RNA-SEQ ANALYSIS OF ALTERATIONS IN HUMAN BRONCHIAL EPITHELIAL CELL TRANSCRIPTOMES FOLLOWING EXPOSURE TO ELECTRONIC (E)-CIGARETTE VAPORS

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Tobacco use is a leading cause of preventable disease and death worldwide

According to the World Health Organization (WHO) *

~ 5 million deaths are caused by tobacco use annually

the estimated death toll from tobacco use associated disease is expected to be

~ 1 billion in the 21st Century

The Centers for Disease Control and Prevention (CDCP) estimates that

~ 1 in 5 deaths in the US each year are attributable to tobacco use or exposure

~ 443,000 people die prematurely from smoking or exposure to second-hand smoke

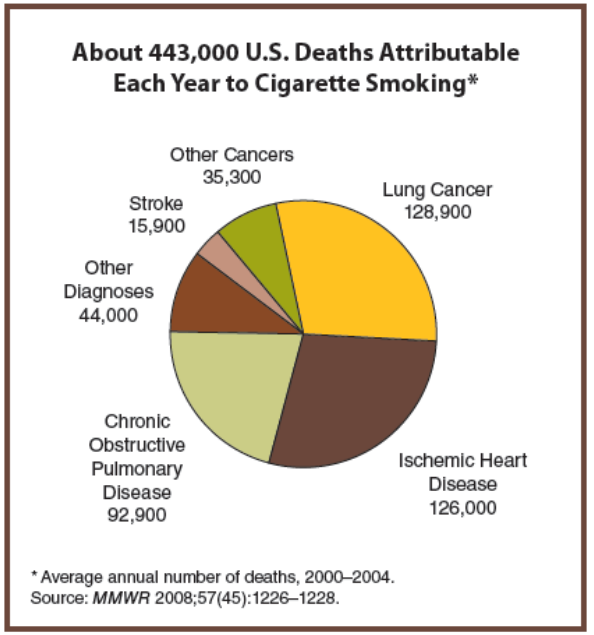
~ 8.6 million people suffer with a serious illness caused by smoking.

Major contributors :

Chronic obstructive pulmonary disease (COPD)

Lung and oral cancers

Related respiratory disorders



* whqlibdoc.who.int/publications/2011/9789241501576_eng.pdf

A significant economic burden is coupled with the enormous health toll worldwide

$96 billion per year in medical expenditures

$100 billion per year resulting from lost productivity

Although health professionals feel it ideal for all smokers is to quit completely, a substantial proportion of smokers either do not want to stop smoking or are unable to do so despite many attempts.

"Harm reduction" strategies are aimed at reducing the adverse health effects of tobacco use in these individuals.

Reduced cigarette consumption

Changing consumption habits to "low tar" cigarettes

Switching to other smoke tobacco products (cigars, pipes)

Use of non-tobacco containing cigarette products

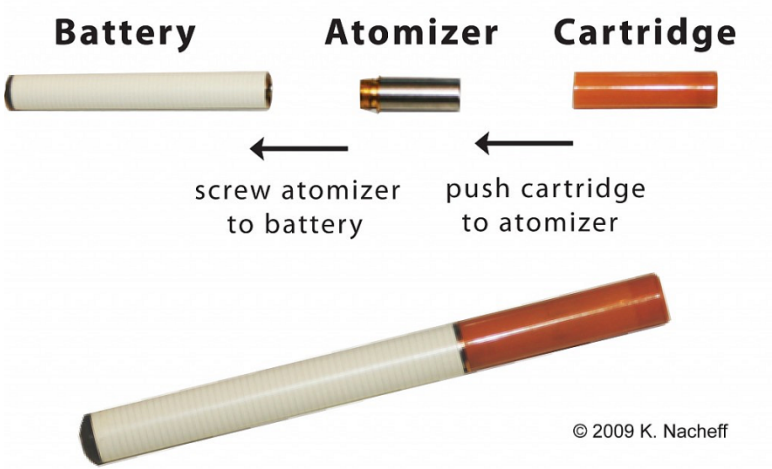
Use of chewing, snus and other smokeless oral tobacco products

Use of nicotine replacement therapy (NRT)

Use of alternative smoking devices and nicotine delivery products ("electronic" cigarettes)

Electronic cigarettes - Nicotine vapor delivery devices

Electronic cigarettes, or "e-cigarettes", are battery-operated electronic devices resembling traditional cigarette products that are designed to deliver nicotine, flavor and other chemicals in a "vapor" that is inhaled by the user.



http://www.e-cigarettepedia.com/tag/definition-of-electronic-cigarette/

Contain, a nicotine-containing reservoir, a battery, and an atomizer or heating element that is triggered by the user inhaling that generates a warm nicotine-containing mist or vapor. The inhaled nicotine vapor usually consists of propylene glycol as a vehicle.

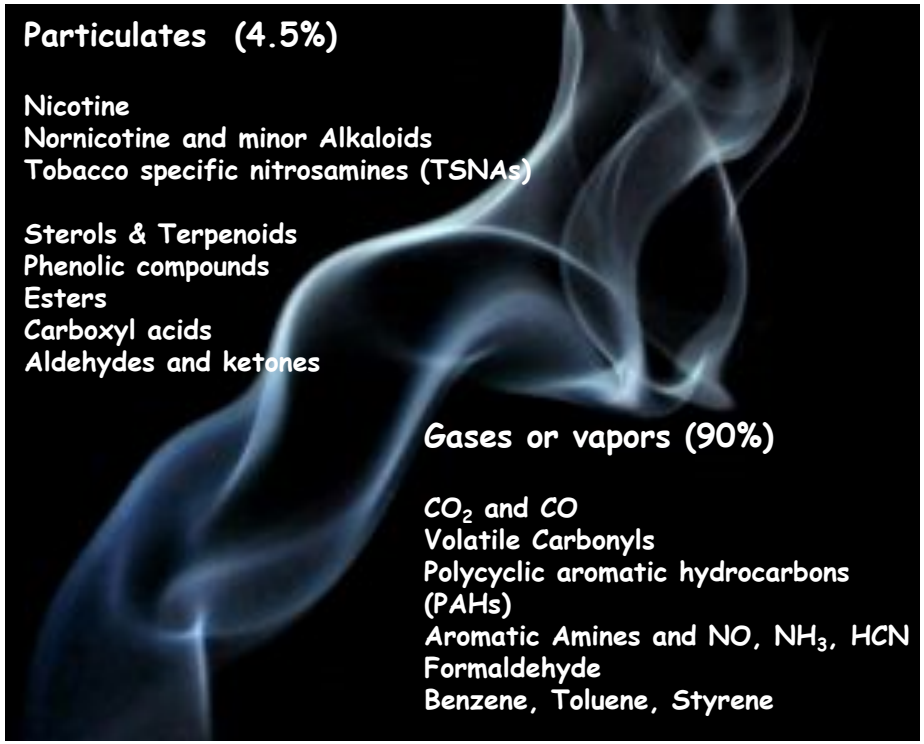
Do not contain actual tobacco leaf, do not emit vapor unless the user is inhaling, and do not require ignition by a lighter or match.

http://www.njoy.com/pages/FAQs.html, accessed on October 21, 2011.

Smoke Composition

A complex mixture of > 5000 chemical constituents both volatile and nonvolatile

Known to contain a variety of FDA /WHO defined harmful components



Vapor Composition

A relatively simple mixture of volatile chemical constituents – primary components known, variable minor components sometimes present; may contain hazardous impurities (e.g., diethylene glycol (DEG)* TSNAs*, anabasine**, myosmine**, and β-nicotyrine**).

*Cahn Z and Siegel M Journal of Public Health Policy (2011) 32, 16–31. doi:10.1057/jphp.2010.41

**Food and Drug Admininstration http://www.fda.gov/NewsEvents/PublicHealthFocus/ucm173146.htm

The Family Smoking Prevention and Tobacco Control Act (Tobacco Control Act) - June 22, 2009 –

Food and Drug Administration (FDA) has the authority to regulate the manufacture, distribution, and marketing of tobacco products to protect public health.

December 2010, an appellate panel of three judges said e-cigarettes did not meet the definition of medical devices under the Federal Food, Drug and Cosmetic Act.

The U.S. Court of Appeals for the D.C. Circuit, in *Sottera, Inc. v. Food & Drug Administration*, 627 F.3d 891 (D.C. Cir. 2010), recently issued a decision with regard to e-cigarettes and other products "made or derived from tobacco" and the jurisdictional line that should be drawn between "tobacco products" and "drugs," "devices," and combination products, as those terms are defined in the FD&C Act. The court held that e-cigarettes and other products made or derived from tobacco can be regulated as "tobacco products" under the Act and are not drugs/devices unless they are marketed for therapeutic purposes.

