Using ANOVA to Analyse Thalidomide's Molecular Mechanisms in Human PBMC Microarrays

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Abstract— **Thalidomide is an anti-inflammatory and immunomodulatory drug that is the treatment of choice for erythema nodosum leprosum (ENL), an inflammatory cutaneous and systemic complication of multibacillary leprosy. Its use in leprosy and other conditions is controversial due to its teratogenic effects. Fifty years after the development of thalidomide, its action mechanism is still not well understood. In the present study we analyzed the in vitro effect of thalidomide on global gene expression in cultured human Peripheral Blood Mononuclear Cells (PBMC) of normal donors. We normalized the background subtracted spotted cDNA microarray data using a locally weighted linear regression and modeled its residual by a two-step analysis of variance to detect differentially expressed PBMC human genes in response to thalidomide. Using the full set of spotted genes, 13 genes were identified as differentially expressed genes. A reduced set was derived with a direct simple filtering approach and this set resulted in eight differentially expressed genes. Among these 21 genes, 15 are involved in the immune regulation processes.**

I. INTRODUCTION

Thalidomide (N-ftalimil-glutarimida) is the treatment of choice for ENL, or type II reaction, an acute inflammatory state occurring in lepromatous leprosy characterized by systemic symptoms, including fever, painful cutaneous lesions, arthritis, glomerulonephritis, and the presence of circulating immune complexes. In October 1957, the German company Chemie Grünenthal introduced Thalidomide as a "safe" over-the-counter sedative/tranquilizer, subsequently marketed in 46 countries for morning sickness during pregnancy. It was banned from commercial use in 1963, after discovering it exerted teratogenic effects if taken within the 34^{th} and 50^{th} day of pregnancy.

The history of thalidomide was changed by serendipitous discovery by Shenskin in 1965, which reported administering thalidomide to an insomniac patient with erythema nodosum leprosum. The patient symptoms disappeared and the skin lesions healed completely [1]. Several immunologic and potentially anti-inflammatory activities of thalidomide have been described, including the inhibition of the proinflammatory cytokine tumor necrosis factor (TNF), inhibition of the angiogenesis and the co-stimulation of interleukin (IL-2) by T cells. ENL has been associated with increased plasma TNF levels, which was reported to be reduced after treatment with thalidomide. Since it is known that thalidomide acts in different diseases it is uncertain whether the efficacy of thalidomide in ENL is exclusively mediated by the inhibition of TNF [2]. This cytokine is an important mediator in process like apoptosis (programmed cell death), cellular proliferation and differentiation, tumorigenesis and viral replication. Due to its action on TNF, thalidomide is used in several experimental treatments of different pathological conditions characterized by increased plasma levels of this cytokine.

Spotted complementary deoxyribonucleic acid (cDNA) microarray is a technique in which the relative level of expression from thousands of genes can be measured concomitantly and is used to study the relative expression between treatment and control samples. Microarrays experiments are very costly precluding the use of large number of replicates. When the number of replications is small, careful design and analysis are required to find meaningful information and to identify, from the hundreds or thousands spotted genes, those that have differential expression between samples. Microarrays are often used as an screening tool, and further molecular assays for identified differentially expressed genes, such as a semi quantitative and Real Time Polimerase Chain Reaction (RT-PCR), are usually required to confirm obtained results. In microarray experiments several sources of systematic variation are present and the most common bias is the labeling difference between the two fluorescence dyes. This bias can hamper the direct interpretation of the data and averaging over replicates may increase variation and noise since they may have different characteristics in dye bias and ratio distribution [3]. Before selecting genes that are differentially expressed between samples, an important step is to eliminate questionable or low quality measurements and use transformations to adjust the measured intensities for appropriate comparisons [4].

The main focus of this study was to identify candidate human genes responsive to thalidomide exposure that may regulate its immune activity. We explore the in vitro effect of this drug on global gene expression of cultured human Peripheral Blood Mononuclear Cells (PBMC) of heath donors using analysis of variance (ANOVA) following a proposal by Kerr et al [5].

