REVIEW

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Considerations on the application of microarray analysis in rhinology*

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SUMMARY

Molecular biological tools are finding their way into modern rhinological research. This overview aims to discuss the merits and pitfalls of micro-array analysis as one of these molecular tools. The outcome of a micro-array experiment will describe which genes are active in a given cell type or tissue, allowing us to investigate healthy and diseased conditions on a large scale and in extreme detail. Although this will deepen our understanding of our disease of interest, we should not expect that micro-array analysis will resolve all our questions.

In this overview we have indicated points that we feel are critical for any application of micro-array analysis in modern rhinological research. Choices on experimental design and patient inclusion will influence the outcome data of the experiment and the extend of the conclusions that can be drawn from these data. A second important issue is the size of the data set, which can comprise of hundreds of different genes, making it difficult to come to grips with the affected processes in a disease. New visualization and analysis tools for micro-array data are discussed in this overview that deal with these complex issues.

Key words: microarray, network analysis, gene ontology, principal component, rhinology

INTRODUCTION

Molecular biology has opened many different new avenues for rhinological research. Among the molecular biological techniques, microarray analysis with its promise of a complete and detailed overview of all genes involved in a particular disease is finding more and more applications. Are these promises realistic and will such a complete overview of the genetic changes in a disease help us to understand the pathogenesis or pathophysiology of the disease? In this review we will discuss the position of microarray analysis in modern rhinology, identify its strengths and weaknesses, and hope to provide a framework for future research in rhinology using microarray technology.

HOW TO STUDY NASAL PATHOLOGY?

If we want to understand a particular disease we first need to understand what defines the normal situation and at what level the normal situation may change into a diseased state. In every cell, with the exception of the red blood cell, the normal function of this cell is directly dictated by the nucleus. Even though in every cell the complete DNA genome is present, only a selection of the genes within the genome are active. Some of these expressed genes could be considered to be constitutively active - the genes that define the cell type or that are necessary for its survival – whereas other genes are active under specific conditions – for instance the genes that respond to hormones or other extracellular signals. This process is well regulated under normal conditions. Tight control defines what genes are transcribed into messenger RNA (mRNA), which of the mRNAs are translated into protein and to what level, and subsequently, even the functional activity of the proteins is under control by specific modifications of the translated proteins. The collection of DNA genome that is actively transcribed into messenger RNA is also referred to as the transcriptosome, while the collection of proteins that are encoded by these messenger RNAs is referred to as the proteosome. At each of these levels something might go wrong leading to a change from the normal situation into a diseased state. In Down's syndrome an extra copy of chromosome 21 disturbs the normal balance of the genes, in cystic fibrosis a mutation in the gene encoding a chloride channel leads to airway dysfunction, and in Creutzfeldt-Jacob's disease incorrect folding of a protein leads to severe brain abnormalities.

Three different molecular biological approaches that each target a different level of regulation can be used to study complex diseases: genomics, transcriptomics, and proteomics. In genomics the search for mutations in the genes specific for the disease are the main target, In transcriptomics differences in

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the mRNA levels between the normal and diseased state are investigated, whereas in proteomics the proteins themselves are the prime target of investigation (Figure 1). For any disease, the study into the molecular mechanism that underlies the disease is greatly facilitated if a fault in a single gene would be responsible for the pathogenesis. This is very unlikely to be the case in allergy, chronic rhinosinusitis, or nasal polyposis to highlight just three of the most common nasal pathologies. Furthermore, we cannot even identify a single cell type that can be held responsible for the disease. Whatever approach is chosen to study these diseases, it should always take into consideration that multiple cell types and complex interaction between these cells can or will be involved in the pathogenesis.

