.7	809.5	2.2
Apple (F49)	32166.7	1266.4	3.9
Timothy grass pollen (G6)	55895.8	628.9	1.1
Rye cultivated pollen (G12)	56283.5	0.0	< 1
Honeybee venom (I1)	60892.4	175.8	< 1
Wasp venom (I3)	60251.6	166.1	< 1
Latex (K82)	31398.5	3311.2	10.5
Penicillium notatum (M1)	19576.8	762.8	3.9
Cladorporium erbarum (M2)	28703.8	1607.5	5.6
Aspergillus fumigatus (M3)	NA	NA	NA
Alternaria tenuis (M6)	14439.0	440.1	3.0
Celery (S901)	29223.6	706.2	2.4
Birch pollen (T3)	51573.0	3167.1	6.1
Mugwort common pollen (W6)	36677.2	1182.4	3.2
English plantain pollen (W9)	48662.0	3390.9	7.0
Bet v 1 (X901)	58855.7	273.8	< 1
Phl p 5 (X903)	56283.5	0.0	< 1
Der p 1 (X904)	59173.0	698.6	1.2
Der p 2 (X905)	57868.0	1917.0	3.3

Table 3.2. Allergen intra-slide signal precision.
All the allergens were printed in four replicates on the same slide. The slides were incubated with a pool serum reactive for all the 27 allergens of the panel with the exception of the mould *Aspergillus fumigatus* (for which the value of precision is not available). For every allergen the mean signal, standard deviation and coefficient of variation are reported.

Allergen	Mean signal (pmc)	St. Dev.	C.V.%
Dermatophagoides pteronyssinus **(D1)**	59710.5	862.2	1.44
Lepidoglyphus destructor **(D71)**	59768.6	892.9	1.5
Cat epithelium (E1)	58943.9	1213.5	2.05
Dog epithelium (E2)	60714.8	585.4	< 1
Horse epithelium (E3)	49786.9	3645.2	7.3
Egg white (F1)	59517.0	921.0	1.5
Cow's milk (F2)	60728.7	590.7	1.0
Peanuts (F13)	56528.4	2015.3	3.6
Soybean (F14)	37586.6	2247.2	6.0
Apple (F49)	34287.9	2292.9	6.7
Timothy grass pollen (G6)	58814.0	2496.8	4.2
Rye cultivated pollen (G12)	59276.7	2333.1	3.9
Honeybee venom (I1)	60630.5	630.6	1.0
Wasp venom (I3)	59491.9	654.1	1.1
Latex (K82)	31038.7	5118.7	16.4
Penicillium notatum **(M1)**	20125.2	2329.3	11.6
Cladorporium erbarum **(M2)**	29384.6	2262.7	7.7
Aspergillus fumigatus **(M3)**	NA	NA	NA
Alternaria tenuis **(M6)**	14760.0	2045.0	13.9
Celery (S901)	30928.8	1678.1	5.4
Birch pollen (T3)	52166.4	5483.4	10.5
Mugwort common pollen (W6)	35938.2	2082.2	5.8
English plantain (W9)	48845.0	4212.4	8.6
Bet v 1 (X901)	59424.1	911.6	1.5
Phl p 5 (X903)	59276.7	2333.1	3.9
Der p 1 (X904)	58024.6	1993.2	3.4
Der p 2 (X905)	59180.5	2331.8	3.9

Table 3.3. Allergen inter-slide signal precision.
All the allergens were printed in four replicates on three different slides so that the inter slide precision was evaluated on 12 replicates. The slides were incubated with the same pool serum used for the intra assay precision. This serum was reactive for all the 27 allergens of the panel with the exception of the mould *Aspergillus fumigatus* (for which the value of precision is not available). For every allergen the mean signal, standard deviation and coefficient of variation are reported.

3.7.4 Clinical validation

48 serum samples were tested with the microarray immunoassay, each slide containing three replicates of all 27 allergens. Figure 3.18 reports the scatter plots generated, for every single allergen, by expressing the microarray signal as a function of the ELISA class scores. The latter had been assessed from Allergopharma, by evaluating for single allergens the specific IgE reactivity of all the serum samples.

The observation of the scatter plots of Figure 3.18 points out that different allergens display different behaviours when tested in the microarray format. In particular the 27 allergens of the test can be clustered in 4 different groups.

The first group, includes most of the allergens of the assay (13 out of 27: *Dermatophagoides pteronyssinus* D1, Horse epithelium E3, Egg white F1, Cow's milk F2, Timothy grass pollen G6, Rye cultivated pollen G12, Honeybee venom I1, *Alternaria tenuis* M6, Birch pollen T3, English plantain pollen W9, Bet v 1 X901, Phl p 5 X903 and Der p 1 X904). These allergens display a very similar dose/response behaviour characterized by a class score interval where a little increment in allergen-specific IgE results in a massive raise in microarray signal, until the saturation is reached. This "steepness" interval is variable from allergen to allergen. It occurs at very low values of allergen-specific IgE for D1 and E3 (in class score 0), or at higher values of specific antibodies for the other allergens (in class score 1 for X901; in class score 2 for F1, F2, G12, I1, M6, W9, X903, X904; in class score 3 for G6; in class score 4 for T3).

The second group is made up by allergens for which the dose/response relationship does not show steep points and a more gradual rise in the signal at the increment in allergen-specific IgE can be observed. 8 out of 27 allergens can be included in this group: *Lepidoglyphus destructor* D71, Cat epithelium E1, Peanuts F13, Latex K82, *Cladosporium herbarum* M2, Celery S901, Mugwort pollen W6 and Der p 2 X905.

In the third group there are only 4 allergens out of 27, with a more peculiar pattern of reactivity. For these allergens (Dog epithelium E2, Soybean F14, Apple F49 and Wasp venom I3) the scatter plots seem to point out at the existence of two families of serum samples. One family displays a very low reactivity and the dose/response relationship is characterized by a slow increment in signal by increasing the allergen-specific IgE dose. The other family of serum samples displays a behaviour that recalls the behaviour of serum samples on allergens of group 1, since there is a class score interval where the dose/response relationship seems to suddenly steepen. This peculiar pattern of reactivity observed for the allergens belonging to this third group could be ascribed to the fact that one or more major or minor allergenic molecules are not correctly immobilized in the microarray platform, or alternatively that these molecules have been partially or completely degraded either during the lyophilization of the extract or during its reconstitution. This could explain why for these allergens some serum samples show a pattern of reactivity similar to the one recorded by the ELISA, whereas for other samples the response is very different. Of course all these consideration

are valid until the Allergopharma ELISA RV 5 is considered a "gold standard" for the microarray test. However the Allergopharma ELISA RV 5 is a very classical ELISA test with all the limitations of this kind of assays. The use of this test as a reference standard was very helpful in setting up all the basic parameters of the allergen microarray and to tune the latter to the specifications required for an allergen immunoassay. However, for a proper clinical validation a third, more reliable, allergen immunoassay (like ImmunoCAP) would be warranted to better establish the actual serum reactivity against the allergens spotted. The use of a third test would have also helped in assessing if the results obtained, especially for this last group of allergens, were false positives and false negatives or if they rather described the real sample reactivity.

A last group of only 2 allergens (*Penicillium notatum* M1 and *Aspergillus fumigatus* M3) could not be properly assessed in their performance because of the lack of serum samples with a reactivity higher than class score 1.

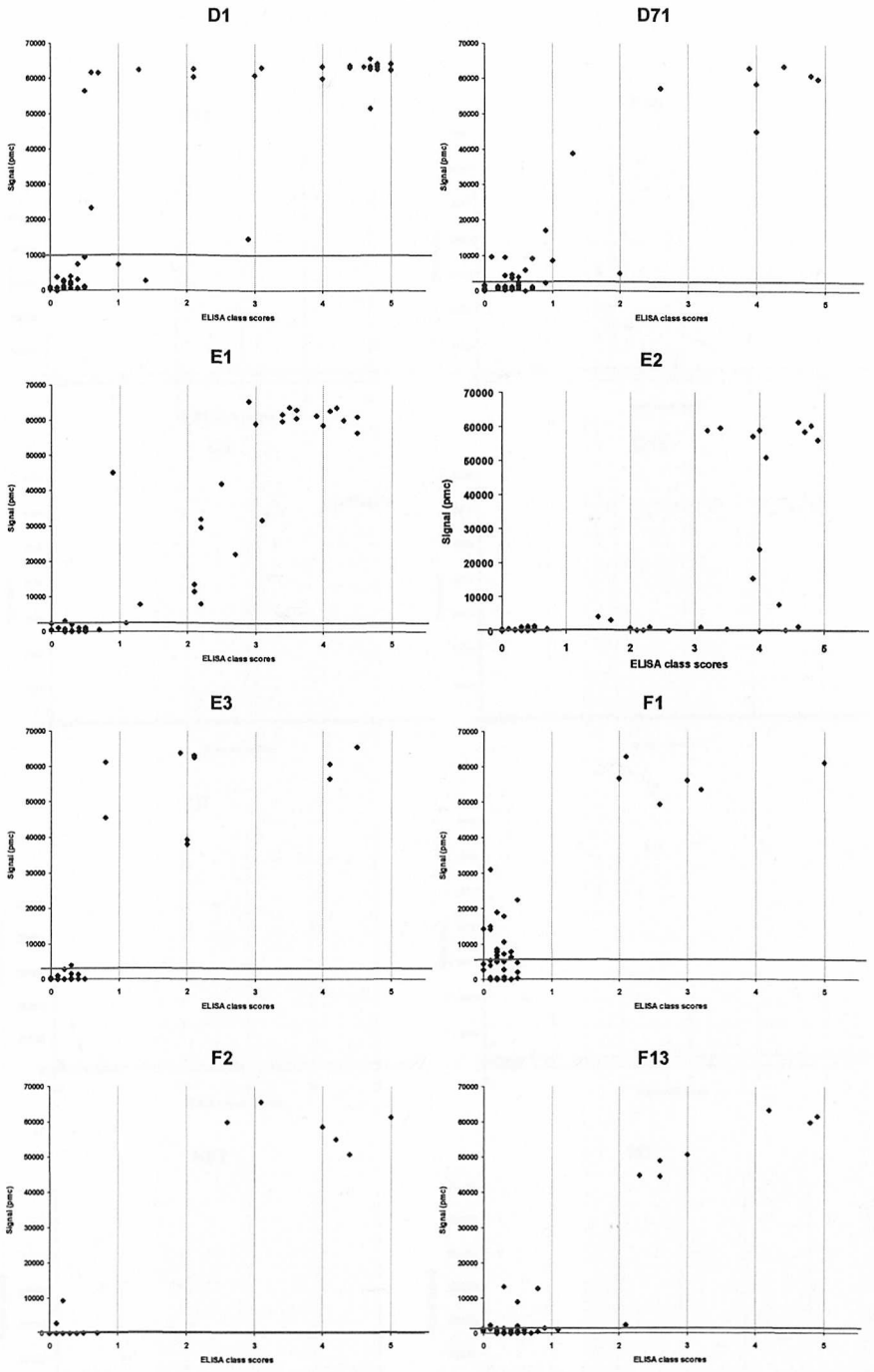


Figure 3.18. Clinical sample reactivity in the microarray immunoassay compared to the ELISA reference system.
From top left to bottom right: serum reactivity to *Dermatophagoides pteronyssinus* (D1), *Lepidoglyphus destructor* (D71), Cat epithelium (E1), Dog epithelium (E2), Horse epithelium (E3), Egg white (F1), Cow's milk (F2) and Peanuts (F13). The signal obtained in the microarray test (pmc) is expressed as a function of the class scores of reactivity as obtained by Allergopharma ELISA RV-5 (reference system). The red line is the cut-off value as calculated for each extract (see Materials and methods, section 2.6.6).

