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Experimental Acute Exposure to Thirdhand Smoke and Changes in the Human Nasal Epithelial Transcriptome in a Randomized Study

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Key Points

Question: Does acute inhalation of thirdhand smoke (THS) alter the transcriptome of human nasal epithelium?

Findings: A 3 hour inhalation exposure of four healthy nonsmoking females to clean air altered the expression of only two genes. When the same four females were exposed to THS at least 21 days later, 389 genes associated with cell stress and survival pathways were differentially expressed, and many affected genes were associated with increased mitochondrial activity, oxidative stress, DNA repair, cell survival, and inhibition of cell death.

Meaning: Acute exposure to THS stresses the human nasal epithelium, a finding that will be valuable to physicians treating exposed patients.
Abstract

Importance: This is the first study to show that acute inhalation of thirdhand smoke (THS) activates stress and survival pathways in human nasal epithelium.

Objective: To evaluate gene expression in the nasal epithelium of nonsmoking human females following acute inhalation of clean air and THS.

Design: Nasal epithelium was obtained from participants in a clinical trial (2011-2015) on the health effects of inhaled THS. In our crossover design, participants were exposed, head-only, to THS and to conditioned, filtered air. The order of exposures was randomized and exposures were separated by at least 21 days.

Setting: Experiments were performed in a controlled laboratory setting.

Participants: RNA in quantities sufficient for analysis was obtained from a subset of four healthy, nonsmoking women.

Exposures: By chance, the females in our subset had all been randomized to receive clean air exposure first and THS exposure second. Exposures lasted 3 hours.

Main Outcomes and Measures: Differentially expressed genes (DEGs) were identified using RNA sequencing with a false discovery rate < 0.1.

Results: Participants were four healthy nonsmoking human females 27-49 years old (mean = 42) with no chronic diseases. 389 DEGs were identified in THS exposed nasal epithelium, while only two genes, which were not studied further, were affected by clean air. Cluster-Profiler identified enriched Gene Ontology terms associated with stress-induced mitochondrial hyperfusion, such as respiratory electron transport chain (q-value = 2.84E-03) and mitochondrial inner membrane (q-value = 7.21E-06). Reactome Pathway Analysis identified terms associated with up-regulation of DNA repair...
mechanisms, such as nucleotide excision repair (q-value = 1.05E^{-2}). Enrichment analyses using Ingenuity Pathway Analysis identified canonical pathways related to stress-induced mitochondrial hyperfusion (e.g., increased oxidative phosphorylation) (p-value = 5.13E^{-4}), oxidative stress (e.g., glutathione depletion phase II reactions) (p-value = 4.36E^{-2}), and cell survival (z-score = 5.026).

Conclusions and Relevance: Acute inhalation of THS caused cell stress leading to the activation of survival pathways. Some responses were consistent with stress-induced mitochondrial hyperfusion and similar to those demonstrated previously in vitro. These data will be valuable to physicians treating THS-exposed patients and will aid in formulating regulations for the remediation of THS contaminated environments.
Introduction

Thirdhand smoke (THS) is a subset of chemicals in secondhand cigarette smoke (sidestream smoke emitted by a burning cigarette and exhaled mainstream smoke) that sticks to indoor surfaces and persists after active smoking has occurred\(^1,2\). THS chemicals accumulate and can react with other compounds or can be re-emitted into the environment\(^1,2,3\). Nonsmokers can be exposed to chemicals in THS months or even years after smoking has stopped\(^3\). Many THS chemicals are toxic volatile and semi-volatile organic compounds\(^2,3,4\). Nicotine, a major chemical in THS, has a high affinity for surfaces\(^3\) and can react with ambient nitrous acid to form tobacco-specific nitrosamines (TSNAs), some of which are carcinogens\(^5,6\). Nicotine-derived nitrosamines in THS include 4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanone (NNK), and N-nitrosonornicotine (NNN)\(^5,6\), which are also found in secondhand smoke and have been associated with the development of lung cancer\(^7\). Ozone can also react with nicotine to form formaldehyde, a known human carcinogen\(^8\).