Although this review will focus on transcriptomics and its most common research tool the microarray, I will briefly discuss the other two approaches. From a theoretical point of view proteomics would be best [1]. Whatever the cause of a disease, nearly all cellular functions are mediated through proteins. However, currently the bottleneck in the application of proteomics is its automation and its inability to deal with large samples sizes. New developments in mass spectrometry and its combination with 2D-gel electrophoresis, solid-phase purification, or affinity protein precipitations are a clear way forward in this field [2]. Genomics and transcriptomics have the advantage that their automations are well established. Large numbers of patient samples can be investigated at the global level. In genomics the main targets are the individual mutations of the genes with the aim of linking specific single nucleotide polymorphisms (SNPs) of genes to a particular disease [3,4]. When such a disease-specific SNP is discovered it can be easily used in the diagnosis or the prediction of a disease. However this only seems to happen when a mutation in a single gene is responsible for the disease. Most often the outcome is that a given SNP found more frequently in sufferers of a given disease than in the normal population, but not that the SNP is specific for this disease. In more complex diseases where more genes interact, multiple SNPs would need to be considered simultaneously and this often runs into the practical problem of sample size: a very large number of patients is required to study SNPs in different genes. This is where transcriptomics has its biggest advantage. If we assume a diseased-state in a cell, then transcriptomics will give a blueprint of this state independent of how this state has originated. So effects of mutations in multiple genes in one individual, or mutations in other genes in different individuals, can still yield the same diseased-state in a given cell. However we must realize that differences in the expression pattern of the genes in a disease can be the consequence of the diseased-state and that it can be hard to find the gene responsible for the disease. Moreover, in the worst case scenario the diseased-state is not reflected in the expression level of the genes at all. Although this does not seem likely, when it would happen, then transcriptomics would not solve the problem.

SELECTION OF THE STARTING MATERIAL

One obvious point that needs to be made from the start is that in the use of transcriptomics, as with any other method, the selection criteria of the patients under investigation are very important. When different diseases would become mixed, the outcome of any analysis method would not be valid. In rhinology this would be especially true for chronic rhinosinusitis and/or nasal polyposis [5]. The clinical criteria for these diseases might not have sufficient discriminatory power to determine whether we are dealing with one single disease or a series of different diseases with identical clinical presentation. Interestingly, as we will discuss in a later section, some analysis methods of microarray data might even be able to deal with this situation and could reveal whether we are dealing with a single disease or a collection of diseases.

As discussed above, micro-array analysis is well suited to describe the diseased state in a cell, independent of the origin of the diseased state. Depending on the choice of the platform used for microarray analysis, up to 55,000 individual probe sets are more then sufficient to cover most, if not all, of the transcriptome. However in tissue this becomes more complicated. When diseased tissue is compared to healthy tissue or to tissue after treatment, the outcome of the microarray will not only depend on disease-specific differences at the gene level, but also on differences in the cellular make-up of the tissue. Under inflammatory conditions (Figure 2B) more, or different, cells will be present in the tissue than compared to control conditions (Figure 2A). When the total messenger RNA is isolated from these different tissues, the microarray data will also reflect these trivial differences. Still such an approach has its merits as it can give us more insight in the overall picture of a disease [6,7]. Moreover, this whole tissue approach could be combined with an estimation of the relative contribution of cell types using (immuno)histochemistry [8], although the general applicability still needs to be demonstrated

At the other side of the spectrum would be to investigate a single cell type associated with the disease and compare the expression profiles under normal and diseased conditions [9-11]. Now the data would reflect the disease-state for the chosen cell type, but would not give the entire picture of the disease. Still this approach would be best when a fair guess could be made of the cell type most likely to play a role in the pathogenesis in the disease. In allergy these could be the epithelium, dendritic cell, or T lymphocyte at the regulatory or initiation phase, while basophils, mast cells, or eosinophils would be the likely candidates at the effector phase. In a recent review from our group a strong case is made to study epithelial cells and (myo)fibroblasts in the case of chronic rhinosinusitis or nasal polyposis [12]. For some form of cancers, like carcinomas, the choice seems self-evident [13,14,15]. When the focus is on a single cell type we need to consider whether the isolation procedure for this cell type may affect the expression profile. Many isola-