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II. MATERIALS AND METHODS

The human oligoarrays were spotted at the Microarray Core Unit at UNAM, Mexico (`http://microarrays.ifc.unam.mx/principal.html`). Three cDNA samples from cultured Peripheral Blood Mononuclear Cell (PBMC) obtained from three healthy donors were stimulated with thalidomide and its diluent dimethyl sulfoxide (DMSO) forming the treatment group (PBMC+DMSO+THALIDOMIDE). The control group comprised cultured PBMC cells stimulated with DMSO for two hours (PBMC+DMSO). We used a reference design comparing the treatment and control group with a reference group consisted of a pool of cultured PBMC non-exposed cells (PBMC). For each of the two conditions, treatment and control, target cDNA mixed with the reference PBMC was hybridized in three microarray slides containing 9,984 human 50mer oligonucleotides (MWGBiotech Company) spotted in duplicate (total of 19,968 spots). In two arrays the treatment and control samples were labeled using red fluorescent, Cy5, and the non-exposed samples were assigned to the green fluorescent dye, Cy3. These arrays are referred here as direct labeling. To control dye labeling differences a third array used reversed dye labeling.

The acquired image of fluorescence intensity was segmented and background corrected to obtain the expression level Y_{ijkg}(for each array $i \in \{1,...,6\}$; dye $j \in \{1,2\}$ for Cy5 (R) and Cy3 (G) dyes, respectively; conditions $k \in \{0,1,2\}$ for reference, control and treatment; and gene $g \in \{1,...,n\}$). Initial data analysis was carried out to evaluate spot quality. Among the different available methods [6] we used here a simple approach considering the ratio of the background correct signal intensities, obtained as the difference between signal intensities and local background, and its background intensity (signal-to-noise ratio). If the signal-to-noise ratio were less than 2, the corresponding spot received a penalty flag in the corresponding array. About 10 thousand spots in all slides were flagged. Since the experiment is not balanced for the dye factor, we select for further analyses the direct array with less flagged spots and the dye-swap array. A second approach was based on eliminating the genes flagged in more than four spots in the selected slides. The experimental design is presented in table I.

TABLE I
EXPERIMENTAL DESIGN

Array	Cy5	Cy3
1	PBMC+DMSO	PBMC
2	PBMC	PBMC+DMSO
3	PBMC+DMSO+ THALIDOMIDE	PBMC
4	PBMC	PBMC+DMSO+ THALIDOMIDE

MA plots is an exploratory tool useful to identify systematic variations due, specially, to different dye labeling efficiencies and differences in concentration of DNA on each array. The MA-plot reveals whether the data exhibits an intensity-dependent structure, and it is based on plotting $M = log_2\left(\frac{R}{G}\right)$ versus $A = 0.5 \times log_2(R \times G)$. Presence of systematic effects impairs a proper analysis of individual slides and comparison of expression levels between slides.These systematic effectc can be revealed by a nonlinear pattern in the MA-plot. Fig. 1 displays MA-plots for both approaches, using all genes and the reduced set.

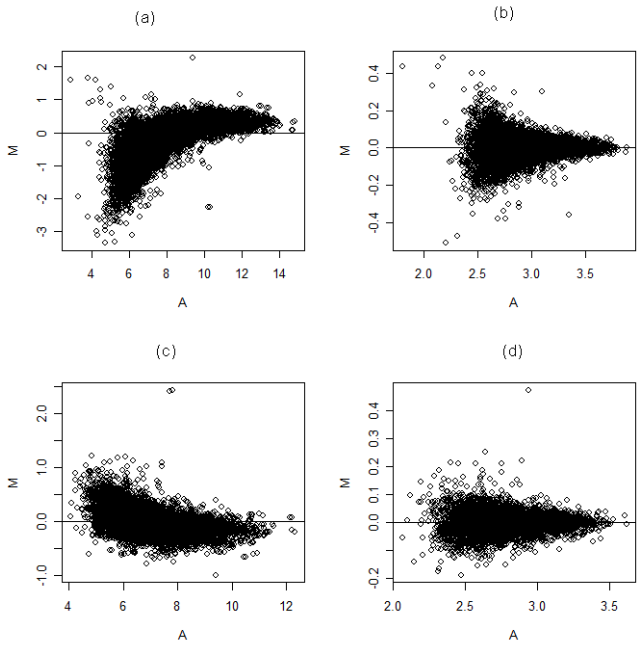

Fig. 1. MAplot for complete (a and b) and reduced (c and d) dataset before and after lowess transformation

The raw intensity data shows a nonlinear pattern in MA-plot, suggesting the need for transformation. For each slide we applied the intensity global lowess transformation, a locally weighted scatter plot smoothing, to the logarithm data (base 2) for normalization and variability reduction of data. The results, after removing the estimated lowess curve, are shown in fig. 1(c and d). The transformed data were analyzed using the mean of each replicate with the second array considered as dye swap experiment, resulting in four experiments, with two dye swaps slides.