Due to the presence of these and other hazardous chemicals, such as acrolein, in THS, it is important to understand if there is a correlation between exposure to THS and human health, especially in nonsmokers. Previous studies have demonstrated that exposure of human cell lines to THS extracts for 24 hours increased DNA strand breaks and oxidative DNA damage\(^9,10\). Mouse neural stem cells undergo blebbing, fragmentation, cytoskeletal disruption, and vacuolization when treated with extracts of THS\(^11\). THS also causes stress-induced mitochondrial-hyperfusion (SIMH), which is accompanied by increased mitochondrial membrane potential, ATP production, and reactive oxygen species (ROS)\(^12\). During SIMH, punctate mitochondria fuse and form tubular networks, which allow exchange of
molecules including mitochondrial DNA as a survival mechanism. Acrolein has been identified as a THS chemical that inhibits cell proliferation. In a metabolomics study using male germ cells, THS exposure is correlated with down-regulation of several molecular pathways, including nucleic acid metabolism, ammonia metabolism and up-regulation of glutathione metabolism.

THS also causes adverse health effects in mice. Three-week old mice that were housed for 6 months in cages containing a THS-impregnated fabric and bedding showed an increase in inflammatory cytokines in lung tissue, impaired wound healing, and were hyperactive compared to controls. Adult mice developed insulin resistance as a consequence of oxidative stress caused by THS and showed increased blood glucose, increased serum insulin, and accumulation of fat in viscera. Oxidative stress in skeletal muscle and accumulation of $H_2O_2$ accompanied by low catalase activity was observed in chronically exposed mice.

After THS exposure, neonatal mice had significantly more eosinophils, increased platelet volume, lower hematocrit, and decreased mean cell volume than controls, while adult exposed mice had a significant increase in the percentage of B-cells and a decrease in myeloid cells.

Elimination of THS can be challenging, as it persists in houses previously owned by smokers even after 2 months of vacancy. Cars previously owned by smokers also retain THS, and new owners may be at risk of exposure. Common household fabrics retained THS chemicals 19 months after smoking had occurred. Individuals absorb nicotine through their skin while wearing THS exposed clothes. Moreover, infants whose mothers smoked outdoors had much higher levels of urine cotinine, a nicotine metabolite, than infants of nonsmoking parents. Other examples of the persistence of THS have been reviewed recently.
Although these prior studies demonstrate humans are at risk of exposure to THS, the molecular effects of such exposure on humans have not been investigated. The purpose of this study was to evaluate the effects of inhalation of THS chemicals on gene expression in humans. Nasal epithelial cells were collected from nonsmokers before and after 3 hours of exposure to either clean air or to THS, subjected to mRNA sequencing (mRNA-seq), and analyzed for differential expression of genes (DEG). Significant changes in gene expression were found following THS exposure, but not exposure to clean air.

Materials and Methods

Ethics: The study was approved by University of California at San Francisco IRB Protocol number 12-09512. Details of participant recruitment, written informed consent screening, selection, compensation and involvement in the study appear in eMethods of the Supplement. The RNA-seq analysis was approved under IRB protocol HS-12-023 from UCR.

Study Population, Generation of THS, and THS exposure: The protocol for the primary study during which the nasal epithelial cell samples were collected appears in the CONSORT Flow Diagram (Figure 1) and eMethods in the Supplement. It was conducted at the University of California, San Francisco between 2011 and 2015. Briefly, 26 healthy nonsmokers who were not exposed to secondhand cigarette smoke (SHS) in daily life, were exposed, head-only to THS aerosol and to conditioned, filtered air for 3 hours, using an exposure chamber described previously\(^2^3\). Of these 26 individuals, 13 (8 women, 5 men) had nasal epithelial cell samples collected before and after each...
exposure. Nasal epithelial samples were collected from the anterior, inferior turbinate using small, sterile plastic curettes (RhinoPro, Arlington Scientific, Inc. Springville, UT, USA). These samples were immediately placed in RNAlater and shipped frozen to the University of California, Riverside, where RNA extraction and subsequent analyses were performed.

RNA Isolation: RNA was isolated from human nasal samples using RNeasy micro kits (Qiagen, Germantown, MD, USA) and stored at -80°C. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples from four participants had RNA concentrations > 3 ng/µL, and these were used for subsequent analysis. Frozen RNA samples were shipped to Cofactor Genomics (St. Louis, MO, USA) for library preparation and sequencing.