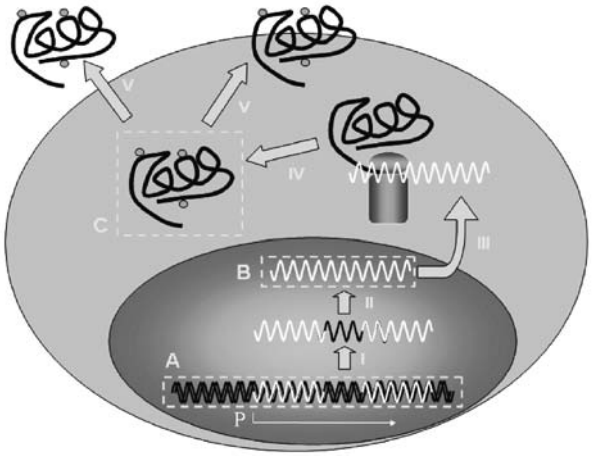

Figure 1. Graphic representation of the three levels of analysis of cellular functions in the nucleus (dark green) or the cytoplasm (light green). Genomics focuses on the changes in the DNA (box A) that affect the messenger RNA level of a gene (box B) or the function of a protein (box C). Transcription (thin hooked arrow) from the promoter region (P) in the DNA of a gene leads in two steps (arrow I and II) to the mature messenger RNA. Messenger RNA levels (box B) of genes that are expressed in different (diseased) conditions are studied with transcriptomics. In the cytoplasm (arrow III) the messenger RNA is translated into a protein on ribosomes and this protein can be further modified by the addition of, for examples, phosphate or sugar groups (orange circles) to form the mature protein (box C). Translocation of the protein (arrow V) to the site of action can be used in proteomics for the analysis of, for instance, membrane fractions of cells or the level of secreted proteins.

tion procedures use an enzymatic approach to disturb the cell-cell contacts within a tissue and this procedure alone may already affect the expression pattern within a cell (unpublished observations). This effect will be less when cells are subsequently cultured *in vitro*, as most of these induced changes are only of a transient nature. However it would be important to validate the outcome of such *in vitro* experiments as the outcome might not correctly reflect the *in vivo* situation.

An interesting development in this respect is the laser dissection microscope [14,15]. This would allow us the isolate specific sections from a tissue biopsy without introducing any isolation artefact. However this does come at a price. Firstly, the overall quality of the messenger RNA isolated is relatively poor, so the difference in microarray dataset may reflect differences in degradation rather than differences in the expression levels of the genes. Secondly, it may still be difficult to obtain a 100% pure population (see Figure 2C where low numbers of eosinophils are still present within the epithelial layer). However, these drawbacks may well be solved as this field further develops.

