Section VIII. Scientific Studies and Analyses

C. Non-Clinical Studies

Table of Contents

List of Tables

VIII. Scientific Studies and Analyses

C. Non-clinical Studies

1. Background

As noted in Section VIII. A. Product Analysis, the SPECTRUM®, PARE® and VLN™ cigarettes are the same cigarette. These products are made to the same specifications, use the same

materials and ingredients, use the same tobacco blend and have the same smoke chemistry. As noted elsewhere, SPECTRUM® cigarettes are research cigarettes made by 22nd Century for NIDA. The results of SPECTRUM® studies come from published articles. Prior to the production of SPECTRUM® cigarettes, NIDA contracted with Ultratech to produce low nicotine cigarettes. The tobacco in the Ultratech cigarettes was extracted to remove the nicotine. PARE® was the subject of 22nd Century's prior 2015 MRTP application. There are no published or 22nd Century-sponsored non-clinical studies with PARE®. VLN™ is the subject of this application. The non-clinical studies on SPECTRUM® serve as the primary data sources. There are additional studies on Quest 3 cigarettes. As shown by the nicotine in the tobacco filler and HPHC data, Quest 3 is similar to VLN™ but not the same. Quest 3 was made using VLN™ tobacco, but used a different blend, filter, flavors, and cigarette materials. The results from the Quest 3 product maybe supportive of the primary studies with SPECTRUM® cigarettes. The SPECTRUM® cigarettes that are the same as VLN™ are identified as NRC 102 (regular king) and NRC 103 (menthol king). These cigarettes are sometimes identified by their nicotine in tobacco levels (0.4 mg/g). There are an additional set of studies conducted using cigarettes that have had the nicotine removed by extraction process. The most prominent product was Philip Morris' Next product made with de-nicotinized tobacco in which the nicotine was chemically extracted from the harvested tobacco leaf. Philip Morris also made test cigarettes for research purposes. These cigarettes were provided principally to Dr. Neil Benowitz and are the subject of many of his publications on VLNC cigarettes. These studies are excluded from this discussion because they are made using tobacco that has been processed in a completely different manner (extraction with super critical CO_2) as compared to the naturally produced VLN™ tobacco. These Next cigarettes are not well characterized, and

their design parameters are not known. There are no bridging studies comparing de-nicotinized tobacco to VLN™ tobacco.

2. Summary

Traditional toxicology studies have not been performed with VLN™ products. 22nd Century believes that VLN™ products are no different (no more toxic) than conventional cigarettes, except that VLN™ cigarettes contain less nicotine. No claims of safety or reduced risk over conventional cigarettes are being made. Non-clinical studies with VLNC products, mostly conducted with Quest 3, are generally limited to special studies related to the effects of nicotine where testing used reduced nicotine cigarettes as one of the controls/treatments. As stated above, the relevance of studies using Quest 3 where the detailed smoke chemistry is different from VLN™ is unknown. There was one *in vitro* investigation using SPECTRUM® cigarettes comparing the effects of cigarette smoke extracts from SPECTRUM® and 3R4F reference cigarettes to nicotine on oxidative stress and inflammation in human blood brain barrier endothelial cells. This study did not demonstrate any unique differences between SPECTRUM® cigarettes and the reference cigarette. Studies with Quest 3 evaluating platelet activation suggest that cigarette smoke activates platelets and that nicotine ameliorates the activation. Cigarettes with lower levels of nicotine potentially could present a risk of thrombolytic disease as compared to cigarettes with normal levels of nicotine. *In vitro* studies with Quest 3 suggest that the condensate is no more cytotoxic or mutagenic than the reference cigarette 2R4F. Quest 3 decreased the gap junction intercellular communication and caused cell cycle arrest. Condensate exposure increased cytoplasmic nucleosomes, sub-G1/G0 population and apoptotic comet tails. Proapoptotic protein Bax increased independent of p53 induction. The authors concluded that

Quest 3 cigarettes do not have less toxicity in human cells. In an *in vitro* model of pre-implantation development in embryonic stem cells, MS and SS smoke solutions from Quest 3, Marlboro Lights, and Advance cigarettes inhibited embryonic cell attachment dose-dependently as did reference cigarettes. In a follow up study, similar activity was seen in human embryonic stem cells. In an *in vivo* study, extract from Quest 3 cigarettes impaired the mechanical strength of healing fractures in rats that was not reversed by nicotine. Another *in vivo* study in mice evaluated the effect of Quest 3 (and other cigarettes) on atherosclerotic plaque formation. Nicotine had a significant impact of lesion area, but tar yield also was important.

The relevance of the results from studies with Quest 3 to VLN™ is unknown since the design and smoke chemistry for Quest 3 is different from VLN™ cigarettes. The platelet activation study with Quest is relevant because it relates specifically to reduced nicotine as having a potential effect.