RNA Sequencing: Cofactor Genomics performed quality control on RNA samples, and RNA integrity was determined using a Bioanalyzer (Agilent 2100). Samples with RIN numbers between 8 -10 were used for library construction. Total RNA was reverse-transcribed using an Oligo(dT) primer, and limited cDNA amplification was performed using the SMARTer® Ultra® Low Input RNA Kit for Sequencing-v4 (Takara Bio USA, Inc., Mountain View, CA, USA). Full-length cDNA was fragmented and tagged, followed by limited PCR enrichment to generate the final cDNA sequencing library (Nextera® XT DNA Library Prep, Illumina, San Diego, CA, USA). Libraries were sequenced as single-end 75 base pair reads using an Illumina NextSeq500 following the manufacturer's instructions. Because the amount of nasal epithelium in each sample was very limited, we were not able to perform confirmatory qPCR.
Bioinformatics Analysis: Fastq files obtained from Cofactor were processed on a High Performance Computing Cluster (HPCC) at the University of California, Riverside. The RNA-Seq analysis workflow implemented by systemPipeR was used to perform all the downstream data processing. Briefly, adapter sequences and low quality tails were removed from the raw reads using the Trimmomatic package. The preprocessed reads were then aligned against the UCSC hg19 human reference genome with Tophat2 (Version 2.0.14). Read counting was performed with the `summarizeOverlaps` function of the GenomicsAlignment package. Only unique reads overlapping the exonic gene regions were counted. Using a cut-off value of at least 1 RPKM average across all samples, raw expression counts of the remaining 10,938 genes passing this filter were used for differential expression analysis with EdgeR. Within each experimental group (Group 1, 2, 3, 4), the read counts from the four biological replicates were combined. For differential expression analysis, Groups 1 & 2 (before and after clean air) and Groups 3 & 4 (before and after THS) were treated as two separate experimental comparisons. Genes were considered to be DEGs if they had a false discovery rate (FDR) < 0.1 by EdgeR. Cluster-Profiler and Reactome PA (RPA) packages were used to identify over represented GO terms and enriched Reactome pathways, respectively, as described in the package manual. Additionally, enrichment analyses of pathways were performed using the Ingenuity Pathway Analysis (IPA) software (Qiagen, Germantown, MD, USA). Briefly, statistically significant transcripts were uploaded to IPA, and human homologs were automatically identified using NCBI’s HomoloGene.

Statistical Analysis: The EdgeR package was used to obtain log-fold changes, p-values, and FDR scores (based on the Benjamini-Hochberg’s method). A gene was
considered significantly differentially expressed if the FDR was less than 0.1.

ClusterProfiler and RPA packages used a Benjamini-Hochberg adjusted p-value of less than 0.05 to identify significantly enriched Gene Ontology terms and Reactome Pathways, respectively. IPA used the Fisher’s Exact Test with a p-value threshold of 0.05 to identify statistically significant pathways; the algorithm considered both direct and indirect relationships using the Ingenuity Knowledge Base (genes only) as the reference set.
Results

Exposure to THS Altered Gene Expression in Human Nasal Epithelium

The samples collected with this method were small and sufficient quantities of RNA for sequencing analysis could only be extracted from four women. By chance, these four participants had all been randomized to receive the clean air exposure first and THS exposure second, thus we were unable to determine the effect of order on RNA expression. After processing RNA-seq reads, data were analyzed to determine if there were differences in gene expression in the groups exposed to either clean air (Group 1 vs Group 2) or THS (Group 3 vs Group 4) (eFigure 1 in the Supplement). The dataset consisted of approximately 10,000 genes of which 2 and 389 were significantly differentially affected (FDR < 0.10) in clean air and THS-exposed subjects, respectively (eTable 1 in the Supplement). The data set is downloadable from SRA/GEO under submission number PRJNA514351/GSE129959. The two down-regulated genes (hemoglobin, alpha 1 and hemoglobin, alpha 2) identified when subjects were exposed to clean air had an absolute fold change of 8.2 and 8.7, respectively (eTable 2 in the Supplement). No genes were significantly up-regulated in the group exposed to clean air (eFigure 2 in the Supplement). Because these results showed that wearing the respirator for 3.5 hours and inhaling clean air did not significantly impact gene expression, clean air was not studied further.

Nasal samples collected after THS exposure had a significant number of DEGs compared to samples collected before exposure (eTable 1 and eFigure 2 in the Supplement). A total of 382 genes were significantly up-regulated (FDR < 0.1), while seven were down-regulated (eTable 1 in the Supplement). The log₂-fold changes for up-regulated genes ranged from 2 to 7, while down-regulated genes
ranged from -2 to -9 (eTable 3 in the Supplement). These data demonstrate that inhalation of THS for a relatively short time significantly altered gene expression in human nasal epithelium.

