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CIGARETTE SMOKE EXTRACTS INDUCE AND REPRESS GENES IN HUMAN EMBRYONIC LUNG CELLS

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ABSTRACT: Disastrous effects of tobacco consumption on health remain in the focus of preclinical and clinical research. Exposures to tobacco smoke are closely connected to acute and chronic smoke lung dysfunctions and the effects are not restricted to airway inflammation processes. The facts that smoke exposure result in aberrant gene expression and changes in cellular phenotype resulting in clinical patterns. Here we pyrolyzed constituents of tobacco smoke, induced a stress response in human embryonic lung (HEL) cells, which respond with an altered expression of a broad spectrum of genes. We systematically analyzed the genetic expression, using the microarray-technology. After exposure of HEL cells to alkaline or acidic extracts of pyrolyzed smoke, already 2h after exposition the most affected genes (*HMOX*1, *CYP1B*1, *ID*3, and *ID*2) were rapidly up-regulated, whereas after 24 hours the genes were almost down-regulated. Using DAVID bioinformatics we detected annotation clusters with significant enrichment scores allowing insight into pharmacological processes and molecular functions. In the alkaline and acidic probes in a ratio 24 h versus 2h we identified annotation clusters with enrichment scores (between 5.77 and 2.89) representing upregulated genes. A negative transcription control leads to the conclusion that a "loss of function" can be possible. STRING tools give insight into the functional network of the gene products of affected genes. Noticeable is the fact that the detected genes with late response encode predicted proteins with unknown function (LOC100134504, LOC645157, LOC653156). With Blast2GO we generated and analysis and graphs of reliable functions.

INTRODUCTION: The WHO reported in 2011 data on the global tobacco epidemic and admonished of the danger and the consequences of the tobacco smoke: Up to half of all tobacco users will die from tobacco-raised disease. Twelve million people world-wide got cancer in one year,about 7 million patients died by cancer per anno, 5.4 million thereof by tobacco smoke [1] The Centers for Disease Control and Prevention (CDC) reported in the "Tobacco Control State Highlights" 2012 tobacco consumption to be the easiest preventable cause of death in the United States and is considered as the most prominent cause of preventable morbidity and premature mortality [2, 3]. Also smoking cigarettes and exposure to second hand smoke causes 443,000 or 1 in 5 deaths each year [4].

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Unfortunately, this horrific tendency seems not to affect the smoking habits significantly. Beyond doubt, tobacco smoke may be considered as a "cocktail of poison" composed of numerous pathogenic components classified as a main reason for cancer. In the cigarette smoke nearly seven thousand different and in part highly toxic constituents were isolated. They act as chemical stressors and affect organs and organ systems. The substances in the tobacco smoke can lead to multiple interactions in organisms and can result in toxic and epigenetic effects [5]. A defined cut-off value, which schedules the innocuousness of the tobacco smoking, is not defined because of the high content of toxic components in the tobacco smoke. Indeed a study evidenced, that the tobacco smoke of three cigarettes per day can enhance the risk for cardiac infarction to 2-3 fold [6, 7].

The fact that the search with "tobacco filter" of 2013 July in the NCBI database PubMed solely found 495 publications from 1953 to 2013 revealing the necessity and motivation for more intensive investigation of the filter development. The substantial potential of cigarette filters and their use is well known, but almost undocumented.

The smoke-caused injury reduction by use of filters was first published in 1953 by Schmidt-Voigt [8]. Recently the Streck group excellently reviewed the well-known facts, which were neglected by the tobacco industry [9]. The choice of the filter material for regulation of the toxicity of cigarette smoke was documented by Laugesen and Fowles in 2005 [10].

A further factor reducing the danger of tobacco smoke ingredients is the introduction of ion exchange resin in the filter material whose surface was amine functionalized. The reduction of aldehydes and hydrogen cyanine in mainstream cigarette smoke was documented by the Branton group in 2011 [11]. In 2012, the Soo group published an analysis of mainstream smoke emission of an especially designed new "super slim" cigarette type [12].

In view of our data in this publication further criterions should be illustrated: The pH-values of different types of tobacco show a wide range and were documented already in 1930: Orient-derived tobacco types generate an acidically reacting main-stream smoke, whereas the tobacco plants cultivated in Havana, Brazil and Java produce a rather alkaline reacting smoke. It should also be noted that all kinds of tobacco, also common tobaccos producing acidic mainstream smoke, generate an alkaline reacting bypass smoke [13].

The tobacco alone seems not to be the origin of additional solid-volatile components, but there are products of smoulder processes of carbohydrates which are either indissoluble (cellulose) or dissoluble in water (sugar). These products increase the amount of acidically reacting particles of the tobacco smoke [13]. The following questions and remarks are to be elucidated and answered in this context:

1. To what extent may the gene expression be impacted by these tobacco smoke constituents?

In which manner "Omics" technologies may permit a toxicological estimation and weighting?

2. Generally it is assumed, that genomics studies should document a change of the differential gene expression as stress response after exposition of cells to tobacco smoke constituents. Is this change accompanied with toxic effects and vice versa by the adverse reactions and their gene expression?

3. Is there a clear demonstration of undesired adverse reactions and detectable effects on organs, like neuronal tissue, liver, kidney and bladder, after exposition to the tobacco smoke components?

We recently demonstrated, to what extent the use of commonly used filters can retain pyrolyzed smoke residues [14]. The tobacco smoke residues, contained in the cigarette filters, were extracted with a neutral solvent and after removal of the solvent the remaining pellet was dissolved in RPMI cell culture medium and immediately applied to human embryonic lung cells (HEL). The cellular stress response was estimated with the change of the differential gene expression using bioinformatics-based micro arrays. Here, additional insight into the gene expression behaviour of the already investigated HEL cells should be given by application of extracts with acidic and alkaline solvents under identical treatment conditions.

EXPERIMENTAL SECTION:

Cell culture: Human embryonic lung cells (HEL) (DKFZ Dept. B040) were cultured and maintained in RPMI (Gibco-BRL, Germany) and 10% fetal calf serum (Gibco-BRL, Germany) at 37°C in a CO_2 atmosphere.

Gene characterization using NCBI genomic databases:

- Reference Sequence database (RefSeq Version 8, www.ncbi.nlm.nih.gov/RefSeq/)

The collection aims to provide a comprehensive, integrated, well-annotated set of sequences, including genomic DNA, transcripts etc. RefSeq provides references for genome annotations, gene identification and characterization, expression studies, and comparative analyses.

- GenBank Version 9.0 (www.ncbi.nlm.nih.gov/genbank/)

This is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences.

Tobacco smoke (TS) constituents' collection: The TS was dissolved in acetonitrile by shaking until the smoke was taken up by the solution. The smoke derived from 100 cigarettes (extract 2.5g). The dissolved residues were concentrated by use of a rotary evaporator (400 mbar, 38 °C) and, after desiccation with N_2 (1.5 - 2 bar) until total removal of the acetonitrile. The dry probe was stored in a freezer at -24°C.

Application of tobacco smoke residues and the filter extracts on HEL cells & RNA Isolation: The pyrolyzed tobacco smoke was kept on the HEL cells for 2 h & 24 h in a final dilution of $1:10^5$ in cell culture medium. As controls untreated HEL cells were used. HEL cells were cultured as described in the methods section above. The pyrolyzed smoke residues were dissolved in medium and applied to the HEL cells in a final dilution of $1:10^5$. The culture medium was removed after 2 h or after 24 h. Cells were washed with Hank´s balanced salt solution.

Total RNA from the HEL cells was isolated using the Qiagen RNeasy Plus Kit (Qiagen Hilden, Germany) according to the manufacture's instruction as follows: The cells were washed twice with Hank´s solution and extracted with 1ml of Qiagen extraction solution. The cells were scraped and transferred into micro centrifuge-tubes, then the cells were homogenized by squeezing though a needle 0.9 × 12 mm followed by centrifugation through a DNA-Eliminator column for 30 sec at 10.000 rpm in a micro centrifuge. An equal volume of 70% ethanol was added to the eluate, mixed with a pipette and applied to the RNeasy spin column. This was eluted with 700 µl RW1-buffer by centrifugation for 15 sec at 10.000 rpm followed by addition of RPE-buffer 2 × 500 µl and centrifugation at 10.000 rpm for 15 sec.

Then, the column was centrifuged to dryness and eluted by centrifugation with water (2 × 30 µl) for 30 sec and 2 min respectively. RNA was resuspended/eluted in water. The quality of total RNA was checked by gel analysis using the total RNA Nano Chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). The samples with RNA index values greater than 7 were selected for expression profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Probe Labeling and Illumina Sentrix BeadChip array Hybridization: Biotin-labeled cRNA samples for hybridization on Illumina Human HT12- BeadChip arrays (Chip IDs: 7196798076/ 8136640040 (illumina_humanht-12_v4_r2) (Illumina, Inc. San Diego, CA) were prepared according to Illumina's recommended sample labeling procedure based on the modified Eberwine protocol[15]. In brief, 500ng of total RNA was used for complementary DNA (cDNA) synthesis, followed by an amplification/labeling step (*in vitro* transcription) to synthesize biotin-labeled cRNA according to the Illumina® Total Prep™ RNA Amplification Kit (Life Technologies). Biotin-16-UTP was purchased from Roche Applied Science, Penzberg, Germany. The cRNA was column purified according to TotalPrep RNA Amplification Kit, and eluted in 60-80 µl of water. Quality of cRNA was controlled using the RNA Nano Chip Assay on an Agilent 2100 Bioanalyzer and spectrophotometrically quantified (NanoDrop).