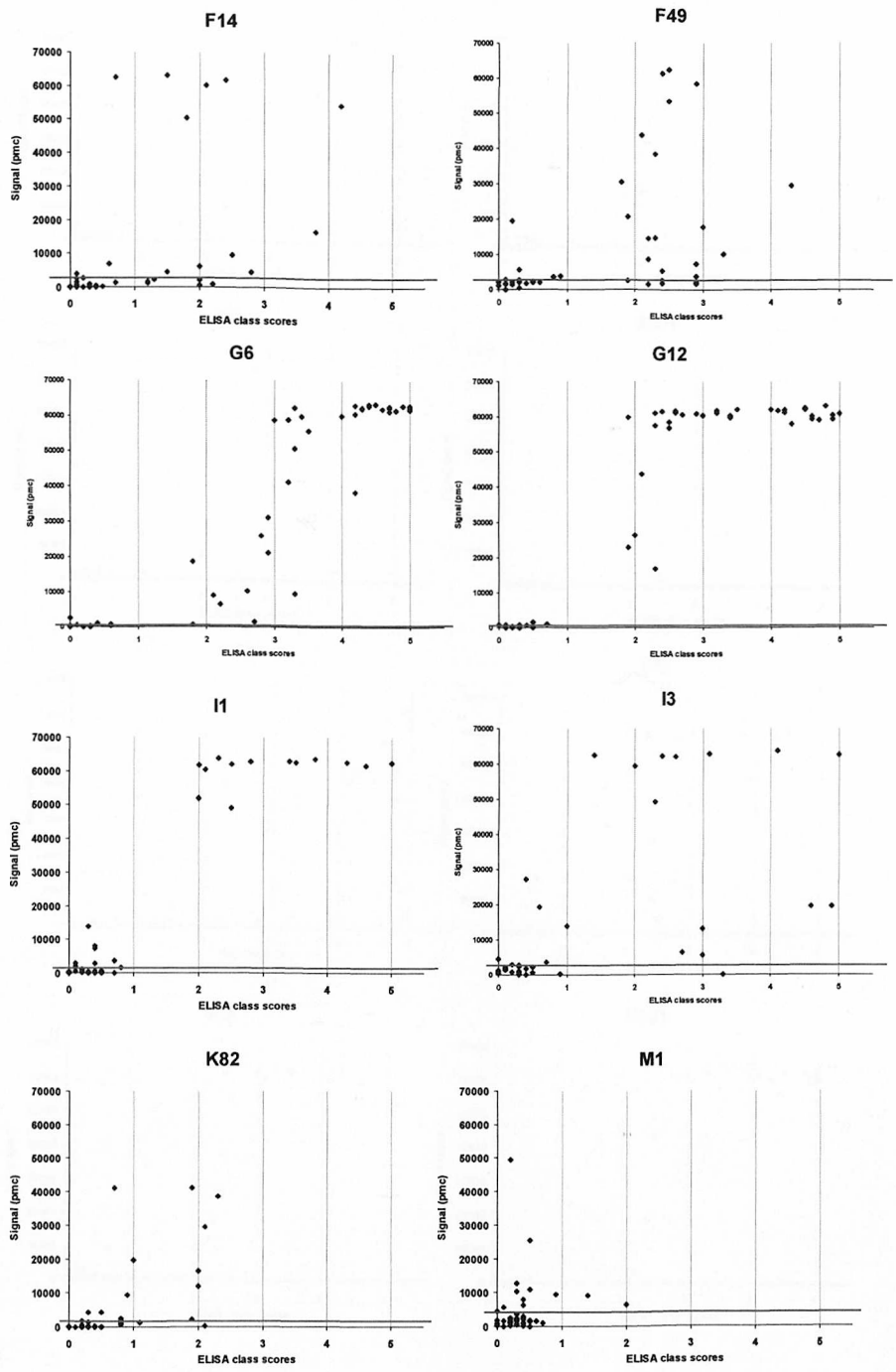


Figure 3.18 (continued). Clinical sample reactivity in the microarray immunoassay compared to the ELISA reference system.
From top left to bottom right: serum reactivity to Soybean (F14), Apple (F49), Timothy grass pollen (G6), Rye cultivated pollen (G12), Honeybee venom (I1), Wasp venom (I3), Latex (K82) and *Penicillium notatum* (M1).

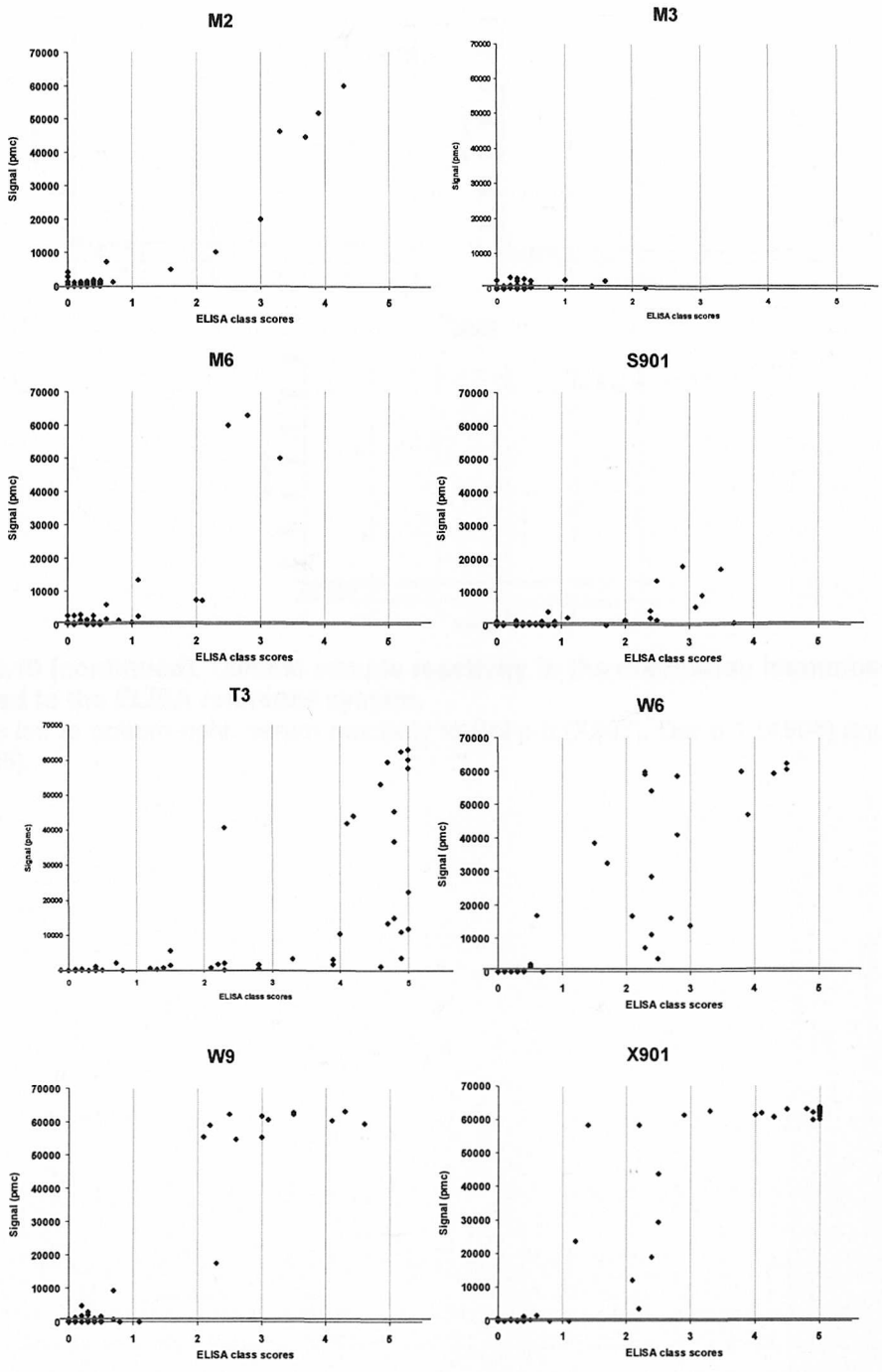


Figure 3.18 (continued). Clinical sample reactivity in the microarray immunoassay compared to the ELISA reference system.
From top left to bottom right: serum reactivity to *Cladosporium herbarum* (M2), *Aspergillus fumigatus* (M3), *Alternaria tenuis* (M6), Celery (S901), Birch pollen (T3), Mugwort common pollen (W6), English plantain pollen (W9) and Bet v 1 (X901).