On January 24, 2011 the U.S. Court of Appeals for the District of Columbia Circuit said it would not review a decision blocking the products from FDA regulation as medical devices.

Classification as drugs/ drug delivery devices would have required clinical trials for e-cigarettes.

Since their introduction in the US in 2008, sales and use have expanded rapidly with an estimated $300 million market in 2011

Are electronic cigarettes harmless?

That is a difficult question to answer!

E-cigarettes and similar devices are still in their infancy
(products are varied in construction and still evolving)

Unlike medicinal inhalers there is no standardization within the industry
-unregulated amounts of delivery
-high variation in product composition (low level contaminants)

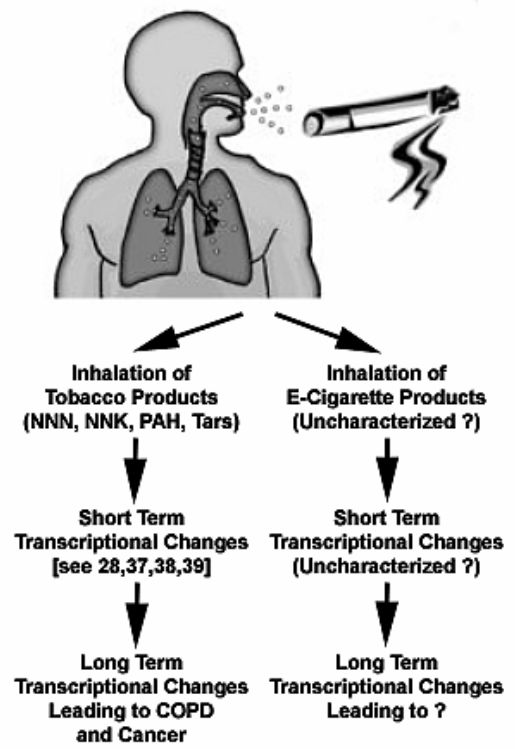
Little (not enough) scientific evaluation has been carried out to date to be conclusive
.

Specific Aims of Study

To determine the effects of exposure to e-cigarette vapor on normal human lung (bronchial) epithelial (NHBE) cell function

Contrast the effects of "vaping" versus MSS exposure

To evaluate whether e cigarettes offer any "harm reduction"



Research Strategy

Establish normal human lung (bronchial) epithelial (NHBE) cell cultures in an *in vitro* model for exposure at a liquid-air interface

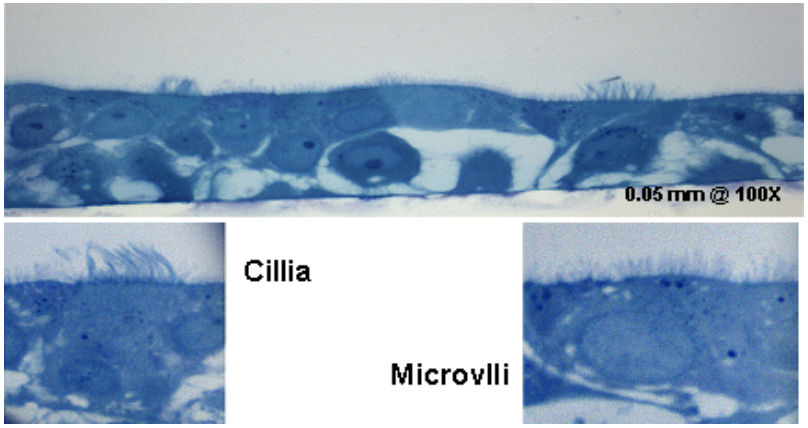
Use transcriptomic profiling (Next Gen sequencing with Illumina RNA-seq technology) to describe alterations NHBE cell function before and after exposure to "e-vapor" and MSS from tobacco cigarettes

Growth of Normal Human Bronchial Epithelial (NHBE) Cell Cultures

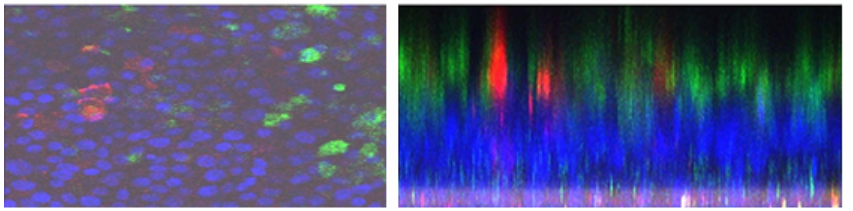
Primary Normal Human Bronchial /Tracheal Epithelial (NHBE) cells were (purchased from Lonza, Walkersville, MD)
Two donor cell lines: healthy non-smoking 16 year-old female and a healthy 26 year -old female non-smoker

Cells (*passage 3*) were seeded onto cell culture inserts (Transwell- Clear, 6.5-mm diameter, 0.4-mM pore size; Corning) at a density of 7.5 x10^4 cells per insert and differentiated as previously described (Maunder et al., 2007) to form a multilayered, apically-ciliated, differentiated epithelium between 21-and 23 days post induction.

Structure and organization

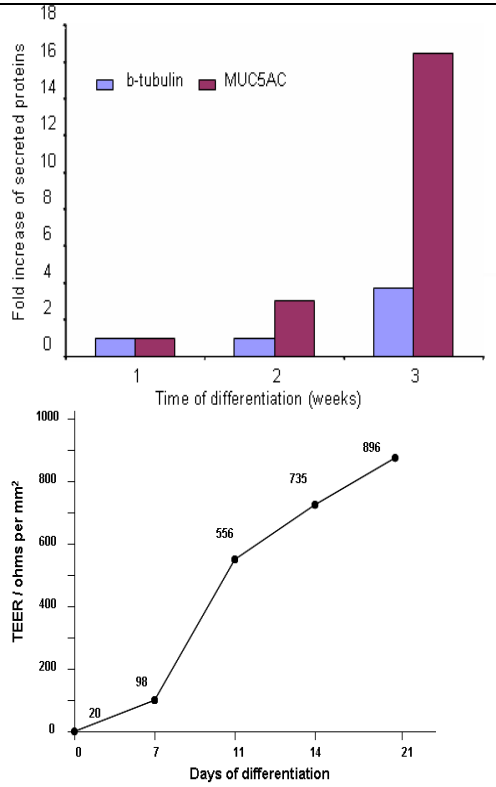


Semithin (0.25 um) plastic embedding . Photograph by T. Kotova & MS Forbes



Goblet cells (microvilli) - ~7.0% Alexa 658 (red) Mouse anti-MUC5AC
Ciliated cells - ~21.1% Alexa 488 (green) Rabbit anti β-tubulin
Columnar epithelium (basal cells) - DAPI stained nuclei (blue)

Mucin and tight junction formation



Immunodetection of MUC5AC and β-tubulin secreted by NHBE cells during lung cell differentiation (left) and acquisition of Trans Epithelial Electrical Resistance (TEER) during differentiation.

Magma Brand Electronic Cigarette

Manufacturer: VolcanoeCigs

http://www.volcanoecigs.com/

Purchased online

Re-usable cartridges

Premium USA V-Liquid – Tobacco flavor

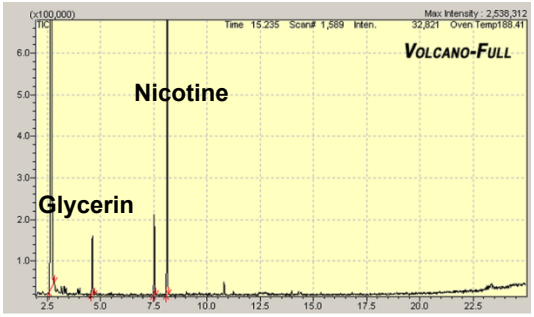
"Tobacco flavoring in it's purest form. Our Tobacco Pure flavor is derived from actual tobacco leaves and is the best representation of a true analog tobacco cigarette you will find anywhere."

Ingredients:

Propylene Glycol (USP Grade),
Glycerine (USP Grade),
Nicotine (optional)
Natural and/or Artificial flavoring.