3. Individual Study Summaries

Summaries of i*n vitro* and animal studies of effects of smoke and other materials derived from VLNC cigarettes made using tobacco from the very low alkaloid variety Vector 21-41, are provided below with links to the publications. **Error! Reference source not found.** lists all of the n on-clinical studies included in this application.

Table VIII.C-1 Non-Clinical Studies Non-Clinical Studies

Product	Title	Subject	Reference
Quest	Activation of platelets exposed to shear stress in the presence of smoke extracts of low-	Platelet Activation	Ramachandran *et al.* 2004 [pg 35]

	nicotine and zero-nicotine cigarettes: the protective effect of nicotine. Discussion on page 8 of this application.		
Quest	*In vitro* model of platelet-endothelial activation due to cigarette smoke under cardiovascular circulation condition Discussion on page 11 of this application.	Platelet Activation	Girdhar *et al.* 2008, Annals of Biomedical Engineering [pg 34]
Quest	Toxicological Analysis of Low-Nicotine and Nicotine-Free Cigarettes Discussion on page 13 of this application.	Mutagenicity/Cytotoxicity	Chen *et al.* 2008 [pg 34]
SPECTRUM	Effect of full flavor and denicotinized cigarettes exposure on the brain microvascular endothelium: a microarray-based gene expression study using a human immortalized BBB endothelial cell line, with the exception of Nicotine, both full flavor (3R4F) and decotinized (ULN) cigarettes activated inflammatory and oxidative stress pathways. Discussion on page 26 of this application.	Oxidative Stress	Naik *et al.* 2015 [pg 35]

Quest	Tobacco extract but not nicotine impairs the mechanical strength of fracture healing in rats Discussion on page 30 of this application.	Bone Strength	Skott *et al.* 2006 [pg 36]
Quest	Potentially Reduced Exposure Cigarettes Accelerate Atherosclerosis: Evidence for the Role of Nicotine Discussion on page 31 of this application.	Atherosclerosis	Catanzaro *et al.* 2007 [pg 34]
Quest	Comparison of toxicity of smoke from traditional and harm-reduction cigarettes using mouse embryonic stem cells as a novel model for preimplantation development Discussion on page 20 of this application.	Pre-implantation	Lin *et al.* 2009 [pg 34]
Quest	Comparison of the Toxicity of Smoke from Conventional and Harm Reduction Cigarettes Using Human Embryonic Stem Cells Discussion on page 22 of this application.	Pre-implantation	Lin *et al.* 2010 [pg 34]

i. In Vitro Studies

(a) Activation of platelets exposed to shear stress in the presence of smoke extracts of low-nicotine and zero-nicotine cigarettes: the protective effect of nicotine.

Cigarette smoke contains thousands of potential toxicants and biological agonists, including nicotine. Cigarette smoke exposure adversely affects platelet function (Ramachandran *et al.* 2004 [pg 35]) causing activation and an increased response to physiological and mechanical agonists, and high thrombogenic potential (Glantz and Parmley 1991[pg 34]); (Rubenstein *et al.* 2004 [pg 35]). While the role of platelets in cardiovascular disease associated with smoking has been well established and described by the Surgeon General, little is known about the effects of nicotine. Indeed, the role of nicotine in smoking-related cardiovascular disease has not been demonstrated (Benowitz and Gourlay 1997 [pg 34]). Earlier studies using platelets alone had shown that smoke-induced platelet activation is largely due to the non-nicotine smoke components and their effects can be modulated in the presence of nicotine. In the current study (Ramachandran et al. 2004 [pg 35]) investigators evaluated platelet function using a more physiologically relevant model including components of vasculature endothelial cells, smooth muscle cells, and extracellular matrix. Ramachandran evaluated the nicotine-dependent and non-nicotine-dependent effects of tobacco smoke on platelets exposed to mainstream and sidestream smoke extracts from high tar, high nicotine commercial cigarettes (Marlboro 100s, 16 mg tar, 1.2 mg nicotine), low-nicotine (Quest1, 10 mg tar, 0.6 mg nicotine), and zero-nicotine cigarettes Quest3 ((10 mg tar, 0.05 mg nicotine) made with reduced and zero nicotine tobacco and with carbon in the filters, under flow conditions that approximately mimicked normal vascular blood flow, and determined the effect of adding nicotine to the zero-nicotine smoke extracts.

Platelets were circulated for 21 minutes in the presence of smoke extracts at an intermittent shear stress of 12 dyne/cm^2, which roughly approximates normal shear exposure in the vasculature. The mean platelet activation rates are shown in Table VIII.C-2 for the various treatment conditions. The results of pairwise comparisons (Table VIII.C-3) demonstrate that platelet activation is inversely related to nicotine content, the lower the nicotine content of mainstream or sidestream smoke, the greater the platelet activation.

