THE MIND-BODY HEALING EXPERIENCE (MHE) IS ASSOCIATED WITH GENE EXPRESSION IN HUMAN LEUKOCYTES

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Abstract

This paper explores the hypothesis that a psychotherapeutic protocol, "The Mind-Body Healing Experience" (MHE), could modulate experience-dependent changes ingene expression in human white blood cells. Peripheral blood was collected just before and immediately followng administrationof the MHE to 18 individuals. Changes in experience-dependent gene expression over 1 hour and 24 hours were assessed through microarray analysis. A total of 200 genes were differentially expressed (>1.2 fold, $p < 0.05$). After 1 hour 46 genes were differentially expressed and after 24 hours 154 genes were differentially expressed. Bioinformatic analysis revealed four significantly enriched GO term pathways (p-value <0.05): acetylation, cytosol, regulation of cell death and negative regulation of apoptosis. To validate the microarray results a subset of genes representative of specific and enriched GO pathways, or with relevant functions, were validated through RT-PCR experiments. This data demonstrated high plasticity of human immune cells

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and a considerable modulation of gene expression following the MHE. Genes apparently expressed in response to the MHE protocol are related to a variety of GO term pathways associated with reducing cellular stress and inflammation while supporting positive immune system functions. These findings may have important applications in therapeutic hypnosis, psychotherapy and rehabilitation.

Keywords

Psychotherapy; Microarray; MAPK signaling; NF-kB; N-Ras gene; IKBKG; Programmed cell death (PCD); PRKAR2B; Experience-Dependent Gene Expression; immune system support.

1. Introduction

In the past decade microarray DNA technology has made it possible to measure thousands of gene expressions simultaneously and validate results through Reverse Transcription Polymerase Chain Reaction (RT-PCR). This experimental approach has revolutionized research in molecular biology and has become a new standard in personalized medicine[1]. Recent research has demonstrated how complex mechanisms modulate gene expression without altering the DNA sequence[2]. It has been found that some genes interact with the environment to modulate behavior and cognition in sickness and health[3]. Furthermore, chronic diseases, anxiety, stress and psychiatric disorders can be understood as multi-factorial interactions between genes and/or gene/environment. These interactions involve a special class of genes, often described as activity or experience-dependent genes, which can be turned on or off by signals from the physical and psychosocial environment to modulate the complex human functions of physiology, psychology and consciousness itself[4–10]. One of the authors[11] has proposed in more than 45 years scientific career a new psychotherapeutic approach called Psychosocial and Cultural Genomics (PCG) to investigate translational mechanisms of healing on all levels from mind and brain to gene[12,13]. In association with extensive clinical and neuro-imaging data PCG considers the study of gene expression and its regulation, as well as genomic aspects for further understanding of brain mechanisms. These epigenetic processes explore the interaction between genes and environment. In the field of psycho-neuro-immunology, for example, many studies demonstrated a consistent and wide communication between the immune system and the central nervous system[7].

Recently, gene expression profiles, principally obtained by means of microarray analysis, and miRNA signatures of white blood cells have been proposed as a convenient alternative to

cerebral markers[14–18]. The study of peripheral white blood cells constitute a highly complex system that communicates with every tissue and organ in the body and share more than 80% of the transcriptome with different organs (e.g. brain, colon, heart, kidney, liver, lung, prostate, spleen, and stomach). Recent research documented how a number of mind-body therapies were able to reduce stress by eliciting the relaxation response (RR) that modulated gene expression[19]. The genome wide transcriptional profiling of leukocytes has shown a common pattern of increased expression of pro-inflammatory genes in the case of diseases and stress. Experimental studies reported that Stress Management reduces gene expression related to pro-inflammatory biochemical pathways in women affected by breast cancer[20]. In our research, we employed DNA microarray and RT-PCR analyses on white blood cells to explore the hypothesis that a carefully specified protocol of therapeutic hypnosis called The Mind-Body Healing Exeperience" could modulate experience

dependent genes to reduce symptoms of the stress related disorders and facilitate mind-body healing[11,21,22]. We hypothesized changes in gene expression over the relatively brief time periods of 1 hour and 24 hours following a single intervention of MHE based on previous research[23]. In fact, the work of Unternaehrer, et al, confirmes our hypothesis regarding time parameters for the therapeutic modulation of stress-associated genes (OXTR, BDNF). This is also consistent with a great deal of previous research that established how stress related distortions of the circadian cycle and the 90-120 minute basic rest-activity cycle are implicated in many psychiatric disorders and psychological dysfunctions[23]. Unternaehrer's research[24] is also entirely consistent with our previous pilot study documenting how the MHE protocol can facilitate brain plasticity and stem cell healing. To confirm our previous pilot study[25], exploring the hypothesis that a top-down creatively oriented positive human experience modulate gene expression, we conducted a further microarray pilot study on a larger number of voluntaries, sufficient to satisfy microarray statistical recommendations[26,27].

2. Materials and Methods

2.1 Subjects and Mind-Body Healing Experience

Eighteen individuals underwent the "Mind-Body Healing Experience" (MHE), originally developed by Rossi and now freely available[22,25], respecting the international standard codes of

ethics in the field of scientific research on human subjects. This group of subjects was formed by 9 females and 9 males, aged from 30 to 50 years. The 18 subjects had advanced academic degrees and were all volunteers. All voluntaries underwent to Hypnotic Treatment gave written informed consent in accordance with the Declaration of Helsinki and with "General Authorisation for the Processing of Genetic Data – 24 June 2011" for the use of blood for research purpose and for processing of genetic data. Biological material (blood) was collected and provided by nurses working at the Benevento hospital in accordance with their ethics code and prior informed consent of the volunteers. Psychological tests to measure the focused attention and mental absorption by The Tellegen Absorption Scale (highly correlated with Standard Scales of Hypnotic Susceptibility), and the Spiritual Intelligence Self Report Inventory (high scorers on the SISRI-24 acknowledge heightened experiences related to critical existential thinking, personal meaning production, transcendental awareness, and conscious state expansion) were administered before the MHE to all volunteers.

2.2 Blood collection and sample processing

Approximately 10 ml of peripheral blood was collected from the 18 volunteers just before (condition A), one hour (condition B) and 24 hours (condition C) after the MHE session. Blood was processed immediately after collection in order to reduce the ex vivo effects on gene-expression. Leukocytes were isolated from 5.0 ml whole blood according to the manual of QIAamp RNA Blood Mini kit (Qiagen, Milano, Italy). Briefly, erythrocytes were selectively lysed in erythrocyte lysis buffer (EL; Qiagen) by incubation on ice and vortexing. Leukocytes were collected by centrifugation at 400g for 10 minutes at 4°C, washed twice with EL buffer and then placed in 0.6 ml of RLT buffer (Qiagen) containing β-mercaptoethanol. Homogenized cell lysate was stored at -80°C until use. The frozen lysates were thawed in a water bath at 37°C until complete melting of the saline buffer. Total RNA was extracted using spin columns (Qiagen). Contaminating DNA was removed from total RNA, while bound to the QIAamp membrane, by means of incubation with DNAse I (Qiagen) following the supplier instructions. After DNA digestion, washing and column elution of RNAs were performed according to the supplied protocol. RNA concentration was determined using the Picodrop Spectrophotometer apparatus (Saffron Walden, United Kingdom) and its quality assessed on agarose gel electrophoresis.