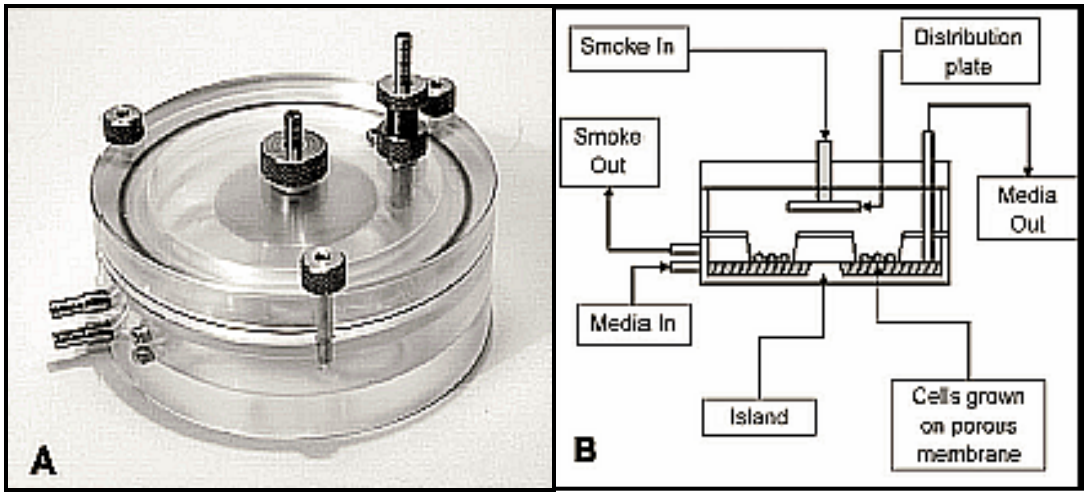
Nicotine levels:

Full Strength - 16 mg
Lights - 8 mg
Zero - 0 mg



Samples dissolved in methanol and separated on a Shimadzu QP2010Plus GC-MS

E- vapor and MSS exposure at a liquid-air interface



British American Tobacco UK and Curbridge Engineering, Ltd. Southhampton, UK
Photo from Maunders et al. (2007) AJP Lung Cell Mol Physiol 292: L1248–L1256

MSS - generated by a TE-10 smoking machine (Teague Enterprises, Davis, CA).

International Organization for Standardization (IOS) parameters:
35-cm^3 puff drawn over 2 sec every 1 min period;
6 min cycle per cigarette (5 min of smoking, 1 min non-smoking)
~ 10 cigarettes smoked per 1 H of treatment

Air/smoke mixture diluted with filtered room air to the desired (constant) TPM and TSP

E-vapor - generated to simulate MSS smoke exposure

35-cm^3 puff drawn over 2 sec every 1 min period; 40-60 total
Cartrige refilled as need to provide 1 H of treatment

No dilution of vapor required to maintain 95% viability over duration of experiment
as determined by Neutral Red cell viability assays and TEER measurements

Evaluating E-vapor and MSS-induced Changes in Gene Expression

Two donor cell lines:

(16, 26 year old
Non-smoking females)

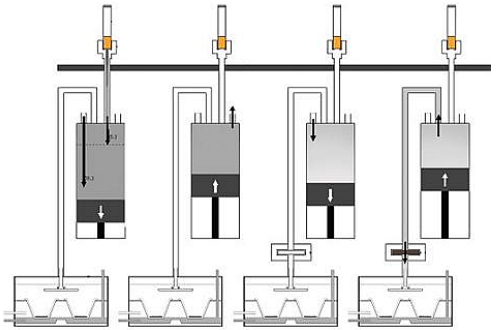
Four treatments:

Air alone (control)
E-vapor – 0 mg Nicotine
E-vapor – 16 mg Nicotine
MSS – 1R5F Reference

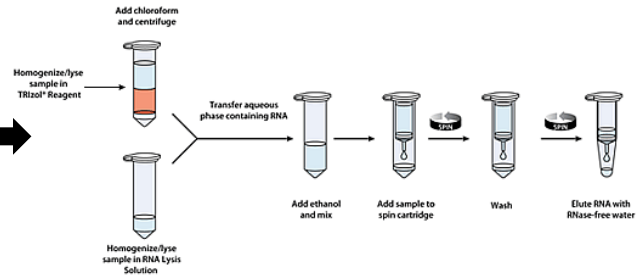
Three time points:

1 H exposure
4 H post-exposure
24 H post exposure

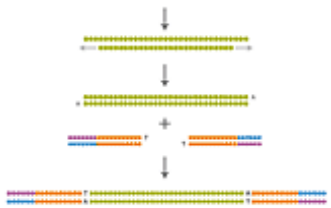
General Flow of Experiments



Exposure of NHBE cells to E-vapor or MSS



RNA isolation (TRIzol® Reagent and Purelink® RNA Mini Kit)



Library preparation using Truseq sample preparation

Sequencing by synthesis on a Illumina GAIIx instrument ~ 1.5 – 8.5 days

Bioinformatics Analysis

Read counts
Quality control metrics (FASTQC)
Read assembly
Align against human RefSeq transcriptome
ANOVA and statistical significance
Ingenuity Pathway Analysis (IPA)

Analytical Methods

Generated 3-5 million reads per sample

• FASTQC used for initial read QC metrics (base quality distribution, etc.); one run (8 samples) repeated due to gross failures

• "Sickle" was used to trim low-quality ends since samples exhibited a drop in quality score boxplots in the last 10-15 bases (of 86);

•"Bowtie" used to align against human RefSeq transcriptome, with "RSEM" based quantification of gene/isoform-level expression

• RSEM counts were transformed to stabilize variance estimates, using "voom"

• Based on PCA plots, four samples deemed to be significant "outliers", thus failing QC

•"Limma"-based moderated-ANOVA used to estimate fold change and statistical significance of various comparisons of interest, adjusting for donor-specific effects

• Per-sample influence on ANOVA was reweighted by per-sample "conformity" to bulk expression

• Filtering: of 39,680 isoforms having counts in any sample, 17,130 isoforms exhibited at least 2 cpm in 10 or more samples.

Raw Sequencing Results

Treatment	Donor	Sample ID	Time Point	Number of Reads	GC Filter
Air treatment	1	Z315	1 H	29951033	
Air treatment	1	Z316	4 H	36563966	
Air treatment	1	Z317	24 H	39897976	
Air treatment	2	Zd328	1 H	40734918	
Air treatment	2	Zd329	4 H	42785060	
Air treatment	2	Zd330	24 H	44336294	
E-vapor 0 mg nicotine	1	Z321	1 H	41151411	
E-vapor 0 mg nicotine	1	Z322	4 H	41577282	
E-vapor 0 mg nicotine	1	Z323	24 H	40545029	
E-vapor 0 mg nicotine	2	Z334	1 H	39548678	
E-vapor 0 mg nicotine	2	Z335	4 H	33465346	
E-vapor 0 mg nicotine	2	Z336	24 H	44490185	
E-vapor 16 mg nicotine	1	Z324	1 H	33887312	PCA fail
E-vapor 16 mg nicotine	1	Zd325	4 H	41802365	
E-vapor 16 mg nicotine	1	Zd326	24 H	39032577	
E-vapor 16 mg nicotine	2	Z337	1 H	29152118	
E-vapor 16 mg nicotine	2	Z338	4 H	40885872	
E-vapor 16 mg nicotine	2	Z339	24 H	28394709	
MSS 1R5F Reference	1	Z318	1 H	30140360	
MSS 1R5F Reference	1	Z319	4 H	41345795	
MSS 1R5F Reference	1	Z320	24 H	40980601	PCA fail
MSS 1R5F Reference	2	Z331r	1 H	41391596	PCA fail
MSS 1R5F Reference	2	Z332	4 H	42510881	PCA fail
MSS 1R5F Reference	2	Z333r	24 H	38913418	

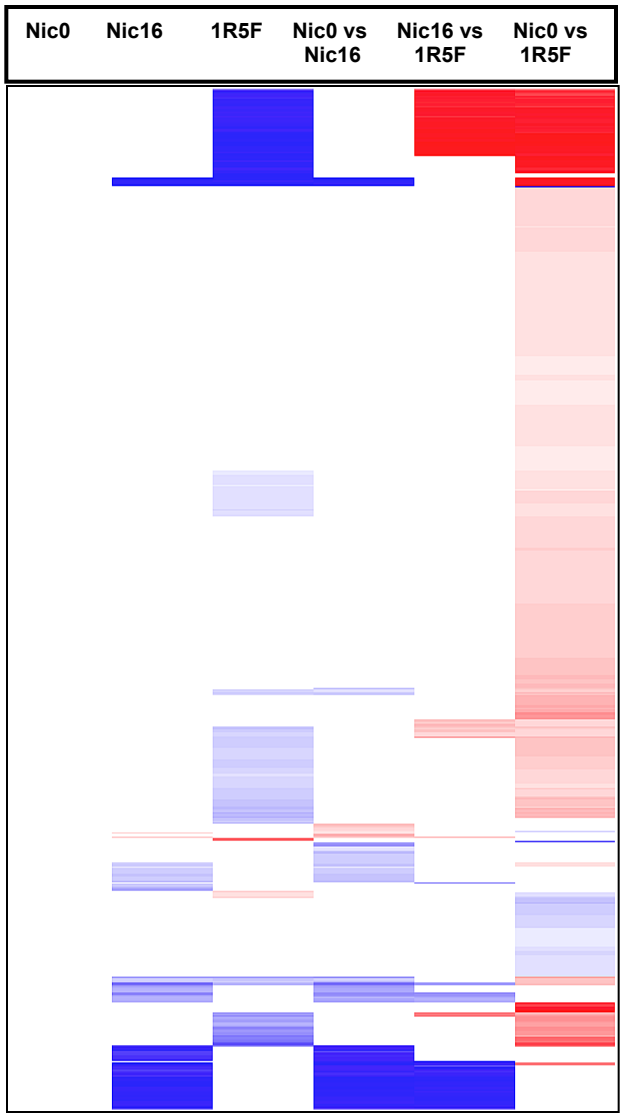
Differentially Expressed Transcript Counts at Different False Discovery Rates (FDRs)