Table VIII.C-2. Mean platelet activation rates (PAR) under intermittent shear stress of 12 dyne/cm2 in the presence of smoke extracts.

Extract Treatment Group	High-nicotine mainstream	Low-nicotine mainstream	Zero-nicotine mainstream	Zero-nicotine sidestream	Zero-nicotine mainstream + nicotine added	Zero-nicotine mainstream - no filters
PAR	0.0084	0.0117	0.0159	0.0179	0.0039	0.0146

Table VIII.C-3. Probabilities (p=) for the individual experimental pair comparisons assuming the null hypothesis that activation rates were identical. Statistically significant differences shown in bold type.

Extract Treatment Group	High-nicotine mainstream	Low-nicotine mainstream	Zero-nicotine mainstream	Zero-nicotine sidestream	Zero-nicotine mainstream + nicotine added	Zero-nicotine mainstream - no filters
Zero-nicotine mainstream	**0.005**	0.2	---	>0.2	**0.007**	>0.2
Low-nicotine mainstream	0.09	---				

Statistically significant differences were observed for high nicotine vs. zero nicotine conditions for mainstream smoke, and zero nicotine condition vs. zero nicotine condition with nicotine added back to the formulation suggesting an effect of the nicotine at the concentrations tested. The authors suggest that in these experiments "...the results demonstrate that for the cigarettes used here, which did not incorporate filter bypass ventilation holes of the type found in light cigarettes, sidestream and mainstream extracts were equally potent in their sensitization of platelets to shear stress."

The equivalence of mainstream and sidestream extracts suggested that the carbon filters used had no effect on the platelet-sensitizing potency of the smoke. To test this hypothesis, the investigators prepared smoke extracts from zero-nicotine cigarettes from which the filters were removed. The rates of platelet activation in the presence of this extract were slightly lower than those seen with the regular mainstream and sidestream smoke extracts of these cigarettes, but the differences were not statistically significant. They concluded that "although the filters of the zero-nicotine cigarettes might reduce slightly the levels of putative inhibitors of platelet activation (perhaps traces of nicotine still present in these cigarettes), the effect is not large." The authors further state that both Quest cigarettes used to prepare the cigarette smoke extracts had identical tar ratings. Such smoke extracts prepared from these cigarettes were more potent in sensitizing platelets to flow-induced activation than were extracts from even high-tar, high-nicotine cigarettes when compared to the most common such cigarette, Marlboro Red, which was used in the experiments. Finally, Ramachandran suggested that smoke extracts from low-nicotine and zero-nicotine cigarettes caused substantially greater activation of platelets than extracts from ordinary high-tar, high-nicotine cigarettes. In support of the conclusion that this is a direct result of nicotine reduction, the addition of a low concentration of nicotine to zero-nicotine smoke extracts was shown to substantially reduce the platelet-activating potential of the extracts.

Conclusion: Even though low-nicotine and zero-nicotine cigarettes may be useful in weaning smokers from cigarettes, they also might cause a significantly increased risk of thrombotic disease in smokers and others exposed to the secondhand smoke from such cigarettes.

(b) *In vitro model of platelet-endothelial activation due to cigarette smoke under cardiovascular circulation condition*

Girdhar (Girdhar et al. 2008, *Annals of Biomedical Engineering* [pg 34]) used a hemodynamic shearing device to investigate platelet activation and endothelial cell (EC) adhesion *in vitro* in a combined system of platelets and cultured human umbilical vein endothelial cells under physiological shear stress by measuring E-selectin expression on cells upon exposure to: (1) platelets and nicotine-free smoke extract, (2) platelets alone, and (3) nicotine free smoke extract alone. Cigarette smoke extract has been shown in the past to increase expression of adhesion molecules in endothelial cells (Shen et al. 1996 [pg35]; Stone, et al., 2002 [pg36]). Cigarette smoke extract was prepared using Quest-3 (nicotine free extract, 10 mg tar, 0.05 mg nicotine), Quest-1 (medium nicotine extract, 10 mg tar, 0.6 mg nicotine), and Marlboro Red (high-nicotine extract, 16 mg tar, 1.2 mg nicotine). A 10% extract from each cigarette condensate in Hepes buffered saline was used in all studies.

Whole blood was taken from human volunteers and platelet-rich plasma was obtained after centrifugation. A combined annular Couette and cone-plate viscometer setup was utilized to study the interaction between cultured endothelial cells and circulating platelets under shear stress simulating *in vivo* dynamic flow conditions, to determine the change in platelet activation upon addition of nicotine free smoke extract, and modulation of this effect by nicotine and expression of E-selectin on ECs upon exposure to platelets and nicotine free smoke extract (NFE). E-selectin expression and platelet-activation rate and platelet activation state were measured for the various exposure conditions under dynamic conditions.