Hybridization was performed at 58°C, in GEX-HCB buffer (Illumina Inc.) at a concentration of

100 ng cRNA/µl, in a wet chamber for 20 h. Spike-in controls for low, medium and highly abundant RNAs were added, as well as mismatch control and biotinylation control oligonucleotides. Microarrays were washed once in High Temp Wash buffer (Illumina Inc.) at 55°C and then twice in E1BC buffer (Illumina Inc.) at room temperature for 5 minutes (in between washed with ethanol at room temperature). After blocking for 5 min in 4 ml of 1% (wt/vol) Blocker Casein in phosphate buffered saline Hammarsten grade (Pierce Biotechnology, Inc., Rockford, IL), array signals were developed by a 10-min incubation in 2 ml of 1 µg/ml Cy3-streptavidin (Amersham Biosciences, Buckingham-shire, UK) solution and 1% blocking solution. After a final wash in E1BC, the arrays were dried and scanned.

Scanning and data analysis: Microarray scanning was carried out using an iScan array scanner. Data extraction was done for all beads individually, and outliers were removed when the absolute difference to the median is greater than 2.5 times the median absolute deviation (2.5 Hampel's method). All remaining bead level data points were quantile normalized[16]. Amplified RNA syntheses from limited quantities of heterogeneous cDNA were performed using the free statistics software environment R. As test for significance the student's t-test was used on the bead expression values of the two groups of interest. In the case of significance of expression against background we carried out a quality control (data not shown here). In both cases the Benjamini-Hochberg correction [17] was applied to the complete set of p-values of all Probe IDs on the chip. The average expression value is calculated as mean of the measured expressions of beads together with the standard deviation of the beads.

We used an integrated genomics profiling and computational biology based strategy to identify the key genes and gene clusters whose expression was altered after exposure to pyrolyzed tobacco smoke residue.

Chip layout & heat maps: With our data we designed heat maps as described as follows:

The quantile normalized probe intensities have been used to calculate the heatmaps using R (heatmap.2 function). Only the probes with fold-change better than 2 and Benjamini-Hochberg corrected p-value smaller 0.001 have been used. To maintain clearness the fold-change cutoff has been adjusted to keep the number of displayed probes below 20 as shown in Figure 1.

The heat map illustrates the analysis of our Illumina Array data reflecting the differential gene expression in HEL cells measured as given in **Table 2- Table 7**. The cluster analysis, established by Eisen [18], describes the genes according to their pattern of gene expression (induced – red; down regulated – blue) with known function. However the array also includes genes with still unknown function.

[Up-regulated genes are also displayed in red; down-regulated genes in blue.

(down-regulated  up-regulated)]

Dendrogram of the normalized data: A dendrogram visualizes the 'Pearson' distance between the measured samples and reflects the experimental setting of the replicates.

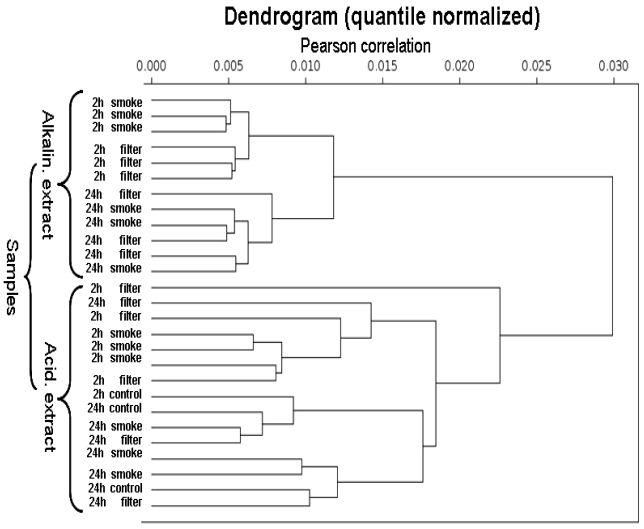


Figure1: The dendrogram illustrates the cluster-presentation of the quantile normalized data of the alkaline and the acidic smoke and filter extracts of HEL cells which showed a differential gene expression profiling. The visualized Pearson correlation coefficient reflects the degree of the correlation of experimental setting the replicates **Table 1.**

Bioinformatic treatment of the sequences:

Differential analyses: Detailed data can be found in supporting information (Fold change, gene loci, size of changes and p-values of groups against controls are listed).

The tables of the major affected genes offering the top fold changes are characterized and were subdivided into sections as shown in the Tables 2 – 7, whose "biological functions" are described in the text according to the Gene Ontology (GO) terms[19-21]. Most of the regulated genes are classified in the annotation category: biological process (BP).

Protein/protein interactions

STRING is a search tool for the retrieval of interacting genes/proteins [22; 23]. It quantitatively integrates interaction data from sources, like genomic context, array-based gene expression data and literature data. The STRING database allows the presentation of the networked information of the identified genes via protein-protein interactions including direct (physical) and indirect (functional) associations. The graphical presentation of the STRING data is shown in **Figure 3.** The significant annotation clusters of the most affected genes were packaged. The normalized data (Benjamini & best p-values; cut off at 1.0E-3) were condensed and represented in **Table 8-11**. The graphical determination and illustration of the affected genes as genetic groups following GO annotations are shown in **Table 12**.

For a deeper insight into the genome network, we used the Blast2GO tool (http://www.blast2go.com/b2glaunch) for functional annotation of FASTA nucleotide sequences (http://husar.dkfz-heidelberg.de/menu/w2h/w2hdkfz/). The tool Blast identifies homologous sequences. MAPPING retrieves GO terms. ANNOTATION performs reliable GO functions and enrichment scores. ANALYSIS produces the graphical display of annotation data with GO graphs (http://www.ncbi.nlm.nih.gov/omim).

The determined gene clusters were defined for annotation, visualization and integrated discovery using the Database for Annotation, Visualization and Integrated Discovery (DAVID). The Database provides functional annotation tools to understand biological meaning behind a large list of genes [24, 25] (http://david.abcc.ncifcrf.gov/home.jsp).

RESULTS: We started with the measurements of the top fold changes which represent the most affected genes in compliance with the chip layout as demonstrated in **Table 1**.

TABLE1: **LIST OF GENE COMPARISON FOR TABLE 2-7**

	Table	Group comparison
Alkaline extract	**2**	smoke_2h / smoke 24h
	3	smoke_2h / control 2h
	4	smoke_24h / control 24h

	Table	Group comparison
Acid extract	**5**	smoke_2h / smoke 24h
	6	smoke_2h / control 2h
	7	smoke_24h / control 24h

The left column of the Table 1, called "Table" lists the numbers (2-7) of the following tables listing the major affected genes.

The tables of the top-10 of the most affected genes are listed according the fold changes with the Benjamini-Hochberg corrected p-values. The Probe IDs are sorted in descending fold change order and correspond to the order as illustrated in the heat mapsfigure. All quality controls (QC) of the Illumina chip expression analysis, hybridization controls, negative controls, etc. of our samples were performed (data not shown).

The differentiation between alkaline and acidic extracts of smoke makes sense as shown in the following coloured graphs. The data show the mean values of the modified gene expression of the 2h probes vs. untreated controls

I. The probes 24h vs. control and

II. The ratio of the 24h versus 2h

III. Probes of the smoke extracts.

Heat map of the smoke extract efficiency:

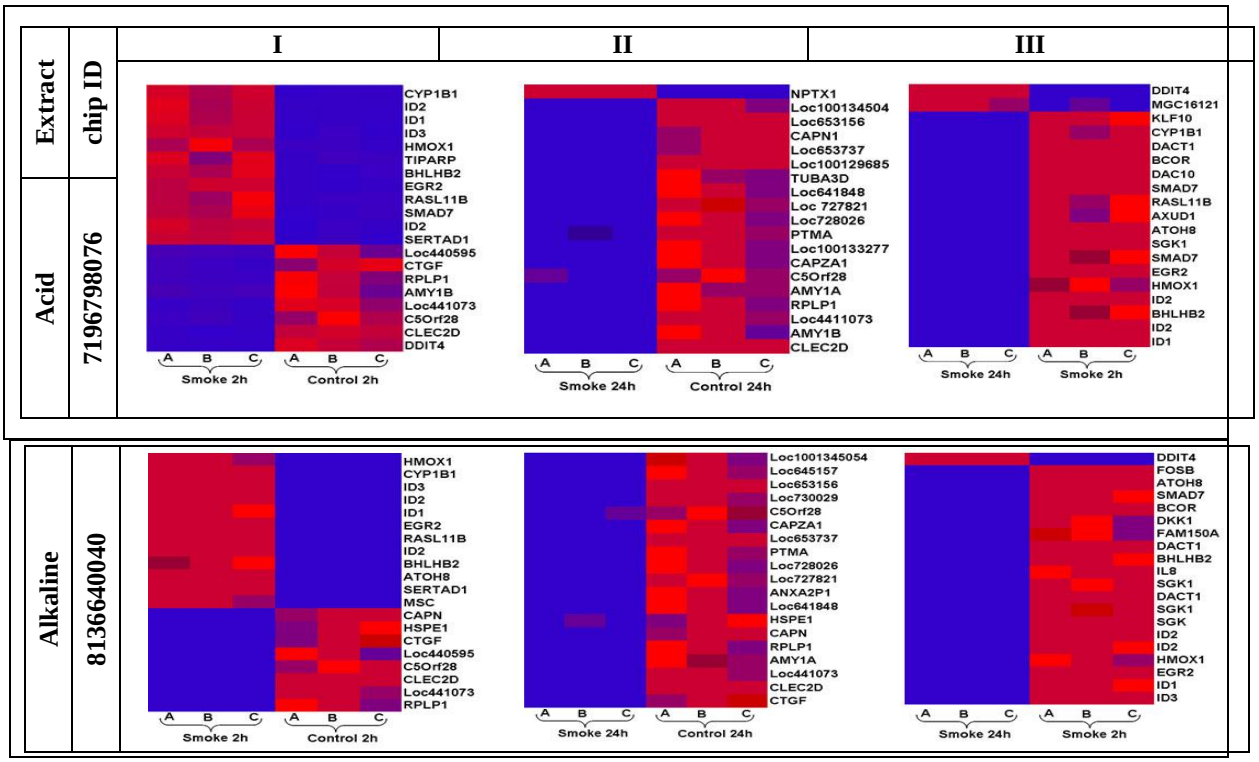


FIGURE 2: THE UPPER/LOWER LINES PRESENT OUR CHIP LAYOUT REPRESENTING THE ACIDIC/ ALKALINE EXTRACTS OF THE SMOKE.