FDR:	20%		10%		5%		2%		1%	
Direction of change:	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
E-vapor 0 mg vs Air (1 H Exposure)	5	9	0	0	0	0	0	0	0	0
E-vapor 16 mg vs Air (1 H exposure)	157	31	86	7	61	2	47	0	34	0
MSS vs Air (1 H exposure)	978	326	356	35	144	5	69	0	49	0
E-vapor 16 mg vs E-vapor 0 mg (1 H exposure)	460	193	142	29	73	7	54	2	35	1
E-vapor 16 mg vs MMS (1 H exposure)	94	155	54	80	31	45	18	34	15	27
E-vapor 0 mg vs MMS (1 H exposure)	960	1811	294	933	45	389	3	118	0	71
E-vapor 0 mg vs Air (4 H exposure)	1	2	1	2	1	2	1	0	1	0
E-vapor 16 mg vs Air (4 H exposure)	0	0	0	0	0	0	0	0	0	0
MSS vs Air (4 H exposure)	54	17	34	10	27	6	9	0	4	0
E-vapor 16 mg vs E-vapor 0 mg (4 H exposure)	0	1	0	1	0	1	0	1	0	1
E-vapor 16 mg vs MMS (4 H exposure)	6	40	3	31	2	23	0	5	0	4
E-vapor 0 mg vs MMS (4 H exposure)	9	42	4	32	3	21	1	18	1	10
E-vapor 0 mg vs Air (24 H exposure)	0	1	0	1	0	1	0	0	0	0
E-vapor 16 mg vs Air (24 H exposure)	2	2	2	2	2	1	1	1	0	1
MSS vs Air (24 H exposure)	577	566	222	237	63	82	27	21	14	8
E-vapor 16 mg vs E-vapor 0 mg (24 H exposure)	2	0	2	0	2	0	0	0	0	0
E-vapor 16 mg vs MMS (24 H exposure)	1326	1483	592	603	170	156	29	37	7	16
E-vapor 0 mg vs MMS (24 H exposure)	469	495	229	189	111	71	27	26	5	14

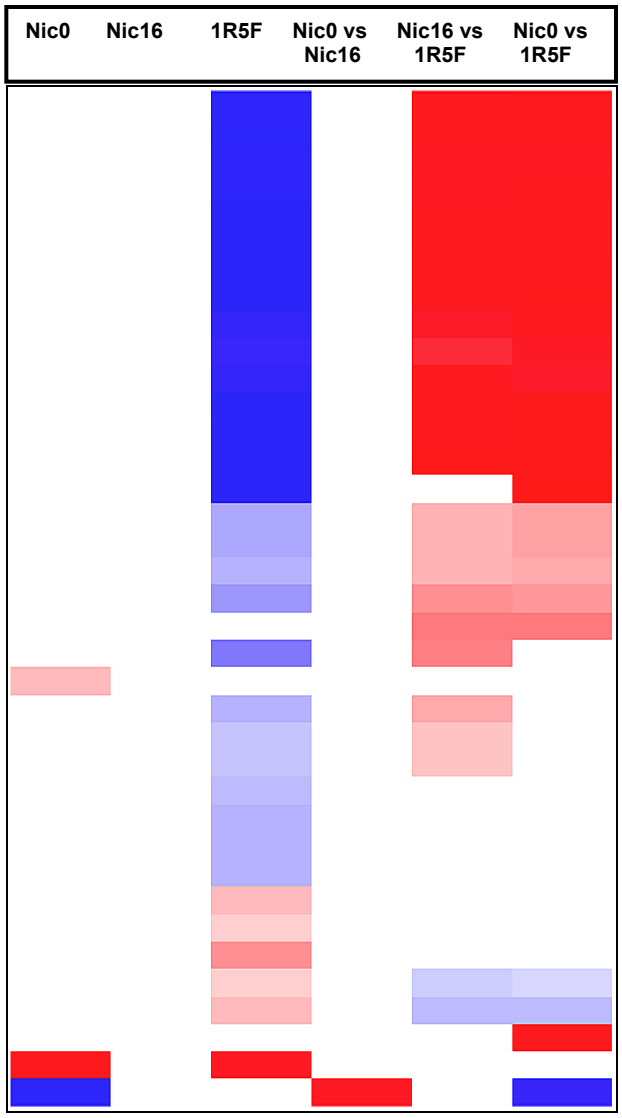
Heat maps showing differentially expressed transcripts

(normalized to Air –treated control)

