ASSESSMENT OF CANDIDATE MODIFIED RISK TOBACCO PRODUCTS ON ORAL HEALTH IN VITRO

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Background

- Smoking causes serious diseases, such as cardiovascular diseases, lung cancer, and chronic obstructive pulmonary disease (U.S. Surgeon General, 2004).

- Smoking cessation remains the most effective approach to minimizing the risk for smoking-related diseases (Godtfredsen et al. 2008; Gepner et al., 2011).

- Providing reduced-risk alternatives to adult smokers who would otherwise continue to smoke cigarettes represents the basis of the “Tobacco Harm Reduction” strategy (IOM, 2002).

- Philip Morris International (PMI) is developing novel products with the potential to reduce individual risk and population harm in comparison with smoking cigarettes.

- To determine whether such reduced-risk products (RRPs*) have the potential to reduce individual risk, we are conducting extensive and rigorous scientific studies comparing their biological impact with that of cigarettes.

*Note: Reduced-Risk Products (“RRPs”) is the term PMI uses to refer to products with the potential to reduce individual risk and population harm in comparison with smoking cigarettes.
Cigarette Smoke vs. Heat-not-Burn

More than 6,000 constituents have been identified in cigarette smoke. Some are harmful and potentially harmful (HPHC), many of which are formed during combustion (burning) of the tobacco.

It is not known which HPHCs are responsible for tobacco-related diseases – selective reduction is not an effective approach.

Lower temperatures reduce constituents in the aerosol

Nicotine is transferred via distillation

Key Principles

- Electrically heated tobacco system version 2.2 (THS 2.2)
  - Tobacco plug
  - Tobacco blends and flavor systems developed to suit lower operating temperature (< 350 °C)

- Heating engine precisely controlled using built-in software
  - Tobacco is heated in a controlled fashion rather than burned, which is intended to prevent generation of HPHCs through pyrogeneration and pyrosynthesis
  - The heater also acts as a temperature sensor
• Compare switching to RRP with continued smoking
• Assess how close switching to RRP is to smoking cessation
Average reductions in formation of HPHCs for THS 2.2 aerosol compared with levels measured in 3R4F reference cigarette smoke*

*Aerosol collection with Health Canada Intense Smoking Regime (55 mL puff volume, 2 second puff duration, 30 second interval puff)
Comparison on a per-stick basis reduction calculations exclude nicotine, glycerin, and total particulate matter.
The PMI 58 list includes the U.S. Food and Drug Administration 18 and the 15 carcinogens of the IARC Groups 1.
Systems Toxicology: A Comprehensive Toxicity Assessment

- Considers biological systems as a whole and aims at elucidating detailed **biological mechanisms** that link exposure to active substances with their adverse consequences.
- Integrates classic toxicology approaches with the quantitative analysis of molecular and functional changes.
- Combines high-throughput methods with advanced computational methods.
- Enables the shift to a new paradigm for risk assessment (21st century toxicology) (*Product Assessment*).

System toxicology enables the shift from regulatory toxicological assessment toward a detailed mechanistic understanding of biological pathways perturbed by exposure to toxicants.
Background: Replacing, Reducing, and Refining

- Human organotypic tissues reconstituted using primary cells cultured in three dimensions, with proper cell-cell contact, recapitulating biological functions (e.g., mucus secretion, mucociliary clearance) and allowing whole cigarette smoke exposure at the air-liquid interface.
- “The Food, Drug, and Cosmetic Act directs the FDA to consult with the Institute of Medicine (IOM) on the design and conduct of studies for the assessment of MRTPs [modified risk tobacco products]. In its 2011 report, Scientific Standards for Studies on Modified Risk Tobacco Products, the IOM identified, as a standard step in this assessment, in vitro tests for cytotoxicity, genotoxicity, proliferation, apoptosis, oxidative stress, inflammation, mucus production, and endothelial cell activation.”
- PETA issued a letter in March 2014 whereby PETA stated the in vitro field has progressed significantly, with many varied technological advances, and the in vitro testing of tobacco products is no longer “limited to a small number of cytotoxicity and genotoxicity assays.”