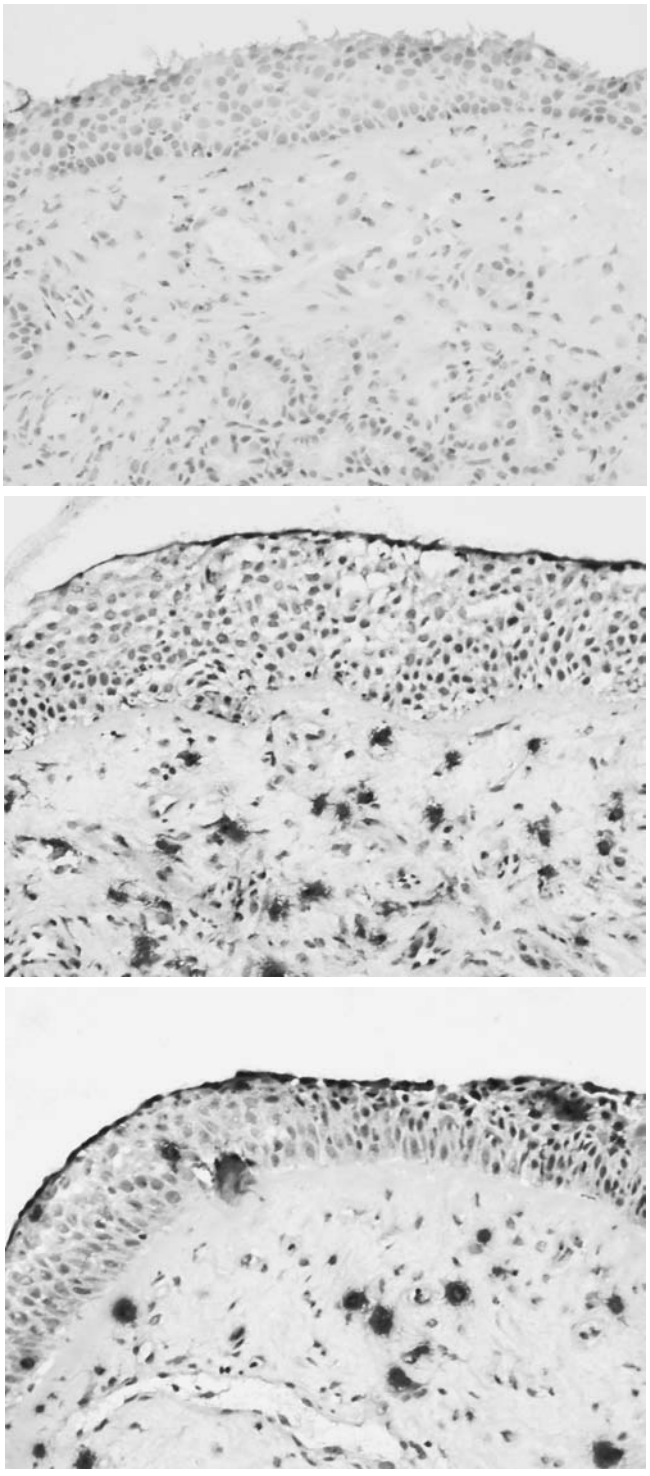

Figure 2. Differences in cellular make-up (eosinophilia) between (A) healthy individuals, (B) patients with allergic rhinitis, or (C) between tissue localizations.

A final choice could be cell lines prepared from the cell type under investigation. However, these cell lines seem far removed from the *in vivo* situation and not always are healthy or diseased cell lines readily available. Two areas seem to be best served by the use of a cell line: mechanistical studies *per se* and the identification of additional components in a disease

mechanism [16]. When a particular disease mechanism has been identified and when this mechanism is present in the cell line then the ease of use and the absence of any intra-individual variation will not only allow to study the mechanism itself, but also to identify genes that change only a little in their expression level.

EXPERIMENTAL SETUP

Several different platforms exist to acquire gene expression profiles and the choice is partly arbitrarily. The first choice is to obtain a more or less complete overview of all active genes or to focus on a sub-class of genes. This choice is highly dependent on the research question. If the question is whether a certain mechanism is involved in a disease or whether a certain mechanism is affected by treatment then a specialized array is clearly cheaper and more convenient. Custom arrays are available for a variety of species (human, mouse, yeast) and topics (metabolism, inflammation, remodelling, immune responses). Such a specialized array can also be custom made and could be used to selectively screen genes in a bigger patient population for which the relevance has first been established in a full genome microarray experiment. Practical examples are mostly found in cancer research where gene profiles have been developed for certain cancers that are linked to subclass identification or prognosis [17,18]. The second choice is to use a single label or dual label approach. In the single label approach RNA from a single condition is applied to the microarray chip, whereas in the dual-label approach the RNAs from two different conditions are mixed and applied to the same microarray chip simultaneously. As both sets of RNA carry distinct colours, the ratio of individual RNAs can be directly compared. In relative simple designs of paired experiments such a dual label approach is convenient, albeit that each comparison is often done twice (with reversed colours) to compensate for potential labelling differences. Three or four way comparisons are more difficult in this approach and in these cases the single label approach is preferred. An example of such a multiple comparison approach is from our work on nasal polyposis (Figure 3) where the aim is to identify differences and similarities between the epithelia in different diseases and between different nasal locations simultaneously [12].