cDNA preparation and gene chip hybridization

Single strand biotinylated cDNA was generated as follows: 100ng of total RNA were subjected to two cycles of cDNA synthesis with the Ambion WT expression Kit (Applera, Milano, Italy). The first cycle – first strand synthesis was performed using an engineered set of random primers that excludes rRNA-matching sequences and includes the T7 promoter sequences. After second-strand synthesis, the resulting cDNA was in vitro transcribed with the T7 RNA polymerase to generate a cRNA. The produced cRNA underwent to a second cycle – first strand synthesis in the presence of dUTP in a fixed ratio relative to dTTP. Single strand cDNA was then purified and fragmented with a mixture of uracil DNA glycosylase and apurinic/apirimidinic endonuclease 1 (Affymetrix Italia, Milano, Italy) in correspondence of incorporated dUTPs. DNA fragments were then labelled by terminal deoxynucleotidyl transferase (Affymetrix) with biotin. The biotinylated DNA was later hybridized to the Human Genechip Gene 1ST Arrays (Affymetrix), containing about 29000 genes selected from human genome databases RefSeq, ENSEMBL and GenBank. Chips were washed and scanned on the Affymetrix Complete GeneChip Instrument System, generating digitized image data (DAT) files.

2.3 Statistical analysis of microarray data

Raw data from .CEL files were imported into Bioconductor R and pre-processed using the AFFY library. Software manual is available from http://cobra20.fhcrc.org/packages/release/bioc/html/affy.html. Different quality control checks such as array images, box plots and relative log expression, were executed before the pre-processing. Robust Multi-array Average (RMA) proposed by Irizarray, et al[28] was used for normalizing data and for obtaining expression measures from Affymetrix .CEL files. Differential gene expression analysis were performed using the LIMMA (Linear Models for Microarray Data) library (software manual available from http://bioinf.wehi.edu.au/limma/). An Empirical Bayes (EB) method to moderate the standard errors of the estimated log-fold changes is used by this software for statistical analysis and assessing differential expression[29,30]. After EB analysis in LIMMA, a false discovery rate (FDR) was carried out using the method of Benjamini and Hochberg[31] to adjust p-values for multiple testing. Genes were ranked then according to B-statistics essentially the log-odds of differential expression. Genes identified as having a fold-

change of 1.2 and an adjusted p-value threshold of 0.05 were considered to be significantly differentially expressed. The list of differentially expressed genes was reported as supplementary materials in Table 1. To investigate the biological meaning of this list of significant genes, DAVID (Database for Annotation, Visualization of Integrated Discovery (http://david.abcc.ncifcrf.gov/) software tool was used to identify enriched gene-GO term and well-known KEGG and BioCarta pathways. For each DAVID result an adjusted p-value, representing the level of significance of that GO term or pathway, is given. Therefore items having an adjusted p-value less than 0.05 were selected.

Table 1. After data collection before administration of the MHE Differentially expressed genes both down or up-regulated at one hour (condition B) and 24 hours (condition C). Genes having fold-change of 1.2 and adjusted p-value threshold of 0.05 were considered differentially expressed at these two conditions.

Figure a: B vs. A (42 down regulated genes)

ID	logFC	adj.P.Val
6622_at	-2.443	1.997e-11
212_at	-2.344	4.056e-10
25793_at	-1.019	1.870e-09
759_at	-2.522	3.150e-09
598_at	-1.080	6.598e-09
3043_at	-3.078	6.598e-09
55363_at	-1.586	1.563e-08
54855_at	-1.107	1.872e-08
3045_at	-0.855	5.791e-08
25853_at	-0.788	1.994e-07
55796_at	-1.000	1.645e-06
51629_at	-1.226	2.387e-06
6521_at	-1.073	5.982e-06
7111_at	-0.632	2.930e-05
669_at	-0.512	3.922e-05
2993_at	-1.066	4.548e-05
2995_at	-0.768	7.212e-05
2038_at	-0.529	1.065e-04
55437_at	-0.546	1.064e-04
2766_at	-1.009	1.185e-04
2235_at	-0.837	1.759e-04
55432_at	-0.490	3.461e-04
148534_at	-0.535	0.001
2039_at	-0.698	0.001
8991_at	-0.708	0.001
23608_at	-0.322	0.003

4635_at	-0.390	0.004
54830_at	-0.325	0.011
51327_at	-0.604	0.012
7504_at	-0.303	0.014
51094_at	-0.484	0.014
8531_at	-0.583	0.016
527_at	-0.431	0.019
26994_at	-0.379	0.022
51759_at	-0.404	0.022
25893_at	-0.463	0.022
1510_at	-0.285	0.023
665_at	-0.344	0.029
439996_at	-0.417	0.029
51203_at	-0.317	0.033
130540_at	-0.435	0.047
9491_at	-0.344	0.050

b) B vs. A (up regulated genes: 4)

ID	logFC	adj.P.Val
100124536_at	1.549	1.113e-04
1376_at	0.291	0.014
677775_at	0.658	0.018
2647_at	0.306	0.030

c) C vs. A (down regulated genes: 122)

ID	logFC	adj.P.Val
6622_at	-2.154	8.427e-10
212_at	-2.169	2.440e-09
598_at	-0.972	1.386e-07
759_at	-2.189	1.386e-07
25793_at	-0.836	3.093e-07
25853_at	-0.748	5.871e-07
54855_at	-0.967	5.871e-07
3043_at	-2.575	5.871e-07
55363_at	-1.363	5.871e-07
55796_at	-0.892	1.922e-05
51629_at	-1.096	2.428e-05
9491_at	-0.532	2.566e-05
3045_at	-0.659	2.940e-05
6521_at	-0.945	6.660e-05
2766_at	-1.020	6.660e-05
669_at	-0.480	8.916e-05
2995_at	-0.712	2.174e-04

2235_at	-0.813	2.200e-04
4635_at	-0.447	2.242e-04
55437_at	-0.513	2.243e-04
51094_at	-0.594	2.561e-04
57610_at	-0.420	3.622e-04
8531_at	-0.691	5.681e-04
55432_at	-0.463	5.681e-04
2993_at	-0.901	6.461e-04
2038_at	-0.464	6.707e-04
665_at	-0.420	7.796e-04
7111_at	-0.513	7.796e-04
93487_at	-0.322	1.919e-03
8303_at	-0.581	2.058e-03
84932_at	-0.495	2.133e-03
5970_at	-0.429	2.133e-03
84919_at	-0.350	2.359e-03
3559_at	-0.636	2.755e-03
2039_at	-0.627	2.755e-03
91300_at	-0.455	3.300e-03
7277_at	-0.430	3.296e-03
8991_at	-0.624	0.004e-03
1072_at	-0.421	5.118e-03
63935_at	-0.287	5.118e-03
9391_at	-0.344	5.494e-03
11230_at	-0.553	7.695e-03
5880_at	-0.377	0.010
10098_at	-0.413	0.010
951_at	-0.320	0.011
23558_at	-0.545	0.011
7314_at	-0.367	0.012
54331_at	-0.430	0.014
5534_at	-0.360	0.014
93380_at	-0.375	0.014
10826_at	-0.838	0.015
9526_at	-0.498	0.015
2342_at	-0.450	0.016
8131_at	-0.425	0.016
10423_at	-0.492	0.017
375743_at	-0.439	0.017
9953_at	-0.554	0.019
527_at	-0.399	0.019
51327_at	-0.532	0.021
2273_at	-0.493	0.022
5089_at	-0.431	0.022