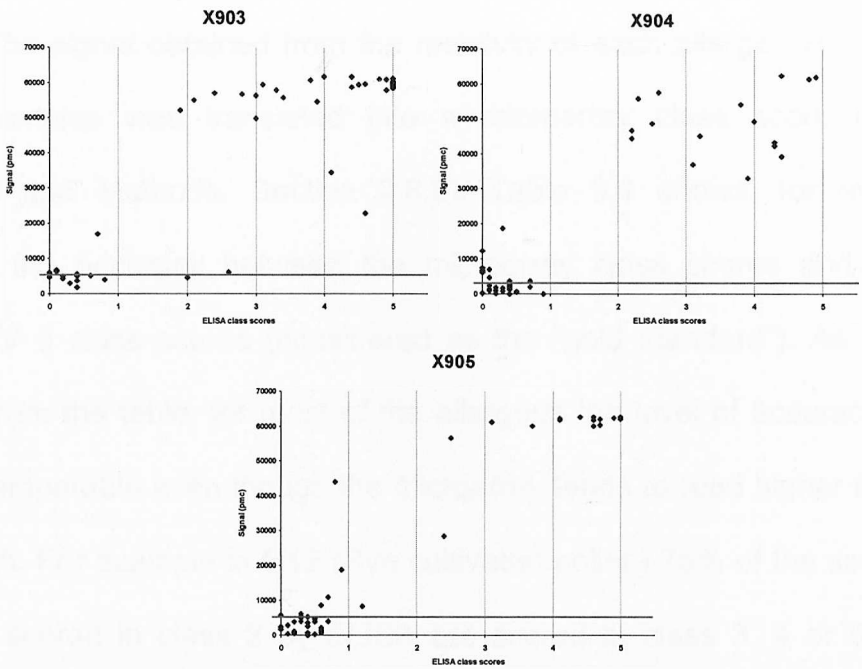


Figure 3.18 (continued). Clinical sample reactivity in the microarray immunoassay compared to the ELISA reference system.
From top left to bottom right: serum reactivity to Phl p 5 (X903), Der p 1 (X904) and Der p 2 (X905).

The signal obtained from the reactivity of each allergen with the serum samples was translated into a microarray class score (see Materials and Methods, Section 2.6.6). Table 3.4 shows, for every allergen, the accuracy between the microarray class scores and the ELISA RV 5 class scores (considered as the "gold standard"). As it is evident from the table, for most of the allergens the level of accuracy is mainly comparable even though the microarray tends to read higher than the ELISA. For example in G12 (Rye cultivated pollen) 75% of the serum samples scored in class 2 by ELISA are scored in class 3, 4 or 5 by microarray; similarly in G6 (Timothy grass pollen) 75% of the serum samples scored in class 3 by ELISA are scored in class 4 or 5 by microarray. These data would indicate that at least for some allergens the microarray is more sensitive than the ELISA. For other allergens (Celery S901 and Birch pollen T3) on the contrary, there is a lower accuracy and the microarray tends to read lower than the ELISA: in Celery 71.4% of the samples scored 2 by ELISA are scored 1 by microarray and all the samples scored 3 by ELISA are scored 1 or 2 by microarray; similarly in Birch pollen 85.7% of the samples scored 2 by ELISA are scored 0 or 1 by microarray and 77% of the samples scored 4 by ELISA are scored 1, 2 or 3 by microarray. For 4 allergens (Dog epithelium E2, Soybean F14, Apple F49 and Wasp venom I3) it appears, once again, that there are two populations of data: some serum samples are ranked higher in the microarray than in the ELISA (and thus the microarray appears to be more sensitive), whereas for another group of serum samples the

behaviour displayed is the opposite. This data would match with the scatter plots of these same 4 allergens where the presence of 2 different populations of serum samples was suggesting itself.

Microarray class scores

ELISA class scores

D1	0 PMC: 0-10035	1 PMC: 10035-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	84.0%	0.0%	4.0%	0.0%	4.0%	8.0%	25
1	66.7%	0.0%	0.0%	0.0%	0.0%	33.3%	3
2	0.0%	0.0%	33.3%	0.0%	0.0%	66.7%	2
3	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	2
4	0.0%	0.0%	0.0%	0.0%	8.3%	91.7%	12
5	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	2

D71	0 PMC: 0-2550	1 PMC: 2550-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	78.4%	21.6%	0.0%	0.0%	0.0%	0.0%	37
1	0.0%	50.0%	0.0%	50.0%	0.0%	0.0%	2
2	0.0%	50.0%	0.0%	0.0%	50.0%	0.0%	2
3	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1
4	0.0%	0.0%	0.0%	20.0%	20.0%	60.0%	5
5	-	-	-	-	-	-	0

E1	0 PMC: 0-2552	1 PMC: 2552-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	87.5%	8.3%	0.0%	4.2%	0.0%	0.0%	24
1	50.0%	50.0%	0.0%	0.0%	0.0%	0.0%	2
2	0.0%	25.0%	37.5%	25.0%	0.0%	12.5%	8
3	0.0%	00%	0.0%	12.5%	0.0%	87.5%	8
4	0.0%	0.0%	0.0%	0.0%	33.3%	66.7%	6
5	-	-	-	-	-	-	0

E2	0 PMC: 0-704	1 PMC: 704-13178	2 PMC: 13179 - 30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	79.2%	20.8%	0.0%	0.0%	0.0%	0.0%	24
1	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	2
2	80.0%	20.0%	0.0%	0.0%	0.0%	0.0%	5
3	16.7%	16.7%	16.7%	0.0%	33.3%	16.7%	6
4	18.2%	18.2%	9.1%	0.0%	27.3%	27.3%	11
5	-	-	-	-	-	-	0

E3	0 PMC: 0-2967	1 PMC: 2967-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	92.5%	2.5%	0.0%	2.5%	0.0%	2.5%	40
1	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1
2	0.0%	0.0%	0.0%	50.0%	0.0%	50.0%	4
3	-	-	-	-	-	-	0
4	0.0%	0.0%	0.0%	0.0%	33.3%	66.7%	3
5	-	-	-	-	-	-	0

F1	0 PMC: 0-5557	1 PMC: 5557-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	64.3%	19.0%	14.3%	2.4%	0.0%	0.0%	42
1	-	-	-	-	-	-	0
2	0.0%	0.0%	0.0%	0.0%	66.7%	33.3%	3
3	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	2
4	-	-	-	-	-	-	0
5	0%	0.0%	0.0%	0.0%	0.0%	100.0%	1

Table 3.4. Classification of sera by ELISA and Microarray for individual allergens. A comparison in the performance of the ELISA reference and the microarray is reported for the allergens *Dermatophagoides pteronyssinus* (D1), *Lepidoglyphus destructor* (D71), Cat epithelium (E1), Dog epithelium (E2), Horse epithelium (E3) and Egg white (F1). The ELISA class scores were provided. The microarray class scores were calculated by interpolating the signals from the blue curve reported in figure 3.19 (see below). n= number of records with a given ELISA class score. As an example: in *Dermatophagoides pteronyssinus* 21 out of 25 samples with ELISA class score 0 (84%) were 0 in the microarray assay as well, 1 (4%) was class score 2 in the microarray and 1 (4%) resulted class score 4 in the microarray.

Microarray class scores

ELISA class scores

F2	0 PMC: 0-289	1 PMC: 289-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	95.2%	4.8%	0.0%	0.0%	0.0%	0.0%	42
1	-	-	-	-	-	-	0
2	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1
3	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1
4	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%	3
5	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1

F13	0 PMC: 0-1463	1 PMC: 1463-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	76.9%	20.5%	2.6%	0.0%	0.0%	0.0%	39
1	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1
2	0.0%	25.5%	0.0%	50.0%	25.0%	0.0%	4
3	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%	1
4	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	3
5	-	-	-	-	-	-	0

F14	0 PMC: 0-2653	1 PMC: 2653-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	90.3%	6.5%	0.0%	0.0%	0.0%	3.2%	31
1	50.0%	16.7%	0.0%	16.7%	16.7%	0.0%	6
2	37.5%	37.5%	0.0%	0.0%	0.0%	25.0%	8
3	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	1
4	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%	1
5	-	-	-	-	-	-	0

F49	0 PMC: 0-2855	1 PMC: 2855-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	83.3%	12.5%	4.2%	0.0%	0.0%	0.0%	24
1	33.3%	0.0%	33.3%	33.3%	0.0%	0.0%	3
2	29.4%	23.5%	11.8%	11.8%	11.8%	11.8%	17
3	0.0%	50.0%	50.0%	0.0%	0.0%	0.0%	2
4	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	1
5	-	-	-	-	-	-	0

G6	0 PMC: 0-561	1 PMC: 561-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	63.6%	36.4%	0.0%	0.0%	0.0%	0.0%	11
1	0.0%	50.0%	50.0%	0.0%	0.0%	0.0%	2
2	0.0%	57.1%	28.6%	14.3%	0.0%	0.0%	7
3	0.0%	12.5%	0.0%	12.5%	37.5%	37.5%	8
4	0.0%	0.0%	0.0%	6.7%	0.0%	93.3%	15
5	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	4

G12	0 PMC: 0-553	1 PMC: 553-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	46.2%	53.8%	0.0%	0.0%	0.0%	0.0%	13
1	0.0%	0.0%	50.0%	0.0%	0.0%	50.0%	2
2	0.0%	8.3%	16.7%	8.3%	25.0%	41.7%	12
3	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	6
4	0.0%	0.0%	0.0%	0.0%	7.1%	92.9%	14
5	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1

Table 3.4 (continued). Classification of sera by ELISA and Microarray for individual allergens.
A comparison in the performance of the ELISA reference and the microarray is reported for the allergens Cow's milk (F2), Peanuts (F13), Soybean (F14), Apple (F49), Timothy grass pollen (G6) and Rye cultivated pollen (G12).