Analysis was done within the R programming environment [7] using the MAANOVA package [8] which consists of an analysis of variance approach for microarray experiments proposed in Kerr et al [5]. Wolfinger et al [9] proposed the 2-stage ANOVA model implemented in the R/maanova. An ANOVA model for microarray experiment can be specified in two stages. The first stage is the normalization model,

$$y_{ijkgr} = \mu + A_i + D_j + AD_{ij} + r_{ijkgr} \qquad (1)$$

where, μ captures the overall mean, the other terms capture the overall effects due to arrays (A), dyes (D) for labeling reactions and interaction between array and dye (AD). Residuals from this first stage are used as inputs for the second stage which models gene-specific effects:

$$r_{ijkr} = G + AG_i + DG_j + VG_k + \epsilon_{ijkr} \qquad (2)$$

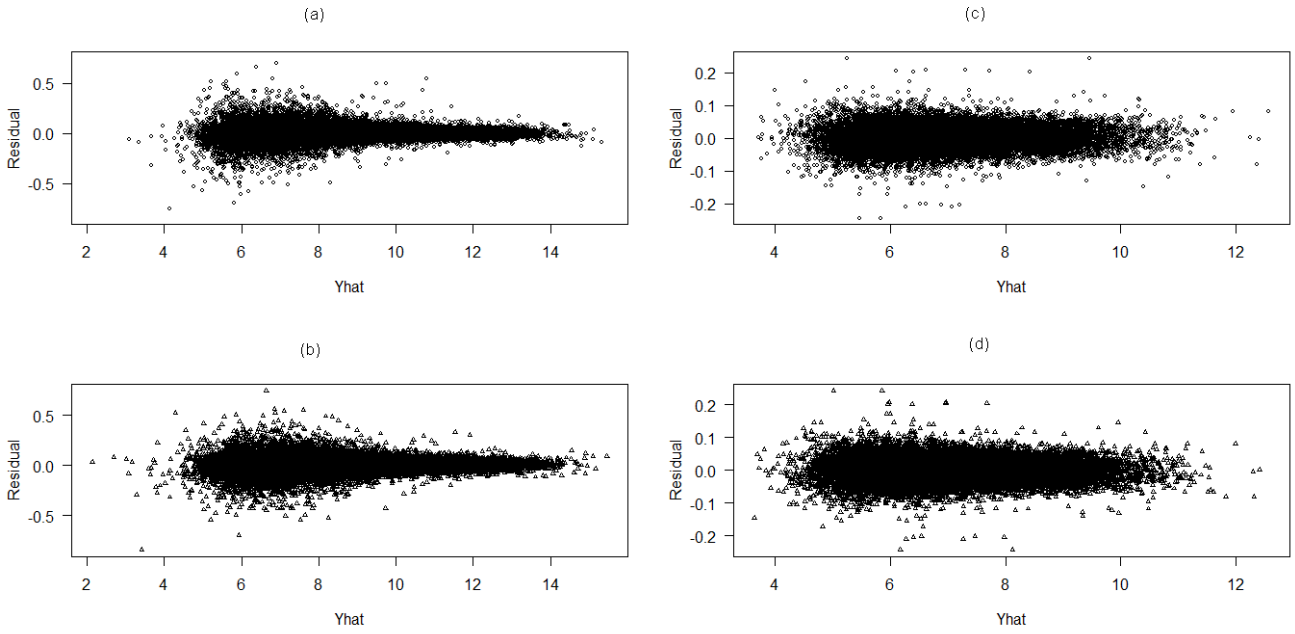

Fig. 2. Residual Plot for the best model in complete dataset with Cy5 dye (a) and Cy3 dye (c) and using the reduced set with Cy5 (b) and Cy3 (d)

here, (G) captures the average effect of the gene, (AG) captures the array by gene variation, (DG) captures the dye by gene variation, (VG) captures the effects for the experimental varieties. In this study, we had three conditions: non-exposed, DMSO exposed and Thalidomide+DMSO treated PBMC. The ϵ term accounts for unexplained factors and is assumed as the error term with mean zero. For the full data set we used the mixed model that treats some factors in an experimental design as random samples from a population. In other words, it assumes that if the experiment were to be repeated, the same effects would not be exactly reproduced, but that similar effects would be drawn from a hypothetical population of effects. In this work, we treated the array effect (AG) as random factor in ANOVA model. For the reduced set we used the fixed effect model that assumes independence among all observations and only one source of random variation. Fig. 2 plots the residuals versus fitted values for each array and shows adequate adjustment of the models.