81619_at	-0.331	0.022
5870_at	-0.367	0.022
25893_at	-0.425	0.023
6737_at	-0.591	0.023
55802_at	-0.333	0.023
89910_at	-0.263	0.024
10116_at	-0.294	0.024
51312_at	-0.308	0.024
51147_at	-0.316	0.024
54891_at	-0.310	0.026
283489_at	-0.337	0.026
860_at	-0.362	0.026
3561_at	-0.344	0.026
25840_at	-0.364	0.026
23383_at	-0.352	0.028
8717_at	-0.298	0.028
8676_at	-0.420	0.028
808_at	-0.409	0.030
51759_at	-0.358	0.030
54820_at	-0.416	0.031
81027_at	-0.813	0.032
90407_at	-0.344	0.032
26986_at	-0.284	0.033
66008_at	-0.297	0.036
90139_at	-0.447	0.036
79144_at	-0.592	0.037
5292_at	-0.267	0.037
4354_at	-0.364	0.037
8349_at	-0.489	0.039
1874_at	-0.307	0.039
130540_at	-0.398	0.040
7572_at	-0.414	0.041
4893_at	-0.418	0.041
6293_at	-0.376	0.041
602_at	-0.380	0.041
3042_at	-0.422	0.041
5606_at	-0.379	0.041
124460_at	-0.384	0.042
11040_at	-0.333	0.043
63905_at	-0.324	0.043
151188_at	-0.302	0.043
9230_at	-0.336	0.043
83658_at	-0.276	0.043
146712_at	-0.330	0.044

8780_at	-0.268	0.045
94241_at	-0.276	0.045
2275_at	-0.442	0.045
8818_at	-0.369	0.046
7538_at	-0.361	0.046
57699_at	-0.287	0.046
1727_at	-0.270	0.046
8517_at	-0.524	0.046
2994_at	-0.321	0.046
55101_at	-0.416	0.046
392_at	-0.296	0.047
84925_at	-0.346	0.047
6563_at	-0.525	0.047
4615_at	-0.298	0.048
10320_at	-0.274	0.049
1861_at	-0.297	0.049
3005_at	-0.448	0.049

d) C vs. A (up regulated genes: 32)

ID	logFC	adj.P.Val
2972_at	0.300	4.631e-04
51651_at	0.351	7.208 e-04
100124536_at	1.359	7.2080 e-04
115416_at	0.376	9.768 e-04
677814_at	0.298	1.221 e-03
677775_at	0.720	2.148 e-03
509_at	0.448	2.486 e-03
1337_at	0.395	3.295 e-03
25983_at	0.425	5.118 e-03
54780_at	0.411	7.767 e-03
3093_at	0.334	8.939 e-03
26157_at	0.451	0.012
8666_at	0.330	0.014
57689_at	0.300	0.016
605_at	0.304	0.017
84992_at	0.352	0.019
51111_at	0.326	0.019
85495_at	0.521	0.020
6741_at	0.510	0.021
10573_at	0.364	0.021
5694_at	0.444	0.024
399717_at	0.293	0.025
55142_at	0.351	0.037

6207_at	0.569	0.039
6605_at	0.297	0.041
55856_at	0.332	0.041
84888_at	0.320	0.041
283106_at	0.360	0.041
692233_at	0.457	0.045
7360_at	0.313	0.045
4987_at	0.268	0.045
55520_at	0.278	0.046

2.4 Real-time quantitative RT-PCR

cDNA synthesis was performed using 100 ng of the purified RNA with the High capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies, Italy) according to the manufacturer's protocol. The cDNA quantity was assessed by the Nanodrop spectrophotometer and 50 ng were used for each real time reaction, using the Power SYBR master mix (Applied Biosystems) in a final volume of 10 ul. The primer list was included in the supplementary material in Table 3. The reaction mixtures underwent 40 cycles of 95°C 15 sec, then 60°C 15 sec, followed by a dissociation curve, on the 7900HT thermal cycler (Applied Biosystems).

3. Results

3.1 Microarray analysis

Leukocyte gene expression of 18 voluntaries was analyzed using Affymetrix high-density oligo arrays. The experimental setup included 3 sets of measurements (conditions a, b and c). Condition a is the genome-wide transcriptional profiles obtained from all the subjects just before the MHE treatment (condition A). Condition bis one hour after administration of the MHE. Condition c is 24 hours after administration of the MHE. A statistical analysis based on linear regression models in the LIMMA package was used to identify genes differentially expressed. Genes having a fold-change cut-off of 1.2 and a multiple-measure adjusted p-value less than 0.05 were considered differentially expressed between two conditions. The Venn diagrams in Figure 1representsthe intersection of 200 genes that weredifferentially expressed in this study.

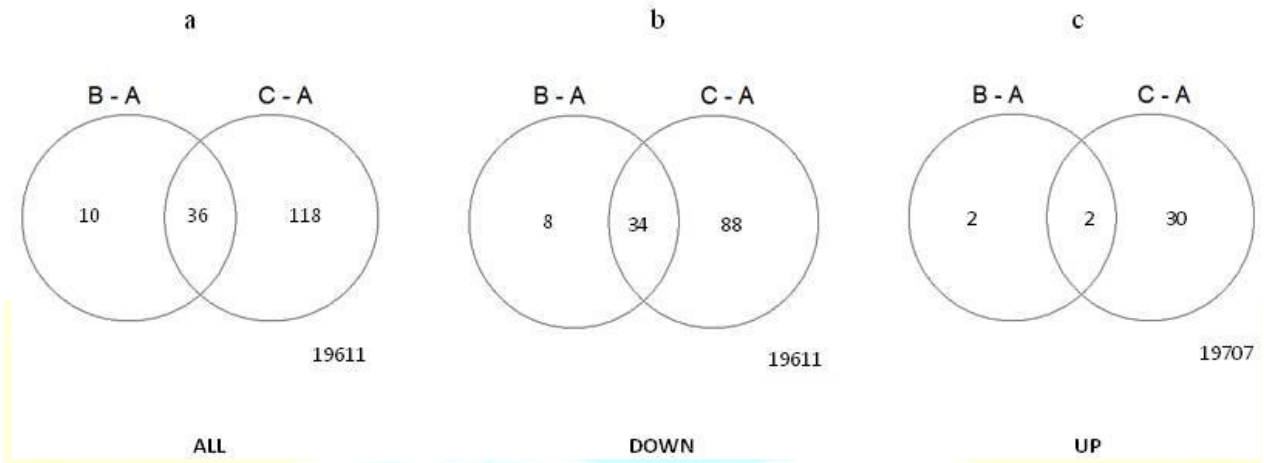

Figure 1:*Venn diagrams of the intersection of 200 genes that werediffferentially expressed in this study.*

Figure 2 illustrates the total up and down regulated genes in this study. Figure 3 illustrates the up and down regulated genes expressed in condition immediately following administration of theMHE. Figure 4 illustrates the up and down regulated genes expressed 24 hours after the administration of the MHE.