Belgium, Estonia, Germany, UK, and Slovakia have banned animal testing for tobacco research
Human Organotypic Culture Models of the Aerodigestive Tract and Exposure System
Systems Toxicology: A Comprehensive Toxicity Assessment

- Transcriptomics (mRNA + miRNA microarray)
- Proteomics (targeted/non-targeted approach)
- DNA/RNA Sequencing
- Histological analysis
- Metabolomics

- Adenylate kinase released assay (cytotoxicity)
- Cytochrome P450 activity
- Profiling of inflammatory mediators
Results from dental color stability study

Effects of cigarette smoking on color stability of dental resin composites.
Zhao X1,3, Zanetti E2, Maged S3, Pan J2, Malinstrom H2, Petrich MG, Hoang J2, Ren Y1

Results from series of studies assessing heated tobacco products

In vitro systems toxicology approach to investigate the effects of repeated cigarette smoke exposure on human buccal and gingival organotypic epithelial tissue cultures.

Systems Toxicology Assessment of the Biological Impact of a Candidate Modified Risk Tobacco Product on Human Organotypic Oral Epithelial Cultures.
Tanzetti E1, Seker A, Markel Z, Jassim A2, Kostadinova H1, Schildge WY1, Maged S, Greulich M, Frentzel S, Tarkela M, Meller C, Gabor N, Hoang J, Petrich MG.

Comparative systems toxicology analysis of cigarette smoke and aerosol from a candidate modified risk tobacco product in organotypic human gingival epithelial cultures: A 3-day repeated exposure study.

Assessment of the impact of aerosol from a potential modified risk tobacco product compared with cigarette smoke on human organotypic oral epithelial cultures under different exposure regimens.
Assessment Studies of Heated Tobacco Aerosols Using Organotypic Oral Cultures (Buccal and Gingival)
Study Design: To Assess the Reduced Impact of an RRP compared with Cigarette Smoke

Buccal study

- 3 experimental repetitions

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Experimental Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal</td>
<td>1</td>
</tr>
<tr>
<td>Gingival</td>
<td>2</td>
</tr>
</tbody>
</table>

- Exposure at comparable nicotine concentrations
- 3R4F reference cigarettes were used as the control to assess the effects of cigarette smoke exposure
Cigarette smoke constituents directly affect immune cells present in gingival tissue.

Cytokines involved in inflammation and repair of periodontal tissues.

Cytokine profiles can potentially be used for diagnosis and prognostic markers.

Cigarette smoke constituents directly affect immune cells present in gingival tissue.
# Inflammatory Mediators in Gingival Crevicular Fluid