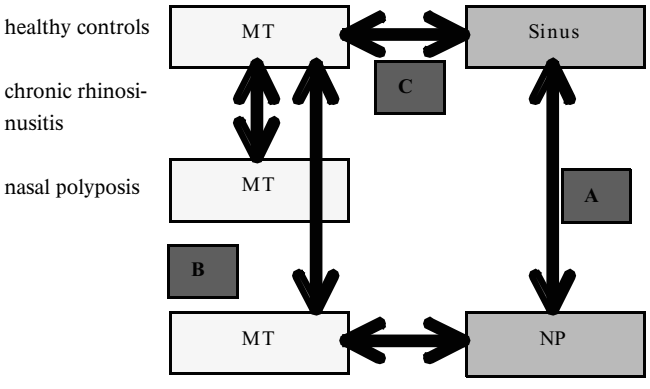

Figure 3. Multiple comparison design to investigate epithelial expression pattern in middle turbinate (MT), ethmoid sinus (Sinus) or nasal polyps (NP) in healthy controls, chronic rhinosinusitis, or nasal polyposis patients. Questions that can be answered with this design are: (A) does diseased polyp epithelium differ from healthy controls, (B) do the epithelia of middle turbinates differ between the different conditions, and (C) is polyposis a local disease or are the middle turbinates also affected. Reproduced with permission [12].

ANALYZING AND INTERPRETING MICROARRAY DATA

The next phase in the microarray experiment is the hybridization of the RNA under investigation to the gene chip and the readout of the initial raw data. Several internal controls and normalization procedures can be used to obtain a reliable expression profile from the raw data, but these technical considerations will not be part of this review. After this analysis a long list of genes whose expression is different between two conditions is obtained, with for each gene the size of the difference (fold change) and their significance level (p-value). This list of genes can be long, varying between a few hundred to a few thousand. An important challenge will be to further develop analysis and visualization tools to help us understand how these genes contribute to the disease.

Usually, such a gene list is made more manageable by using some arbitrary fold change as a cut-off. There is no scientific reason to look at genes that change by more than two-fold only or to think that genes that change the most are more important than genes that only change a little. Such a restriction will severely limit the usefulness of microarray analysis. A step forward is the introduction of the gene ontology concept [19]. In this approach genes are clustered in functional groups. Such groups can be related to “metabolism”, “signal transduction”, “response to stress”, or “apoptosis” to name but a few. A statistical approach will determine whether the genes from a given ontology group are represented more frequently in the data set of the microarray experiment than can be expected on the basis of chance. A clear advantage of this approach is that a context is provided that will help to define processes affected by the disease.

Although gene ontology may help to us to understand a disease better, it is not well suited to identify targets for treatment in a particular disease. For this purpose we would need to know more about the functional interactions between the genes and identify possible key regulators among those genes. The tool best suited for this purpose is network analysis [20]. In network analysis, *a priori* knowledge about the genes is used. Using available literature known regulatory or physical interactions are plotted between the genes from the microarray dataset. In the regulatory network these depict when a gene has a reported action on the transcription of another gene. Important to realize

is that this interaction does not need to be direct and that this interaction may have been described for a particular cell type other than the one under investigation. Similarly, the interaction network only focuses on the physical interaction of the proteins related to the genes in the dataset and this interaction may not affect the function of either protein. Both approaches are able to help to identify potential new target or establish expected targets for medication. The regulatory network analysis of the effect of house dust mite extract on primary nasal epithelia helped us to define a core network (Figure 4) that seemed to be under the transcriptional control of family members of Nuclear Factor kappa B (NFKB1 and NFKB2) and Activator Protein 1 (JUN and ATF3) [21]. However, network analysis of the genes from a microarray dataset can only find interactions that have been described previously, and is not suited to identify new players or new interaction pathways.