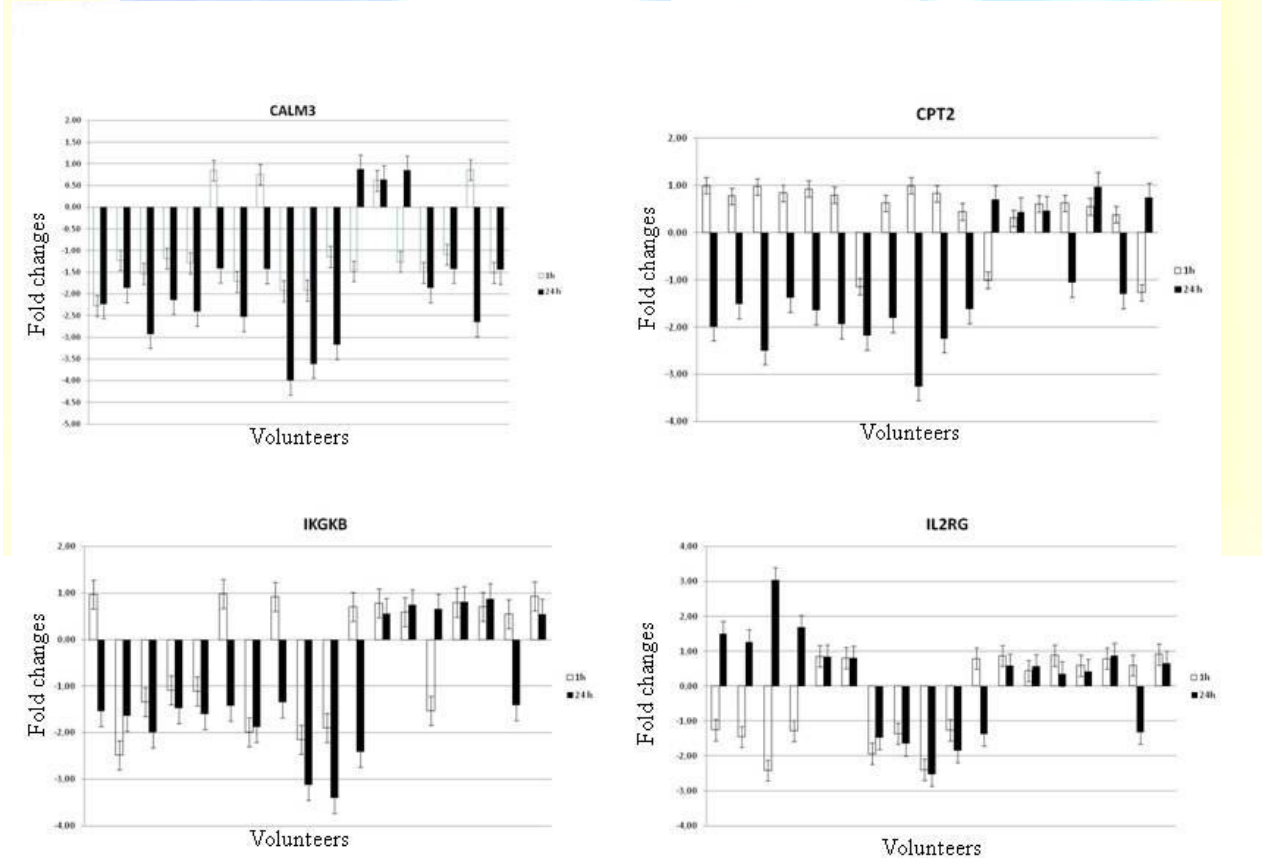

Figure 2.*Thetotal up and down regulated genes in this study.*

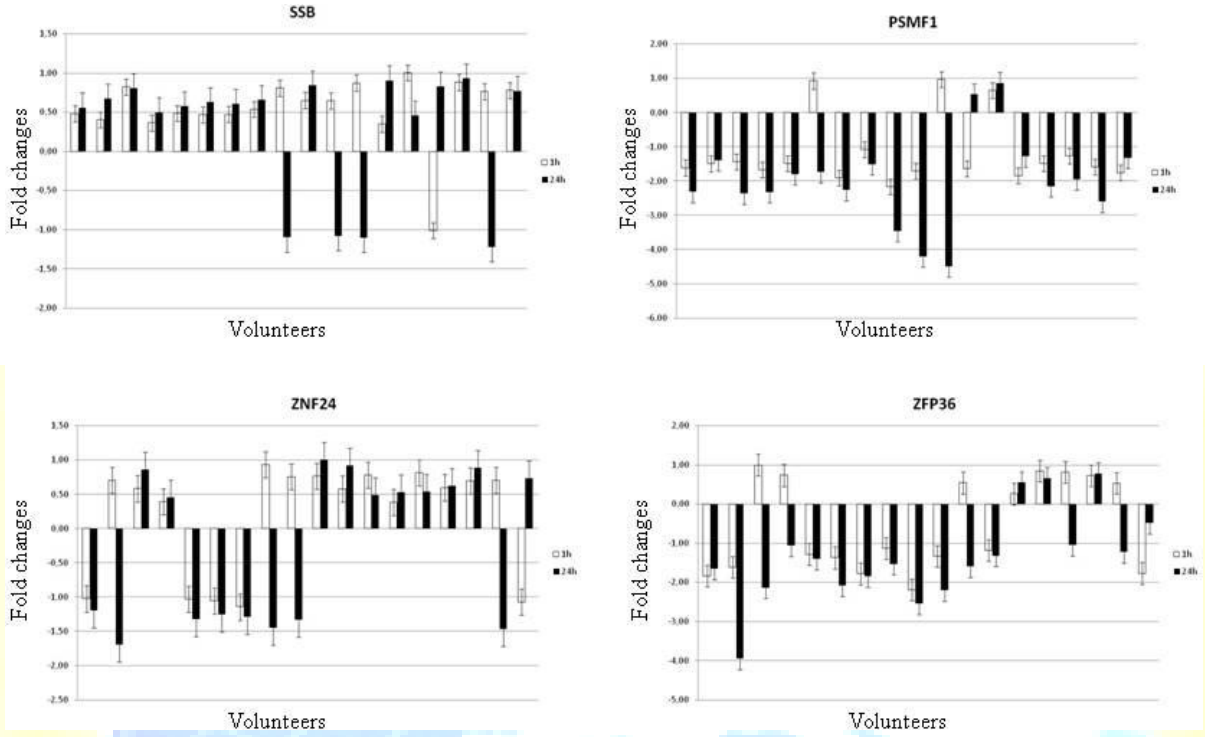

Figure 3:*The up and down regulated genes expressed in condition immediately following administration of the MHE.*