The F test is designed to detect any pattern of differential expression among several conditions by comparing the variation among replicated samples within and between conditions. MAANOVA provides four types of F tests that can be used individually or in conjunction: The gene-specific F test (F1), a generalization of the gene-specific t test, is the usual F test and it is computed gene-by-gene. As with t tests, we can also assume a common error variance for all genes, which results in the global variance F test (F3). A middle ground is achieved by the F2 test, analogous to the regularized t test which uses a weighted combination of global and the gene specific variance estimates in the denominator [10].

The test statistic Fs uses a variance estimator that makes no prior assumptions about distributions of the variance across genes. It behaves well when variances are constant and also if they vary from gene to gene. In the present study, we used the suggested Fs test, since it does not require assumption of a common variance. A statistic like Fs should be more efficient with limited information to estimate the gene specific variance components [11]. Nominal *p*-values can be obtained for the F test, from standard tables, but the F2, F3 and Fs statistics do not follow the tabulated F distribution and critical values should be established by permutation analysis.

Permutation analysis is a nonparametric approach to establish the null distribution of a test statistic. The key to developing a permutation strategy is to identify units in the experiment that are exchangeable under the null hypothesis. In microarray experiments, if we allow for gene-specific variance heterogeneity, then the unit must be the whole arrays. Furthermore, the arrays that are to be shuffled will depend on the design of the experiment and the factor(s) being tested. Two-color arrays are slightly more complex than single color systems as the pairing between the two channels of the array must be maintained in the permuted units. To execute the permutation analysis, MAANOVA generates random shuffles ($p = 1, \ldots, P$) of whole array units and compute a new set of statistics $Fg(p)(g = 1, \ldots, G)$. We did only 100 permutations. In MAANOVA, to reduce the granularity of the gene-specific null distribution, a common null distribution for each test statistic is established. This distribution is obtained using the entire collection of Fg(p) values over indices p and g based on the assumption that the F statistics have common null distributions across genes [12].

Relative fold change was calculated for all genes as the ratio between treatment sample and corresponding control

value. If this number was less than one the (negative) reciprocal is listed (e.g., 0.75, or a drop of 25% from control is reported as -1.3 fold change).

III. RESULTS

The proposed procedure generates a series of *p*-values, one for each gene in the experiment. Using a significance level of 0.001 for the Fs statistic we selected a total of 21 genes differentially expressed, 13 for the full set (table II) and 8 for the reduced set (table II). The Volcano plots for both results are shown in fig. 3 (a and b). This plot arranges genes along dimensions of biological and statistical significance. The *x*-axis is the expression ratio between the two groups (on a log scale) representing the biological impact of the change. The *y*-axis represents the *p*-value for a statistic test of differences between samples (on a negative log scale) and represents the statistical evidence, or reliability of the change.

TABLE II

DIFFERENTIALLY EXPRESSED GENES USING THE COMPLETE DATASET.

Acc.	Symbol	*p*-value	FC
NM_018378	FBXL8	0.0001	2.0
NM_002718	PPP2R3A	0.0003	2.2
NM_000665	ACHE	0.0003	2.1
NM_014499	P2RY10	0.0004	-1.7
NM_000264	PTCH1	0.0004	-2.3
NM_014230	SRP68	0.0004	-2.1
NM_000673	ADH7	0.0004	2.9
NM_022118	RBM26	0.0003	-1.7
NM_003678	C22orf19	0.0004	-2.1
NM_022163	MRPL46	0.0005	2.7
NM_025094	hypothetical protein FLJ22184	0.0005	-1.6
NM_004455	EXTL1	0.0007	-1.9
NM_003192	TBCC	0.0009	2.9

TABLE III

DIFFERENTIALLY EXPRESSED GENES USING THE REDUCED DATASET.