**GO Term Enrichment Analysis**

We performed Gene Ontology (GO) enrichment analysis on the up-regulated DEGs to identify biological functions affected by THS (Figure 2A and B; eTables 4-6 in the Supplement). The GO database categorizes genes into different ontologies that represent biological knowledge. Our analysis identified 11 functions enriched within the Biological Processes, 13 within Cellular Components, and 1 within Molecular Function. All the processes were significantly enriched (q-value of < 0.05) (eTables 4-6 in the Supplement). Most of the affected biological processes and cellular components in THS-exposed subjects involved mitochondrial function or RNA metabolism. The top GO Biological Process terms included Ribonucleoprotein Complex Biogenesis (GO:0022613), Cellular Respiration (GO:0045333), Respiration Electron Transport Chain (GO:0022904), and Mitochondrial ATP Synthesis Coupled Electron Transport (GO:0042775) (Figure 2A). Most of the remaining GO Biological Processes included oxidative phosphorylation-related functions (eTable 4 in the Supplement). The top enriched GO Cellular Components terms included Mitochondria Protein Complex (GO:0098798), Mitochondrial Membrane Part (GO:0044455), Ribosomal Subunit (GO:0044391), Inner Mitochondrial Membrane Protein Complex (GO:0098800), Respiratory Chain (GO:0070469), Large Ribosomal Subunit (GO:0015934) and Respiratory Chain Complex (GO:0098803) (Figure 2B).

All the remaining GO terms involved mitochondrial functions except for the two that were related to Ribosomal Subunit (eTable 5 in the Supplement). No enriched GO terms could be identified for the down-regulated genes in the THS experimental
group, most likely due to the small number of genes in this set.

### Reactome Enrichment Analysis

The Reactome enrichment analysis was used to further evaluate the up-regulated DEGs after THS exposure. This analysis yielded a total of 25 pathways that were significantly enriched (eTable 7 in the Supplement). The top six pathways (Figure 2C) included the Citric Acid Cycle (TCA) (R-HSA-1428517), Respiratory Electron Transport (R-HSA-611105), Translation (R-HSA-72766), Mitochondrial Protein Import (R-HSA-1268020), mRNA Splicing-Minor Pathway (R-HSA-72165), and Nucleotide Excision Repair (R-HSA-5696398). Figure 2C shows the genes associated with each pathway and their overlap, when they belong to multiple pathways. Also shown are the approximate fold-change values of each gene.

### Ingenuity Pathway Analysis

IPA was also performed using upregulated genes in the THS-exposed group. The top pathways identified included Sirtuin Signaling Pathways, EIF2 Signaling, Mitochondrial Dysfunction, and Oxidative Phosphorylation (Table 1). Some pathways identified in IPA overlapped with those identified using Reactome enrichment analysis, including Mitochondrial Related Pathways and DNA repair-related pathways. The top toxicological pathways identified included mainly processes related to mitochondrial activity, such as Mitochondrial Dysfunction, Increases Transmembrane Potential of Mitochondria & Mitochondrial Membrane, and Decreases Permeability Transition of Mitochondria & Mitochondrial Membrane. In addition, genes were linked to Glutathione Depletion Phase II Reactions (Table 1).
IPA identified Diseases and Functions that are associated with the DEGs after THS exposure (Table 2). These data were filtered and only functions with activated z-scores that predict transcriptional activation or inhibition based on literature reports are presented (Table 2). The identified functions included decreased cell death and increased cell viability, homologous recombination, and cell proliferation. eFigure 3 in the Supplement shows up-regulated genes associated with inhibition of cell death. The figure includes gene names and whether their expression could activate (orange lines) or inhibit (blue lines) cell death. For cell death, the majority of the up-regulated genes predict inhibition (blue lines). Based on each gene’s biological role, IPA predicted that cell death had an activation z-score of -3.117 (overall process decreased) (Table 2). Complementary to cell death, cell viability (z-score = 5.026) (eFigure 4 in the Supplement) and homologous recombination (z-score = 2.828) (eFigure 5 in the Supplement) both had increased activation states (Table 2).