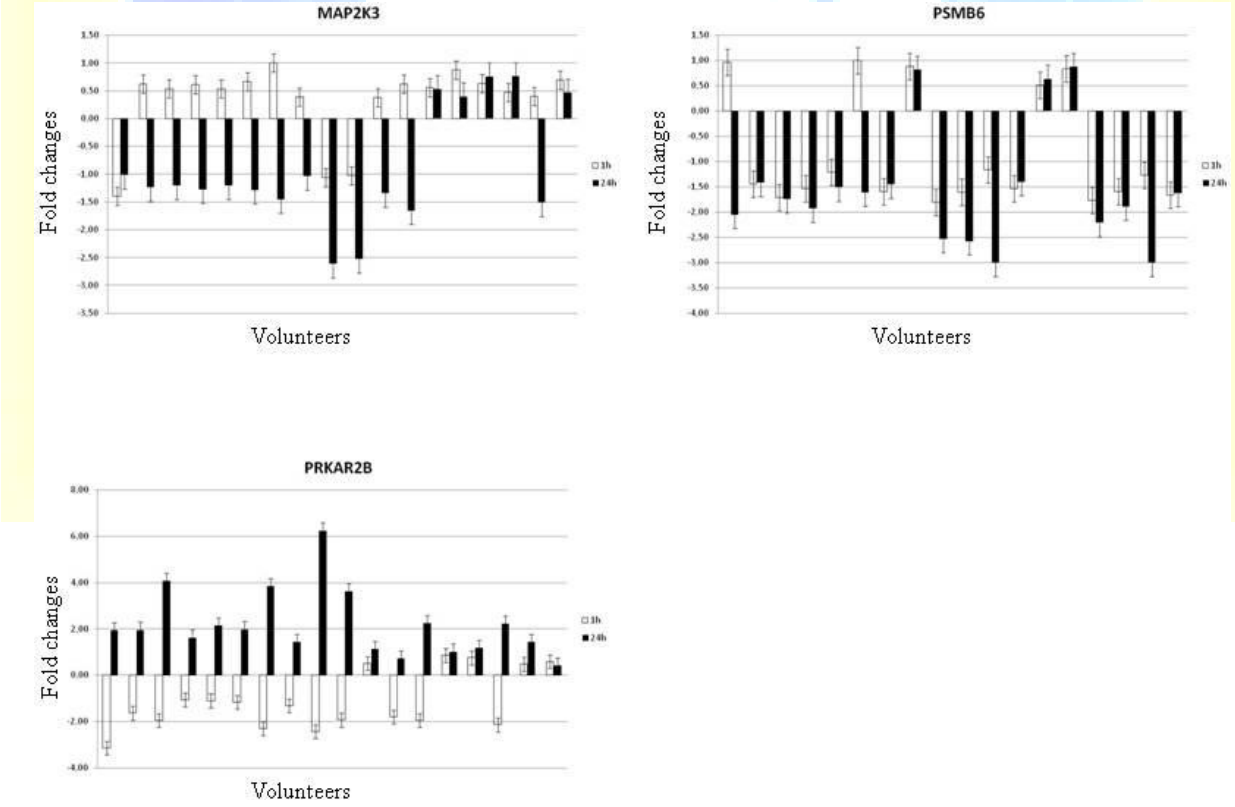

Figure 4:*The up and down regulated genes expressed 24 hours after the administration of the MHE.*

In order to minimize the effect of gene redundancy and to investigate functionally coherent gene-sets, such as biochemical pathways, an enrichment analysis was performed to find statistically over-represented significant genes of our lists and their biological meanings. The bioinformatics tools DAVID, KEGG and BioCarta were used to parse the data of this study. A gene list of enrichment GO terms and BioCarta pathways is given in Table 1. Bioinformatics analysis revealed four GO term significantly enriched pathways (corrected p-value <0.05): acetylation (39 genes), cytosol (27 genes), regulation of cell death (13 genes) and negative regulation of apoptosis (13 genes).

3.2 Real-time PCR analysis

In order to validate the microarray analysis, a series of RT-PCR experiments was performed using a subset of 11 genes in Table 2. Some genes were chosen because representative of specific and enriched GO pathways, with a preference for acetylation, cell cycle and regulation of cell death categories, or because they have specialized functions with a potential link with the mind-body relationship studied here.

Table 2. General annotation for differentially expressed genes identified after the HyT session.

Gene Name	EntrezGene	Ensembl* Chr	Start (bp)	End (bp)	KEGG
SNCA	6622	10	90405987	90428552	hsa01510 hsa05010 hsa05020
ALAS2	212	X	55052213	55074136	hsa00260 hsa00860
FBXO7	25793	chr22	31217773	31224798	
CA1	759	8	86427709	86451265	hsa00910
BCL2L1	598	11	129445011	12951991 0	hsa01510 hsa04210 hsa04630 hsa05030 hsa05212 hsa05220 hsa05222
HBB	3043	chr11	5203276	5204861	
HEMGN	55363	9	99728894	99746959	
FAM46C	54855	1	117950127	11797253 4	
HBD	3045	11	5203272	5212434	
WDR40A	25853	9	34076386	34124183	
MBNL3	55796	X	131339862	13145167 7	
SLC25A39	51629	17	39752549	39757722	
SLC4A1	6521	17	39682566	39700993	
TMOD1	7111	9	99303742	99403357	
BPGM	669	7	133982095	13401510 5	hsa00010
GYPA	2993	4	145249906	14528129 6	hsa04640
GYPC	2995	2	127130149	12717071 5	
EPB42	2038	15	41276720	41300773	
ALS2CR2	55437	2	202024637	20205369 5	
SNORA38B	100124536	17	63167247	63167377	
GMPR	2766	6	16346790	16403757	hsa00230
FECH	2235	10	3811260	3817455	hsa00860
YOD1	55432	1	205283817	20529294 8	hsa00632 hsa00903
EPB49	2039	8	21971322	21995982	
SELENBP1	8991	1	149603405	14961180 7	
MKRN1	23608	7	139799329	13982577 1	
MYL4	4635	17	42641427	42656044	
NUP62CL	54830	X	106253367	10633631 9	
ERAF	51327	16	31446680	31447624	
XK	7504	1	153445114	15345024 6	
CPT2	1376				hsa00071 hsa03320 hsa04920
ADIPOR1	51094	chr1	201176576	20118054 7	hsa04920
CSDA	8531	12	10742958	10767471	hsa04530
SCARNA5	677775	2	233849112	23384938 7	

Two histograms of the relative changes for all validated genes expressid are given in Figure 5. As well as microarray analysis, gene expression was detected for each voluntary at both conditions (condition B=1 h and condition C=24 h after MHE). At condition B, in the majority of the individuals, five genes (CALM3, PRKAR2B, PSBM6, PSMF1, ZFP36) were down-regulated (1-2 folds), while other four genes (CPT2, MAP2K3, SSB, ZNF24) were slightly up-regulated around one fold. The remaining genes (IKBKG and IL2RG) did not show any particular variation in their gene expression pattern at this condition (1 hour around 50% up regulated). At condition C, two out of the 11 selected genes (PRKAR2B, SSB) were over-expressed in all subjects. On the contrary, in the majority of the voluntaries, seven genes (ZFP36, CPT2, MAP2K3, PSMF1, PSMB6, CALM3 and IKBKG) were down-regulated, some of which up to three - four folds (CALM3, PSMB6), compared to condition A. IL2RG and ZNF24 genes showed a similar trend to that observed at condition B for IL2RG gene. Among the RT-PCR assayed genes, it is noteworthy that MAP2K3, IKBKG, CPT2 and PRKAR2B showed a characteristic trend, they inverted in fact their gene expression profiles. In particular, the first three genes were up-regulated at condition B, while they resulted down regulated at condition C. PRKAR2B gene, vice versa, was down-regulated at condition B while over expressed at condition C in Figure 1. The modulation of gene expression (up or down-regulation) of the selected differentially RT-PCR assayed genes, detected by microarray analysis, was confirmed for the majority of them (8 out of 11), in particular at condition C. Moreover, fold change values, revealed by RT-PCR analysis, were in several cases higher than those observed with microarray analysis.