Acc.	Symbol	*p*-value	FC
NM_022163	MRPL46	<0.0001	-2.6
NM_002032	FTH1	<0.0001	-2.1
XM_052761	Hypothetical protein 52761	0.00001	-1.8
NM_000336	SCNN1B	0.00007	1.7
NM_000581	GPX1	0.0001	-1.6
NM_001020	RSP16	0.0001	-1.6
XM_069860	similar to similar to ribosomal protein l31 (h. sapiens) loc136380	0.0001	-1.6
NM_024597	MAP7D3	0.0001	1.6

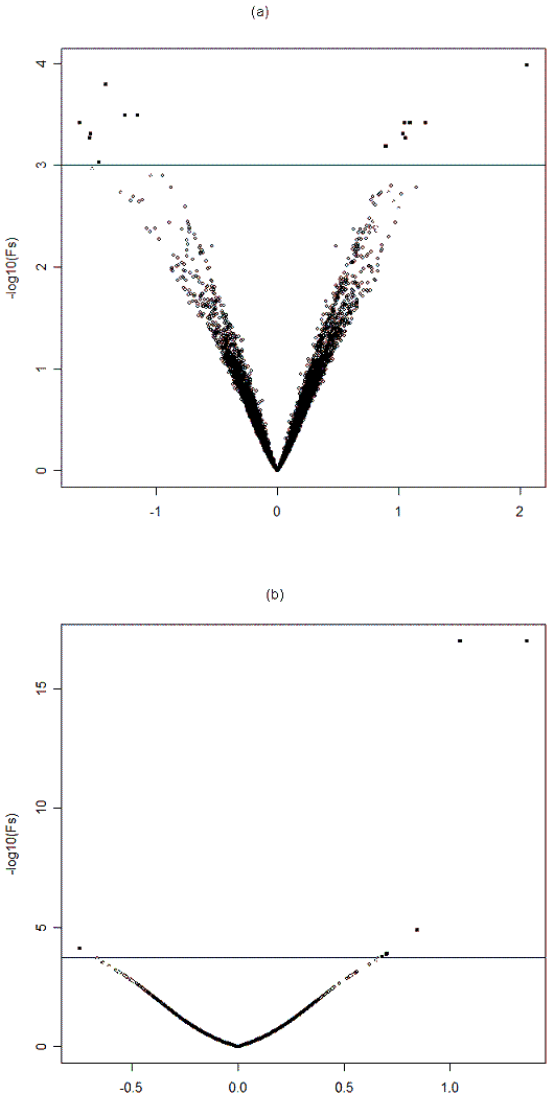

Fig. 3. Volcano plot with results of Fs test. Differentially expressed genes are shown above the line for complete dataset (a) and reduced set (b). In reduced set, two genes had same values for expression ratio and *p*-value.

IV. DISCUSSION

In the present study, PBMCs were tested for thalidomide treatment using microarrays containing 9,984 oligonucleotides spotted in duplicate to detect differentially expressed genes. It is well known in the literature that using the raw intensity ratios to infer differentially expressed genes is an inefficient procedure, since the inherent variability affects the measured expression as shown in fig. 1 (a and c). It is common practice in microarrays experiments to apply appropriate transformations to deal partially with this problem. Here, we applied lowess to the log_2-ratio of stimulated and control intensities to obtain a more symmetrical distribution, correcting the curvature of the MA-plot (fig. 1c and 1d). This study screen changes in gene expression between two conditions using a reference model. This has been achieved using ANOVA modeling for the transformed data. The sample term in the model was the focus of interest to select a smaller set of genes that need to be further tested with real time PCR probing. The ANOVA model generates estimates of *p*-values for the relative expressions used to rank the observed differential expressions.

We used the mixed model for the complete database, with array as a random effect. The fixed model was used for the second approach with eliminated genes based on the signal-to-noise ratio. For the analysis using all 9,984 genes, the list of differentially expressed genes belongs to the flagged set. Therefore, the resulting list from the reduced

set does not show intersection with this result. Analysis of the fitted model showed that the residuals have heavier-tailed distributions than normal distribution (data not shown) precluding the use of standard F distribution, therefore, *p*-values were estimated via bootstrapping, as suggested in the MAANOVA package [8], with the knowledge that it has lower statistical power.

The results presented in table II and III, show the unadjusted *p*-value, the fold-change and a summary of the function of genes that showed differential expression for both cases, full dataset and filtered dataset. We opt not to use multiple test adjustments for two main reasons. First, the microarray experiment was set up as an exploratory tool to find among a large set of genes a smaller set for further confirmatory study using RT-PCR. Second, a preliminary analysis showed that the majority (99%) of the genes had fold-changes below 2. According to some authors [13], [14], in a exploratory analysis when sensitivity is the major goal of a study, multiple comparison correction is not strictly necessary. In this work we found only 21 genes differentially expressed and if we apply even a less stringent multiple test correction, such as the pFDR, we would find only one gene as statistically significant.