The columns show the data of the mean values of the 2h probes (I) and the probes 24h (II) vs. the corresponding untreated controls, the right columns presents the modified gene expression ratio of the 24h versus 2h (III) probes of the smoke extracts.

As shown in **Table2** in the alkaline extract probes displaying the comparison of the early response (2h) with the late (24h) gene expression we detected in the 10 most affected genes only one gene, which was upregulated with the highest fold change *DDIT0* 9.79. The following 9 genes (*FOSB*, *ATOH*8, *SMAD7 BCOR*, *DKK1*, *FAM150*A, *DACT*1, *BHLHB*2, *IL*8) show down regulated expression profiles (foldchange between 0.36 and 0.32).

TABLE 1: **DIFFERENTIAL GENE EXPRESSION BY ALKALINE EXTRACTS RATIO 24H/2H**

Physical map	Symbol	Mean		Fold change	Definition
		24h	2h	24h vs. 2h	
10q22.1[R]	***DDIT4***	1774,09	181,15	**9,79**	Homo sapiens DNA-damage-inducible transcript 4 (DDIT4), mRNA.
19q13.32[G]	***FOSB***	101,32	279,68	**0,36**	Homo sapiens FBJ murine osteosarcoma viral oncogene homolog B (FOSB), mRNA.
1p11.2[R]	***ATOH8***	168,59	465,07	**0,36**	Homo sapiens atonal homolog 8 (Drosophila) (ATOH8), mRNA.
18q21.1[R]	***SMAD7***	144,1	414,8	**0,35**	Homo sapiens SMAD family member 7 (SMAD7), mRNA.
Xp11.4[R]	***BCOR***	174,79	519,43	**0,34**	Homo sapiens BCL6 co-repressor (BCOR), transcript variant 1, mRNA.
10q11.2[G]	***DKK1***	1280,42	3878,31	**0,33**	Homo sapiens dickkopf homolog 1 (Xenopus laevis) (DKK1), mRNA.
8q11.23[R]	***FAM150A***	158,03	498,35	**0,32**	Homo sapiens family with sequence similarity 150, member A (FAM150A), mRNA.
14q23.1[G]	***DACT1***	122,94	386,92	**0,32**	Homo sapiens dapper, antagonist of β-catenin, homol 1 (*Xenopus laevis*) (DACT1), mRNA.
3p26.1[G]	***BHLHB2***	589,61	1842,46	**0,32**	Homo sapiens basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.
4q13.3[G]	***IL8***	170,27	529,18	**0,32**	Homo sapiens interleukin 8 (IL8), mRNA.

The rapid response (2h) measurements after alkaline extracts treatment compared to the gene expression of untreated control cells **Table 3** show a clear up-regulation of the 10 genes (*HMOX1*, *CYP1B1*, *ID3*, *ID2*, *ID1*, *EGR*2, *RASL11B*, *BHLHB*2, *ATOH*8, *SERTAD*1, and *MSC* with a fold change between 9.38 and 3.57. The following identified genes (*CAPN*1, *HSPE*1, *CTGF*) showed a down-regulated expression profile (fold change between 0.27 and 0.26).

TABLE 2: **DIFFERENTIAL GENE EXPRESSION BY ALKALINE EXTRACTS RATIO 2H/CONTROL**

Physical map	Symbol	Mean		Fold change	Definition
		2h	control	2h vs. control	
22q13.1	***HMOX1***	2472,9	263,54	**9,38**	Homo sapiens heme oxygenase (decycling) 1 (HMOX1), mRNA.
2p22.2[R]	***CYP1B1***	791,59	127,96	**6,19**	Homo sapiens cytochrome P450, family 1, subfam B, polypeptide 1 (CYP1B1), mRNA.
1p36.13[R]	***ID3***	6759,34	1182,31	**5,72**	Homo sapiens inhibit of DNA bind 3, domin neg helix-loop-helix prot (ID3), mRNA.
2p25.1[R]	***ID2***	3186,72	564,13	**5,65**	Homo sapiens inhibit of DNA bind 2, domin neg helix-loop-helix prot (ID2), mRNA.
20q11.21[R]	***ID1***	3942,24	780,83	**5,05**	Homo sapiens inhibit of DNA binding 1, dom neg helix-loop-helix prot (ID1), mRNA.
10q21.3[R]	***EGR2***	471,5	101,2	**4,66**	Homo sapiens early growth response 2 (Krox-20 homolog, Drosophila) (EGR2), mRNA.
4q12[R]	***RASL11B***	613,07	143,3	**4,28**	Homo sapiens RAS-like, family 11, member B (RASL11B), mRNA.
3p26.1[G]	***BHLHB2***	1842,46	475,26	**3,88**	Homo sapiens basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.
1p11.2[R]	***ATOH8***	465,07	120,5	**3,86**	Homo sapiens atonal homolog 8 (Drosophila) (ATOH8), mRNA.
19q13.1[G]	***SERTAD1***	2852,79	755,51	**3,78**	Homo sapiens SERTA domain containing 1 (SERTAD1), mRNA.
8q21[R]	***MSC***	851	238,2	**3,57**	Homo sapiens musculin (activated B-cell factor-1) (MSC), mRNA.
11q13.1[R]	***CAPN1***	252,54	947,09	**0,27**	Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA.
2q33.1[R]	***HSPE1***	292,52	1069,08	**0,27**	Homo sapiens heat shock 10kDa protein 1 (chaperonin 10) (HSPE1), mRNA.
6q23.1[G]	***CTGF***	1484,05	5745,51	**0,26**	Homo sapiens connective tissue growth factor (CTGF), mRNA.

The **Table 4** displays the probe with late gene expression stress response (24h). It is important to note that all detected genes offer a clear down-regulation (fold change between 0.32 and 0.29). Moreover, the major part of these genes transcribes mRNA coding for proteins with still unknown function (predicted).

The comparison of the early response (2h) with the late (24h) gene expression shows that it becomes apparent, that the acidic extract probes give an expression profile similar to the corresponding alkaline probe.

The list of the 10 most affected genes (arranged in descending fold change order) displays two up-regulated genes, *DDIT*4 and additionally *MGC16121*, with the fold change 9.83, and 3.65. Also detected in the acidic probe (**Table 5**) were (*SLC30A*1, *KLF*10, *CYP1B*1, *DACT*1, *BCOR*, *SMAD*7, *RASL*11B, *AXUD*1, *ATOH*8), with down-regulated expression profiles (fold change between 0.34 and 0.29).

TABLE 3: **DIFFERENTIAL GENE EXPRESSION BY ALKALINE EXTRACTS RATIO 24H/CONTROL**

Physical map	Symbol	Mean		Fold change	Definition
		24h	control	24h vs. control	
7	***LOC100134504***	539,45	1684,81	**0,32**	PREDICTED: Homo sapiens hypothetical protein LOC100134504 (LOC100134504), mRNA.
6p22.3	***LOC645157***	845,71	2617,41	**0,32**	PREDICTED: Homo sapiens misc_RNA (LOC645157), miscRNA.
4q28.1	***LOC653156***	749,98	2454,97	**0,31**	PREDICTED: Homo sapiens similar to hCG1782414 (LOC653156), mRNA.
1p21.1	***LOC730029***	906,46	3031,38	**0,3**	PREDICTED: Homo sapiens similar to hCG1997137, transcript variant 2 (LOC730029), mRNA.
5p12[G]	***C5orf28***	357,86	1188,05	**0,3**	Homo sapiens chromosome 5 open reading frame 28 (C5orf28), mRNA.
1p13.2[G]	***CAPZA1***	205,44	677,99	**0,3**	Homo sapiens capping protein (actin filament) muscle Z-line, alpha 1 (CAPZA1), mRNA.
16p13.2	***LOC653737***	146,11	481,54	**0,3**	PREDICTED: Homo sapiens hypothetical LOC653737 (LOC653737), mRNA.
2q37.1[R]	***PTMA***	367,7	1211,42	**0,3**	Homo sapiens prothymosin, α (PTMA), transcript variant 1, mRNA.
9q22.32	***LOC728026***	328,87	1136,17	**0,29**	PREDICTED: Homo sapiens hypothetical LOC728026 (LOC728026), mRNA.
4p14	***LOC727821***	463,39	1572,79	**0,29**	PREDICTED: Homo sapiens misc_RNA (LOC727821), miscRNA.