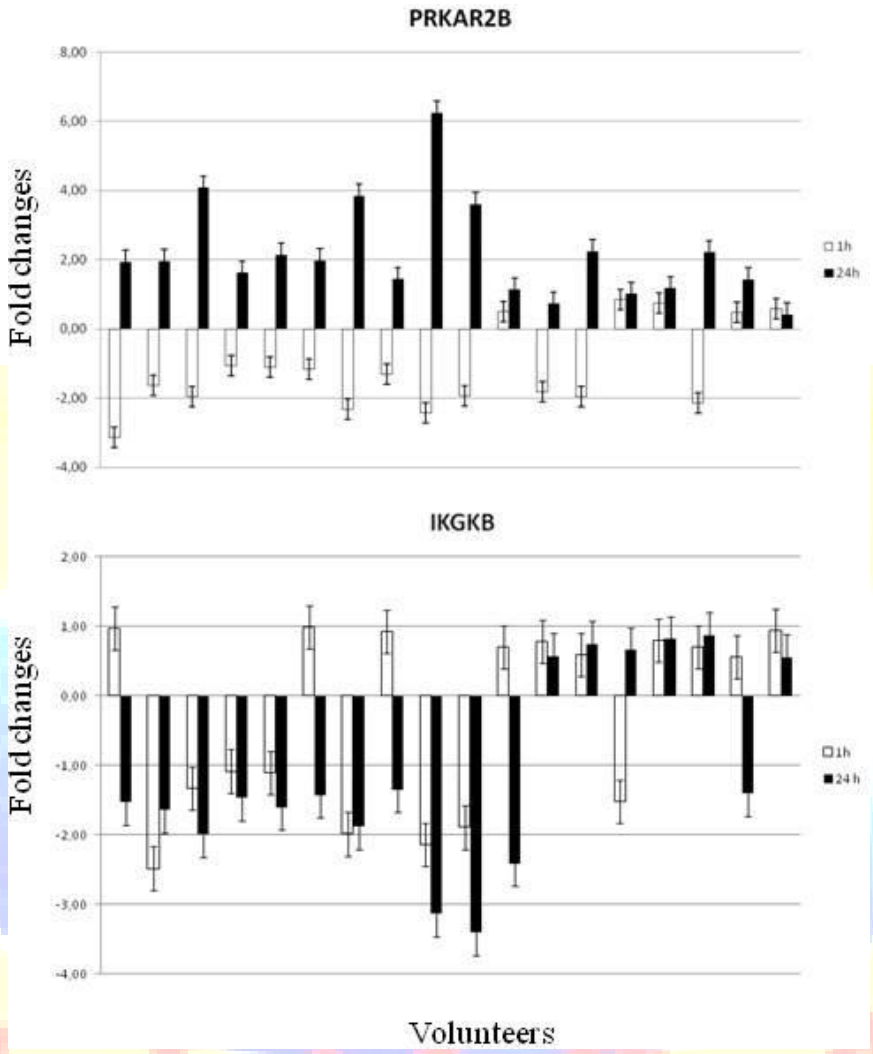

Figure 5:*Two histograms of the relative changes in gene expression.*

3.3 Functional analysis of differentially expressed genes

To facilitate interpretation of the general response pattern, the differentially expressed 200 genes including those assessed by RT-PCR, were assigned to specific cellular pathways according to their best-characterized KEGG biological function, in order to identify those pathways where the genes could be active. This analysis allowed us to identify 5 KEGG pathways: MAPK-, NFKB-, Ca-, T cell-signaling and cell death. As reported in Table 3, some genes were present and differentially expressed in more than one pathway. Since physiological processes are not

separated within cells, active genes, in more than one pathway, suggest a possible connection between KEGG pathways at biochemical level in Figure 5.

Sequences of oligonucleotide primers used for Real-time quantitative RT-PCR

Primer name	**Sequence** (5'-3')
RN18S For	ATGGCCGTTCTTAGTTGGTG
RN18S Rev	CGCTGAGCCAGTCAGTGTAG
IKBKG2 For	GGAGCTTCTGCATTTCCAAG
IKBKG2 Rev	CCTTCTGCCTCTTCAGATCG
IL2RG2 For	CTGGGGGAGCAATACTTCAA
IL2RG2 Rev	TCCGTTCCAGCCAGAAATAC
MAP2K32 For	GGAGCTCATGGACACATCCT
MAP2K32 Rev	CGCACGATAGACACAGCAAT
PRKAR2B1 For	GAGGGCACTTGGCAATTAAA
PRKAR2B1 Rev	CCAAGGCCAGCACATAACTT
PSMB62 For	CCTATTCACGACCGCATTTT
PSMB62 Rev	TTAAAGAGGCTGGCTGCTGT
PSMF12 For	GCCAAGCCTATGAAATTGGA
PSMF12 Rev	GGCAAGAACCACACAAGGTT
CALM3 For	GCATTCCAGAAGGACTGAGG
CALM3 Rev	CCAGTGGGATGTGAGTGTTG
CPT2 For	TGTGCCTTCCTCTCTGTCCT
CPT2 Rev	AGGTGTCTGGCCTTGTCATC
ZFP36 For	GGGACTTGGGGGACAGTAAT
ZFP36 Rev	ATCCCCCACCATCATGAATA
ZNF24 For	ACAGGTGGGCAAGTATCAGG
ZNF24 Rev	CACAGTGCCTTGTCGGTAGA
SSB For	ATCCAAGGCAGAACTCATGG
SSB Rev	AGTTGCATCAGTTGGGAAGC

Table 3:*Oligonucleotide primers used for Real-time quantitative RT-PCR used in this study.*

4. Discussion

The study of gene expression by microarray analysis has been useful to examine the whole genomic profile of leukocytes in voluntaries subjected to Mind-Body Healing Experience (MHE). Several studies have demonstrated that exists a straight communication between immune and central nervous system[15,16,32]. Microarray technology provides the characterization of gene expression in human blood and represents an interesting and readily available source of information on genes and their associated molecular pathways involved in the signaling between

physical and psychosocial environment and brain human functions. In our study we found several differentially expressed genes, demonstrating that MHE, a top-down creatively oriented positive human experience (via MHE protocol) can modulate gene expression in white blood cells. Our data are consistent with those produced by other teams demonstrating the high plasticity of circulating immune cells and the large effect on overall gene expression caused by stress, depression, disorders or neural diseases[33,34]. The analysis of our microarray data sets revealed that, at condition B, 90% of differentially expressed genes were down-regulated in all the MHE voluntaries, while only 10% were up- regulated. At condition C, a slight increase of those up-regulated (20%) was observed when compared with condition B. Therefore we can conclude that the MHE exerted a strong down-regulation of the gene expression as observed in many other studies[19,20]. Modulation of gene expression caused by MHE in leukocyte cells confirmed the hypothesis that a carefully specified protocol of therapeutic hypnosis (MHE) called MHE could modulate the expression of activity or experience-dependent genes to reduce symptoms of the stress related disorders and facilitate mind-body healing[11,21,22] as we previous reported in our pilot study[35] where we documented limited changes in the expression of few early responsive genes, within one hour after MHE. We hypothesized that the expression of early responsive genes by MHE induced a subsequent cascade of gene expression 24 hours later. Unternaehrer, et al[23] reported the modulation of two stress-related candidate genes, oxytocin receptor (OXTR) and brain-derived neurotrophic factor (BDNF), after acute psychosocial stress. Short-term DNA methylation changes in OXTR target sequences, but not in the target sequences of BDNF, were observed pre-stress, post-stress and 90-min follow-up stress measurement, suggesting a rapid and reversible modulation of gene expression in the blood white cells. These results are consistent with previous research that established how stress related distortions of the circadian cycle and the 90-120 minute basic rest-activity cycle are implicated in many psychiatric disorders and psychological dysfunctions[24].