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Oral biofilm plaque</td>
<td>Initiate the host immune response</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>Oral vascular and junctional epithelium</td>
<td>Represent the high cell turnover of the gingival sulcus</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>Gingival blood vessel plexus</td>
<td>Polymorphonuclear neutrophils are involved in innate immunity. Monocytes/macrophages and lymphocytes are involved in cell-mediated immunity</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Gingival blood vessels</td>
<td>Result from damage to small blood vessels and capillaries</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Fibroblasts, osteoblasts, osteoclasts, neutrophils</td>
<td>Plays a role in superoxide generation and in the first line of defense</td>
</tr>
<tr>
<td>Cartilage II</td>
<td>Macrophages</td>
<td>Active enzyme in proteolysis</td>
</tr>
<tr>
<td>Collagenase-2 (matrix metalloproteinase-9)</td>
<td>Neutrophils</td>
<td>Active enzyme associated with collagenic activity</td>
</tr>
<tr>
<td>Gelatinase (matrix metalloproteinase-9)</td>
<td>Neutrophils</td>
<td>Hydrolysis of intercellular matrix</td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>Neutrophils</td>
<td>Cleavage of elastin, collagen and proteoglycans</td>
</tr>
<tr>
<td>Macrophage elastase</td>
<td>Macrophages</td>
<td>Cleavage of elastin, collagen and proteoglycans</td>
</tr>
<tr>
<td>Carboxyterminal telopeptide of type I collagen</td>
<td>Fragment of bone type I collagen</td>
<td>Highly correlated with bone turnover</td>
</tr>
<tr>
<td>Interleukin-1beta</td>
<td>Macrophages</td>
<td>Regulates immune and inflammatory reactions. Stimulates bone resorption.</td>
</tr>
<tr>
<td>Tissue inhibitors of metalloproteinases</td>
<td>Neutrophils, macrophages, fibroblasts, keratinocytes</td>
<td>Inhibits matrix metalloproteinases</td>
</tr>
<tr>
<td>Tumor necrosis factor-alpha</td>
<td>Neutrophils, macrophages, lymphocytes</td>
<td>Delays neutrophil apoptosis</td>
</tr>
<tr>
<td>Transforming growth factor-beta</td>
<td>Macrophages</td>
<td>Modulates proinflammatory cytokine production</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>T-cells, macrophages, osteoblasts</td>
<td>Regulator of T- and B-cell growth. Stimulates osteoblast formation</td>
</tr>
<tr>
<td>Interleukin-8</td>
<td>Macrophages, epithelial cells</td>
<td>Recruitment and activation of neutrophils</td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>Macrophages</td>
<td>Recruitment and activation of neutrophils</td>
</tr>
<tr>
<td>Interleukin-13</td>
<td>Macrophages</td>
<td>Recruitment and activation of neutrophils</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Neutrophils, acinar cells</td>
<td>Antibacterial. Creates iron-limiting environment.</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Neutrophils, macrophages</td>
<td>Hydrolysis of peptidoglycans of bacterial cell walls</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>Osteoblasts</td>
<td>Decoy receptor for RANKL. Inhibits osteoclast formation</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>Osteoblasts</td>
<td>Calcium binding</td>
</tr>
<tr>
<td>Prostaglandin E2</td>
<td>All cell types</td>
<td>Proinflammatory and immunomodulatory effects</td>
</tr>
<tr>
<td>Transforming growth factor-alpha</td>
<td>Macrophages, keratinocytes</td>
<td>Regulation of tissue repair, cell proliferation, chemotaxis, differentiation and matrix synthesis</td>
</tr>
</tbody>
</table>

Barros et al., 2016
Greater Changes in the Concentrations of Secreted Inflammatory Mediators Were Detected Following Cigarette Smoke than THS 2.2 Aerosol Exposure

Assessment of THS 2.2 using human organotypic gingival cultures

<table>
<thead>
<tr>
<th></th>
<th>I exposure</th>
<th>II exposure</th>
<th>III exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL8: released in gingival crevicular fluid of smokers and periodontitis patients</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
</tr>
<tr>
<td>MMP-1: potential marker of tissue repair in periodontitis</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
</tr>
<tr>
<td>IL1A: shown to increase bone resorption and collagen turnover and stimulate other inflammatory cytokines</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
</tr>
<tr>
<td>TNFA: upregulated in gingival tissues of smokers</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
</tr>
<tr>
<td>IL6: decreased in smokers’ saliva</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
<td>0.8 0.8</td>
</tr>
</tbody>
</table>

1Giannopoulou et al., 2003  
2Romanelli et al., 1999  
3Kim et al., 2006  
4De Nardin et al., 2001  
5Bostrom et al., 1998; Bostrom et al., 1999; Ojima and Hanioka, 2010,  
6Tymkiw et al., 2011
Tissue Damage Was Not Observed in Buccal and Gingival Cultures Following CHTP 1.2 Aerosol Exposure
Decreased Expression of E-cadherin Was Detected Following Cigarette Smoke but Not CHTP 1.2 Aerosol Exposure

A destabilization of E-cadherin expression by CS was also demonstrated in oral mucosa cells\textsuperscript{1,2}