TABLE 4: **DIFFERENTIAL GENE EXPRESSION BY ACIDIC EXTRACTS RATIO 24H/2H**

Physical map	Symbol	mean		Fold change	Definition
		24h	2h	24h vs. 2h	
10q22.1[R]	***DDIT4***	1717,33	174,68	**9,83**	Homo sapiens DNA-damage-inducible transcript 4 (DDIT4), mRNA.
Xq26.3[G]	***MGC16121***	949,12	260,04	**3,65**	PREDICTED: Homo sapiens hypothetical protein MGC16121 (MGC16121), mRNA.
1q32.3[R]	***SLC30A1***	183,93	544,59	**0,34**	Homo sapiens solute carrier family 30 (zinc transporter), member 1 (SLC30A1), mRNA.
8q22.2[R]	***KLF10***	151	451,85	**0,33**	Homo sapiens Kruppel-like factor 10 (KLF10), transcript variant 1, mRNA.
2p22.2[R]	***CYP1B1***	314,74	997,93	**0,32**	Homo sapiens cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1), mRNA.
14q23.1[G]	***DACT1***	119,52	375,66	**0,32**	Homo sapiens dapper, antagonist of β-catenin, homol. 1 (Xenopus laevis) (DACT1), mRNA.
Xp11.4[R]	***BCOR***	163,83	512,45	**0,32**	Homo sapiens BCL6 co-repressor (BCOR), transcript variant 1, mRNA.
18q21.1[R]	***SMAD7***	134,5	471,86	**0,29**	Homo sapiens SMAD family member 7 (SMAD7), mRNA.
4q12[R]	***RASL11B***	197	685,14	**0,29**	Homo sapiens RAS-like, family 11, member B (RASL11B), mRNA.
3p21.3[R]	***AXUD1***	373,18	1291,3	**0,29**	Homo sapiens AXIN1 up-regulated 1 (AXUD1), mRNA.
1p11.2[R]	***ATOH8***	129,6	447,87	**0,29**	Homo sapiens atonal homolog 8 (Drosophila) (ATOH8), mRNA.

The rapid response (2h) measurement after cell treatment with the acidic extracts was compared to the untreated control cells **Table 6.** It shows a clear up-regulation of 10 genes (*CYP1B*1, *ID*3, *ID*2, *ID*1, *HMOX*1, *TIPARP*, *BHLHB*2, *EGR*2, *RASL*11B, and *SERTAD*1, similar to the corresponding alkaline expression profile with a fold change between 7.8 (*CYP1B*1) and 3.94 (*SERTAD*1). The identified

gene, *CTGF* already detected in the alkaline probe, is described here as *LOC440* encoding the mRNA with still unknown function, with a down-regulated expression profile (fold change between 0.25 and 0.24).

TABLE 5: **DIFFERENTIAL GENE EXPRESSION BY ACIDIC EXTRACTS RATIO 2H/CONTROL**

Physical map	Symbol	Mean	Fold change		Definition
		2h	Contr.	2h vs. Contr.	
2p22.2[R]	***CYP1B1***	997,93	127,96	**7,8**	Homo sapiens cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1), mRNA.
2p25.1[R]	***ID2***	4029	564,13	**7,14**	Homo sapiens inhibit of DNA binding 2, domin neg helix-loop-helix protein (ID2), mRNA.
20q11.21[R]	***ID1***	5052,93	780,83	**6,47**	Homo sapiens inhibit of DNA binding 1, domin neg HLH protein (ID1), mRNA.
1p36.13[R]	***ID3***	7348,14	1182,31	**6,22**	Homo sapiens inhibit of DNA binding 3, dominant negative HLH protein (ID3), mRNA.
22q13.1	***HMOX1***	1598,9	263,54	**6,07**	Homo sapiens heme oxygenase (decycling) 1 (HMOX1), mRNA.
3q25.31[R]	***TIPARP***	4056,27	688,55	**5,89**	Homo sapiens TCDD-inducible poly(ADP-ribose) polymerase (TIPARP), mRNA.
3p26.1[G]	***BHLHB2***	2529,67	475,26	**5,32**	Homo sapiens basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.
10q21.3[R]	***EGR2***	498,19	101,2	**4,92**	Homo sapiens early growth response 2 (Krox-20 homolog, Drosophila) (EGR2), mRNA.
4q12[R]	***RASL11***	685,14	143,3	**4,78**	Homo sapiens RAS-like, family 11, member B (RASL11B), mRNA.
18q21.1[R]	***SMAD7***	640,72	134,84	**4,75**	Homo sapiens SMAD family member 7 (SMAD7), mRNA.
19q13.1[G	***SERTAD***	2976,96	755,51	**3,94**	Homo sapiens SERTA domain containing 1 (SERTAD1), mRNA.
7q21.3	***LOC440***	437,45	1745,93	**0,25**	PREDICTED: Homo sapiens misc RNA (LOC440595), miscRNA.
6q23.1[G]	***CTGF***	1375,87	5745,51	**0,24**	Homo sapiens connective tissue growth factor (CTGF), mRNA.

Error! Reference source not found.**Table 7** shows the differential late gene expression (24h) and exhibits a similarity to the equivalent alkaline probe. It is demonstrative and important to note that all detected genes offer a clear down-regulation (fold change between 0.36 and 0.34) with the exception of *NPTX*1, which offers an up-regulated expression (fold change 2.82). We also established here, that the major part of genes transcribes mRNA coding for predicted proteins with still unknown function.

It is quite conspicuous, that the probes (alkaline as well as acidic) of the late response (24 h/ control) probe contain a high proportion "predicted genes". Despite the fact that DAVID gene analysis methodologies cannot characterize the "predicted" genes; all these findings should become more intensively investigated and interpreted.

Gene analyses using DAVID: DAVID bioinformatics resources then allow insight in biological and pharmacological mechanisms and account for a better understanding of the association of aberrant gene expression with gene-based changed phenotypes and diseases[26-27].

The corresponding DAVID cluster annotations are listed according to the GO terms dependent on their classification stringency "high". The DAVID tool identified two annotation clusters with significance (enrichment scores 3.66; and 3.1) of annotations (GOTERM_BP_FAT) in the list of affected genes in the 2 h probe extract versus untreated control

Table 8. The score annotation cluster 2 "biological process" (BP) detected genes acting as negative regulators. We used the established BlastiX program (NCBI Blast2GO, Version 2.6.2), for the appraisal of the FASTA sequences generated by HUSAR software.

Listed "gene bundles" with the Blast Expect Value with the cut off 1.0E-3 of the most affected genes were analyzed.

Clusters of the affected genes: The acidic extract probes (**Table 10**) contain annotation clusters with the highest enrichment scores and Benjamini values. The DAVID tool identified two annotation clusters with significant enrichment scores 3.66 and 3.1 of the annotation (DOTERM_BP_FAT) listed as affected genes in the 2h probe versus untreated control (Table 8).

TABLE 6: **DIFFERENTIAL GENE EXPRESSION BY ACIDIC EXTRACTS RATIO 24H/CONTROL**

Physical map	Symbol	Mean		Fold change	Definition
		24h	control	24h vs. control	
17q25.3[R]	***NPTX1***	815,95	289,03	**2,82**	Homo sapiens neuronal pentraxin I (NPTX1), mRNA.
7	***LOC100134504***	604,13	1684,81	**0,36**	PREDICTED: Homo sapiens hypothetical protein LOC100134504, mRNA.
4q28.1	***LOC653156***	880,98	2454,97	**0,36**	PREDICTED: Homo sapiens similar to hCG1782414 (LOC653156), mRNA.
11q13.1[R]	***CAPN1***	327,76	947,09	**0,35**	Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA.
16p13.2	***LOC653737***	166,76	481,54	**0,35**	PREDICTED: Homo sapiens hypothetical LOC653737 (LOC653737), mRNA.
17p13.2	***LOC100129685***	1193,91	3438,55	**0,35**	PREDICTED: Homo sapiens hypothetical protein LOC100129685, mRNA.
2q21.1[G]	***TUBA3D***	434,44	1235,3	**0,35**	Homo sapiens tubulin, α 3d (TUBA3D), mRNA.
7	***LOC641848***	298,11	847,04	**0,35**	PREDICTED: Homo sapiens similar to ribosomal protein S3a (LOC641848), mRNA.
4p14	***LOC727821***	556,46	1572,79	**0,35**	PREDICTED: Homo sapiens misc_RNA (LOC727821), miscRNA.
9q22.32	***LOC728026***	385,77	1136,17	**0,34**	PREDICTED: Homo sapiens hypothetical LOC728026 (LOC728026), mRNA.

TABLE 7: **CLUSTER ANALYSIS (DAVID) OF GENETIC RESPONSE BY THE ALKALINE EXTRACT 2H/CONTROL**

Annotation Cluster 1	**Enrichment Score: 3.66**			
Category	**Term**	**Count**	**p_value**	**Benjamini**
GOTERM_BP_FAT	blood vessel morphogenesis	5	5.1E-5	3.3E-3
GOTERM_BP_FAT	blood vessel development	5	9.1E-5	5.1E-3
GOTERM_BP_FAT	vasculature development	5	1.0E-4	5.0E-3

The table lists the annotation clusters 1and 2 with the enrichment scores 3.66 and 3.1 of the most prominent entries which offer the highest fold change values of the probe 2 h after tobacco smoke ingredients treatment in DAVID functional annotations analysis which gave clusters of GO term enrichment.

The DAVID tool identified in the 24 h probe, versus untreated control one annotation cluster (enrichment score 0.45) of the annotation "molecular function" (GOTERM_MF_FAT) in the list of affected genes with the terms "calcium ion binding", "metal ion binding", "cation binding", and "ion binding". But they do not fulfil the demands of the range of p_value and the Benjamini

cut offs, therefore we made no tabular mention of this data. In the alkaline extract probes 24 h versus 2h, the DAVID tool detected three annotation clusters with significance (enrichment scores 5.62; 4.12; and 3.55) (**Table 9**).