**Discussion**

The adverse health effects of THS have been studied in cultured cells and animal models, but similar investigations have not been previously performed on human subjects. Our study provides the first insight into the transcriptional responses of human respiratory epithelium to acute THS exposure. Remarkably, we found changes in gene expression in healthy nonsmokers following a 3-hour exposure to THS. The absence of an effect following clean air exposure provides evidence that the changes in gene expression following THS exposure are caused by THS per se and are not by the respirator worn during exposure. Because gene expression in the nasal epithelium is similar to the bronchial epithelium, our data are also relevant to the cells deeper in the respiratory system.
Our analyses demonstrate that brief exposure to THS affects mitochondrial activity. We previously reported that cultured mNSC undergo SIMH following exposure to THS extracts. This process was originally described during treatment of mouse embryonic fibroblasts with UV light and cell cycle inhibitors, such as actinomycin D. SIMH is characterized by fusion of mitochondria and subsequent increased production of ATP and superoxide. We found an enrichment in pathways and biological processes related to increased mitochondrial activity and oxidative stress after THS exposure, such as mitochondrial ATP synthesis coupled electron transport chain (GO:0042773), respiratory electron transport (R-HSA-611105) and oxidative phosphorylation (IPA). Increased expression of these pathways is also consistent with an increase in ATP synthesis, as occurs in SIMH. Some genes related to the TCA cycle were also upregulated, which could also increase ATP production. Several studies have shown that cigarette smoking also induces activation of mitochondrial pathways similar to those found in our study.

While SIMH results in increased ATP production, it also increases ROS. Our IPA analysis showed that Glutathione Depletion Phase II Reactions were upregulated after THS exposure. Specifically, there was an increase in glutathione synthetase (GSS) expression, which was also increased in a male germ cell line exposed to THS. This gene is part of the glutathione (GSH) synthetase pathway, which scavenges ROS, suggesting the increase of the GSS gene is a cellular response to high levels of ROS.

In prior studies, increased ROS was associated with oxidative stress and damage of proteins, lipids and DNA, while THS treatment was correlated with DNA damage in vitro. Our IPA-based enriched pathway analysis included up-regulation of the Nucleotide Excision Repair Pathway in THS exposed subjects. Two of the
genes affected in this pathway included Xeroderma pigmentosum group C (XPC) and RNA polymerase II. The former is essential for recognition of DNA damage and plays a role in the early steps of the Nucleotide Excision Repair Pathway. Upregulation of RNA polymerase II has also been associated with a response to increased DNA damage. IPA also identified an increased activation of homologous recombination. This pathway provides a repair mechanism for double stranded DNA breaks. Activation of the DNA repair pathways is also a cellular mechanism to facilitate survival. In addition, an in vitro study showed that THS induces oxidation of mitochondrial proteins. The increase in ROS as evidenced by upregulation of ROS scavenging genes in our data could also result in oxidation of mitochondrial proteins by high local concentrations of superoxide.

Our data further demonstrate that there is an overall increase in processes related to cell viability, which includes some genes involved in cell proliferation. Our results are consistent with previous in vitro studies showing increased proliferation of cultured mouse neural stem cells and human lung cancer cells exposed to THS extract. Nicotine, a major component of THS and a chemical in our exposure chamber, can activate alpha nicotinic acetylcholine receptors (nAChRs) in normal human airway epithelial cells, leading to phosphorylation (activation) of serine/threonine kinase Akt, which is involved in many cellular survival pathways. Akt can be activated within minutes of exposure to nicotine or NNK, further demonstrating that chemicals in THS could produce a rapid response. Nicotine is also associated with increased proliferation of human cancer cell lines by activating the 7α nAChR. Considering that nicotine stimulates cell proliferation, it is possible that nicotine in THS contributes to the increase in cell viability pathways that we observed.
Nicotine is also involved in inhibiting apoptosis. In our study, the increased expression of genes involved in inhibiting cell death (IPA) may have been associated with nicotine, which was present in the THS at a concentration of 0.03 mg/m$^3$. Consistent with our study, cells exposed to THS *in vitro* showed decreased expression in pro-apoptotic genes. The mechanism by which nicotine inhibits apoptosis has been studied in mouse liver cells. Activation of 7α nAChRs in the mitochondrial outer membrane by nicotine inhibited hydrogen peroxide induced apoptosis by impairing Ca$^{2+}$ accumulation in mitochondria and cytochrome C release. However, this suppression of cell death may be transitory. Bahl et al. showed that cells exposed to THS for 30 days had a decrease in cell proliferation and lost mitochondrial membrane potential, indicating that cells were entering apoptosis.