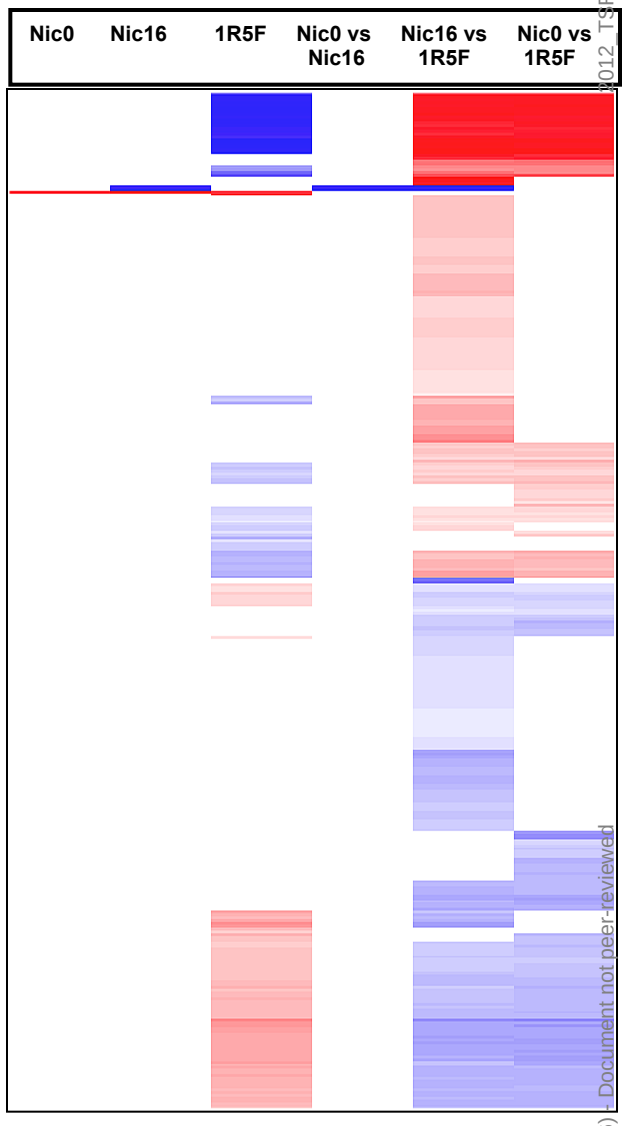
1 H exposure



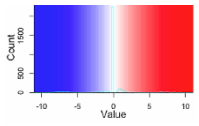
4 H post-exposure

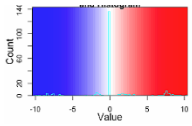


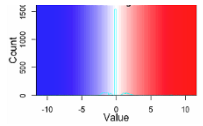
24 H post-exposure



Gene count

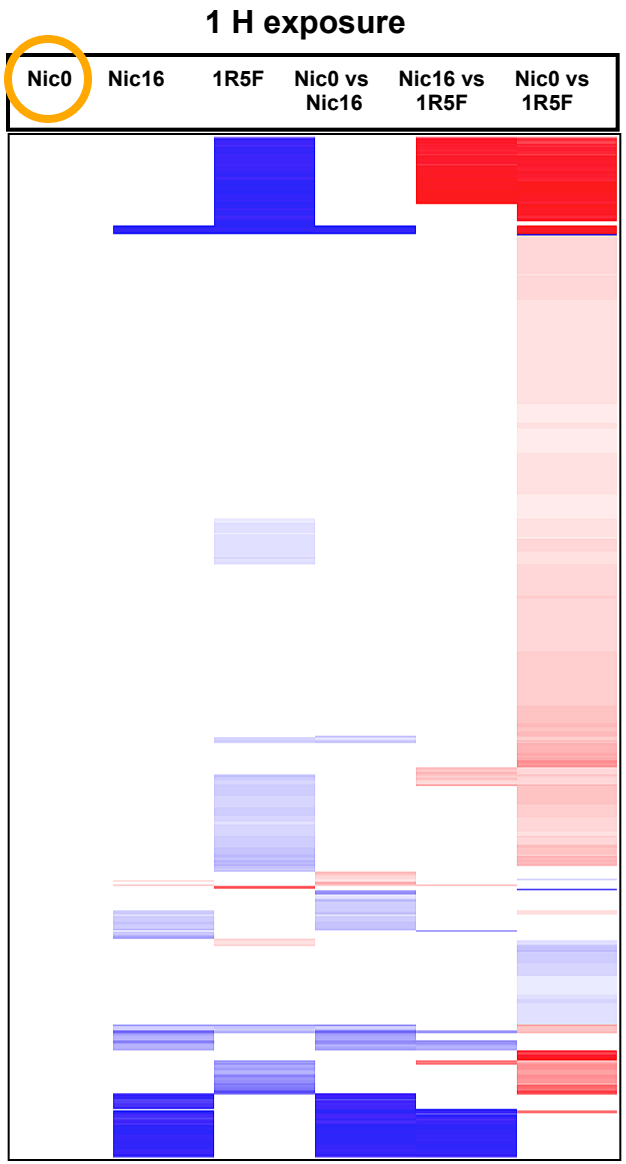






After 1 H of exposure E-vapor alone has little to no effect on NHBE transcriptome composition

FDR:	20%		10%	
Direction of change:	Neg	Pos	Neg	Pos
E-vapor 0 mg vs Air (1 H Exposure)	5	9	0	0
E-vapor 16 mg vs Air (1 H exposure)	157	31	86	7
MSS vs Air (1 H exposure)	978	326	356	35
E-vapor 16 mg vs E-vapor 0 mg (1 H exposure)	460	193	142	29
E-vapor 16 mg vs MMS (1 H exposure)	94	155	54	80
E-vapor 0 mg vs MMS (1 H exposure)	960	1811	294	933
E-vapor 0 mg vs Air (4 H exposure)	1	2	1	2
E-vapor 16 mg vs Air (4 H exposure)	0	0	0	0
MSS vs Air (4 H exposure)	54	17	34	10
E-vapor 16 mg vs E-vapor 0 mg (4 H exposure)	0	1	0	1
E-vapor 16 mg vs MMS (4 H exposure)	6	40	3	31
E-vapor 0 mg vs MMS (4 H exposure)	9	42	4	32
E-vapor 0 mg vs Air (24 H exposure)	0	1	0	1
E-vapor 16 mg vs Air (24 H exposure)	2	2	2	2
MSS vs Air (24 H exposure)	577	566	222	237
E-vapor 16 mg vs E-vapor 0 mg (24 H exposure)	2	0	2	0
E-vapor 16 mg vs MMS (24 H exposure)	1326	1483	592	603
E-vapor 0 mg vs MMS (24 H exposure)	469	495	229	189



Nicotine (16 mg) and other flavor components in the E-vapor leads to significant changes in NHBE transcriptome composition following 1 H of exposure

FDR:	20%		10%	
Direction of change:	Neg	Pos	Neg	Pos
E-vapor 0 mg vs Air (1 H Exposure)	5	9	0	0
E-vapor 16 mg vs Air (1 H exposure)	157	31	86	7
MSS vs Air (1 H exposure)	978	326	356	35
E-vapor 16 mg vs E-vapor 0 mg (1 H exposure)	460	193	142	29
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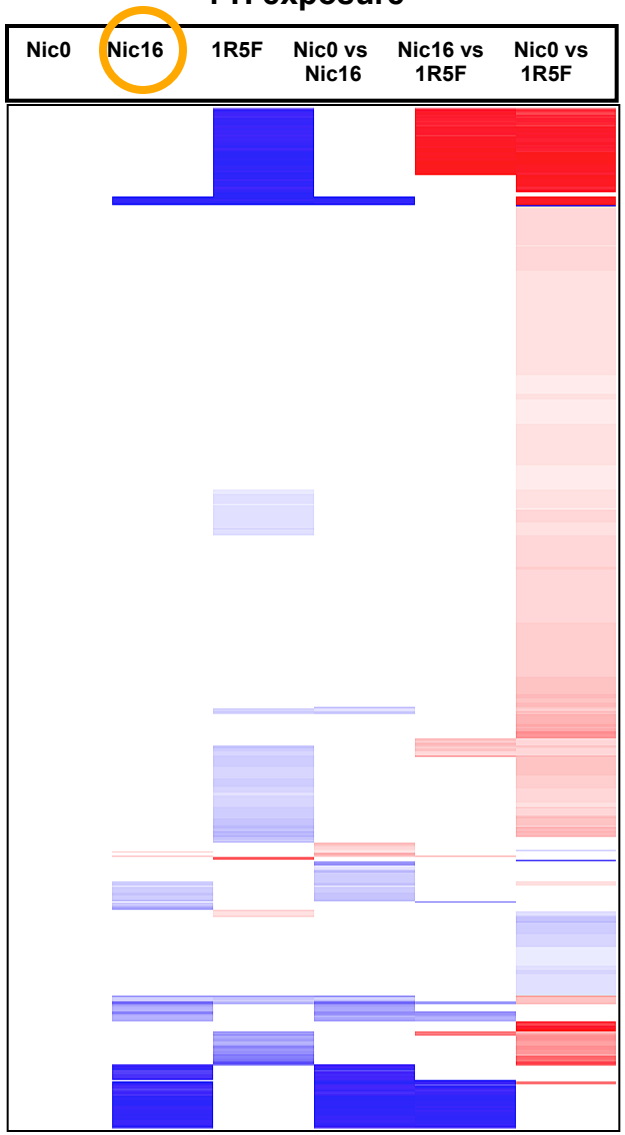
E-vapor 0 mg vs MMS (1 H exposure)	960	1811	294	933
E-vapor 0 mg vs Air (4 H exposure)	1	2	1	2
E-vapor 16 mg vs Air (4 H exposure)	0	0	0	0
MSS vs Air (4 H exposure)	54	17	34	10
E-vapor 16 mg vs E-vapor 0 mg (4 H exposure)	0	1	0	1
E-vapor 16 mg vs MMS (4 H exposure)	6	40	3	31
E-vapor 0 mg vs MMS (4 H exposure)	9	42	4	32
E-vapor 0 mg vs Air (24 H exposure)	0	1	0	1
E-vapor 16 mg vs Air (24 H exposure)	2	2	2	2
MSS vs Air (24 H exposure)	577	566	222	237
E-vapor 16 mg vs E-vapor 0 mg (24 H exposure)	2	0	2	0
E-vapor 16 mg vs MMS (24 H exposure)	1326	1483	592	603
E-vapor 0 mg vs MMS (24 H exposure)	469	495	229	189



Compared to E-vapor alone, E-vapor containing Nicotine (16 mg) and other flavor components has a substantially greater impact on transcriptome composition