TABLE 8: **CLUSTER ANALYSIS (DAVID) OF GENETIC RESPONSE BY THE ALKALINE EXTRACT 24H/2H**

Annotation Cluster 1	**Enrichment Score: 5.62**			
Category	**Term**	**Count**	**p_Value**	**Benjamini**
GOTERM_BP_FAT	negat. regul. of transcription factor activity	5	1.0E-7	4.5E-5
	negative regulation of DNA binding	5	1.7E-7	3.8E-5
	negative regulation of binding	5	3.2E-7	4.6E-5
	regulation of transcription factor activity	5	3.0E-6	3.2E-4
	regulation of DNA binding	5	5.7E-6	3.5E-4
	regulation of binding	5	1.4E-5	7.8E-4
Annotation Cluster 2	**Enrichment Score: 4.12**			
Category	**Term**	**Count**	**p_Value**	**Benjamini**
GOTERM_BP_FAT	neg. regul. transcr. RNA polym. II promotor	6	4.9E-6	3.5E-4
	negat. regul. of transcript., DNA-dependent	6	2.0E-5	9.7E-4
	negat. regulation of RNA metabol. process	6	2.2E-5	9.5E-4
	heart development	5	5.5E-5	2.0E-3
	negative regulation of transcription	6	6.8E-5	2.3E-3
	negative regulation of gene expression	6	1.1E-4	3.3E-3
	neg. regulat. of nucleic acid metabol. process	6	1.1E-4	3.3E-3
	neg. reg. of nitrogen compd. metab. process	6	1.2E-4	3.3E-3
	neg. reg. of macromol. biosynthetic process	6	1.6E-4	4.0E-3
	neg. regul. of cellular biosynthetic process	6	1.8E-4	4.0E-3
Annotation Cluster 3	Enrichment Score: 3.55			
Category	**Term**	**Count**	**p_Value**	**Benjamini**
BP	regul. of transcript. RNA polym. II promotor	8	3.1E-6	2.7E-4

The Table lists the annotation clusters 1-3 with the enrichment scores between 5.62 and 3.55 of the most prominent entries which offer the highest fold change values of the alkaline extract probes of the ratio 24h versus 2h after tobacco smoke ingredients treatment.

The alkaline probe comparison of the ratio of the rapid and late gene expression detected three annotations cluster of the category "biological process" (BP) with the enrichment scores between 5.62 and 3.55 containing both terms "negative regulators" of transcription, metabolic (nucleic acid, nitrogen compound, macromolecular, and biosynthetic) processes.

In the acidic extract probes 2 h versus untreated control (**Table10**), the DAVID tool detected 3 annotation clusters which one significant enrichment score 3.16.

TABLE 9: **CLUSTER ANALYSIS (DAVID) OF GENETIC RESPONSE BY THE ACIDIC EXTRACT 2H/CONTROL**

Annotation Cluster 1	**Enrichment Score: 3.16**			
Category	**Term**	**Count**	**p_Value**	**Benjamini**
BP	negat. regul. of transcription factor activity	4	1.2E-5	4.4E-3
	negative regulation of DNA binding	4	1.8E-5	3.2E-3
	negative regulation of binding	4	2.8E-5	3.3E-3

The table shows the annotation cluster 1 with the enrichment score 3.1 which offers the highest fold change values of the probe 2h after treatment with acidic extracts of tobacco smoke ingredients versus untreated control.

In the acidic extract probes 24 h versus untreated control, the DAVID tool could not identify annotation clusters with significant enrichment scores. All annotations of the detected scores with "molecular function" are beyond the Benjamini Cut Off range (as described in the methods part).

The following annotation clusters with the enriched annotation "biological process" were identified in the probes 24 h versus 2h (**Table11**). The acidic probe which compared the ratio of the early and late gene expression detect three annotations cluster of the category with the enrichment scores between 5.77, 4.29, and 3.82 containing both terms "negative regulators" of transcription, metabolic (nucleic acid, nitrogen compound, macromolecular, and biosynthetic) processes, and "regulator" of transcription and DNA binding.

TABLE 10: **CLUSTER ANALYSIS (DAVID) OF GENETIC RESPONSE BY THE ACIDIC EXTRACT 24H/2H**

Annotation Cluster 1	Enrichment Score: 5.77			
Category	**Term**	**Count**	**p_Value**	**Benjamini**
BP	negat. regul. of transcription factor activity	5	7.5E-8	3.3E-5
	negative regulation of DNA binding	5	1.2E-7	2.8E-5
	negative regulation of binding	5	2.3E-7	3.4E-5
	regulation of transcription factor activity	5	2.1E-6	1.9E-4
	regulation of DNA binding	5	4.1E-6	2.6E-4
	regulation of binding	5	1.0E-5	5.8E-4
	negative regulation of molecular function	5	2.2E-4	5.1E-3

Annotation Cluster 2	**Enrichment Score: 4.29**			
Category	**Term**	**Count**	**p_Value**	**Benjamini**
GOTERM BP FAT	neg. reg. of transcr. RNA polym. II promotor	6	3.2E-6	2.4E-4
	neg. regul. of transcript. DNA-dependent	6	1.3E-5	6.6E-4
	neg. regul. of RNA metabolic process	6	1.4E-5	6.4E-4
	heart development	5	4.0E-5	1.6E-3
	negative regulation of transcription	6	4.5E-5	1.7E-3
	negative regulation of gene expression	6	7.1E-5	2.4E-3
	neg. reg. nucleic acid metabolic process	6	7.6E-5	2.4E-3
	neg. reg. of nitrogen compd. metabolic process	6	8.1E-5	2.4E-3
	neg. reg. of macromol. biosynt. process	6	1.0E-4	2.9E-3
	neg. regul. of cellular biosynthetic process	6	1.2E-4	3.1E-3
	negative regulation of biosynthetic process	6	1.3E-4	3.2E-3
	neg. reg. of macromol. metabolic process	6	4.1E-4	9.2E-3

Annotation Cluster 3	**Enrichment Score: 3.82**			
Category	**Term**	**Count**	**p_Value**	**Benjamini**
BP	regul. of transcript. RNA polym. II promoter	8	1.6E-6	1.8E-4

The table lists the annotation clusters 1-3 with the enrichment scores from 5.77 to 3.82 of the most prominent entries which offer the highest fold change values of the acidic extract probes of the ratio 24h versus 2h after tobacco smoke ingredients.

We consider that large groups of detected gene products with the term "negative regulator" are down regulated, which can result in a "loss of function" in case of tumor suppressor properties. This can lead to a reciprocally scenario documented as a "gain of function" is case of proto-oncogenic activation, as shown in Table 8 - Table 11. As a summary of the data of Table 8 - Table 11, we graphically designed a comparison of gene activation at the different time points with alkaline and/or acidic extracts.

The alkaline probe (**Table 12**; 2h/control, left column, upper line) graph shows the annotation – molecular function with the terms "sequence-specific DNA binding transcription factor activity" (GO:0003700) and "transcription regulator activity" (GO:0030528) which are represented by the genes *ATOH8*, *BHLHE40*, *EGR2*, *ID1*, *ID3*, *MSC*.

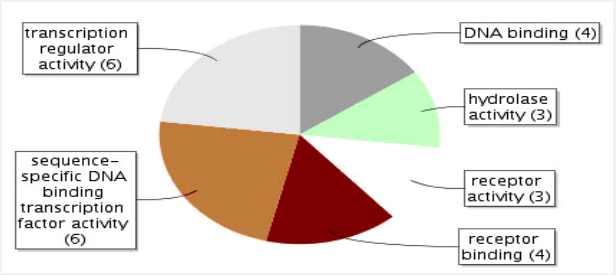
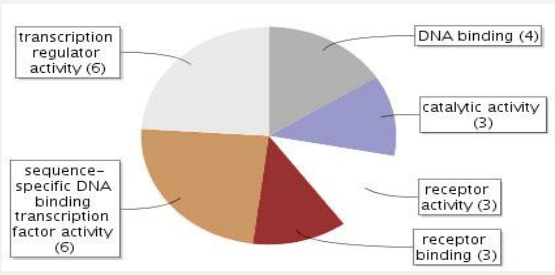
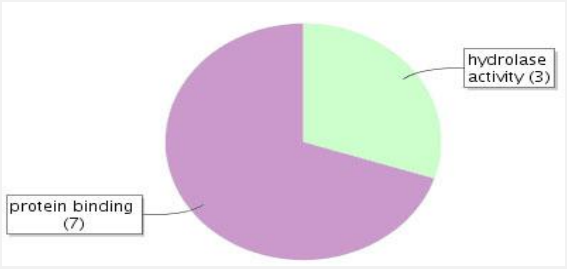
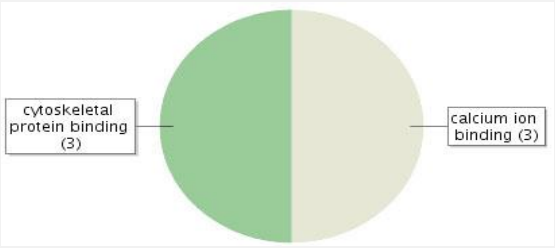
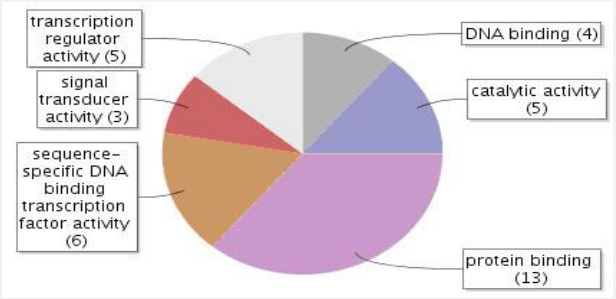
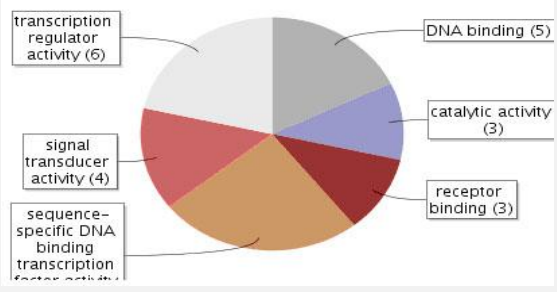
The corresponding acidic probe (**Table 12**; 2h/control, right column, upper line) graph shows the annotation – molecular function with the terms "sequence-specific DNA binding transcription factor activity" (GO: 0003700) and "transcription

regulator activity" very similar to the alkaline probe. Here three genes were involved in "catalytic activity" (GO: 0003824) *HMOX*1, *CYP1B1*, *CAPN1*. The long term probes (**Table 12**; 24h/control, left column, middle line) show the terms "hydrolase activity" (GO: 0016787) and "protein binding" (GO:0005515) (alkaline) with the affected genes *AMY1A*, *CAPN1*, and *CLEC2D*, *CTGF*, *CAPZA1*, *AMY1A*, *ANXA2*, *CAPN1*, *HSPE1*, *RPLP1* and "cytoskeletal protein binding" as well as "calcium ion binding" (acidic) with partially identical genes *CAPZA1*, *ANXA2*, *CAPN1*.