Some of the differentially expressed genes (200) we detected throughout microarray analysis were related to relevant pathways, such as: MAPK signaling, NF-KB signaling, Ca signaling, cell death, T-cell signaling. Most of RT analysed genes were included in the designed pathways; other genes, though were not validated by quantitative RT-PCR, might play an important role in these pathways. The most enriched pathway in the present analysis was MAPK signaling. The mitogen-activated protein kinase (MAPK) cascade is a highly conserved module, that controls

eukaryotic gene expression programs in response to extracellular signals. This pathway transduces a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis[36]. Four main MAPK pathways have been recognized: ERK1/2, c-Jun, p38 (MAPK14), and ERK-5 (MAPK7). MAPK signaling is crucial during neuronal maturation and plasticity and, ultimately, to learning and memory[37,38]. Mutations in the genes of MAPK signaling pathway cause developmental dysfunctions during neural development, which could have an impact on the formation and functioning of the brain. They were detected in several syndromes associated to mental deficits. Recently, McMillan. et al[39] reported that Down and Fragile X syndromes, both associated to mental retardation and to mental disability, share similar dysregulation of specific genes, involved in some signal transduction cascades, during the development of early human neural progenitor cells (hNPCs). In particular, alterations in signaling pathways might affect the differentiation, growth, proliferation of hNPCs, with serious implications in brain development. Among genes participating to MAPK pathway, MAP2K3 (mitogen-activetd proein kinase-kinase 3) belongs to a dual specificity kinase group and is a specific upstream activator of the p38 MAPK protein. In particular, MAP2K3 phosphorylates and activates p38 during skeletal muscle development, chondrocyte differentiation and neuronal regulation. An expression profiling study on autistic patients, in fact, revealed some alterations of immune system-related genes, including MAP2K3 gene, recognizing them as autism susceptibility genes[40]. In our experiment, MAP2K3 gene inverted its gene expression at the two analysed conditions after MHE (1 hour, condition B and 24 h, condition C): it was down- and up-regulated at conditions B and C, respectively. Recent studies found that MAP2K3 up-regulation was involved in invasion and progression of tumors[41,42], however a down-regulation of MAP2K3 gene, together with others, was associated to a depletion of MYST4 gene (histone acetyltransferase MYST histone acetyltransferase 4), that induces a dysregulation of ERK1/2 signaling and, in general, leads to Noonan syndrome–like features, causing a mental retardation[43]. Even N-Ras gene (one of down-regulated gene identified by microarray), together with H-Ras and K-Ras, participated to MAPK pathway. N-Ras represents one of the members of the Ras gene superfamily, discovered in human tumors more than 30 years ago. Ras genes codified for GTPase proteins[44]. These small proteins are essential players in many cellular signaling networks placing as link-molecules between upstream and downstream cellular pathways, and regulating a large variety of cell functions, such as cycle

progression, growth, migration, senescence and apoptosis. Somatic activating mutations in Ras are associated with a growing number of cancers and pathological conditions[45]. Ras pathway mutations cause syndromes typically associated with severe developmental anomalies in various combinations of facial abnormalities, heart defects, short stature, skin and genital abnormalities, and mental retardation[46]. Abnormalities in N-Ras, H-Ras and K-Ras have been identified in several other syndromes, associated with a mental retardation, supporting the importance of Ras signaling in the development of intellectual processing[47–49]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is a family of transcription factors involved in the rapid regulation of many genes of the innate immune response, inflammation such as tumor necrosis factor-□ (TNF□ and interleukins), and antiapoptotic and development processes. The genes of NF-kB pathway involved the expression of inflammatory cytokines and chemokines and, in turn, the NF-kB is induced by them[50,51], establishing a positive feedback mechanism[52], that, if aberrantly active, can determine a chronic or excessive inflammation associated with several inflammatory diseases. Inflammation, in fact, potentially plays a major role in a number of neurological and psychiatric conditions. Direct evidence for an innate inflammatory response has been demonstrated in Alzheimer's disease (AD) [53], Parkinson's disease[54], multiple sclerosis and many others[55]. The activation of NF-kB was modulated by the inhibitor complex of kappaB kinase (IKK), which consists of different catalytic subunits, including IKBKG also known as NEMO gene. The NEMO protein lies at the top of this pathway and functions as a molecular conduit, connecting signals transmitted from upstream sensors to the downstream NF-kappaB transcription factor and subsequent gene activation. The identification of several effectors that link NF-kappaB signaling to additional pathways and the role of NEMO within this pathway was recently reported[56]. Mutations in the NEMO gene on chromosome Xq28 cause Incontinentia pigmenti[57], a severe X-linked dominant genodermatosis that is lethal in males; in females, it has a highly variable and often severe clinical presentation always associated with skin defects, but also multiple and neurological abnormalities[58]. In the present study, a significant difference in gene expression levels was detected for IKGKB gene at both condition (B and C). NF-kappaB signaling can also modulate the expression of genes involved in inflammation response, inducing either up-regulation or down-regulation of cytokine receptors. Inflammation is part of the innate immune response triggered by infection or injury. Numerous different processes, including activation of cell death mechanisms apoptosis, the removal of dead cells and the promotion of

wound healing, aimed cell and tissue homeostasis and protection by stimulating the release of cytokines[59]. In the periphery, cytokines coordinate complex components of the immune response including the innate and adaptive immune responses. In the brain, cytokines are responsible for neuronal and neuroendocrine pathways: the central nervous system regulates the immune system and, in turn, the immune system signals modulate the brain through neural and humoral routes. Among cytokines, the interleukin (IL)-2 family consists of at least six members and shares the common gamma chain codified by IL2RG gene[60]. This gene is located on the X chromosome in mammals and its defects cause X-linked severe combined immunodeficiency[61]. Several studies report peripheral immune alterations in patients with neurological and neuropsychiatric diseases and that dysregulation of cytokine pathways was involved in many human diseases such as depression[62], schizophrenia, and can affect many behaviors, including effects on sleep, appetite, sex, memory. Furthermore, the observation that neuro-inflammation induces a robust up-regulation of IL2Rγ emphasized the role of IL2Rγ in the brain and its important role in human behavior[63].

Cells have a Ca signaling toolkit with many components that can be mixed and matched to generate a wide range of spatial and temporal signals. This versatility is exploited to control processes as diverse as proliferation, development, learning and memory. In particular, Ca2+ function is well illustrated in lymphocytes responding to antigens trough increasing of proliferation. Ca2+ influx from the environment, or release from internal stores, causes a very rapid and dramatic increase of cytoplasmic Ca concentrations, as signal transduction. Some proteins bind directly and sense Ca2+. However, in other cases Ca2+ is sensed through intermediates such as calmodulin (CALM). This Ca2+-binding protein is most pervasive in mediating the control of a large number of enzymes, ion channels and other proteins, as kinases and phosphatases, activating cascades essential for many common physiological processes. In our study, CALM3 gene involved in Ca2+ signaling pathway has been detected by microarray analysis and also assayed in RT PCR. CALM3 gene was strongly down-regulated after MHE at both condition (B and C). The misregulation of Ca2+/CALM-dependent kinases can lead to a variety of disease states, such as cell proliferation and apoptosis, or cancer. Moreover, neuronal growth and function related to brain development, synaptic plasticity as well as memory formation and maintenance can be modified as result of gene cascade alteration[64]. Some reports indicated that alterations in Ca2+/CALM signaling pathway are involved in the control of

proliferation and survival of immortalized lymphocytes from Alzheimer's disease (AD) patients. The disruption of Ca signaling causes the neuronal dysfunction and ultimately apoptosis[65]. A recent study reports that the genes codifing CALM proteins are not strongly modulated in their expression, although the lymphocytes of the patients were characterized by high level of CALM protein. Probably, CALM degradation in AD patients was impaired, and thus Ca2+/ CALM-dependent signaling pathway may be important in the etiopathogenesis of the AD[66].