FDR:	20%		10%	
Direction of change:	Neg	Pos	Neg	Pos
E-vapor 0 mg vs Air (1 H Exposure)	5	9	0	0
E-vapor 16 mg vs Air (1 H exposure)	157	31	86	7
MSS vs Air (1 H exposure)	978	326	356	35
E-vapor 16 mg vs E-vapor 0 mg (1 H exposure)	460	193	142	29
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E-vapor 0 mg vs Air (4 H exposure)	1	2	1	2
E-vapor 16 mg vs Air (4 H exposure)	0	0	0	0
MSS vs Air (4 H exposure)	54	17	34	10
E-vapor 16 mg vs E-vapor 0 mg (4 H exposure)	0	1	0	1
E-vapor 16 mg vs MMS (4 H exposure)	6	40	3	31
E-vapor 0 mg vs MMS (4 H exposure)	9	42	4	32
E-vapor 0 mg vs Air (24 H exposure)	0	1	0	1
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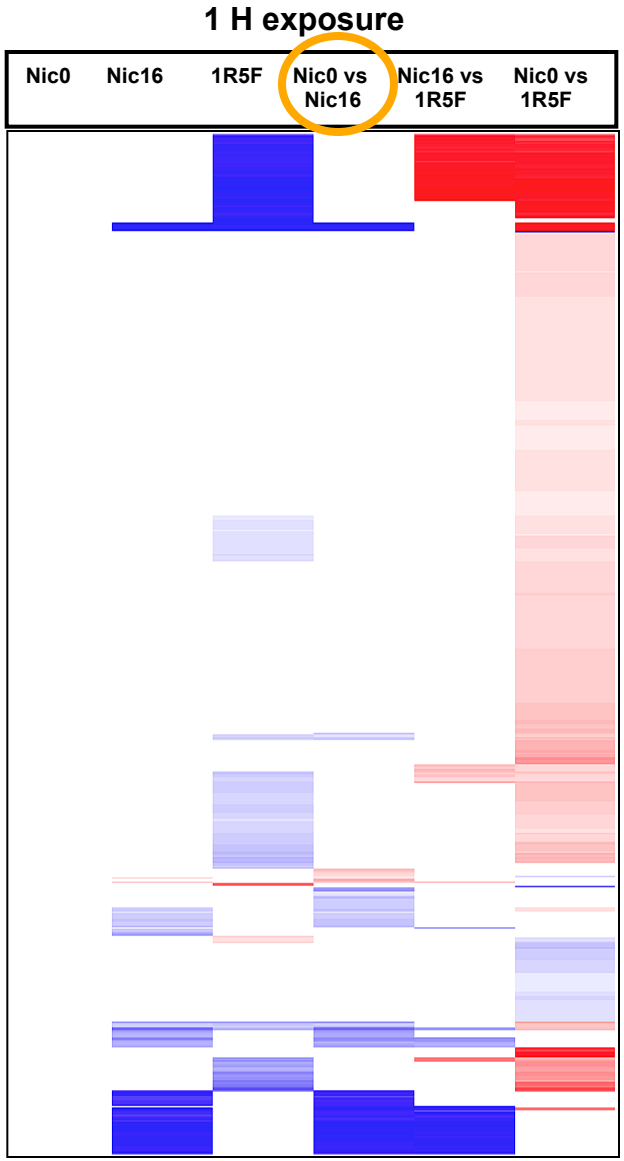
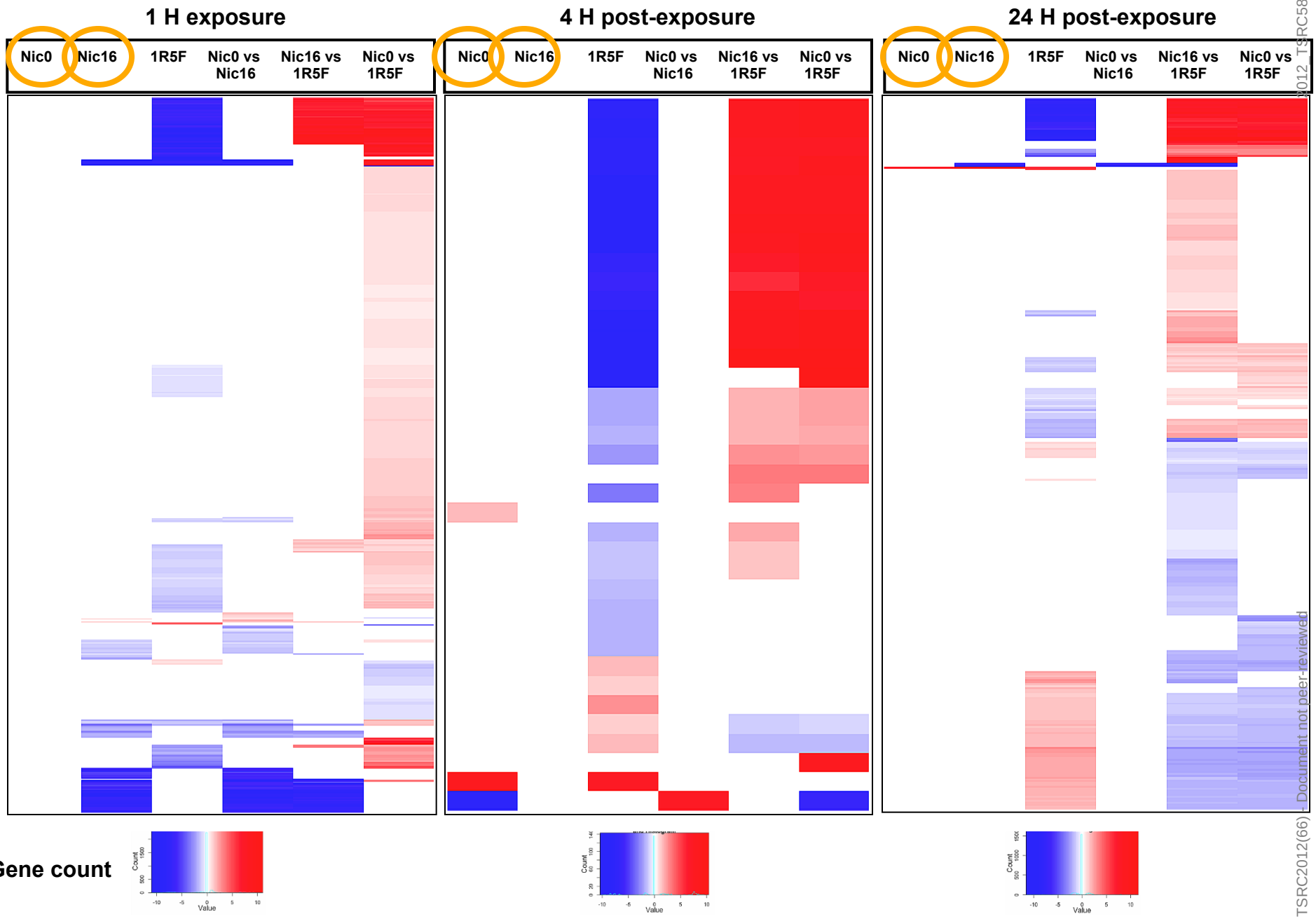
MSS vs Air (24 H exposure)	577	566	222	237
E-vapor 16 mg vs E-vapor 0 mg (24 H exposure)	2	0	2	0
E-vapor 16 mg vs MMS (24 H exposure)	1326	1483	592	603
E-vapor 0 mg vs MMS (24 H exposure)	469	495	229	189



NHBE cells show a very rapid recovery to baseline following exposure to E-vapor (with or without additive)

FDR:	20%		10%		5%		2%		1%	
Direction of change:	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
E-vapor 0 mg vs Air (1 H Exposure)	5	9	0	0	0	0	0	0	0	0
E-vapor 16 mg vs Air (1 H exposure)	157	31	86	7	61	2	47	0	34	0
MSS vs Air (1 H exposure)	978	326	356	35	144	5	69	0	49	0
E-vapor 16 mg vs E-vapor 0 mg (1 H exposure)	460	193	142	29	73	7	54	2	35	1
E-vapor 16 mg vs MMS (1 H exposure)	94	155	54	80	31	45	18	34	15	27
E-vapor 0 mg vs MMS (1 H exposure)	960	1811	294	933	45	389	3	118	0	71
E-vapor 0 mg vs Air (4 H exposure)	1	2	1	2	1	2	1	0	1	0
E-vapor 16 mg vs Air (4 H exposure)	0	0	0	0	0	0	0	0	0	0
MSS vs Air (4 H exposure)	54	17	34	10	27	6	9	0	4	0
E-vapor 16 mg vs E-vapor 0 mg (4 H exposure)	0	1	0	1	0	1	0	1	0	1
E-vapor 16 mg vs MMS (4 H exposure)	6	40	3	31	2	23	0	5	0	4
E-vapor 0 mg vs MMS (4 H exposure)	9	42	4	32	3	21	1	18	1	10
E-vapor 0 mg vs Air (24 H exposure)	0	1	0	1	0	1	0	0	0	0
E-vapor 16 mg vs Air (24 H exposure)	2	2	2	2	2	1	1	1	0	1
MSS vs Air (24 H exposure)	577	566	222	237	63	82	27	21	14	8
E-vapor 16 mg vs E-vapor 0 mg (24 H exposure)	2	0	2	0	2	0	0	0	0	0
E-vapor 16 mg vs MMS (24 H exposure)	1326	1483	592	603	170	156	29	37	7	16
E-vapor 0 mg vs MMS (24 H exposure)	469	495	229	189	111	71	27	26	5	14

NHBE cells show a very rapid recovery to baseline following exposure to E-vapor (with or without additive)



The effects of MSS on NHBE transcriptome composition are significant, robust and persistent