The probes which show the ratio; 24h versus 2h, lower line) of the differentially expressed genes reveal a nearly similar graphical distribution of the gene terms. The main difference lies in the content of the term "protein binding" (GO: 0005515) in the alkaline probe harbouring the genes *SGK1*, *ATOH8*, *BCOR*, *BHLHE40*, *DACT*1, *EGR2*, *FOSB*, *HMOX1*, *ID1*, *ID2*, *ID3*, *SMAD7*, *DKK1*, *IL8* (lower line, left column), whereas the "receptor binding" (GO: 0005515) term shows three genes, absent in the acidic probe *DKK1*, *IL8*, *SMAD7* (lower line, right column).

In **Table 12**, graphics were generated according to the GO annotations like "molecular function". With these data we tested the strongest affected functional gene groups of the alkaline and the acidic extract probes.

TABLE 12: GO ANNOTATION OF THE MOLECULAR FUNCTION OF THE MOST AFFECTED GENES

	Alkaline	Acidic
2h / control		
24h / control		
24h / 2 h		

The table displays the graphical mapping of the sequence distribution and the annotation "molecular function" the most affected genes of the score bundles of the most affected functional gene groups of the alkaline and the acidic extract probes, generated by Blast2GO (cut off at 1.0E-3).

Differentially expressed genes and the predicted protein interactions: The STRING database allows the presentation of a network information of the identified genes via protein-protein interactions with inclusive direct (physical) and indirect (functional) associations.

NETWORK ANALYSIS OF THE REGULATED GENES AFTER SMOKE EXTRACTS APPLICATION

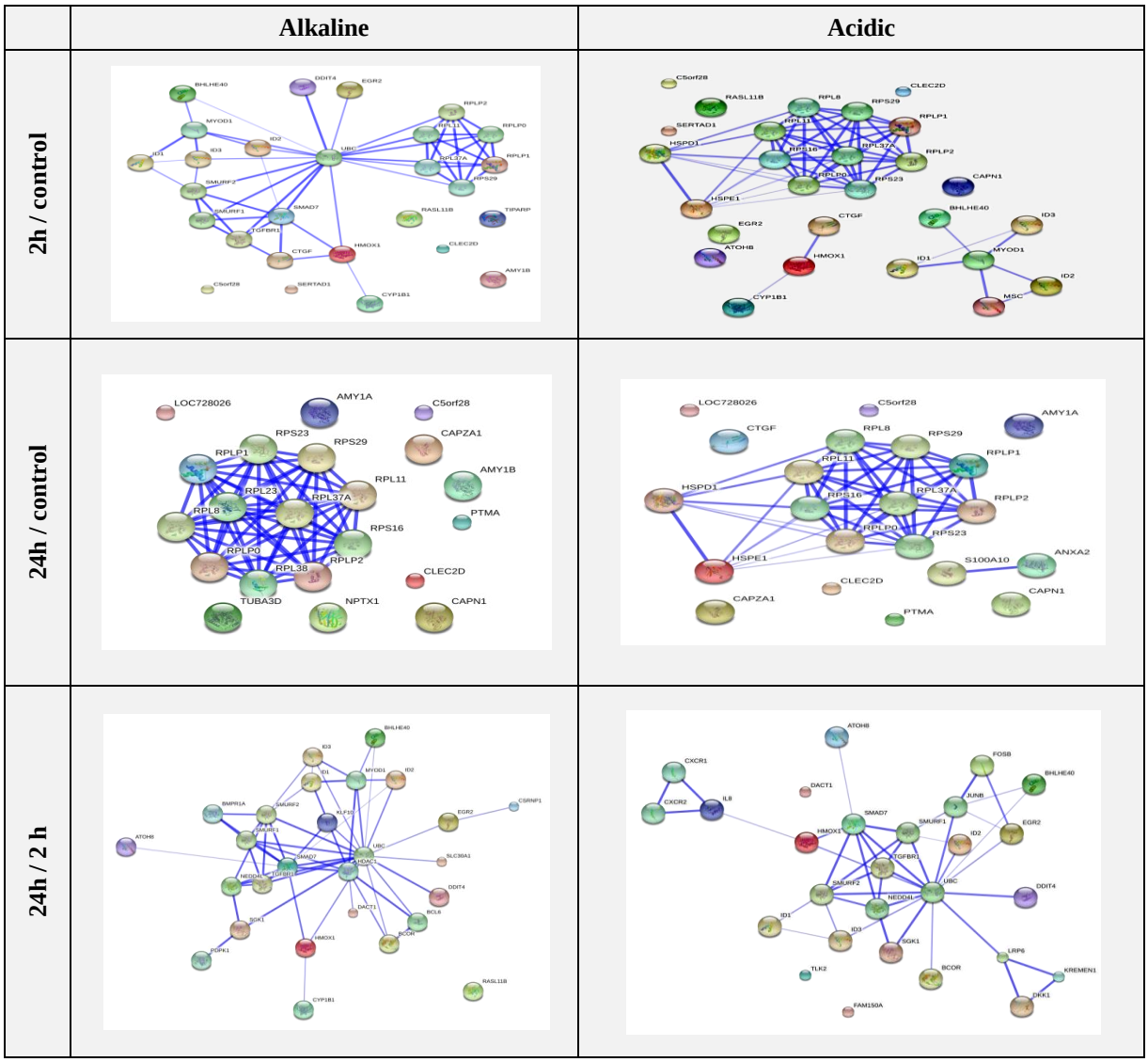


FIGURE 3: SHOWS THE GRAPHICAL PRESENTATION OF THE STRING DATA.

The upper/lower lines present the predicted protein interactions as stress response in HEL cells, induced by the acidic/alkaline extracts of the tobacco smoke ingredients. The left column describes the rapid cellular stress response (2h) in comparison to the untreated control cells; the middle column displays the response 24 h after exposition. In the right column the ratio of the 24 h versus 2 h is diagramed. The data correspond to the alignment of the heat maps (Figure 2) and the graphical mapping of the annotations (Table 12).

2h / control - Alkaline extract: The HMOX1 and CYP1B1 offer the highest fold change factor of the induced genes in the 2 h probe and both can influence combined with *EGR*2 and *DDIT*4 via *UBC* the ribosomal gene network, as well as the *ID*1, *ID*2, and *ID*3 genes, which are additionally influenced by *BLHE*40 and *MYOD*1. The rapid response after tobacco smoke extracts of *CYP1B*1 can be explained with these monooxygenases properties which catalyze many redox reactions like oxidation of drugs and other cytotoxic substrates under reduction of water [28]. T his oxygenase applies also to *HMOX*1, which catalyzes not alone heme under biliverdin formation, but

also various nonheme substances which can act as a substrate, and can be the reason for the up regulation as shown here [29].

Acidic extract: This probe detected the *CYP1*B1, *ID*2, *ID*1, and *ID*3 as well with the highest fold changes similar to the alkaline extract probe but in a dissenting order, presenting a different graphical image of the association of the corresponding gene products. The products of the ID 1, 2, 3-gene family acts, isolated from the *HMOX*1, *CYP1*B1 network, as inhibitors of DNA binding. The corresponding HLH gene products are considered as negative regulator proteins and a high-expression is associated in cancer progression [30-33].

24h / control - Alkaline / acidic extracts: Genes with LOC-numbers are unaccounted in the STRING software. Due to the high content of predicted proteins and of genes with still unknown function a compilation of the compelling graphical display of a network is not possible.

Comparison 24h vs. 2h - Alkaline acidic extracts: The *DDIT*4 offers in both probes the highest fold change (9.79 and 9.83 respectively) and is pivotally involved in DNA repair processes after damage and is induced by stressors like reactive oxygen species (ROS) [34]. As shown in **Error! Reference source not found.**, it regulates directly *HDAC*-mediated chromatin-based gene regulation [35]. The increased fold change indicates an induction of repair procedures, comparet to fold change rates less than one in *FOS*B, *SMAD*7, *DKK*1, and *BHLHB*2. In case of the down regulated *BCOR*, we detect a repressor gene [36] in the alkaline probe. In the acidic probe the *KLF*10, *CYP1B*1, *DACT*1, and *AXUD*1 are involved, similar to *DDIT*4, in the stress induced regulation of *HDAC*; all these detected genes, except *DDIT*4, are down regulated, which suggest a decreased cellular stress response against DNA damages tobacco smoke components-caused.