To find new partners in a disease process one can assume that genes that are regulated in a similar fashion are involved in the same or a related process. So comparing the behaviour of genes for which the relevance to disease process has been established, to other genes in the microarray dataset will help to identify such new players. However a direct comparison is often difficult as baseline expressions or the absolute effect of the change for genes can be so very different. One way of dealing with this situation is K-mean clustering [20,22]. An example of this application comes again from our work on the effect of house dust mite extract on the primary nasal epithelium from healthy and allergic individuals [23]. In this experiment the expression in both primary epithelia was compared both before and after allergen exposure. The four sets of expression data (Figure 5) are first normalized using Z-scores. In Z-scoring the expression of individual genes under the 4 conditions are expressed as a unit of the overall standard deviation from a normalized expression mean of zero. The next step is to group these normalized expression patterns. In K-mean clustering this is done unsupervised and does not use any *a priori* knowledge. The only input of the researcher is number of clusters the research wants to use. There are no strict rules regarding the number of clusters and this is often determined interactively. Using this approach we were able to determine that most of the genes (clusters 11 and 12 in Figure 5) show a similar expression pattern with the expression level of the genes in the healthy population going up from a low baseline expression, while the expression in the allergic population is already high at baseline and stays high after house dust mite exposure. Interestingly some of the genes in these clusters have a clear documented relationship with allergy (e.g. PLAU, PLAUR), for others it could explain the relationship between viral infections and allergy (eg TLR3, TICAM1), while for others (BMP2, DUSP1) the relationship is (still?) unknown [24-27]. The clustering procedures can also be used in combination with network analysis [15,28-30], where the colour scheme of the clusters can be used in the network (Figure 4).

The analysis and visualization methods described above focuses on individual genes, processes, or regulatory mechanisms. Principal Component Analysis (PCA) is radically different in this respect as it will describe overall features of the dataset. The technical description of PCA is a mean to reduce the dimensionality of the microarray data set. Figure 6A shows this in simplified form. When a microarray chip would only have two genes than all the microarray chips of an experiment can be mapped in two dimensional space; the X-axis would show the expression of gene A, the Y-axis the expression of gene B. In the example of Figure 6A it is clear that all the data points microarray chips are mostly positioned on one line. Nearly all the variation in the data can be described along a new X-axis (dashed) that follows the data points and a minute fraction of the variation can be seen perpendicular to this axis (the points deviate a little bit from this line). So, in practical terms can the data set also be depicted in a linear fashion resulting in a reduction of the dimensionality from two to one. Please note that we do not know what this new axis represents. In the original two-dimensional representation each of the axes is a measure of the expression of a gene, but this is not the case for the new axis. In a real microarray experiment, the dimensionality of the original 45,000 genes is far bigger than in this example and are more "new" axes necessary to describe the data set. Still practise shows that 3-4 dimensions describe 80 to 90 % of the variation in this original data set.

Interestingly, the data points on the new axis can often be separated on the basis of an easily identifiable feature, like treatment effect or disease state. An example can be seen in Figure 6B, again from our work on the effect of house dust mite extract on the primary nasal epithelium from healthy and allergic individuals [23]. Here it is evident that PC1 (X-axis), the principal component that describes the biggest part (57%) of the variation in the experiment, turns out to be the difference between the healthy (on the right hand side) and the allergic individuals (on the left hand side). Moreover the PC2 (Y-axis) turns out to be the variation (12%) in the data of the healthy individuals, a variation that is much smaller in the allergic individuals. Such an analysis method would also identify subgroups. When we would not have known whether we were screening allergic or healthy individuals the clustering of the data points in PCA would have suggested that such a bipartition could exist. In our experiment this would be trivial, but in other experiments this could identify different types of tumours, different treatment responses, or hint at other not expected subdivisions.

THE WAY FORWARD FOR EXPRESSION PROFILING IN RHINOLOGY

In this review we have described different aspects of the application of micro-array technology in rhinology; the importance of a good clinical characterization of patients and the various system biological tools available for the analysis of complex data sets.

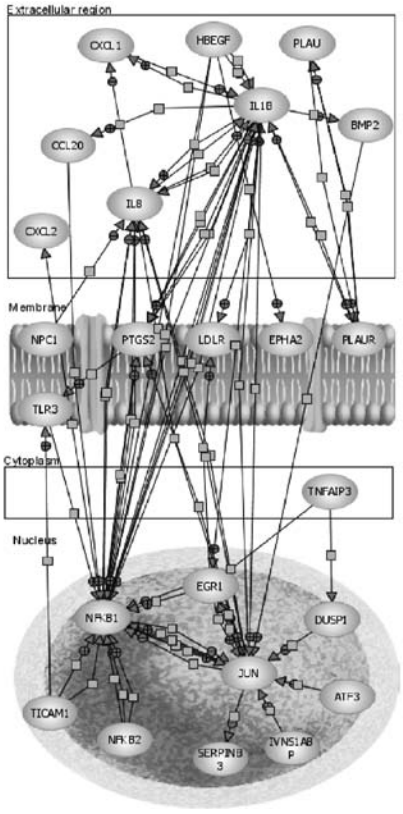

Figure 4. Overview of the regulatory network that describes the effect of house dust mite on primary nasal epithelium and a bronchial epithelial cell line [21]. Reproduced with permission