FDR:	20%		10%		5%		2%		1%	
Direction of change:	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
E-vapor 0 mg vs Air (1 H Exposure)	5	9	0	0	0	0	0	0	0	0
E-vapor 16 mg vs Air (1 H exposure)	157	31	86	7	61	2	47	0	34	0
MSS vs Air (1 H exposure)	978	326	356	35	144	5	69	0	49	0
E-vapor 16 mg vs E-vapor 0 mg (1 H exposure)	460	193	142	29	73	7	54	2	35	1
E-vapor 16 mg vs MMS (1 H exposure)	94	155	54	80	31	45	18	34	15	27
E-vapor 0 mg vs MMS (1 H exposure)	960	1811	294	933	45	389	3	118	0	71
E-vapor 0 mg vs Air (4 H exposure)	1	2	1	2	1	2	1	0	1	0
E-vapor 16 mg vs Air (4 H exposure)	0	0	0	0	0	0	0	0	0	0
MSS vs Air (4 H exposure)	54	17	34	10	27	6	9	0	4	0
E-vapor 16 mg vs E-vapor 0 mg (4 H exposure)	0	1	0	1	0	1	0	1	0	1
E-vapor 16 mg vs MMS (4 H exposure)	6	40	3	31	2	23	0	5	0	4
E-vapor 0 mg vs MMS (4 H exposure)	9	42	4	32	3	21	1	18	1	10
E-vapor 0 mg vs Air (24 H exposure)	0	1	0	1	0	1	0	0	0	0
E-vapor 16 mg vs Air (24 H exposure)	2	2	2	2	2	1	1	1	0	1
MSS vs Air (24 H exposure)	577	566	222	237	63	82	27	21	14	8
E-vapor 16 mg vs E-vapor 0 mg (24 H exposure)	2	0	2	0	2	0	0	0	0	0
E-vapor 16 mg vs MMS (24 H exposure)	1326	1483	592	603	170	156	29	37	7	16
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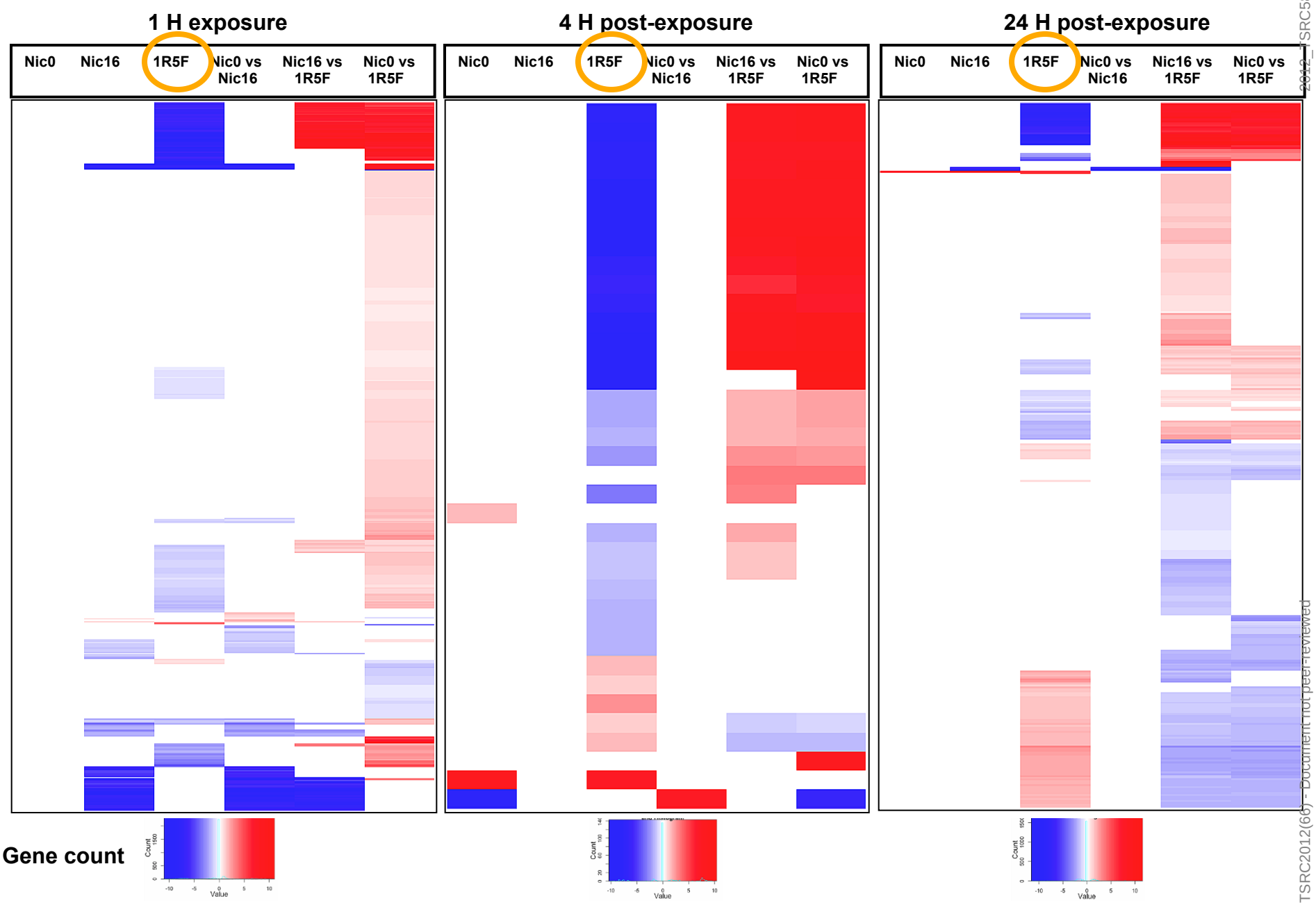
The effects of MSS on NHBE transcriptome composition are significant, robust and persistent



Exposure to E-vapor containing Nicotine (16 mg) and other flavor components is more comparable to MSS

FDR:	20%		10%		5%		2%		1%	
Direction of change:	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
E-vapor 0 mg vs Air (1 H Exposure)	5	9	0	0	0	0	0	0	0	0
E-vapor 16 mg vs Air (1 H exposure)	157	31	86	7	61	2	47	0	34	0
MSS vs Air (1 H exposure)	978	326	356	35	144	5	69	0	49	0
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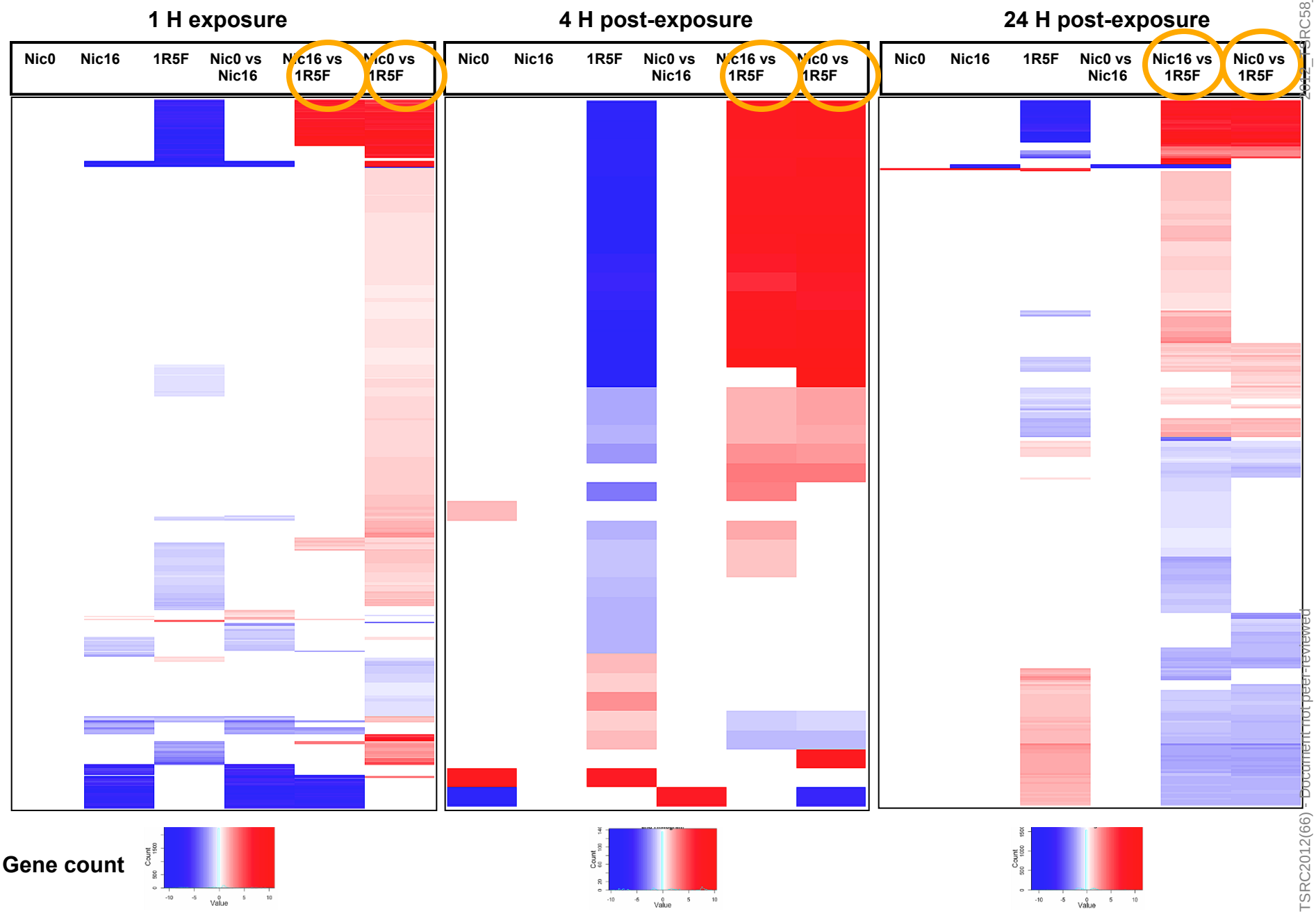
E-vapor 16 mg vs MMS (1 H exposure)	94	155	54	80	31	45	18	34	15	27
E-vapor 0 mg vs MMS (1 H exposure)	960	1811	294	933	45	389	3	118	0	71
E-vapor 0 mg vs Air (4 H exposure)	1	2	1	2	1	2	1	0	1	0
E-vapor 16 mg vs Air (4 H exposure)	0	0	0	0	0	0	0	0	0	0
MSS vs Air (4 H exposure)	54	17	34	10	27	6	9	0	4	0
E-vapor 16 mg vs E-vapor 0 mg (4 H exposure)	0	1	0	1	0	1	0	1	0	1
E-vapor 16 mg vs MMS (4 H exposure)	6	40	3	31	2	23	0	5	0	4
E-vapor 0 mg vs MMS (4 H exposure)	9	42	4	32	3	21	1	18	1	10
E-vapor 0 mg vs Air (24 H exposure)	0	1	0	1	0	1	0	0	0	0
E-vapor 16 mg vs Air (24 H exposure)	2	2	2	2	2	1	1	1	0	1
MSS vs Air (24 H exposure)	577	566	222	237	63	82	27	21	14	8
E-vapor 16 mg vs E-vapor 0 mg (24 H exposure)	2	0	2	0	2	0	0	0	0	0
E-vapor 16 mg vs MMS (24 H exposure)	1326	1483	592	603	170	156	29	37	7	16
E-vapor 0 mg vs MMS (24 H exposure)	469	495	229	189	111	71	27	26	5	14