Microarray class scores

ELISA class scores

I1	0 PMC: 0-1570	1 PMC: 1570-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	71.4%	25.7%	2.9%	0.0%	0.0%	0.0%	35
1	-	-	-	-	-	-	0
2	0.0%	0.0%	0.0%	0.0%	28.6%	71.4%	7
3	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	3
4	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	2
5	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1

I3	0 PMC: 0-2665	1 PMC: 2665-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	81.8%	12.1%	6.1%	0.0%	0.0%	0.0%	33
1	0.0%	0.0%	50.0%	0.0%	0.0%	50.0%	2
2	0.0%	20.0%	0.0%	0.0%	20.0%	60.0%	5
3	25.0%	25.0%	0.0%	0.0%	25.0%	25.0%	4
4	0.0%	0.0%	66.7%	0.0%	0.0%	33.3%	3
5	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1

K82	0 PMC: 0-1874	1 PMC: 1874-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	84.2%	13.2%	0.0%	2.6%	0.0%	0.0%	38
1	25.0%	25.0%	25.0%	25.0%	0.0%	0.0%	4
2	16.7%	16.7%	50.0%	16.7%	0.0%	0.0%	6
3	-	-	-	-	-	-	0
4	-	-	-	-	-	-	0
5	-	-	-	-	-	-	0

M1	0 PMC: 0-4263	1 PMC: 4263-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	78.3%	17.4%	2.2%	0.0%	0.0%	0.0%	46
1	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	1
2	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	1
3	-	-	-	-	-	-	0
4	-	-	-	-	-	-	0
5	-	-	-	-	-	-	0

M2	0 PMC: 0-1038	1 PMC: 1038-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	61.0%	39.0%	0.0%	0.0%	0.0%	0.0%	41
1	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	1
2	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	1
3	0.0%	0.0%	25.0%	50.0%	25.0%	0.0%	4
4	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1
5	-	-	-	-	-	-	0

M3	0 PMC: 0-826	1 PMC: 826-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	59.1%	40.9%	0.0%	0.0%	0.0%	0.0%	44
1	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	3
2	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1
3	-	-	-	-	-	-	0
4	-	-	-	-	-	-	0
5	-	-	-	-	-	-	0

Table 3.4 (continued). Classification of sera by ELISA and Microarray for individual allergens.

A comparison in the performance of the ELISA reference and the microarray is reported for the allergens Honeybee venom (I1), Wasp venom (I3), Latex (K82), *Penicillium notatum* (M1), *Cladosporium herbarum* (M2) and *Aspergiullus fumigatus* (M3).

Microarray class scores

ELISA class scores

M6	0 PMC: 0-964	1 PMC: 964-13178	2 PMC: 13179-30426	3 PMC: 30427 -47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	67.5%	32.5%	0.0%	0.0%	0.0%	0.0%	40
1	33.3%	33.3%	33.3%	0.0%	0.0%	0.0%	3
2	0.0%	50.0%	0.0%	0.0%	0.0%	50.0%	4
3	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%	1
4	-	-	-	-	-	-	0
5	-	-	-	-	-	-	0

S901	0 PMC: 0-495	1 PMC: 495-13178	2 PMC: 13179-30426	3 PMC: 30427 - 47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	64.7%	35.3%	0.0%	0.0%	0.0%	0.0%	34
1	50.0%	50.0%	0.0%	0.0%	0.0%	0.0%	2
2	0.0%	71.4%	28.6%	0.0%	0.0%	0.0%	7
3	0.0%	75.0%	25.0%	0.0%	0.0%	0.0%	4
4	-	-	-	-	-	-	0
5	-	-	-	-	-	-	0

T3	0 PMC: 0-339	1 PMC: 339-13178	2 PMC: 13179-30426	3 PMC: 30427 -47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	78.6%	21.4%	0.0%	0.0%	0.0%	0.0%	14
1	20.0%	80.0%	0.0%	0.0%	0.0%	0.0%	5
2	14.3%	71.4%	0.0%	14.3%	0.0%	0.0%	7
3	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	3
4	0.0%	30.8%	15.4%	30.8%	7.7%	15.4%	13
5	0%	20.0%	20.0%	0.0%	20.0%	40.0%	5

W6	0 PMC: 0-764	1 PMC: 764-13178	2 PMC: 13179-30426	3 PMC: 30427 -47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	89.7%	6.9%	3.4%	0.0%	0.0%	0.0%	29
1	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	2
2	0.0%	27.3%	27.3%	9.1%	18.2%	18.2%	11
3	0.0%	0.0%	33.3%	33.3%	33.3%	0.0%	3
4	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	3
5	-	-	-	-	-	-	0

W9	0 PMC: 0-880	1 PMC: 880-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	73.5%	26.5%	0.0%	0.0%	0.0%	0.0%	34
1	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1
2	0.0%	0.0%	20.0%	0.0%	40.0%	40.0%	5
3	0.0%	0.0%	0.0%	0.0%	20.0%	80.0%	5
4	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	3
5	-	-	-	-	-	-	0

X901	0 PMC: 0-339	1 PMC: 339-13178	2 PMC: 13179-30426	3 PMC: 30427 -47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	76.5%	23.5%	0.0%	0.0%	0.0%	0.0%	17
1	33.3%	0.0%	33.3%	0.0%	33.3%	0.0%	3
2	0.0%	28.6%	28.6%	14.3%	14.3%	14.3%	7
3	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	1
4	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	8
5	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	12

Table 3.4 (continued). Classification of sera by ELISA and Microarray for individual allergens.
A comparison in the performance of the ELISA reference and the microarray is reported for the allergens *Alternaria tenuis* (M6), Celery (S901), Birch pollen (T3), Mugwort common pollen (W6), English plantain pollen (W9) and Bet v 1 (X901).

Microarray class scores

ELISA class scores

X903	0 PMC: 0-5616	1 PMC: 5616-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	66.7%	33.3%	0.0%	0.0%	0.0%	0.0%	15
1	0.0%	0.0%	50.0%	0.0%	50.0%	0.0%	2
2	20.0%	20.0%	0.0%	0.0%	60.0%	0.0%	5
3	0.0%	0.0%	0.0%	0.0%	71.4%	28.6%	7
4	0.0%	0.0%	9.1%	9.1%	18.2%	63.6%	11
5	0.0%	0.0%	0.0%	0.0%	12.5%	87.50%	8

X904	0 PMC: 0-3172	1 PMC: 3172-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	71.9%	25.0%	3.1%	0.0%	0.0%	0.0%	32
1	-	-	-	-	-	-	0
2	0.0%	0.0%	0.0%	40.0%	60.0%	0.0%	5
3	0.0%	0.0%	0.0%	75.0%	25.0%	0.0%	4
4	0.0%	0.0%	0.0%	42.9%	14.3%	42.9%	7
5	-	-	-	-	-	-	0

X905	0 PMC: 0-5009	1 PMC: 5009-13178	2 PMC: 13179-30426	3 PMC: 30427-47674	4 PMC: 47675-58925	5 PMC: >58926	n
0	74.2%	19.4%	3.2%	3.2%	0.0%	0.0%	31
1	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	1
2	0.0%	0.0%	50.0%	0.0%	50.0%	0.0%	2
3	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	2
4	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	9
5	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	3

Table 3.4 (continued). Classification of sera by ELISA and Microarray for individual allergens.
A comparison in the performance of the ELISA reference and the microarray is reported for the allergens Phl p 5 (X903), Der p 1 (X904) and Der p 2 (X905).

Table 3.5 summarizes the accuracy of the allergen microarray immunoassay versus the ELISA RV 5 by grouping all the tests (i.e. all the allergens tested with all the serum samples). This table suggests that the overall trend is for the microarray to assign higher class scores than the ELISA, pointing out, once again, at the possible higher sensitivity of the former, although with exceptions when considering single allergens. As for the results of the scatter plots reported in Figure 3.18, these data would be more consistent by running the same experiment with a third allergen immunoassay.

Microarray class scores

ELISA class scores	*0* PMC: 0-CutOff	*1* PMC: CutOff-13178	*2* PMC: 13179-30426	*3* PMC:30427-47674	*4* PMC: 47675-58925	*5* PMC: >58926	*n*
0	76.2%	20.6%	1.9%	0.6%	0.2%	0.5%	835
1	25.9%	35.2%	14.8%	11.1%	5.6%	7.4%	54
2	10.8%	26.4%	14.9%	10.8%	16.2%	20.9%	148
3	2.5%	12.7%	7.6%	10.1%	24.1%	43.0%	79
4	1.5%	4.5%	5.2%	7.5%	12.7%	68.7%	134
5	0.0%	2.6%	2.6%	0.0%	5.1%	89.7%	39

Table 3.5. Classification of sera by ELISA and Microarray for all allergens.
The ELISA class scores were provided. The microarray class scores were calculated by interpolating the signals from the blue curve reported in figure 3.19 (see below). n= number of records with a given ELISA class score. As an example: for ELISA class score 0, the total number of allergens whose ELISA class score is 0 with one or more serum samples is 835. This total represents all the different combinations of allergens and serum samples clustered according to their lack of reactivity in ELISA.

Using individual cut-off values for every allergen (as described in Material and Methods, Section 2.6.6) in order to discriminate between positive and negative samples in the microarray test, has made possible to evaluate the clinical sensitivity and clinical specificity. The values of these clinical parameters for all the allergens are reported in Table 3.6. As a general consideration, it appears that the allergen microarray is more clinically sensitive than clinically specific. The clinical sensitivity values range from 100% for Lepidoglyphus destructor D71, Horse epithelium E3, Egg white F1, Cow's milk F2, Timothy grass pollen G6, Rye cultivated pollen G12, Honeybee venom I1, Penicillium notatum M1, Cladosporium herbarum M2, Mugwort pollen W6, Der p 1 X904 and Der p 2 X905, down to 62.5% for Soybean F14, with a median value of 97%. The clinical specificity's highest value has been recorded for Cow's milk F2 (92.5%) and values above 90% have been obtained also for Cat epithelium E1 (91.7%) and Horse epithelium E3 (92.5%). In general, the values of clinical specificity were lower than the values of clinical sensitivity, with a median value of 77%. However, with the exception of Rye cultivated pollen G12 (whose specificity was 46.2%) this parameter was always above the 60% for all the allergens. Once again, this analysis was tuned on the Allergopharma ELISA RV 5; it may be that the use of a different reference test will improve the overall picture and will raise the values of both these clinical parameters.