To improve the exploratory results, we obtained the function of the differentially expressed genes from Entrez Gene (`www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene`), a gene-specific database that has tracked unique identifiers (GeneID) for genes and reports information associated with these identifiers for unrestricted public use [15]. The search was carried out using the access number of the gene. Table II shows the differentially expressed genes that could provide some information about thalidomide's mechanism of action found using all genes. Here we describe for some genes the related information found at Entrez Gene and that deservers further consideration for RT-PCR confirmatory analysis.

The gene F-box and leucine-rich repeat protein 8 is connected to the ubiquitin system. This system is related to the path of degradation of cellular proteins, a high complex, temporally controlled, and tightly regulated process that plays major roles in a variety of basic pathways during cell life and death. It includes the breakdown of muscle protein (cachexia), which occurs in diseases such as cancer and the Acquired Immune Deficiency Syndrome (AIDS). This system is also involved in processes related to immune and anti-inflammatory responses, since processing of most known MHC class I antigens is mediated by the ubiquitin-proteasome pathway. Thalidomide presents important response in these processes: immune and anti-inflammatory responses and loss of muscle protein [16], [17], [2]. Despite the observed low-fold change, our analysis suggests an increased expression in relation to DMSO, with *p*-value less than 0.0002.

The results also showed the gene PTCH1 as differentially expressed, with a fold-change lower than one, showing that this gene is suppressed. This gene acts in the formation of embryonic structures and it is well known that the principal thalidomide's side effect is teratogenicity [16]. Besides these effects, thalidomide can inhibit the replication of HIV-1 in vitro [18], but the mechanism has not been elucidated.

Our analysis found that the gene PP2R3A, that codes Protein Phosphatase 2, subunit regulatory B", alpha, is differentially expressed, with fold-change of 2.29 (*p*-value of 0.0003). Protein Phosphatase 2 is one of the four major Ser/Thr phosphatases and is implicated in the negative control of cell growth and division. It functions as an inhibitor of G0 to M transition of the cell cycle and is involved in other key cellular processes such as the control of RNA transcription. In many cell types, Protein Phosphatase 2A exists as two forms, with differents substrate specificities, a holoenzime with two regulatory sub-units (A and B) and catalytic subunit C, and a enzime core, with A and C sub-units. The balance between this two enzymatic forms is important to phosphatase activity and affects HIV-1 gene expression and viral replication. [19]. Furthermore, the observation that okadaic acid (OKA), a potent inhibitor of PP2A and protein phosphatase 1, induces NF-κB binding and activates the HIV-1 promoter suggested that PP2A might inhibit HIV-1 transcription and, hence, replication [20].

Filtering out bad spots caused by adverse experimental conditions is a useful way to reduce variability, allowing the biological differences to come the fore [21]. Filtering is a pre-processing step used to identify and remove spots where measured intensities are not distinguished from the background noise. A filtering procedure must provide a balance between reliable measurements and minimimal loss of information [22]. The simple filtering criteria used in this work is a conservative approach, eliminating genes that could be differentially expressed despite their low signal-to-noise-ratio. This might explain why all genes selected using the complete set were flagged.

In the reduced set (table III) we found 8 differentially expressed genes. Two genes could be directly involved in thalidomide's mechanisms of action. The gene Ferritin Heavy Chain 1 (FTHC1) acts during inflammation antagonizing apoptosis induced by tumor necrosis factor (TNF) through NF-κB transcription factors action. Pham et al [23] identified FTCH1 as an essential mediator of the antioxidant and protective activities of NF-κB. FTHC1 is induced downstream of NF- κB and is required to prevent apoptosis triggered by TNF. In our findings this gene is downregulated (table III) and this could be a result for thalidomide's action on TNF. Another important gene is the ITH5, a member of the inter-α-trypsin inhibitor (ITI) family formed by a group of proteins built up from one light chain and a variable set of heavy chains. Originally identified as plasma protease inhibitors, recent data show that ITI plays a role in the extracellular matrix stabilization and in prevention of tumor metastasises [24]. In this analysis this gene is upregulated and this could help to explain the antitumoral activity of thalidomide.

V. CONCLUSION

The ANOVA model is a flexible technique for detecting differentially expressed genes. The appropriate use of

this technique allows the selection of putative differentially expressed genes among several variants, even in adverse conditions as with restricted replication. Microarrays are potentially powerful tools for investigating the mechanism of drug action. Clearly, a deeper investigation will provide evidence for using gene expression profiles to understand the molecular basis of thalidomide's mechanism of action and to enable the development of new drugs that reduce its adverse effects.

VI. ACKNOWLEDGMENTS

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