Exposure to E-vapor containing Nicotine (16 mg) and other flavor components is more comparable to MSS



Ingenuity Pathway Analysis

RefSeq isoform lists (10% FDR; 7 /9 with nonzero counts) were uploaded to Ingenuity® Knowledge Base

[IPA uses a repository of biological interactions and functional annotations created from millions of individually modeled relationships between proteins, genes, complexes, cells, tissues, metabolites, drugs, and diseases.]

Many significant pathways/functions associated with smoke treatment; very few for e-vapor treatments, and minimal overlap

Analysis	Upstream Regulator	Log Ratio	Molecule Type	Predicted Activation State	Activation z-score
Observation 3	KRAS	↓-0.462	enzyme	Activated	2.000
Observation 9	5-fluorouracil		chemical drug	Activated	2.000
Observation 9	miR-1 (and other miRNAs w/seed GG		mature microRNA	Activated	2.185
Observation 3	tanespimycin		chemical drug	Activated	2.186
Observation 9	miR-17-5p (and other miRNAs w/see		mature microRNA	Activated	2.224
Observation 3	miR-155-5p (miRNAs w/seed UAAUC		mature microRNA	Activated	2.578
Observation 3	miR-302d-3p (and other miRNAs w/		mature microRNA	Activated	2.815
Observation 3	miR-124-3p (and other miRNAs w/se		mature microRNA	Activated	2.979
Observation 3	Vegf		group	Inhibited	-3.505
Observation 3	HGF		growth factor	Inhibited	-3.353
Observation 9	HGF		growth factor	Inhibited	-2.740
Observation 3	Tgf beta		group	Inhibited	-2.557
Observation 9	bleomycin		chemical drug	Inhibited	-2.449
Observation 2	PDGF BB		complex	Inhibited	-2.414
Observation 3	kainic acid		chemical toxicant	Inhibited	-2.384
Observation 2	EGF	↑0.285	growth factor	Inhibited	-2.373
Observation 2	forskolin		chemical toxicant	Inhibited	-2.370
Observation 9	Vegf		group	Inhibited	-2.359
Observation 3	FSH		complex	Inhibited	-2.287
Observation 9	bucladesine		chemical toxicant	Inhibited	-2.219
Observation 9	EGF	↑1.643	growth factor	Inhibited	-2.215
Observation 9	SP1	↑0.528	transcription regulator	Inhibited	-2.201
Observation 2	Vegf		group	Inhibited	-2.200
Observation 2	HGF		growth factor	Inhibited	-2.173
Observation 2	CREB1	↑0.403	transcription regulator	Inhibited	-2.170
Observation 2	cocaine		chemical drug	Inhibited	-2.133
Observation 3	SOX2-OCT4-NANOG		complex	Inhibited	-2.000
Observation 9	Retnlb		other	Inhibited	-2.000
Observation 9	TNFSF11		cytokine	Inhibited	-2.000

Highlights of Ingenuity Pathway Analysis

e-Vapor Nicotine (16 mg) versus Air (1 hr post exposure)

Inhibition: PGDF BB, Vegf, HGF, CREB1, forskolin, cocaine

PDGF-BB - platelet-derived growth factor / mitogenic factors for cells of mesenchymal origin; required for cellular repair; blocking of action associated with smoke exposure.

Vegf – well known to be reduced in response to cigarette smoke extract, reduced VEGF implicated in the destruction of alveolar wall components including microvasculature

HGF – over-expression previously correlated with and tumor stages; nicotine activated HGF expression found in lung cancer cells.

MSS versus Air (1 hr exposure)

Activation: **KRAS, miR-155-5p, miR-302d-3p, miR-124-3p, Tanespimycin**

KRAS - mutations in KRAS have been widely hypothesized to be related to direct tobacco exposure and associated with non-small cell lung cancers (NSCLCs)

miR-155 - an oncogenic microRNA that has been shown to increase in various types of human malignancy, including different forms of B cell lymphoma and carcinoma of breast, lung, colon, head/neck, and kidney.

miR-302d-3p -reprograming/monitoring - Human induced pluripotent stem cells (hiPSCs)

miR-124-3p -A well-known epigenetically silenced miRNA in human carcinogenesis

Inhibition of **Vegf, HGF, TGFβ, Kainic acid, FSH, SOX2-OCT4-NANOG**

Vegf – well known to be reduced in response to cigarette smoke extract, reduced VEGF implicated in the destruction of alveolar wall components including microvasculature

HGF – over-expression previously correlated with and tumor stages; nicotine activated HGF expression found in lung cancer cells.

TGFβ -increased expression by smoking irritation may interfere with the repair response

Sox2, Oct4 and Nanog - vital for the development and maintenance of pluripotent stem cells

Highlights of Ingenuity Pathway Analysis

MSS versus e-Vapor Nicotine (16 mg) (24 hr exposure)

Activation: miR-1, miR-17-5p, 5-fluorouracil

miR-1 - regulation of muscle, cardiovascular development

miR-17-5p - regulates MLL leukemia stem cell potential

Inhibition: Vegf, EGF, SP1, Retnlb,TNFSF11, Bleomycin, Bucladesine

Vegf – well known to be reduced in response to cigarette smoke extract, reduced VEGF implicated in the destruction of alveolar wall components including microvasculature

HGF – over-expression previously correlated with and tumor stages; nicotine activated HGF expression found in lung cancer cells.

SP1 – key regulator of cigarette smoke-induced *MUC5AC* mRNA transcription in lung epithelial cells.

Retnlb - IL-13–regulated genes associated with airway inflammation, remodeling, and mucus production

Conclusions

1. Electronic cigarettes (e-cigarettes) are increasingly popular devices for smoking cessation. Their overall effectiveness and safety remain to be determined.

2. Analysis of transcriptome profiles in e-vapor and MSS exposed NHBE cells revealed unique and overlapping gene expression signatures.

3. E-vapor alone showed few or no significant alterations in gene expression after 1 H of exposure compared to Air-treated controls, and NHBE cells showed rapid recovery to pre-treatment phenotypes.

4. The presence of Nicotine (and flavor components) resulted in transcriptomic changes similar to that of MSS, but the NHBE cells NHBE cells showed rapid recovery to pre-treatment phenotypes.

5. MSS elicited clear and persistent changes in NHBE cell transcriptome profiles.

6. Exposure of NHBE cells to E-vapor containing nicotine elicited a subset of alterations found in MSS exposed cells.

7. Additional studies are clearly needed and we need to use caution in extrapolating the *in vitro* findings to complex *in vivo* situations.

Timko Lab

Tatiana Kotova, MD	NHBE cell culture
Michael J. Wolkowicz, PhD	MSS and e-vapor exposure
S. Neil Holby	E-vapor exposure
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