Allergen	Clinical sensitivity	Clinical specificity
Dermatophagoides pteronyssinus	90.9%	80%
Lepidoglyphus destructor	100%	76.3%
Cat epithelium	95.8%	91.7%
Dog epithelium	68.2%	79.2%
Horse epithelium	100%	92.5%
Egg white	100%	64.3%
Cow's milk	100%	95.2%
Peanuts	88.9%	79.5%
Soybean	62.5%	87.5%
Apple	73.9%	83.3%
Timothy grass	100%	63.6%
Rye cultivated	100%	46.2%
Honeybee venom	100%	71.4%
Wasp venom	93.3%	81.8%
Latex	77.8%	84.2%
Penicillium notatum	100%	78.3%
Cladosporium herbarum	100%	61%
Aspergillus fumigatus	75%	61.4%
Alternaria tenuis	87.5%	67.5%
Celery	92.3%	64.7%
Birch pollen	93.9%	76.9%
Mugwort pollen	100%	89.7%
English plantain pollen	92.9%	73.5%
Bet v 1	96.8%	76.5%
Phl p 5	96.9%	62.5%
Der p 1	100%	71.9%
Der p 2	100%	77.4%

Table 3.6. Clinical sensitivity and clinical specificity of the allergen microarray immunoassay.
The clinical sensitivity is calculated by expressing the number of samples positive by microarray immunoassay as a percentage of the number of samples positive for the disease (i.e. positive by ELISA RV-5). The clinical specificity is calculated by expressing the number of samples that are negative by the microarray immunoassay as a percent of the number of samples negative for the disease (i.e. negative by ELISA RV-5).

A comparision of ELISA and microarray IgE dose-response curves generated with the same standard preparations shows very similar curve shapes although the microarray curve is steeper and thus more sensitive (Figure 3.19).

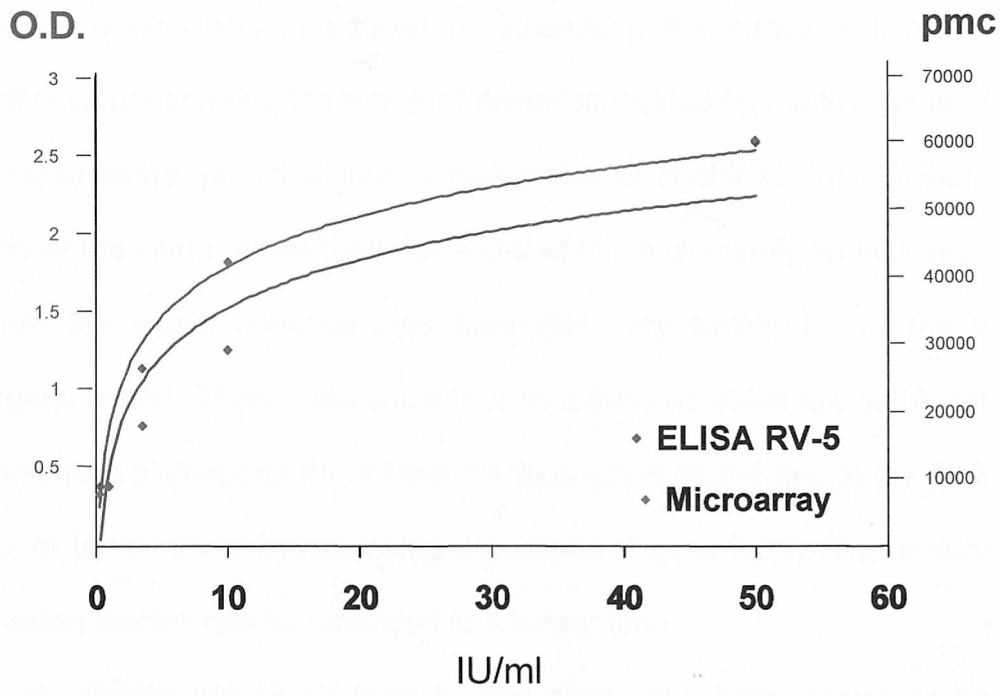


Figure 3.19. ELISA and Microarray IgE dose-response.
The red curve was generated in the reference test Allergopharma ELISA RV 5; the blue curve was generated with the microarray assay. Both curves used the same reference standards provided in the Allergopharma ELISA RV 5 kit.

3.7.5 Stability of printed and processed arrays

According to the NCCLS guidelines the assay is considered stable when the percentage of change in the signal over time remains within 10% (Matsson et al., 1997). In the present study only a small number of representative allergens were tested for the stability and only over 90 days. However for 4 out of 6 allergens the coefficient of variation remained well below the recommended 10%. For the remaining 2 allergens a bias was introduced by incubating the arrays with a low reactive serum sample: the standard deviation divided by the low value of the mean signal provided thus a high value of coefficient of variation. However the value of standard deviation, which is ultimately an indication of how the signal changes over time was very similar for all the 6 allergens tested. These data show thus that arrayed slides are stable for representative allergens for at least 90 days when stored dry, in the dark at room temperature before being processed (Figure 3.20). These very promising studies can be extended to a longer time.

In addition the signal from treated slides has been shown to be stable for at least 138 days after being processed (Figure 3.21). This is in marked contrast to ELISA tests where the plate has to be read within 2 hours because the signal fades very rapidly.

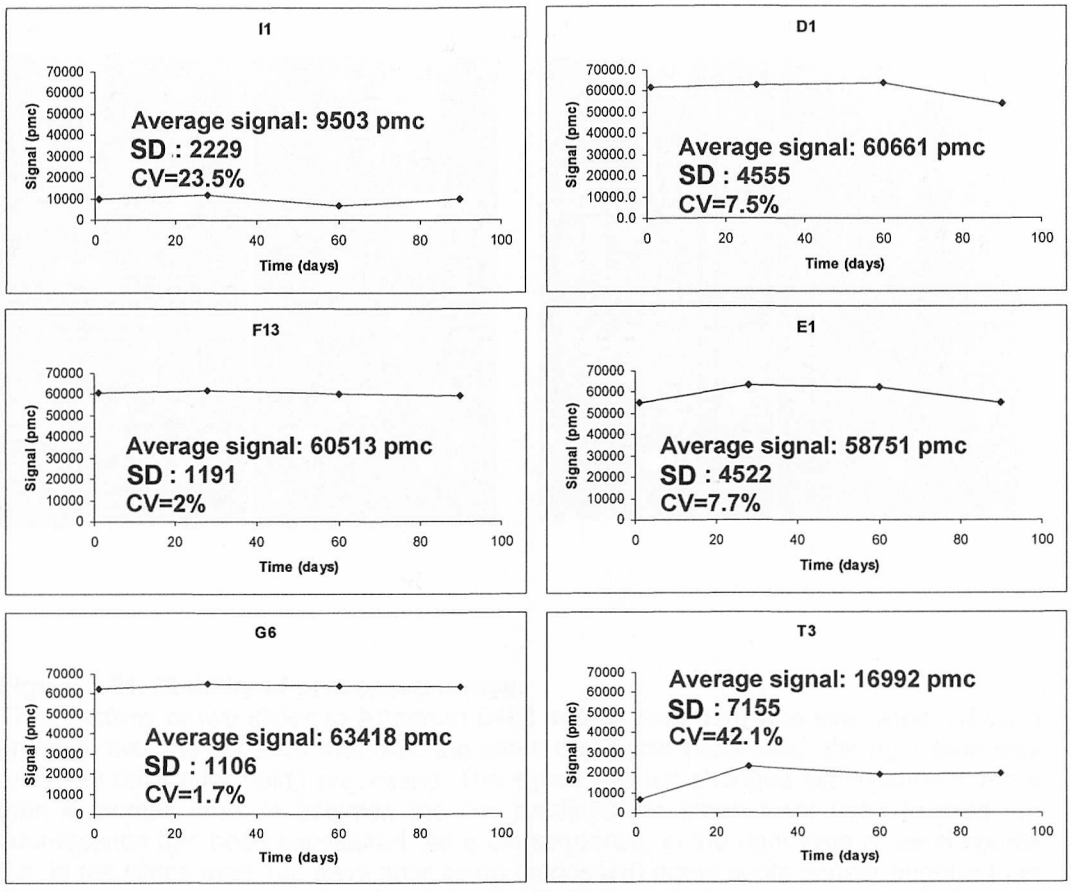


Figure 3.20. Stability of the printed arrays.
The reactivity of the printed allergens was evaluated over a 90 days period and the signal collected expressed as a function of time. As shown by the low values of coefficients of variation (and where this parameter is high, by the values of standard deviation) the signal from the allergens remained stable over time regardless of their high or low reactivity to the serum sample employed for the study.

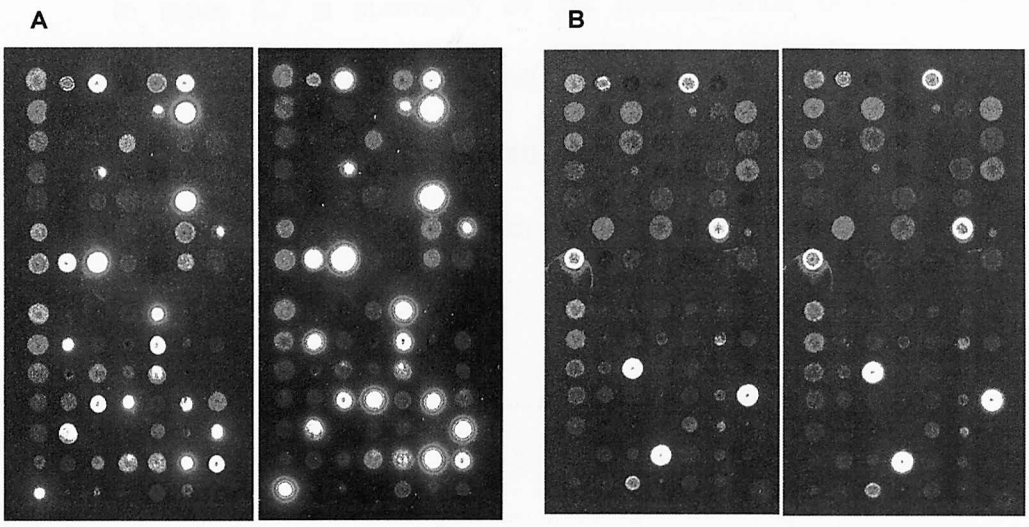


Figure 3.21. Stability of processed arrays.
The reactivity of two slides to **A:** serum 5492 and **B:** serum 16 was evaluated. Of each group of two, the left slide was read the same day it was processed; the right slide was read 138 days after being processed. The signal has not changed significantly in more than 4 months time. In between the two readings the green laser used to read the fluorescence has been substituted. As a consequence, in the right slide of each couple (i.e. in the slides read 138 days after being processed) some spots appear brighter than the correspondent ones in the slides read on the same day of processing. Therefore this difference is to be ascribed only to the use of a newer laser to read off the signal.