The platelet activation state (PAS) was increased with the addition of nicotine free extract, with and without the presence of endothelial cells (Table VIII.C-4). Platelet activation rate (PAR) was also elevated upon addition of the nicotine free extract. E-selectin expression obtained upon concurrent exposure of ECs to the nicotine free extract and platelets was also affected. E-selectin expression was increased in the presence of endothelial cells, endothelial cells and platelets, endothelial cells and nicotine free extract, and was greatest with platelets, endothelial cells and nicotine free extract combined.

Table VIII.C-4. Platelet activation state and platelet activation rate in combination with endothelial cells and nicotine free extract using the hemodynamic sheer stress device under the same sheer stress conditions.

Condition	Platelet Activation State (at 54 min)	Platelet Activation Rate (X 10^{-5})
Platelets	0.06 ± 0.01	1.00 ± 0.60
Platelets + NFE	0.13 ± 0.03	3.02 ± 1.0
Platelets, EC	0.02 ± 0.00	0.02 ± 0.15
Platelets, EC + NFE	0.08 ± 0.01	3.00 ± 1.00

NFE -nicotine free extract, EC - endothelial cells

In experiments substituting cigarette smoke extracts, smoke extracts were prepared from two commercially available cigarettes Marlboro Red (1.2 mg nicotine) and Quest-1 (0.6 mg nicotine) in buffer as already described. Addition of these extracts (10%) in place of the NFE, reduced platelet activation and PAS was changed in the presence of ECs but this difference was not influenced by the smoke extracts or nicotine. The authors further conclude that despite the higher tar content of Marlboro cigarettes (16 mg) relative to the Quest cigarettes (10 mg), platelet activation was significantly reduced by the 2-fold higher nicotine content in the Marlboro Red extract. The authors observed the PAS measured with Marlboro Red extract was less than that measured with the NFE by 48% (platelets only) and 47% (platelets + ECs). The PAS measured with the Quest1 extract was also significantly lower compared to the NFE extract ($p = 0.03$).

Conclusions: These findings support the role of nicotine as a key modulator of platelet activation in smokers. The authors concede that their experiments are short duration and it is unknown whether these responses would occur in the same timeframe *in vivo* if they occur at all. This *in vitro* study evaluated a combined system of endothelial cells and platelets under simulated sheer stress conditions over a short timeframe owing to the tendency of the platelets to spontaneously activate even in the absence of known agonists. Other physiological mechanisms must be operating in *vivo* to prevent such activation, and these mediators are clearly missing and as of now still undescribed in this *in vitro* model.

(c) *Toxicological Analysis of Low-Nicotine and Nicotine free Cigarettes*

In vitro toxicological evaluations of Quest cigarettes were performed by Chen *et al.* (2008 [pg 34]) who studied the toxicological effects of low nicotine and nicotine free Quest cigarettes compared to the Kentucky reference cigarette, 2R4F, using the modified Ames test, and cytotoxicity evaluations. In an effort to understand the potential toxicities, they also performed a detailed smoke chemistry evaluation of 2R4F research reference conventional cigarette (0.8 mg nicotine yield) and reduced nicotine cigarette smoke from Quest 3 nicotine-free (< 0.05 mg nicotine) and low-nicotine yield (Quest 1 0.6 mg nicotine) cigarettes for smoke constituents which are of general interest as either direct toxicants or indicators of a class of compounds that may be toxicants (Roemer et al. 2012 [pg35]; Smith and Hansch 2000 [pg36]).

1) Smoke Chemistry

Borgwaldt Smoking machines were used for sample generation and collection of total particulate matter on glass fiber pad, followed by a solvent filled impinger for volatile

constituents, or a combination of the two (semi-volatile constituents). Cigarette samples were conditioned prior to smoking by exposure to a controlled environment (60% relative humidity, 23.5 °C) for 48h. Cigarettes were tested using smoking conditions specified by the Federal Trade Commission (FTC; 35 ml puffs with 2 second duration every 60 seconds, smoked down to a specified butt length).

The tobacco-specific nitrosamines were significantly lower in nicotine free Quest, and aldehydes, and volatile organic compounds were also reduced in the Quest cigarettes. However, aromatic amines were elevated in nicotine free Quest compared to the 2R4F as shown in the Table VIII.C-5. Phenols and volatile carcinogens were also reduced for nicotine free Quest.

Table VIII.C-5. Yields of smoke constituents under FTC condition (mean ± S.D.) of reference cigarette, Quest low-nicotine and nicotine free cigarettes.