Although micro-array analysis should not be seen as the holy grail of research that will answer all unanswered questions in rhinology, it can be a valuable tool to gain some insight into the pathophysiological mechanisms that underlie (non)-allergic rhinitis, chronic rhinosinusitis, or nasal polyposis. This optimism stems from our observations in allergic rhinitis where the analysis of the interaction of house dust mite allergen with nasal epithelial cell has yielded important new data with emerging new concepts. When we would have the data for other aeroallergens we might be able to shed some light on the question why a given individual has become allergic to one particular allergen and not to another, or why some individuals will only develop allergic rhinitis while others progress towards asthma. Currently we only have data in adult individuals with established allergic rhinitis and no data in young children that may develop allergic rhinitis later in life. A better understanding of this critical period in the start of the "atopic march" may not only help us to stop this "atopic march", but may even help to prevent the first manifestations of allergic disease in young children. In allergic rhinitis we are still far removed from this ultimate goal, but as we have indicated before the principal component analysis tool may already help us to get a reliable diagnosis of allergic rhinitis in these young children [23]. Alternatively, our analysis method could also be applied to non-allergic rhinitis or mixed forms of rhinitis, for which we do not yet have a clear understanding of the pathophysiology.

In chronic rhinosinusitis and nasal polyposis micro-array analysis may help us identify potential subgroups of patients. In particular it should prove useful to study the role of comorbidities in the pathogenesis of these diseases or shed some light on why some individuals respond to treatment while other do not. Especially in these diseases we need to consider the heterogeneous groups of patients and the complex nature of the biological samples used for micro-array analysis very carefully, as this may block the development of clear new concepts from such

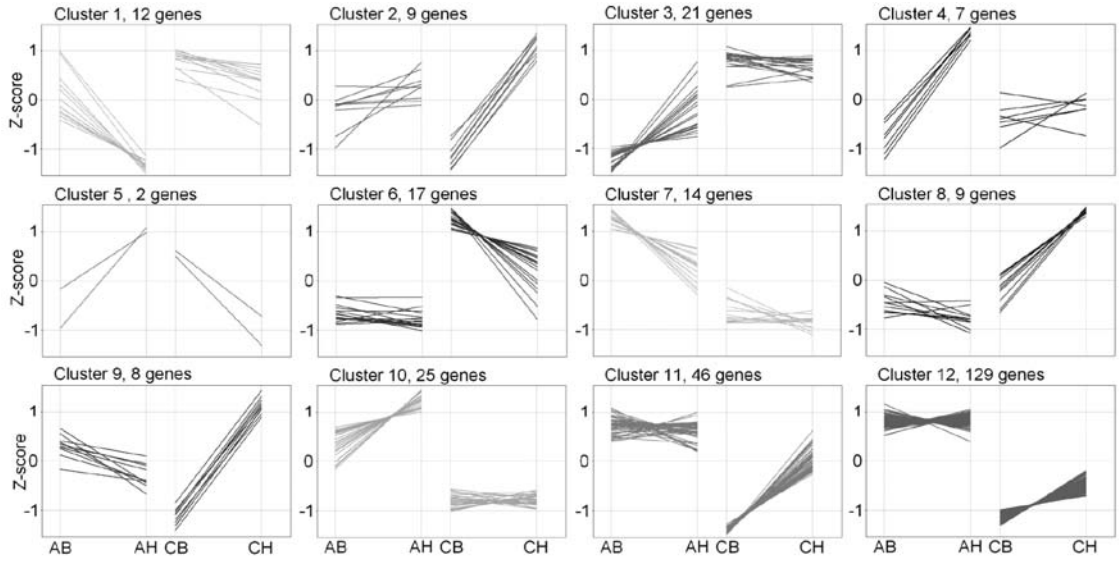

Figure 5. K-means clustering of differentially expressed primary nasal epithelial genes. AB = allergic at baseline, AH = allergic after HDM exposure, CB = healthy control at baseline, CH = healthy control after HDM exposure. Reproduced with permission [23].