In table 3.7 a summary of the performance of the allergen microarray immunoassay, compared to the results obtained with the Allergopharma ELISA RV 5, used as reference method, and the specifications provided by NCCLS for IgE immunoassays (Matsson et al., 1997) is given.

Parameter	Targeted	Achieved	NCCLS specifications
Serum Requirements	< 50 µL per test	100 µL per assay	< 100 µL per test
Analytical Sensitivity	≤ 0.35 IU/mL	0.15 IU/mL	Assay and unit dependent
Analytical Specificity	95%	> 99%	> 99%
Measuring Range	0.35 IU/mL – 50 IU/mL	0.15 IU/mL – 50 IU/mL	Assay and unit dependent
Intra-slide Precision (reproducibility)	≤ 12%	< 10.5% (typically < 5%)	< 10%
Inter-slide Precision (repeatability)	≤ 15%	< 16.1% (typically < 8%)	< 15% or < 20%
Stability (after printing)	NA	≥ 90 days (typically < 10% change over time)	< 10% change over time
Clinical Sensitivity	NA	68% - 100% (typically > 97%)	No specifications indicated
Clinical specificity	NA	46% - 95% (typically > 77%)	No specifications indicated

Table 3.7. Performance of the allergen microarray immunoassay compared with the reference method and the NCCLS specifications.

4.0 Discussion

To date the cutaneous reactions, particularly the skin prick test, are the gold standards for the diagnosis of allergy. They are inexpensive, the results are quickly accessible and they provide a very high clinical sensitivity, so that no allergen reactivity is missed. However these tests cannot be performed on patients under anti-histamine or anti-depressants treatment. In these patients the levels of histamine are reduced, leading thus to false negative results. Also most patients (especially children) will tolerate only a limited number of tests. The possibility of anaphylactic shock, occurring after cutaneous tests, cannot be ruled out, and for this reason patients often have to be monitored for some time after the test. Finally the results provided by these tests are only a "yes-or-no" type (i.e. allergic or non-allergic) and the variability in results is very frequent, due to the lack of standardization of the allergen extracts employed.

Most of these drawbacks can be overcome by using the numerous *in vitro* diagnostic tests commercially available, such as RAST, ELISA and CAP. They can be performed on patients under any kind of therapy because they are not affected by the levels of histamine in blood, the patient does not undergo the risk of anaphylactic shock, and the test response is not purely qualitative but it provides a semi-quantitative or a quantitative information on the concentration of total and/or allergen-specific IgE antibodies in blood. However these tests cannot be used to screen against a wide panel of allergen specificities, given the costs in terms of reagents and the high volumes of serum required from the patient. Since

only a limited number of allergen specificities can be tested, these *in vitro* methods have always to be performed in accordance with clinical history and physical examination. In addition the time-to-result is longer than in the cutaneous reactions and the clinical sensitivity is poorer, leading thus to frequent false negative results.

The use of microarray as a tool in the allergy diagnosis has the potentiality to overcome most of the drawbacks of cutaneous reactions and *in vitro* tests, embracing the advantages of both. The miniaturization offered by the microarray enables to screen a big number of allergens in one single test, reducing the requirements in terms of allergen extracts and reagents necessary for the processing, especially the serum sample. The latter is very important particularly when the allergy diagnosis is carried out in children. This means that, after the initial costs necessary for being set up, the microarray system proves to be highly economical when considering the number of results that can be provided with just one test. In addition it does not require suspension of any therapy from the patient and the risk of anaphylactic shock is avoided.

However the setting up of such a system is not simple. This is shown by the fact that the initial attempts to produce allergen microarrays to be employed for clinical purposes resulted in immunoassays with a poor analytical sensitivity and not reproducible calibration system (Kim et al., 2002), poor clinical sensitivity (Hiller et al., 2002), poor intra-assay and inter-assay precision (Jahn-Schmidt et al., 2003) or with clinical parameters variable from allergen to allergen (Wiltshire et al., 2000). As a

general consideration, there are many technical issues to be taken into account when producing allergen microarrays.

When the microarray was initially introduced in research as a tool to study gene expression profiling, an initial challenge had to be faced in establishing the right spotting conditions for the DNA molecules. Then, once set up, these conditions could be universally applied to any DNA microarray because whichever gene is printed, the biochemical properties of DNA molecules are the same, regardless their length and nucleotide sequences.

To immobilize proteins is far more a challenging task since proteic material is very heterogeneous in terms of amino acidic composition, isoelectric properties and three-dimensional arrangements in subunits. In addition many proteins undergo post-translational modifications such as attachment of carbohydrate moieties or other biomolecules and this will further add to their biochemical complexity.

One of the main challenges when producing allergen microarrays is thus the correct immobilization of the allergens on the solid surface of choice, since most of the epitopes on the allergenic molecules are recognized by the allergen-specific antibodies only when they are arranged in a very precise tridimensional conformation. It follows that to retain their ability to bind serum IgEs the allergens have to preserve their tridimensional structure and their original folding. In addition the term "allergenic molecules" indicates a very heterogeneous collection of proteins and/or glycoproteins, each with their own conformation,

biochemical properties and structure. Therefore in order to bind every single allergen, a miscellaneous collection of spotting buffers (differing in their chemical composition, pH and presence or absence of additives) had to be employed. This wide variety of spotting diluents accounts for the above mentioned heterogeneous biochemistries of the allergenic molecules.

It follows that some allergens will require a spotting diluent with an alkaline pH, whereas others could be better oriented when diluted in acidic or neutral spotting buffers.

Furthermore some proteins can require the presence of additives, such as Tween 20 and SDS, at very low concentrations. Inclusion of these detergents at low concentrations in salt-based spotting solutions can improve pin loading, spot morphology and the homogeneous distribution of the sample within the spot.

The reducing agent dithiotreitol was tested on epidermal extracts, given their high content in keratin, a protein rich in cysteine residues and thus in disulphide bonds. However this spotting diluent was effective only for the printing of horse epithelium extract, whereas no improvements were observed on the printability of cat and dog epithelia extracts. This is probably due to the fact that the major allergens of these three epithelia are not skin proteins, but salivary proteins belonging to the family of lipocalins (Konieczny et al., 1997; Smith et al., 2004; Guida et al., 2005). Therefore the beneficial effect of dithiotreitol on horse epithelium printability is likely to be ascribed to the particular aminoacidic sequence

or protein folding of one or more of the major allergens of the extract, rather than to a feature common to all the epidermal extracts.

A spotting buffer containing propan-1-ol and Tween 20 was initially chosen for some allergen extracts with a high content in carbohydrate moieties (like the moulds). However the partial or complete evaporation of the alcohol from the spotting solution led to a change in the chemical composition of the solution itself, either before the spotting of the allergenic extracts or before the allergen could properly bind to the slide surface, accounting thus for the variable signal detected in different prints. These data clearly showed the unsuitability of such a spotting diluent for the allergen microarray.

The concentration at which the material is spotted can greatly affect the results as well. When a sub optimal mass of material is deposited onto the slide surface, the resulting signal will be low or absent and, as a consequence, the reactivity from serum samples with a low content of allergen-specific IgEs can be completely missed out. This will lead to a drop in clinical sensitivity, and consequently to the classification of some positive samples as negative. In contrast, when the spotting concentration is too high one of two things can happen: if the allergen is a purified protein the amplification is such that a loss in clinical specificity occurs, i.e. some negative samples will be ranked as positives; alternatively if the allergen is part of an extract, the allergenic molecules (spotted in parallel with other non-allergenic components occurring in the

extracts) will be so tightly packed that less epitopes will be accessible to be bound by the allergen-specific IgE.

Allergen immobilisation can influence the test performance because of conformational change or degradation of the allergen itself that may occur when the spotted allergen does not bind correctly to the solid surface. In parallel it is critical to minimize the non specific binding of other proteins to the surface in subsequent steps. Many different slide surface modifications are available for the immobilization of proteins for array applications, but none of them has been established as the surface of choice for protein microarrays. This is one of the main challenges to their commercial development.

Amongst the solid surfaces that have been tested, the amino-silane glass slides and the Black Polymer MaxiSorp™ plastic slides bind allergens via passive absorption which involves various types of weak bonds (electrostatic forces and/or ionic bonds), making this kind of binding difficult to control. This results in either poor specific signal or low-quality spot morphology.

On the other hand, aldehyde and silylated slides are able to covalently bind allergenic molecules to the glass surface of a microscope slide. Covalent binding results in diminished loss of immobilized molecules during the course of experiments and storage of printed arrays. Use of covalent immobilization techniques thus permits harsher wash steps, which in turn reduce background signals and clearly enhances sensitivity (Cel Associates Inc. http://www.cel-1.com/). However the high degree of

background and non specific signal observed with the silyl immobilization chemistry rendered this surface unsuitable for clinical applications. Consequently, covalent attachment of allergenic molecules to slide surfaces via aldehyde chemistry was the immobilization method of choice for this study. The binding of the proteins to the aldehyde slides takes place via the Schiff base aldehyde-amine chemistry. The aldehydes on the glass surface react readily with primary amines on the proteins to form a Schiff's base linkage. Because typical proteins display many lysines on their surface as well as the generally more reactive alpha-amine at their amino-termini, they can attach to the slide in a variety of orientations, allowing different epitopes of the protein to interact with their specific antibodies. Following attachment of the proteins to these slides, the unreacted aldehydes are quenched, and non-specific binding minimized, by immersing the slides in a blocking solution containing bovine serum albumin. Given the high content of amino-groups in lysine residues of the bovine serum albumin, the BSA reacts with the aldehydes via the Schiff base aldehyde-amine chemistry as well (figure 4.1).