The smoke particles-based cellular injuries can in the last resort lead to a change of the balance of the interaction between protein networks and result in developmental disorders in children and in different disease patterns in adulthood [37-42].

DISCUSSION: In the cultural history, it becomes apparent, that the tobacco plant is among the oldest cultivated plants.

It is documented in the WHO reports about the global tobacco consumption 2009, that the "tobacco epidemic" is "one of the greatest disasters of the human history" [43]. It seems to be difficult to find arguments for tobacco smokers, to change from the smoking to the non-smoking habit. A further study shows the broad effect of tobacco smoke on the human organism and gives insight into epigenetic processes and also reveals that quitting tobacco smoking allows regaining the methylation state of the DNA of never smokers, as recently documented by the Zeilinger group [44].

Tobacco smoke components cause a wide spectrum of cardiovascular and chronic obstructive pulmonary diseases as well as various types of cancer including lung cancer which is thought to occur through mechanisms that include DNA damage, inflammation, and oxidative stress [45-51].

Furthermore, different smoking-related aberrant methylation processes are well documented [52-56].

There is considerable evidence that inhaled toxicants such as cigarette smoke can cause both irreversible changes to the genetic material (DNA mutations) and putatively reversible changes to the epigenetic landscape (changes in the DNA methylation and chromatin modification state).

It is also documented, that tobacco smoke-genomics, including analyses of gene expression for the individual genotypes may contribute to the prediction of the phenotype of the illness due to multiple parameters. Such parameters are age, nutritional status, diseases and different medications [57]. The diseases which are believed to involve genetic and epigenetic perturbations include lung cancer, chronic obstructive pulmonary disease (COPD) [58], and cardiovascular diseases (CVD) [59].

All mentioned clinical entities are epidemiologically strongly linked to cigarette smoking. In these reviews, the significance of

genomics and epigenomics in these major smoking-related diseases is highlighted.

Efforts to reduce tobacco consumption in overall population delivered very modest results and it is unlikely that the smoking habits will significantly change in a positive way in the near future.

Reasons for this are unknown but likely include the fact that humans were exposed to tobacco for much of their history. Although initially taken as very promising, graphic health warning labels on tobacco packages and mass media campaigns turned out to reduce the consumption of tobacco products only marginally. Although the battle seems to be lost a capitulation is inacceptable and careful considerations are required

Securing a cigarette smoke-free environment for children is of particular importance. The loss of the balance in gene expression resulting in a slight imbalance of the protein networks can result in developmental disorders in children [60-63] and ultimately in different disease patterns in adulthood [64-67]

Our DAVID data show clearly, that negative regulators of transcription are down-regulated by tobacco smoke ingredients. A strongly decreased level of expression in the treated cells compared to the level of expression in their control cells suggests that gene products have tumor suppressor gene (TSG) function. Here we show a scenario of a possible "Loss of function" of the TSG *AXUD1* mapped at 3p21.3 [68]. The GO annotation biological process (BP) indicates an involvement in the post-embryonic development (GO: 0009791) [69, 70].

A further down regulated TSG candidate, detected here, is the *BCOR* gene, acting as a repressor of the regulation of the *BCL*6-oncogene mediated apoptosis network. It's down regulation results in loss of inhibition of the function of the *HDAC* gene (as first published by Bardwell in 2000 [71]) regaining the function of the *BCL*6-oncogene, first found in B-cell lymphomas and documented by the McKeithan group [72].

Despite the variety of the acidic and alkaline reacting ingredients of pyrolyzed tobacco smoke in our experiments the classification in these two chemical groups should facilitate and contribute to the development of technologies by design of new filter materials with differential functionalization of the filter surface. This offers a high potential of both absorption and adsorption of smoke particles to the filter material. The data clearly reveal that the effective reduction of tobacco smoke components harmful to health is indispensable.

The prevention of tobacco-related diseases is an important issue that exceeds efforts of natural science community and clinical medicine and requires substantial involvement of different segments of the society including education and legislature. According to current knowledge, smoking remains to be the leading preventable cause of death in the industrialized world [1]. There are evidences that reduction of the tar yield of a cigarette in spite of compensatory smoking habits reduce conspicuously the risk of sickness caused by tobacco smoke [73]. Further the rationale of all efforts to minimize these horrible sustainable effects on the genome must be approached by methodologies, like the choice of the kind of tobacco plants, the cigarette rolling paper and the use of effective and appropriate filter materials.

Here technologies deriving from the ligation chemistry could produce relief. Coupling via "Click Chemistry") of reaction partners of the Diels Alder Reaction with inverse electron demand (DAR_{inv}) [74-77] can be considered exemplarily as a promising tool to realize the ligation of functional groups eligible for the catalytic filter detoxification of volatile tobacco smoke ingredients.

The authors remind that processing industry holds responsibility for the benefit of their customers. Additionally to all activities, documented in the WHO report, mentioned above, the content of the pyrolyzed tobacco constituent parts must be drastically minimized by their immobilization at the surface of the filter material.

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Conflict of interest: The authors declare herewith no financial or commercial conflict of interest.

REFERENCE:

1. WHO Report on the Global Tobacco Epidemic, 2011 -Warning about the dangers of tobacco-. In: Geneva: World Health Organization, 2011;1-150.
2. MacKenzie TD, Bartecchi CE, Schrier RW. The human costs of tobacco use (2). N Engl J Med 1994; 330(14): 975-980.
3. Bartecchi CE, MacKenzie TD, Schrier RW. The human costs of tobacco use (1). N Engl J Med 1994; 330(13): 907-912.
4. CDC.Tobacco Control State Highlights 2012. In: Atlanta U.S.: Department of Health and Human services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health., 2013;1-477.
5. Hou L, Zhang X, Wang D, *et al.* Environmental chemical exposures and human epigenetics. Int J Epidemiol 2012; 41(1): 79-105.
6. Pope CA, III, Burnett RT, Krewski D, *et al*. Cardiovascular mortality and exposure to airborne fine particulate matter and cigarette smoke: shape of the exposure-response relationship. 2009; 120(11): 941-948.
7. Pope CA, III, Ezzati M, Dockery DW. Fine-particulate air pollution and life expectancy in the United States. N Engl J Med 2009; 360(4): 376-386.
8. Schmidt-Voigt J. [Reduction of injuries through filter-smoking]. Arztl Wochensch 1953; 8(17): 416-420.
9. Pauly JL, Mepani AB, Lesses JD, *et al*. Cigarettes with defective filters marketed for 40 years: what Philip Morris never told smokers. Tob Control 2002; 11 Suppl 1: I51-I61.
10. Laugesen M, Fowles J. Regulation of cigarette smoke toxicity. N Z Med J 2005; 118(1213): U1400.
11. Branton PJ, McAdam KG, Winter DB, *et al*. Reduction of aldehydes and hydrogen cyanide yields in mainstream cigarette smoke using an amine functionalised ion exchange resin. Chem Cent J 2011; 5(1): 15.
12. Siu M, Mladjenovic N, Soo E. The analysis of mainstream smoke emissions of Canadian 'super slim' cigarettes. Tob Control 2012.
13. Wenusch A. Beiträge zum Entnikotinisierungsproblem.V - The Chemical Components of Tobacco and Tobacco Smoke. Fachliche Mitt Österr Tabakregie 1930(1) 13-19 1930; 1: 13-19.
14. Braun K, Müller G, Schick M, *et al*. Expressions profiling project of Human embryonic lung cells exposed to Pyrolyzed Cigarette smoke. 2013; 4(11): 4203-4222.
15. Eberwine J, Spencer C, Miyashiro K, *et al*. Complementary DNA synthesis in situ: methods and applications. Methods Enzymol 1992; 216: 80-100.
16. Bolstad B. Probe Level Quantile Normalization of High Density Oligonucleotide Array Data. 2001; 1-8.
17. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate - A Practical and Powerful Approach to Multiple Testing. 1995; 57(1): 289-300.
18. Eisen MB, Spellman PT, Brown PO, *et al*. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998; 95(25): 14863-14868.
19. Ashburner M, Ball CA, Blake JA, *et al*. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000; 25(1): 25-29.
20. Karp PD. Ontology for biological function based on molecular interactions. Bioinformatics 2000; 16(3): 269-285.
21. Jenssen TK, Laegreid A, Komorowski J, *et al*. A literature network of human genes for high-throughput analysis of gene expression. Nat Genet 2001; 28(1): 21-28.
22. Franceschini A, Szklarczyk D, Frankild S, *et al.* STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Res 2013; 41(Database issue): D808-D815.
23. Snel B, Lehmann G, Bork P, *et al.* STRING: a web-server to retrieve and display the repeatedly occurring neighbourhood of a gene. Nucleic Acids Res 2000; 28(18): 3442-3444.
24. Sherman BT, Huang dW, Tan Q, *et al*. DAVID Knowledgebase: a gene-centered database integrating heterogeneous gene annotation resources to facilitate high-throughput gene functional analysis. BMC Bioinformatics 2007; 8: 426.
25. Huang dW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4(1): 44-57.
26. Huang dW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009; 37(1): 1-13.
27. Huang dW, Sherman BT, Tan Q, *et al*. The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. Genome Biol 2007; 8(9): R183.
28. Shen Z, Wells RL, Elkind MM. Enhanced cytochrome P450 (Cyp1b1) expression, aryl hydrocarbon hydroxylase activity, cytotoxicity, and transformation of C3H 10T1/2 cells by dimethylbenz(a)anthracene in conditioned medium. Cancer Res 1994; 54(15): 4052-4056.
29. Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Sci U S A 1989; 86(1): 99-103.
30. Maruyama H, Kleeff J, Wildi S, *et al*. Id-1 and Id-2 are overexpressed in pancreatic cancer and in dysplastic lesions in chronic pancreatitis. Am J Pathol 1999; 155(3): 815-822.
31. Schindl M, Oberhuber G, Obermair A, *et al*. Overexpression of Id-1 protein is a marker for unfavorable prognosis in early-stage cervical cancer. Cancer Res 2001; 61(15): 5703-5706.
32. Chaudhary J, Schmidt M, Sadler-Riggleman I. Negative acting HLH proteins Id 1, Id 2, Id 3, and Id 4 are expressed in prostate epithelial cells. 2005; 64(3): 253-264.
33. Yuen HF, Chiu YT, Chan KK, *et al*. Prostate cancer cells modulate osteoblast mineralisation and osteoclast differentiation through Id-1. Br J Cancer 2010; 102(2): 332-341.
34. Ellisen LW, Ramsayer KD, Johannessen CM, *et al*. REDD1, a developmentally regulated transcriptional target of p63 and p53, links p63 to regulation of reactive oxygen species. Mol Cell 2002; 10(5): 995-1005.
35. Turgeon N, Blais M, Gagne JM, *et al*. HDAC1 and HDAC2 Restrain the Intestinal Inflammatory Response by Regulating Intestinal Epithelial Cell Differentiation. PLoS One 2013; 8(9): e73785.
36. Bertos NR, Wang AH, Yang XJ. Class II histone deacetylases: Structure, function, and regulation. 2001; 79(3): 243-252.
37. Johannessen A, Bakke PS, Hardie JA, *et al*. Association of exposure to environmental tobacco smoke in childhood with chronic obstructive pulmonary disease and respiratory symptoms in adults. Respirology 2012; 17(3): 499-505.
38. Liu Y, Dai M, Bi Y, *et al*. Active smoking, passive smoking, and risk of nonalcoholic fatty liver disease (NAFLD): a population-based study in China. J Epidemiol 2013; 23(2): 115-121.
39. Cupul-Uicab LA, Skjaerven R, Haug K, *et al*. Exposure to tobacco smoke in utero and subsequent plasma lipids, ApoB, and CRP among adult women in the MoBa cohort. Environ Health Perspect 2012; 120(11): 1532-1537.
40. Cupul-Uicab LA, Skjaerven R, Haug K, *et al.* In utero exposure to maternal tobacco smoke and subsequent obesity, hypertension, and