Constituents	(Units)	Reference 2R4F	Test Cigarette Quest Low-nicotine	Quest Nicotine free
FTC parameters				
TPM	mg	11.13±0.41	10.32±0.34	8.50±0.22*
Tar	mg	9.62±0.38	8.99±0.26*	7.87±0.16*
Nicotine	mg	0.80±0.03	0.53±0.01*	0.032±0.001*
Water	mg	0.71±0.00	0.80±0.13*	0.60±0.06*
Carbon-monoxide	mg	12.05±0.17	10.69±0.21*	9.97±0.57*
Puff count	puff	9.2±0.1	6.6±0.1*	6.0±0.0*
Aldehydes(carbonyls)				
Acrolein	µg	57.2±6.6	22.0±3.5*	19.8±3.0*
Acetaldehyde	µg	617±75	337±45*	363±53*
Formaldehyde	µg	20.3±0.6	21.0±1.7	9.5±2.1*
Aromatic amines				
2-Naphthylamine	ng	6.30±0.07	5.51±0.71	8.32±0.86*
4-Aminobiphenyl	ng	1.25±0.01	1.11±0.08	1.93±0.14*
N-Nitrosamines				
NNK	ng	132.0±4.0	24.8±4.1*	10.3±1.9*
NNN	ng	140.0±12	49.2±2.4*	67.9±3.3*

Phenols				
Catechol	µg	43.7±2.7	35.3±1.9*	20.0±0.6*
Phenols	µg	8.33±0.62	11.6±0.7*	9.47±0.43
Resorcinol	µg	0.67±0.01	0.74±0.05	0.37±0.02*
PAH				
Benzo(a)anthracene	ng	13.9±0.8	10.5±0.4*	8.66±0.29*
Benzo(a)pyrene	ng	5.82±0.25	4.51±0.27*	3.75±0.21*
Volatiles				
Benzene	µg	49.4±1.6	15.0±1.0*	14.8±1.5*
Isoprene	µg	371±3	149±8*	130±7*
1,3-butadiene	µg	32.7±1.1	15.7±0.9*	15.7±0.9*

*, p<0.05 compared with Reference 2R4F

2) Salmonella/microsome plate incorporation Assay (Ames Test)

This assay was conducted as previously described (Mortelmans and Zeiger 2000 [pg 35]). The Salmonella TA98 or TA100 strain were evaluated. Test samples along with positive (2-anthramine, 1.0 µg/plate) and vehicle controls were mixed with Arochlor-induced Sprague Dawley rat S9 fraction, then top agar and bacteria were poured directly onto the agar plates.

In the TA98 and TA100 strains, with activation by S9 all exhibited a dose-dependent revertant response ($p<0.01$) with significantly positive slopes. The revertant colonies at a dose of 0.15 mg cigarette smoke condensate (CSC) CSC/plate were more than 10 times the spontaneous background for the three test cigarette smoke condensates. When compared on a revertants per mg TPM basis, there was no statistical difference ($p>0.05$) in the dose response slopes of the three CSCs. Nicotine alone had no mutagenic activity and when nicotine was added back to Quest CSC at levels similar to conventional “low tar” yield cigarettes, the slope of the dose response curve was not altered.

3) LDH release assay, WST-8 conversion assay and cell growth assay

The acute cytotoxicity of CSC or nicotine was examined using lactate dehydrogenase (LDH) release method. Normal human bronchial epithelial (NHBE) cells were placed into a 96-well plate at a density of 5000 cells/well overnight, and then treated with CSC and/or nicotine at 37°C for 3 hours. LDH release reflects direct damage to the cell plasma membrane and is the most sensitive cytotoxic method for short-time exposures. Quest and 2R4F CSCs all caused a dose-dependent elevation in LDH release at doses > 60–80µg/ml, but there was no significant difference among them at any dose level ($p>0.05$) indicating that they are similar with respect to cytotoxicity.

The WST-8 (WST-8 is a water-soluble tetrazolium dye) conversion assay was also used to determine cytotoxic activity of CSC or nicotine on normal human bronchial epithelial (NHBE) cells. NHBE cells were seeded into 96-well plate at a density of 1 × 104 cells/well and treated with CSC with or without nicotine overnight. WST-8 conversion was then assessed using a one-step Cell Counting Kit-8 (CCK-8), which only generates a signal from viable cells. The CCK-8 assay quantifies total cell number and viability by assaying mitochondrial dehydrogenase activity and revealed that although all three CSC treatments suppressed WST-8 conversion, CSC from Quest nicotine free at doses of 80, 120 and 160 µg/ml was apparently more toxic than CSC Quest low nicotine or CSC from 2R4F ($p<0.05$). That means that besides the similar acute cell killing mechanism evidenced by the LDH release assay, other toxic mechanisms may exist as there were differences between Quest and reference cigarette smoke condensates.