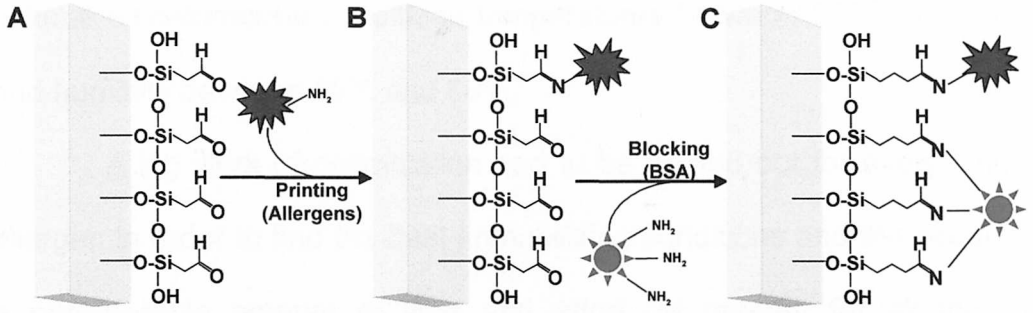


Figure 4.1. Covalent binding of allergens to the aldehyde treated glass slides.
A: An allergen is printed on an aldehyde-activated glass slide. **B:** The aminogroups of the lysine residues of the allergenic molecules react with the aldehydic groups and the result is a covalent binding via the formation of a Schiff base. **C:** The unreacted aldehyde groups are then quenched by blocking the slides with bovine serum albumin.

Another important feature affecting the performance of microarrays, and thus to be taken into account, are the environmental conditions, especially humidity and temperature. Low humidity leads to premature drying of the sample in the pins and degrades the uniformity of individual spots. "Donut" shaped spots are often the result of low humidity during the spotting. On the other side, humidity higher than 65% can lead to problems with the pins sticking in the printhead and can cause the spots to spread out to a larger diameter.

The effect of temperature is important as well as the effect of humidity, at least for protein microarrays. A temperature higher than 25°C can lead to excessive evaporation of the sample prior to its collection by pins and to its correct binding to the slide surface. On the other side, too low temperatures can affect the yet unblocked slide surface by moisturising it (especially if the low temperature occurs in combination with high humidity). The experience acquired in this field led me to set up as

optimal environmental conditions temperatures between 20°C and 25°C and humidity between 40% and 60%.

A big work of optimization had to be carried out for every single allergen in order to find the best immobilizing conditions and this required a considerable amount of time and effort. At present for all the 27 allergens tested (and for an additional amount of 73 allergens that are undergoing optimization right now in the laboratory) the optimal spotting conditions have been found. However, given the great heterogeneity of allergen extracts and allergenic molecules, it could be that for some of them such optimization will not be achieved. This would be clearly a disadvantage for this kind of assay. A possible alternative to circumvent such a problem would be to use a common capture surface, like a streptavidin-coated slide, a strategy frequently employed today for many microarray-based assays (Collett et al., 2005). However the application of this surface requires the biotinylation of the spotted molecules and this would be a challenge for a system like the one described here. In this project crude extracts, rather than purified allergenic molecules, have been arrayed. Therefore, in order to use a streptavidin-coated surface a fractionation of the raw allergenic material should be performed first, in order to separate allergenic from non-allergenic molecules, and secondly a biotinylation of the relevant allergenic molecules should be carried out. An alternative to the use of streptavidin-coated surfaces could be the expression of recombinant allergens with a tag, like GST (Glutathione-S-Transferase) or histidine. However, despite the time and costs required

for these procedures, for many allergen extracts no specific allergenic molecules have been identified yet and these allergen specificities would thus be excluded from such a test.

Another challenge to tackle when making allergen microarrays is given by the method chosen to process them. Previous studies conducted by our group (Bacarese-Hamilton et al., 2002) showed that a processing protocol making use of the indirect immunofluorescence is not suitable for the allergen microarray. This is due to the fact that allergen-specific IgE antibodies are normally 1000 times less concentrated in blood than IgG antibodies. Consequently, a protocol based on the indirect immunofluorescence is not able to detect the IgE reactivity in a clinically significant range (i.e. 1 - 100 ng/mL). For this reason a multi-step protocol had been initially developed that made use of the biotin-avidin system, followed by the tyramide non-linear amplification to raise the signal from the low concentrated analytes (i.e.: serum IgEs). With this protocol the biotin was coupled to a monoclonal anti-human IgE produced in mouse. This particular antibody was chosen because of its extremely high specificity for human IgE antibodies and its little or absent affinity for other antibodies occurring in serum when compared to the polyclonal previously used in the microarray test (Table 4.1). The choice of biotinylating this anti-human IgE was based on the very high affinity occurring between the biotin and the streptavidin conjugated to the HRP enzyme. Not only is the affinity between biotin and streptavidin much stronger than the affinity between an antigen and its specific antibody, but

since a molecule of biotin can bind up to four molecules of streptavidin, a first step of amplification is provided (Guesdon et al., 1979). However every biotinylation procedure resulted in a different average number of biotin molecules per antibody molecule. This accounted for a lack of reproducibility in the system and to a loss in clinical sensitivity when the biotin incorporation was low, notwithstanding the amplification provided by the tyramide.

Coated protein	Polyclonal anti-IgE (KPL) – O.D.	Monoclonal anti-IgE (Allergopharma) – O.D.
IgE myeloma (Calbiochem)	> 3.0	> 3.0
IgE myeloma (Allergopharma)	> 3.0	> 3.0
IgG (gammaglobulin)	**0.579**	0.108
IgG1	**0.508**	0.102
IgG2	0.114	0.100
IgG3	**0.510**	0.102
IgG4	**0.377**	0.103
IgA1	**0.191**	0.107

Table 4.1. Specificities of polyclonal and monoclonal anti-human IgEs to other classes of antibodies (Allergopharma – personal communication).
A comparison between the polyclonal and monoclonal anti-human IgE antibodies shows that the polyclonal cross-reacts with IgG1, IgG3, IgG4 and IgA1 antibodies, whereas the monoclonal is very specific only for human IgE. The background optical density (O.D.) is between 0.100 and 0.120. Therefore O.D. values < 0.120 are considered negative.

An alternative strategy, in order to keep the Allergopharma monoclonal antibody in the detection protocol, was to change the processing steps successive to the incubation with the secondary antibody and to replace the biotin-avidin system. A tertiary antibody anti-mouse IgG produced in goat and conjugated with HRP was thus introduced. This allowed to circumvent the problem of the variable biotinylation procedure and at the same time to maintain the specificity of

the monoclonal secondary antibody and the amplification provided by the tyramide (for which the presence of the HRP enzyme is essential). In addition this protocol modification provided a further increment in the sensitivity of the system, as it is shown in the results (section 3.4).

In the final array, containing triplicates of the 27 allergens and positive and negative controls, an anti-human IgE dilution curve was incorporated and it was used as a signal control for the IgE. The anti-IgE dilution curve was designed to contain a mixture of two different monoclonals, recognizing different epitopes on the IgE molecules. This strategy was intended to maximize the capture of human IgE from myeloma by the spotted capture antibodies. The blocking solution was spiked with a high concentration of human IgE from myeloma so that a control signal could be generated in every slide and allergen-specific IgEs were free to react with the immobilized allergens.

The calibration of the immunoassay, as described in Materials and Methods (section 6.6), notwithstanding having been achieved in an indirect way, has allowed me to carry on the clinical trial. A more direct method to correlate the microarray signal to a given IgE concentration in serum is currently under evaluation in the lab.

The greatest challenge to optimisation of IgE assays is given by the complex nature of allergen extracts. Decades have been spent in the attempt to identify the so-called major allergens, i.e. the antigens to which a majority of allergic individuals produce IgE antibodies (Matsson et al.,

1997). Once identified, the next step has been to clone the gene, sequence it and then produce recombinant allergens in unlimited quantities. Although progress has been made, it will take years before the principal allergens of even the most important allergen groups are identified and recombinant allergens are generated. The importance of recombinant allergens is the possibility to have continuous and reproducible quantities of allergenic proteins in purified form for use in *in vitro* assays or for immunotherapy. One example of the potential relevance of recombinant allergens in IgE antibody assays involves the major allergen from natural rubber latex Hev b 5 (Slater et al., 1996; Bernstein et al., 2003). This acidic protein is labile and potentially lost when natural rubber latex extracts are insolubilized as allergosorbents. Purified recombinant Hev b 5 has been used to enrich natural latex extracts resulting in detection of higher levels of IgE antibody and, more importantly, increased diagnostic sensitivity of an IgE assay for latex-specific IgE by 10% with no loss in diagnostic specificity. It could be that other IgE immunoassays can be engineered to achieve higher sensitivity, relative to skin testing, by means of supplementation of low-level but important allergens with recombinant sources.

Despite the advantages that in this and other situations may be offered by employing recombinant allergens, for this project the use of crude extracts, rather than purified or recombinant allergenic molecules, has been chosen because in this way the epitopes recognized by the IgE are more representative of the complex allergen epitopes *in vivo*. When

purified allergens were spotted on the same slide in parallel with their source of origin, i.e. the whole extracts, the signal from the latter was always lower (see Results, Figure 3.10). This is due to the presence of allergenic and non allergenic components in the extracts and the latter ultimately have the effect of "diluting" the allergenic ones. However by printing only the major allergens (either purified or in recombinant form) the risk is to miss out the reactivity of a number of patients who raise IgE antibodies to minor allergens or to molecules whose allergenic power is still unknown and that, for this reason, are not available in a purified or recombinant form on the market (Figure 4.2).

Finally, recombinant allergenic molecules produced in bacteria often lack the carbohydrate moieties that are occurring in the natural molecule as a result of post-translational modifications and that in some allergens are an essential part of the epitopes recognized by IgE antibodies. This is particularly true for allergens sourced from plants (especially fruits and vegetables) and for moulds. In these situations the use of natural allergens should be preferred over the use of the recombinant ones expressed in prokaryotic organisms (Foetisch et al., 2003).