**Limitations.** This is an initial study based on four participants. Future work should be done to determine if similar data are obtained with a larger number of subjects that includes both genders.

In summary, this is the first exposure study to document an association between THS and gene expression in human subjects. Our results show that THS induced cell survival responses, which included up-regulation of genes involved in DNA repair, activation of cell viability, increased mitochondrial activity, and inhibition of cell death (Figure 3). These changes are very similar to those reported previously for in vitro cultured cells. Importantly, the changes in gene expression in the current study were seen following a relatively short (3 hr) exposure, indicating that humans respond rapidly to THS. Future studies on long-term exposure in conjunction with our study would help complete our understanding of the effects of THS on human health. Our study provides an important foundation.
for physicians treating patients exposed to THS and for future development of regulations dealing with remediation of indoor environments contaminated with THS.

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Access to Data and Data Analysis
PT and GP had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

**Originality of the Content**

All information and materials in the manuscript are original.

**Data Sharing Statement:** The data discussed in this publication have been deposited in NCBI’s BioProject database and are available through the SRA accession number PRJNA514351 (https://www.ncbi.nlm.nih.gov/sra/PRJNA514351) and GEO accession GSE129959.

**Figure Legends**

**Figure 1. CONSORT Flow Diagram of Parent Study and Subset Sample.** Of 26 participants included in the parent study, nasal epithelial samples from four had sufficient RNA to be included in the subset sample.

**Figure 2. Gene Ontology (GO) and Reactome pathway enrichment analysis of the DEGs in THS exposed human nasal epithelium.** Bar charts showing the most highly enriched Biological Process (A) and Cellular Component (B) GO terms. Each bar represents the number of genes identified in our study that are associated with each process or component. All Biological Process and Cellular Components identified had an adjusted p-value for multiple testing < 0.05. (C) Network plot showing the top six enriched pathways and the associated genes using Reactome PA. Also shown are the approximate fold change values of each gene (fold-change 2 = yellow; fold-change 3 = green; fold-change 4 = blue; fold-change 5 = purple; fold-
The colored lines show the link between the genes and pathways identified. Abbreviations for bar graph (A): RNP= Ribonucleoprotein; ETC= Electron Transport Chain; ET= electron transport; Mt= Mitochondria. Abbreviations for bar graph (B): IMM= Mitochondrial inner membrane; MM= Mitochondrial membrane; Mt=Mitochondria; RC= Respiratory Chain; LSU= Large Subunit; MRC= Mitochondrial Respiratory Chain; NADH-DN= NADH Dehydrogenase Complex.