4) Gap junction intercellular communication (GJIC)

Normal bronchial epithelial cells were grown to 70-80% confluence in flasks. Cells designated as "donor cells" were exposed to 40 µg/ml of CSC or nicotine overnight and then sequentially labeled with the gap junction–permeable dye calcein AM for 30 minutes and PKH26, a nontransferable membrane dye, for 7 minutes. The double-stained donor cells were then plated onto monolayers of unstained "recipient" cells at ratio of 1:10 (donor : acceptor) and incubated for 2 hr at 37 °C. The co-cultures were then harvested, washed, re-suspended and subjected to FACS (Becton Dickinson FACStar Plus dual laser system, Heidelberg, Germany) analysis. GJIC results in the transfer of calcein from donor cells to recipient cells, presented as a coupling ratio CR, which is given by the number of calcein-transferred recipient cells which are labeled green, per donor cell (labeled with red and green).

Quest CSCs inhibited GJIC significantly more than CSC from the 2R4F reference cigarette. There was, however, no statistically significant difference between the two Quest CSCs, and the addition of nicotine at 10-50 µM did not significantly affect the gap junction intercellular communication.

5) Effects of CSC and nicotine on apoptosis

NHBE cells were exposed overnight to the apoptosis-inducer VP-16 or CSCs at either low dose (50 µg/ml) or high dose (100 µg/ml). Cytoplasmic nucleosome levels were then determined using the sensitive ELISA method. CSC from 2R4F and both Quest CSCs exposed at doses of 50 µg/ml did not trigger a significant apoptotic response. In contrast, at higher doses, the cytoplasmic nucleosome level was significantly increased ($p<0.05$) after exposure to CSC from

Quest nicotine free (100 µg/ml), but not to CSC from Quest low nicotine or CSC from 2R4F indicating that the nicotine free CSC could induce an apoptotic effect. When the equivalent amount of nicotine (10 µM) was added to the CSC from Quest nicotine free, this increasing effect was abolished, indicating that nicotine in the CSC suppressed the CSC-induced apoptosis.

6) Cell cycle analysis

NHBE cells were placed in cell culture flasks at 50% confluence and incubated with CSC and/or nicotine at 37°C for different times. Following incubation and trypsinization, DNA content of individual cells was analyzed with a fluorescence-activated cell sorter. Cells with less DNA than that of G1/G0 cells (sub-diploid DNA peak) were considered to be apoptotic. Both Quest CSC's caused a dose-dependent cell cycle arrest early (16 h) at G1 checkpoint and later (48 h) at the G2/M checkpoint when CSC concentrations were greater than 40 µg/ml. Both Quest CSC's showed similar effects. Nicotine itself in the range from 1 -100 µM did not affect the cell cycle suggesting that components other than nicotine from Quest CSC resulted in cell cycle arrest effects. The data also showed that both Quest CSC treatments (nicotine free, and low nicotine) resulted in more sub-G1/G0 cells than control which is recognized as an apoptotic cell response.

7) Comet assay

The Comet assay was conducted using a Comet Assay kit under neutral conditions, which detects mainly double strand DNA breaks and can be useful for assessing the DNA fragmentation associated with apoptosis. The cells were viewed with an epifluorescence microscope and photographed for scoring the comet pattern. Typical apoptotic cells are defined as those with

diffuse fan-like tails and very small heads. Scores more than 2 (i.e. relative tail intensity >50%) are attributed to apoptosis.

When NHBE cells were exposed to 50 µg/ml of CSCs for 18 hours and were electrophoresed under neutral conditions, there were more frequent and longer comet tails (oligonucleosomal DNA fragmentation) with small comet heads (chromosomal condensation) compared with control cells. The percentage of nuclei that yielded comet scores >2 were 1%, 43%, 15%, 12%, 17% for medium control, positive control VP-16, CSC-2R4F, CSC-Quest low nicotine, and CSC-Quest nicotine free, respectively.

8) Western blotting

Protein levels in treated cells were assayed by Western blot analysis. NHBE cells were plated in 6-well plates at 80% confluence and treated with CSC and / or nicotine at 37°C overnight. After treatment, cells were harvested and lysed and the protein concentration determined, then immuno-stained with primary antibodies against either p53, PARP, Bcl2, Bax, or Caspase 3. The immunopositive bands were visualized with Super Signal West Pico Chemiluminescent substrate system. None of three CSC treatments upregulated the expression of p53 protein, But CSC-Quest nicotine free increased the Bax expression even at the low dosage, but it did not change the Bcl-2 expression. Therefore, the ratio of Bax/Bcl-2 increased after exposure to Quest nicotine free. The authors found no expression of caspase-3 in NHBE cells before and after any CSC treatments.