Figure 6A. Scatter plot representing 8 individual microarray chips plotted as the expression of the two genes on the chip (solid axes), or plotted on axes (dashed lines) representing the (orthogonal) directions of the largest variance.

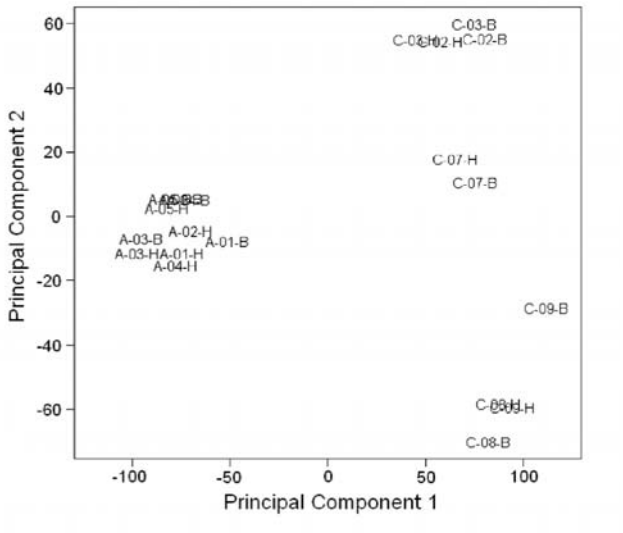

Figure 6B. Scatter plot of 20 arrays from healthy (C-number-H/B) and allergic (A-number-H/B) epithelium showing the contribution to principal component 1 (PC1) and principal component 2 (PC2). Reproduced with permission [23].

experiments. Perhaps more can be gained from a focussed analysis on for the disease potentially relevant cell types. In a recent position paper we have postulated that a potential vicious circle between damaged epithelial and activated (myo)fibroblast in nasal polyposis or chronic rhinosinusitis could underlie the pathophysiological mechanisms in these diseases [12]. This vicious cycle, which is also thought be involved in asthma remodelling, may go some way to explain the increased prevalence of nasal polyposis in asthmatics.

CONCLUDING REMARKS

With proper care microarray experiments are a valuable tool in trying to understand underlying pathophysiological mechanisms, even in more complex diseases. A priori knowledge of the basic mechanisms in complex sino-nasal disease is still required before microaray research can answer the burning rhinologist's question: "Could you please explain this disease to me?"

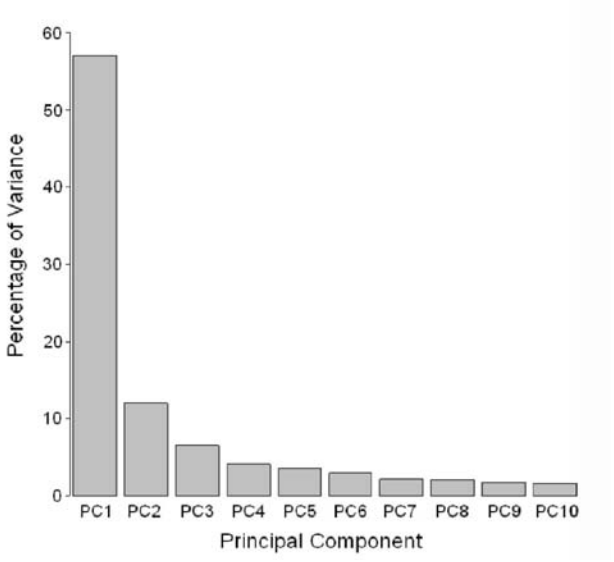

Figure 6C. Relative contribution of the first 10 principal components to the variance in the house dust mite extract data set. Reproduced with permission [23].

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ANNOUNCEMENT