Figure 3. Schematic diagram summarizing the responses of human nasal epithelium to THS. THS induced cellular stress leading to activation of cell survival responses including activation of DNA repair pathways, increased cell proliferation, activation of DNA repair pathways, and increased mitochondrial activity in human nasal epithelium. Previous in vitro studies have shown similar results where THS causes DNA damage\textsuperscript{9,10}, increase proliferation\textsuperscript{11,43}, increase mitochondria activity\textsuperscript{12} and increase ROS\textsuperscript{12}. 
| IPA Pathways                                      | p-Value   | p-Value > 0.01 | p-Value < 0.01 | # of Genes |
|--------------------------------------------------|-----------|----------------|---------------|------------|
| **Canonical Pathways**                           |           |                |               |            |
| Sirtuin Signaling Pathway                        | 1.23E-02  | X              |               | 11         |
| EIF2 Signaling                                   | 4.57E-03  |                | X             | 10         |
| Mitochondrial Dysfunction                       | 2.69E-03  |                | X             | 9          |
| Oxidative Phosphorylation                        | 5.13E-04  |                | X             | 8          |
| Hereditary Breast Cancer Signaling               | 3.89E-02  | X              |               | 6          |
| Oncostatin M Signaling                           | 2.88E-03  |                | X             | 4          |
| Nucleotide Excision Repair Pathway               | 3.24E-03  |                | X             | 4          |
| Colanic Acid Building Blocks Biosynthesis        | 1.74E-03  |                | X             | 3          |
| Methionine Degradation I (to Homocysteine)       | 5.01E-03  |                | X             | 3          |
| Cysteine Biosynthesis III (mammalia)             | 6.61E-03  |                | X             | 3          |
| Glutathione-mediated Detoxification              | 8.32E-03  |                | X             | 3          |
| Superpathway of Methionine Degradation           | 2.34E-02  | X              |               | 3          |
| Serine Biosynthesis                              | 3.02E-03  |                | X             | 2          |
| Superpathway of Serine and Glycine Biosynthesis I| 6.17E-03  |                | X             | 2          |
| γ-glutamyl Cycle                                 | 2.45E-02  | X              |               | 2          |
| UDP-N-acetyl-D-galactosamine Biosynthesis I       | 1.78E-02  |                | X             | 1          |
| Spliceosomal Cycle                               | 3.47E-02  |                | X             | 1          |
| L-DOPA Degradation                               | 3.47E-02  |                | X             | 1          |
| GDP-L-fucose Biosynthesis I (from GDP-D-mannose) | 3.47E-02  |                | X             | 1          |
| **Top Toxicological Pathways**                   |           |                |               |            |
| Mitochondrial Dysfunction                        | 3.03E-03  |                | X             | 9          |
| Increases Transmembrane Potential of Mitochondria and Mitochondrial Membrane | 5.82E-02 |                | X             | 3          |
| Decreases Permeability Transition of Mitochondria and Mitochondrial Membrane | 6.16E-03 |                | X             | 2          |
| Glutathione Depletion Phase                      | 4.36E-02  |                | X             | 2          |
| Categories                  | Diseases or Functions Annotation | Predicted Activation State | Activation z-score | # Molecules | p-Value   | p-Value > 0.01 | p-Value < 0.01 |
|----------------------------|----------------------------------|----------------------------|--------------------|-------------|-----------|----------------|----------------|
| Cell Death and Survival    | Cell death                       | Decreased                  | -3.117             | 77          | 1.97E-03  |                |                |
| Cell Death and Survival    | Apoptosis                        | Decreased                  | -3.686             | 63          | 1.00E-03  |                |                |
| Cell Death and Survival    | Necrosis                         | Decreased                  | -2.641             | 59          | 3.59E-02  |                |                |
| Cell Death and Survival    | Cell death of tumor cell lines   | Decreased                  | -3.029             | 50          | 2.80E-02  |                |                |
| Cell Death and Survival    | Apoptosis of tumor cell lines    | Decreased                  | -2.617             | 41          | 2.20E-02  |                |                |
| Cell Death and Survival    | Cell viability                   | Increased                  | 5.026              | 38          | 1.45E-02  |                |                |
| Cell Death and Survival    | Cell viability of tumor cell lines| Increased                  | 4.59               | 32          | 2.16E-02  |                |                |
| Cell Death and Survival    | Cell viability of breast cancer cell lines | Increased | 3.094 | 10 | 1.84E-02 | | |
| Cell Death and Survival    | Cell viability of blood cells    | Increased                  | 2.195              | 6           | 1.58E-02  |                |                |
| Cell Death and Survival    | Cell viability of leukocytes     | Increased                  | 2.2                | 5           | 3.34E-02  |                |                |
| Infectious Diseases        | Viral infection                  | Increased                  | 5.315              | 54          | 2.00E-03  |                |                |
| Infectious Diseases        | Infection by RNA virus           | Increased                  | 4.494              | 31          | 2.56E-02  |                |                |
| Infectious Diseases        | Infection of cells              | Increased                  | 4.594              | 29          | 9.26E-03  |                |                |
| Infectious Diseases        | Infection by HIV-1               | Increased                  | 4.301              | 23          | 2.34E-02  |                |                |
| Infectious Diseases        | Replication of RNA virus         | Increased                  | 3.087              | 19          | 4.12E-03  |                |                |
| Infectious Diseases        | Infection of cervical cancer cell lines | Increased | 3.772 | 18 | 1.03E-02 | | |
| Infectious Diseases        | Replication of Influenza A virus | Increased                  | 2.824              | 13          | 5.64E-03  |                |                |
| Cell Cycle, DNA            | Homologous                       | Increased                  | 2.828              | 8           | 1.63E-01  |                |                |
| Replication, Recombination, and Repair | recombination of cells |          | 05 |          |
|---------------------------------------|-----------------------|----------|----|----------|
| Cellular Development, Cellular Growth and Proliferation | Cell proliferation of breast cancer cell lines | Increased | 2.811 | 18 | 3.37E-02 | X |