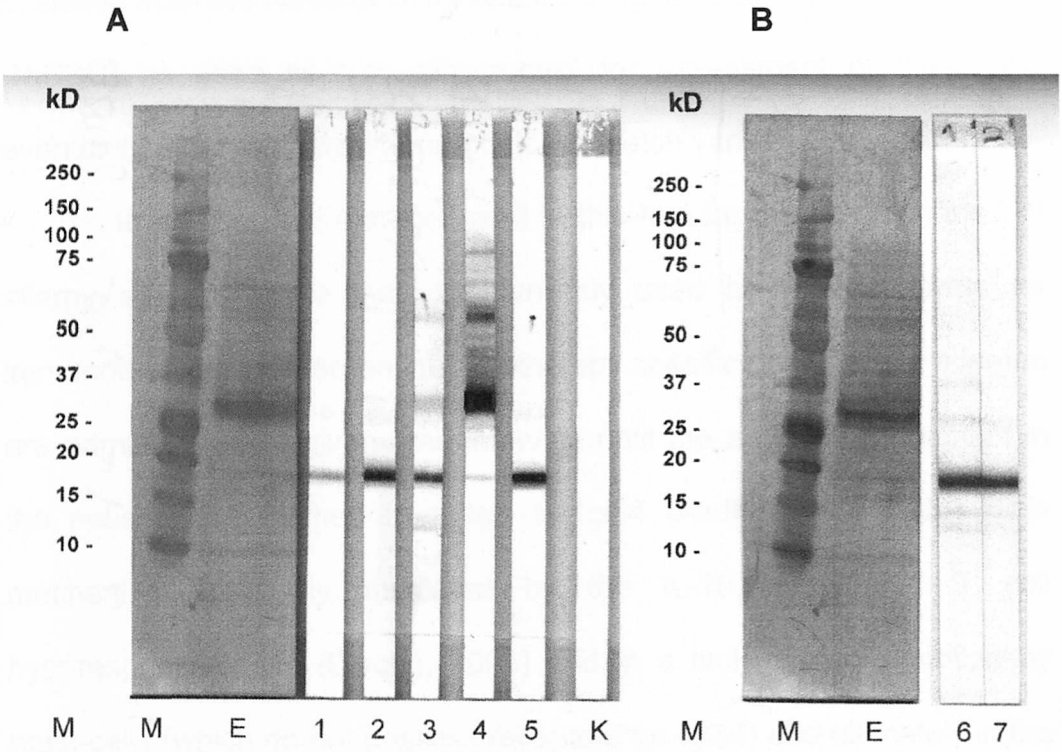


Figure 4.2. Serum reactivity to different allergenic molecules of apple extract (personal communication – Allergopharma).
A: Western blot performed to assess the reactivity to apple extract of 5 serum samples collected from patients allergic to apple. Serum samples in Lanes 1, 2, 3 and 5 show reactivity mostly or exclusively in correspondence of a 18 kDa band, corresponding to the major apple allergen, Mal d 1. Serum sample in Lane 4 shows very little IgE reactivity to Mal d 1 and much more reactivity to other components of the extract. **B:** Monoclonal antibodies 2B2 (Lane 6) and 2C9 (Lane 7) against the major apple allergen Mal d 1 are used as positive controls. M: Bio-Rad Dual Colour marker. E: Apple extract. K: Negative control.

A drawback that can occur in the use of raw extracts rather than purified allergens is the risk of higher batch-to-batch variability, as it happens in the skin prick test. However in the present study well-characterized allergen extracts have been used and the problem of inter-batches variability has been experienced only for two extracts: Dog epithelium and Latex. This clearly indicates that even the use of raw material can bring to a reproducible immunoassay. For extracts such as Dog epithelium and Latex it is recommended that the reference material

available from the National Institute for Biological Standards and Controls (NIBSC) be used as a quality control for assessment of these and eventually other extracts where a batch-to-batch variation may occur.

In addition the extracts used in this test for the serodiagnosis of allergy are the same that are currently used by Allergopharma for immunotherapy. In allergen immunotherapy specific doses of the allergen are administered at given time intervals until the antibody production in the patients is switched from IgE to IgG4 (Akdis et al., 1998). This mechanism, probably mediated by the IL-10, results in T cell hyporesponsiveness (Larché, 2005) and in a lack of degranulation by mast-cells (which do not possess receptors for IgG4) and ultimately in the improvement of the clinical condition of the patient. By using the same extract for the immunotherapy and the serodiagnosis could provide more accurate results without the bias introduced if allergen extracts produced by different manufacturers and undergone to different quality controls were employed. The peptide immunotherapy, recently suggested as a valid alternative to the use of whole allergen extracts, can be applied only in those cases when molecules capable to sensitize and elicit allergic responses are known (like Fel d 1 for Cat epithelium, Phospholipase A2 for Honeybee venom, and so on). At present the use of the extracts, especially for allergens for which allergenic molecules have not been identified yet, is the only immunotherapeutic possibility and the use of the same extract for both diagnosis and therapy will likely allow a more

precise monitoring in the progressing of the immunotherapy on improvement of the clinical symptoms.

The aim of this work has been the application of the allergen microarray as a novel and powerful tool to screen the antibody reactivity against the whole range of allergenic molecules (the allergome) capable to elicit an immune response in sensitized or atopic people. Such a tool has the potentiality to be employed for both research and diagnostic purposes.

The diagnostic applications are quite obvious: the screening for a large number of allergen specificities is a long and expensive process; therefore a typical request for allergy testing includes a total serum IgE and a limited number (4 or 5) of allergen-specific IgE determinations based on specific clinical symptoms and/or to particular seasons of the year (especially for pollens). However by using this strategy important and/or clinically relevant allergen specificities can be missed (Sinclair et al., 2004). The allergome, contained on the surface of a chemically-activated microscope slide, allows the clinician to investigate a large number of potential allergens in a short time and employing a minute sample. Such a test will prove particularly useful in those situations when a type I hypersensitivity is suspected but no obvious allergen suggests itself, and in patients in whom the clinician wants to demonstrate that an allergic disease is not the cause of the clinical symptoms. In addition this test can become the most powerful *in vitro* device to date to be coupled to

the medical examination and clinical history to precisely locate and characterize the allergen specificities to which a given patient is sensitized within short time and without the need of trained personnel (necessary for the skin prick test) to carry it out. The prospect for this test is its applicability outside the practitioner clinics and its setting up in a simple nursery or in a Pharmacy where the patient just gives a few drops of blood (ideally via a simple skin prick) and a nurse or a personnel with a minimal training can carry out an automated test and hand out the results within a couple of hours. Despite these additional features, this test retains the potentiality to be configured also for big hospital laboratories (like all the currently used *in vitro* tests) showing thus the possibility of wide-scale applications for the allergome microarray.

The applications of the allergome microarray in research are attractive as well: a system like this, with the advantages of the miniaturization, can accommodate on the restricted area of a glass slide thousands of allergenic spots. This can be potentially useful to screen against allergenic sources yet unknown (e.g. molecules in genetically modified foods) or to detect patterns of allergen reactivities that, to date, are not possible because of the limitations in cost, time and personnel offered by the current systems. Like for the well established pattern of cross reactivity between the major birch pollen allergen, Bet v 1, and other very similar allergenic molecules present in the pollens of the order of *Fagales* (Aln g 1 in Alder, Cor a 1 in Hazel, Car b 1 in Hornbeam) as well as in some plant-derived foods (Dau c 1 in Carrot, Mal d 1 in Apple,

Api g 1 in Celery) which share very similar sequence in conserved pathogenesis-related proteins (Ebner et al., 1995; Hirschwehr et al., 1992; Hoffmann-Sommergruber et al., 1999a; Hoffmann-Sommergruber et al., 1999b; Breiteneder et al., 1995; Neudecker et al., 2001; Vanek-Krebitz et al., 1995), it could be that other similar (and maybe wider) patterns of reactivity can be discovered. All this information will ultimately allow a better targeting immunotherapy and/or pharmacological treatment. If this pathway will be thoroughly investigated, it may be that, due to extensive cross-reactivities among certain allergens it will not be necessary, for the future, to test for an enormous number of allergens. It is rather likely that several specific epitopes for each allergen can additionally be included to allow for a more refined diagnostic analysis. In addition this wide-scale analysis for serum reactivity against so many allergens in one single test has the potentiality to discern unique patterns of reactivity in individual patients. This will allow to target in a very precise manner the immunotherapy for each individual patient.

The data generated by comparing the performance of the microarray immunoassay with the reference ELISA RV 5 and the specifications provided by the NCCLS for the IgE immunoassays demonstrate the feasibility of using a micro-array immunoassay as the basis of a test for the determination of allergen-specific IgE in human serum.

The serum volume requirements are the main advantage of the allergome microarray immunoassay: for other tests, 50 μL to 100 μL of serum are requested for each test, i.e. for each allergen specificity. The microarray-based immunoassay requires only 100 μL of serum per assay, where an assay can include up to 300 allergen specificities.

The performance of the allergen microarray in terms of analytical sensitivity and analytical specificity was better than in the ELISA reference, allowing thus a better detection, even of very low IgE concentrations (below the detection limit of 0.35 IU/mL normally accepted by most IgE immunoassay) and in a more specific manner, without problems of cross-reactivity with other antibodies.

The NCCLS guidelines for IgE immunoassays (Matsson et al., 1997) recommend that the intra assay precision never exceeds 10% of coefficient of variation, regardless the reactivity of the sample analysed. The inter assay precision must stay within 15% of coefficient of variation for samples of high and medium reactivity, and within the 20% of coefficient of variation for samples of low reactivity (i.e. closest to the lowest calibrator). The results obtained in the allergome microarray show thus values well within the specifications for these two parameters.

The stability of the array, for at least 90 days after being produced, is another major advancement, given the problems normally occurring in keeping proteins in stable conditions.

The clinical parameters showed an excellent performance as well. The slightly lower median value of clinical specificity (77%) compared to one

for the clinical sensitivity (97%) is likely to be ascribed to the fact that for some allergens the microarray reads higher than the reference ELISA, bringing thus to a bias in the values of this parameter.

Other microarray-based assays for the determination of allergen-specific IgEs have been reported in the literature (see Introduction, section 2.4). However all these tests have some not trivial drawbacks like poor analytical (Hiller et al., 2002; Kim et al., 2002) or clinical performance (Wiltshire et al., 2000). The assay described in the present study can be configured to contain all known allergens and combines the advantages of high sensitivity and a time-to-result of less than 4 hours. The performance of the allergen microarray here described has been demonstrated to be equal or superior to that of the reference system in terms of analytical and clinical parameters.

In conclusion this microarray-based allergen immunoassay, used in combination with signal amplification procedures, has the capability to circumvent most of the current limitations affecting allergy diagnosis (number of analytes determined in a single assay, time-to-result and cost) by allowing the simultaneous and multiparametric analysis of serum reactivity against a large repertoire of allergens. In addition, the possibility of detecting low concentrations of allergen-specific IgE allows the monitoring of earlier stages of allergic disease as well as its development and management with therapeutic treatment.

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