Programmed cell death (PCD) is genetically controlled mechanism that may balance cell death with survival of normal cells. Three main forms of PCD can be easily distinguished: apoptosis, autophagy and programmed necrosis[67]. Apoptosis, in particular, was first described by Kerr, et al[68]. It can be associated to specific morphological and biochemical changes of cells, including chromosomal DNA cleavage, nuclear condensation and fragmentation, alterations of dynamic membrane and loss of adhesion caused by proteolysis. Four genes (BCL2L1, PRKAR2B, IKBKG=NEMO, RELA) of PCD pathway were evidenced by microarray analysis in our voluntaries. All of them are strongly modulated by MHE suggesting an important role in the regulation of the life cycle of leukocytes. Other than NEMO (see above), another gene PRKAR2B (Protein kinase, cyclic adensine monophosphate-dependent, regulatory, type II beta) was assayed in RT PCR. A strong down-regulation in almost all voluntaries was detected just after 1h MHE (condition B), whilst its expression showed an opposite trend reaching in some voluntaries four-six folds up-regulation. PRKAR2B participates as regulator to the cAMP-dependent protein kinase system that controls the cellular effects of cAMP, a second messenger in many signaling cascades. Recently, a genome-wide study identified 21 markers related to antipsychotic-induced side effects, involving also PRKAR2B gene[69]. This gene results an important mediator both in the regulation of metabolism and the transducing of the antipsychotic effect. Moreover, it is well established that cAMP signaling regulates behavior and several evidences strongly suggest that the aberrant pathway was correlated to biochemical phenotypes associated with bipolar disorders[70]. Many of the previous described genes are also involved in the T-cell signaling pathway, that plays a key role in the immune system. The T cell receptors (TCR) constitute a complex of integral membrane proteins that participates in the activation of T cells and in the formation of the immunological synapses in response to antigen exposure. The specific recognition and binding lead to activation of transcription and commitment of the T cells, resulting in a series of signaling cascades.

5. Conclusions

The MHE modulated the expression of many genes primarily through down-regulation. The data obtained through the analysis KEGG has shown a close correlation between the various pathways. We hypothesize a regulative cascade in consequence of MHE as shown in Figure 6. Of the 10 genes involved in the four pathways seven genes fall within the MAPK signalling. Three out of the seven are specific to the MAPK signalling pathway, two are shared with the T cell pathway (IL2RG and N-ras) and two with everyone. There are four genes involved in the cell death pathway, one is specific to this pathway (PRKAR2B) and three are shared with the NFKB pathway (BCL2L1, NEMO and RelA). Moreover two of the last three, NEMO and RELA, as previously said, are present in all four pathways. In addition Ca pathway could be directly connected to the other four pathways, and the gene calm3 is central in the regulation of this pathway. Therefore, we can assume that the Ca pathway plays a fundamental role in the modulation of the other four pathways it is connected to. In particular, this observation would suggest a close correlation between the modulation of the expression of the gene calm3 and that of NEMO and RELA genes, shared by the remaining four pathways, and therefore these three genes play a fundamental and central role in the response of leukocytes to MHE.

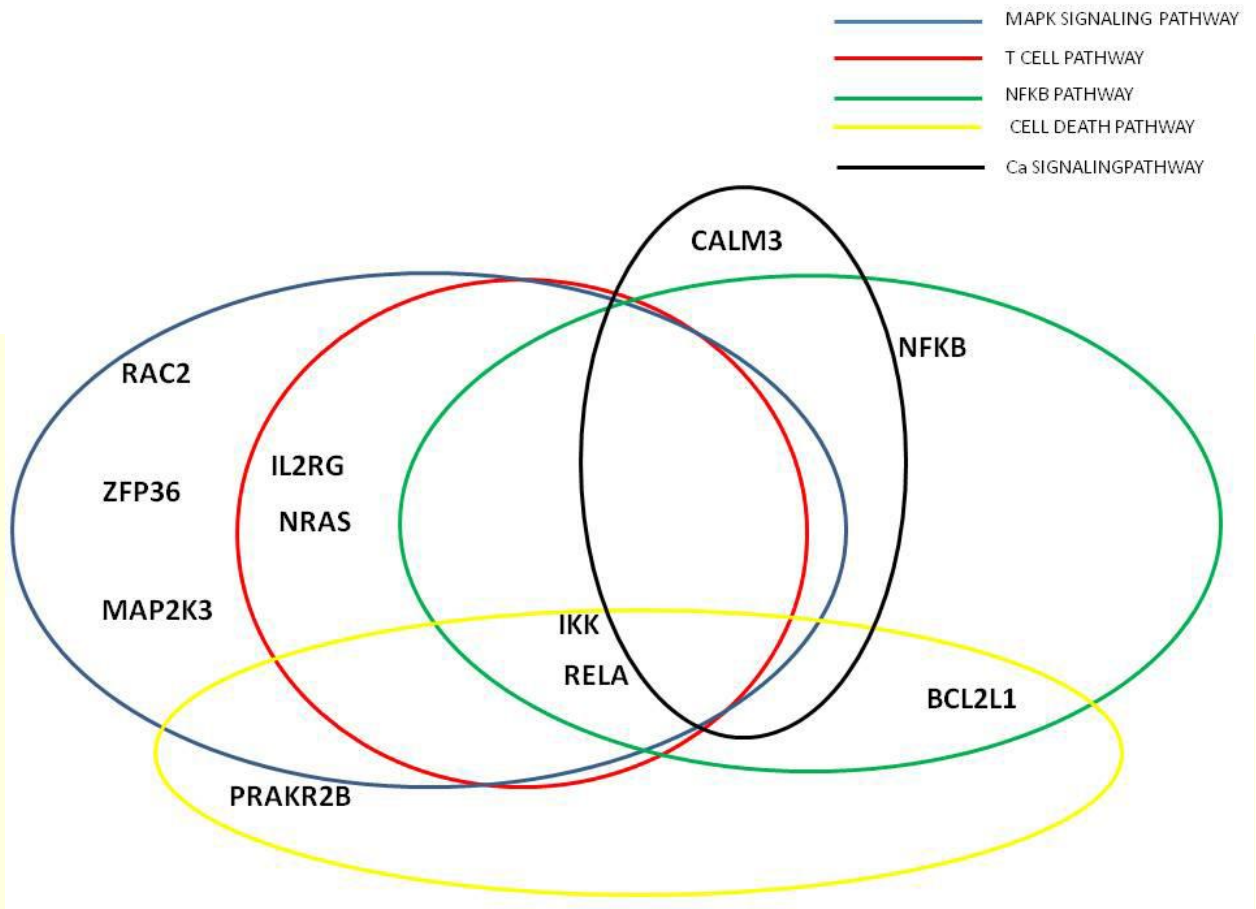

Figure 6:*A Venn diagram of the pathways associated with MHE.*

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