A significant reduction in E-cadherin levels was reported in periodontal disease compared with healthy conditions\textsuperscript{3}

\textsuperscript{1}Hasegawa et al., 2002. \textsuperscript{2}Coppe et al., 2008. \textsuperscript{3}Arun R et al., 2010.
Quantitative Mechanism-Based System Impact Assessment

Transcriptomics data

Exposed Sample vs. Control

Causal models of biological processes relevant for respiratory tissues

Xenobiotic Metabolism Response
Apoptosis
Tissue Damage
Cell Cycle

(http://www.causalbionet.com/)
Network models are encoded using OpenBEL

Quantitative measure of exposure impact on various networks

Network Family
- Cell Fate (CFA)
- Cell Proliferation (CPR)
- Cell Stress (CST)
- Inflammatory Process Network (IPN)

Network

NPA

Quantitative measure of exposure impact on various networks

Network Perturbation Amplitude (NPA) Score

Exposed Sample vs. Control

* significant NPA score

Hoeng et al., 2012
The Causal Biological Networks are composed of more than 120 manually curated and well-annotated biological network models that can be accessed at [http://causalbionet.com](http://causalbionet.com).

BEL-scripted causal network models that describe signaling pathways relevant in diseased and non-diseased pulmonary and vascular tissues.
A Network-Based Enrichment Analysis on the Transcriptome Data Demonstrated Reduced Impact on Biological Processes Following Exposure to THS 2.2 Aerosol in Buccal Cultures Compared with Cigarette Smoke Exposure
A Network-Based Enrichment Analysis on the Transcriptome Data Demonstrated Reduced Impact on Biological Processes Following Exposure to THS 2.2 Aerosol in Buccal Cultures Compared with Cigarette Smoke Exposure

- Inflammation, oxidative stress, and xenobiotic metabolism are known to be impaired by cigarette smoke\(^1\)
- Important network models related to periodontal diseases\(^2\)
Results: Metabolomics Investigations Allowed Detection of a Lower Number of Metabolites Impacted by THS 2.2 Aerosol than by Cigarette Smoke

Gingival THS 2.2 Study
Dental Color Stability Study
Collaboration with the University of Rochester - Prof. Yanfang Ren
Nicotine and TAR are responsible for the following smoke-related teeth alterations:

- Tooth discoloration
- Increased build up of plaque and tartar on the teeth
- Gloss alteration
- Surface roughness alterations

http://www.kenzdental.com/blog/tips-to-prevent-tooth-discoloration-after-teeth-whitening

https://twitter.com/hashtag/discoverdental
Study Design

Aerosol inlet
Vitrocell plate well
Insert
Composite resin disc
Cigarette Smoke Exposure Causes a Higher Discoloration of Composite Resins than THS 2.2 Aerosol

ΔE values (mean and standard deviation) of composite resins after three weeks of smoking challenges

<table>
<thead>
<tr>
<th></th>
<th>DVS</th>
<th>TEC</th>
<th>FSU</th>
</tr>
</thead>
<tbody>
<tr>
<td>THS2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3R4F</td>
<td>4.0 ± 0.6</td>
<td>23.0 ± 1.2</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>3R4F</td>
<td>5.3 ± 1.5</td>
<td>30.4 ± 1.4</td>
<td>30.0 ± 2.5</td>
</tr>
<tr>
<td>THS2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3R4F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3R4F</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

http://www.fsw.cc/color-spaces/
Conclusions

• Acute and repeated cigarette smoke exposure impacted organotypic buccal and gingival culture biology.
• THS 2.2 aerosol exposures minimally affected organotypic buccal and gingival cultures compared with cigarette smoke.
• A series of experimental repetitions ensured statistical robustness and reproducibility of results.
• Systems toxicology comprises several endpoints and allows a better understanding of biological processes with a mechanistic-multi-omics approach.
• Composite resin color alterations exerted by THS 2.2 aerosol are minimal and below the clinical threshold ($\Delta E < 3.3$) for the FSU product.
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