Conclusions: The authors reported that in addition to nicotine, some tobacco-specific nitrosamines (TSNAs), aldehydes, and volatile organic compounds were reduced in the Quest

cigarettes compared to the 2R4F. Aromatic amines were slightly lower in CSC from Quest 1 and slightly higher in CSC from Quest 3. CSC from all three types of cigarettes had a similar mutagenic potency in the Ames test, produced a similar concentration-dependent increase in lactate dehydrogenase in the cell culture medium of normal human bronchial epithelial cells, and increased cytoplasmic nucleosomes, the sub-G1/G0 population of cells, and the frequency of apoptotic comet tails. CSC from either of the Quest cigarettes inhibited cell growth more than CSC from the reference cigarette and adding nicotine to the CSC from Quest cigarettes attenuated this inhibition. CSC from Quest cigarettes also decreased gap junction intercellular communication, caused cell cycle arrest, and increased pro-apoptotic protein Bax independent of p53 induction. The authors concluded that nicotine attenuates cytotoxicity in the assays possibly in part through inhibition of apoptotic pathways

(d) *Comparison of toxicity of smoke from traditional and harm-reduction cigarettes using mouse embryonic stem cells as a novel model for preimplantation development*

Lin *et al.* (2009 [pg 34]) used embryonic stem cells from the inner cell mass of mouse blastocysts to test the effects of toxicants on preimplantation development. Embryonic stem cells were used to compare the toxicity of mainstream (MS) and sidestream (SS) cigarette smoke on cell attachment, survival and proliferation by comparing the effects of exposure to extracts of smoke from a full flavor traditional commercial cigarette (Marlboro Red; 15 mg tar, 1.1 mg nicotine) with smoke extracts from three so-called harm-reduction brands Marlboro Lights (10 mg tar, 0.8 mg nicotine); Advance Lights (10 mg tar, 0.8 mg nicotine); and Quest 3 (10 mg tar, 0.05 mg nicotine) and reference research cigarettes (2R1 and 1R4F). Mainstream and sidestream smoke solutions were prepared using an analytical smoking machine and tested at three doses

using murine embryonic stem cells (mESC) plated on 0.2% gelatin and evaluated at 6 and 24-hour intervals. At 6 and 24 h, images were taken, and the number of attached cells evaluated. Marlboro Red, Marlboro Lights, Advance and Quest MS and SS cigarette smoke solutions were tested for their effects on embryonic stem cells attachment at 6 h, using three doses of 0.1, 0.01 and 1.0 puff equivalents (PE).

1) Effects on Embryonic cell attachment

MS and SS smoke solutions from all test cigarettes inhibited embryonic cell attachment dose dependently. In all four brands of cigarettes, non-filtered MS smoke was more inhibitory than filtered MS smoke, showing that the filter removed toxicants, while SS smoke and non-filtered MS smoke had similar inhibitory activity. Attachment was also inhibited dose dependently by MS and SS smoke from 2R1 and 1R4F research brand cigarettes.

2) Effects on Embryonic cell proliferation and survival

MS and SS smoke inhibited embryonic cell proliferation and survival dose dependently. Filtered MS smoke from all brands of harm-reduction cigarettes significantly inhibited proliferation dose dependently. In contrast, filtered MS smoke from Marlboro Red cigarettes did not have a significant effect at any dose. At 1.0 PE, the highest dose tested, MS smoke from the harm-reduction brands either caused cell death (Marlboro Lights) and or enabled survival without significant proliferation (Advance and Quest). MS smoke from 2R1 and 1R4F research cigarettes produced results similar to Quest cigarettes. When experiments were repeated using cigarettes from which the filter had been removed, non-filtered MS smoke from all four brands had similar effects on embryonic cell survival and proliferation. All non-filtered MS smoke

solutions were more potent than filtered MS smoke at producing reduced cellular proliferation and survival. Sidestream smoke solutions from all four cigarette brands showed detrimental effects on embryonic stem cell survival and proliferation too. At 1.0 PE, cells did not survive in any treatment group. At 0.1 PE, cells died in SS smoke from Marlboro Light cigarettes, survived in smoke from Quest and proliferated at a slower rate than the control in Marlboro Red and Advance SS smoke. In general, SS smoke and non-filtered MS smoke were similar in their effects on survival and proliferation, while filtered MS smoke was the least potent of the three types of smoke tested. SS smoke from 2R1 and 1R4F research cigarettes produced results similar to Marlboro Lights.

The authors concluded that for all endpoints (cell attachment, survival, proliferation, and death), harm-reduction cigarette smoke (Marlboro Lights, Advance Lights and Quest) was as potent as, or more potent than, traditional cigarette smoke (Marlboro Red). In addition, the data showed that for all brands tested, SS smoke solutions were considerably more potent than filtered MS smoke solutions. However, removal of the filter increased the toxicity of MS smoke to the levels observed for SS smoke.

Conclusion: Quest was as potent as, or more potent than, traditional cigarette smoke at inhibition of cell attachment, survival, and proliferation.