gestational diabetes among women in the MoBa cohort. Environ Health Perspect 2012; 120(3): 355-360.
41. Harding R, Maritz G. Maternal and fetal origins of lung disease in adulthood. Semin Fetal Neonatal Med 2012; 17(2): 67-72.
42. Bakker H, Jaddoe VW. Cardiovascular and metabolic influences of fetal smoke exposure. Eur J Epidemiol 2011; 26(10): 763-770.
43. WHO Report on the Global Tobacco Epidemic, 2009: Implementing smoke-free environments. In: Geneva: World Health Organization, 2010.
44. Zeilinger S, Kuhnel B, Klopp N, *et al*. Tobacco smoking leads to extensive genome-wide changes in DNA methylation. PLoS One 2013; 8(5): e63812.
45. Feltes BC, de Faria PJ, Notari DL, *et al*. Toxicological effects of the different substances in tobacco smoke on human embryonic development by a systems chemo-biology approach. PLoS One 2013; 8(4): e61743.
46. Kobayashi Y, Bossley C, Gupta A, *et al*. Passive smoking impairs histone deacetylase-2 in children with severe asthma. 2013.
47. Mills AL, Messer K, Gilpin EA, *et al*. The effect of smoke-free homes on adult smoking behavior: A review. Nicotine Tob Res 2009; 11(10): 1131-1141.
48. Mackay J. Implementing tobacco control policies. 2012; 102(1): 5-16.
49. Bono R, Bellisario V, Romanazzi V, *et al*. Oxidative stress in adolescent passive smokers living in urban and rural environments. Int J Hyg Environ Health 2013.
50. Yamamoto ML, Chapman AM, Schiestl RH. Effects of side-stream tobacco smoke and smoke extract on glutathione- and oxidative DNA damage repair-deficient mice and blood cells. Mutat Res 2013; 749(1-2): 58-65.
51. Lowe FJ, Luettich K, Gregg EO. Lung cancer biomarkers for the assessment of modified risk tobacco products: an oxidative stress perspective. Biomarkers 2013; 18(3): 183-195.
52. Breitling LP, Yang R, Korn B, *et al*. Tobacco-smoking-related differential DNA methylation: 27K discovery and replication. Am J Hum Genet 2011; 88(4): 450-457.
53. Flom J, Ferris J, Gonzalez K, *et al*. Prenatal Tobacco Smoke Exposure and Genome-wide Methylation in Adulthood. 2011; 20(4): 720-721.
54. Flom JD, Ferris JS, Liao Y, *et al*. Prenatal smoke exposure and genomic DNA methylation in a multiethnic birth cohort. Cancer Epidemiol Biomarkers Prev 2011; 20(12): 2518-2523.
55. Ferris JS, Flom JD, Tehranifar P, *et al*. Prenatal and childhood environmental tobacco smoke exposure and age at menarche. Paediatr Perinat Epidemiol 2010; 24(6): 515-523.
56. Wan ES, Qiu W, Baccarelli A, *et al*. Cigarette smoking behaviors and time since quitting are associated with differential DNA methylation across the human genome. Hum Mol Genet 2012; 21(13): 3073-3082.
57. Sexton K, Balharry D, BeruBe KA. Genomic biomarkers of pulmonary exposure to tobacco smoke components. Pharmacogenet Genomics 2008; 18(10): 853-860.
58. Talikka M, Sierro N, Ivanov NV, *et al*. Genomic impact of cigarette smoke, with application to three smoking-related diseases. Crit Rev Toxicol 2012; 42(10): 877-889.
59. Breitling LP. Current genetics and epigenetics of smoking/tobacco-related cardiovascular disease. Arterioscler Thromb Vasc Biol 2013; 33(7): 1468-1472.
60. Chelchowska M, Ambroszkiewicz J, Jablonka-Salach K, *et al*. Tobacco Smoke Exposure During Pregnancy Increases Maternal Blood Lead Levels Affecting Neonate Birth Weight. Biol Trace Elem Res 2013.
61. Strong C, Chang LY. Family socioeconomic status, household tobacco smoke, and asthma attack among children below 12 years of age: Gender differences. J Child Health Care 2013.
62. Arora M, Mathur MR, Singh N. A framework to prevent and control tobacco among adolescents and children: introducing the IMPACT model. Indian J Pediatr 2013; 80 Suppl 1: S55-S62.
63. Rush D, Callahan KR. Exposure to passive cigarette smoking and child development. A critical review. Ann N Y Acad Sci 1989; 562: 74-100.
64. Wan ES, Qiu W, Baccarelli A, *et al*. Systemic steroid exposure is associated with differential methylation in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2012; 186(12): 1248-1255.
65. Szyfter K, Banaszewski J, Jaloszynski P, *et al*. Carcinogen: DNA adducts in tobacco smoke-associated cancer of the upper respiratory tract. Acta Biochim Pol 1999; 46(2): 275-287.
66. Szyfter K, Jarmuz M, Giefing M, *et al*. [Chromosome alterations in tobacco smoke-associated tumors]. Przegl Lek 2007; 64(10): 865-870.
67. Zeskind JE, Lenburg ME, Spira A. Translating the COPD transcriptome: insights into pathogenesis and tools for clinical management. Proc Am Thorac Soc 2008; 5(8): 834-841.
68. Wang K, Ling T, Wu H, *et al*. Screening of candidate tumor-suppressor genes in 3p21.3 and investigation of the methylation of gene promoters in oral squamous cell carcinoma. Oncol Rep 2013; 29(3): 1175-1182.
69. Ishiguro H, Tsunoda T, Tanaka T, *et al*. Identification of AXUD1, a novel human gene induced by AXIN1 and its reduced expression in human carcinomas of the lung, liver, colon and kidney. 2001; 20(36): 5062-5066.
70. Glavic A, Molnar C, Cotoras D, *et al*. Drosophila Axud1 is involved in the control of proliferation and displays pro-apoptotic activity. Mech Dev 2009; 126(3-4): 184-197.
71. Huynh KD, Fischle W, Verdin E, *et al*. BCoR, a novel corepressor involved in BCL-6 repression. Genes Dev 2000; 14(14): 1810-1823.
72. Baron BW, Nucifora G, McCabe N, *et al*. Identification of the gene associated with the recurring chromosomal translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in B-cell lymphomas. Proc Natl Acad Sci U S A 1993; 90(11): 5262-5266.
73. Wynder EL. Preventive approach to tobacco-related diseases. Cancer Detect Prev 1982; 5(4): 457-460.
74. Wiessler M, Kliem C, Lorenz P, et al. EU Patent: Ligation reaction based on the Diels Alder Reaction with invers electron demand. In: Anonymous. 2006.
75. Wiessler M, Waldeck W, Kliem C, *et al*. The Diels-Alder-reaction with inverse-electron-demand, a very efficient versatile click-reaction concept for proper ligation of variable molecular partners. Int J Med Sci 2009; 7(1): 19-28.
76. Wiessler M, Waldeck W, Pipkorn R, *et al*. Extension of the PNA world by functionalized PNA monomers eligible candidates for inverse Diels Alder Click Chemistry. Int J Med Sci 2010; 7(4): 213-223.
77. Hassert R, Pagel M, Ming Z, *et al*. Biocompatible Silicon Surfaces through orthogonal Click Chemistries and a high Affinity Silicon Oxide Binding Peptpide. Bioconjug Chem 2012.

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