(e) *Comparison of the Toxicity of Smoke from Conventional and Harm Reduction Cigarettes Using Human Embryonic Stem Cells*

In a follow-up study, Lin *et al.* (2010 [pg34]) utilized a similar study design and methodology to evaluate the same panel of conventional and reduced harm cigarettes for biological effects in

H9 human embryonic stem cells (hESC). Using BioStation technology to create time-lapse videos of cells during treatment in various in vitro assays with video bioinformatics analysis (automated processing and data mining of biological spatiotemporal data), the authors obtained quantitative data for attachment, colony growth, and survival end points in hESC.

Mainstream and sidestream smoke solutions were prepared for each test cigarette using a University of Kentucky smoking machine that was set up to generate mainstream and sidestream smoke, then bubbled through mTeSR medium to create the smoke extract. One cigarette was used to achieve a concentration of 10 puffs of smoke dissolved in 5 ml of medium (10 puffs/cigarette, 2 puffs/1 ml of medium). Concentrations of the smoke in solution were expressed in puff equivalents (PE) where one PE is the amount of smoke in one puff that dissolves in 1 ml of medium. Serial dilutions of smoke solution were made to achieve the PE concentrations used for testing, which were 0.0, 0.01, 0.1, and 1.0 PE. Smoke solutions were made with conventional (Marlboro Red) and so-called harm reduction (Marlboro Lights, Advance Lights, and Quest) cigarettes.

1) Evaluation of Cytotoxicity of MS and SS Smoke from Conventional and Harm Reduction Cigarettes

MS and SS smoke solutions from conventional and harm reduction cigarettes were evaluated for their cytotoxic effects on hESC colony morphology (loss of cells from colonies, colony elevation, and granularity in colonies) after 6 h of treatment with doses of 0.01, 0.1, and 1.0 PE. MS smoke at 0.01 PE and 0.1 PE did not affect hESC colony morphology or survival. However, 1.0 PE of both Marlboro Red and Advance Lights MS smoke caused some cells to detach

from the plate after 6 h of treatment. Although colonies did not detach in 1.0 PE of Marlboro Lights MS smoke, cells were elevated and granular, and colonies had irregular edges unlike the negative control without smoke extract. Quest MS smoke did not affect hESC colony morphology at 1.0 PE. SS smoke solutions at 0.01 PE and 0.1 PE did not affect hESC colony morphology after 6 h of treatment. However, at 1.0 PE, SS smoke from all four brands caused loss of cells from colonies and in some cases complete rounding up of colonies. SS smoke from Advance Lights was the most potent followed by Marlboro Red, Quest, and Marlboro Lights. Cells treated with Advance Lights SS smoke solutions completely detached from the plate and formed small round clumps. Colonies treated with Marlboro Red and Quest SS smoke solutions were partly detached. Cells that remained attached were elevated and granular. With the morphological assay, cytotoxic effects were observed with both MS and SS smoke at 1.0 PE, but not at lower doses, and the effects were stronger with SS smoke than with MS smoke. For all brands except Quest, MS smoke was slightly cytotoxic at 1.0 PE. Using a Magic Red stain and a fluorescence endpoint the authors were also able to show that cell death when occurring was due to apoptosis. In fact, all groups treated with 1.0 PE of MS smoke showed some fluorescence, whereas all groups treated with 1.0 PE of SS smoke solutions were markedly fluorescent indicating that at this dose, cell death was induced apoptotically.

2) Conventional and Harm Reduction SS Cigarette Smoke Inhibited hESC Colony Attachment at a Noncytotoxic Dose (0.1 PE)

For all brands tested, SS smoke from both conventional and harm reduction cigarettes significantly inhibited colony attachment to the gel substrate when compared with the untreated control. Although control and treated colonies reached their maximum attachment by 2 h, the

proportion of colonies that attached was significantly lower in all treatment groups (20–60%) than in the control (70–90%). As was observed in earlier work with mESC (Lin et al, 2009 [pg 34]), the harm reduction brands were more inhibitory in this assay than Marlboro Red, the conventional brand. As shown in the previous study, the hESC were more sensitive to SS smoke exposure than the MS.

3) MS and SS Smoke from Conventional and Harm Reduction Cigarettes Inhibited hESC Colony Growth at a Noncytotoxic Dose (0.1 PE)

Kinetic analysis of colony growth using the video bioinformatics software showed that treatment of hESC colonies with 0.1 PE of MS smoke did not alter the rate of growth when compared with the untreated control. When analyzed by ANOVA, the mean percentage increase in colony size at the final frame was not significantly different among groups. In contrast, hESC colonies treated with SS smoke solutions had slower growth rates than the untreated control. Quest and Advance produced the greatest inhibition in rate of growth of the four brands tested. When the mean percentage increase in colony size was compared over a 48-h interval, all four types of SS smoke solutions significantly inhibited colony growth when compared with the untreated control. Both the Advance and Quest brands were the most inhibitory by this criterion. When growth characteristics were compared with earlier data with mESC, MS smoke was generally more inhibitory in the mouse system than in the human. However, Marlboro Red and Advance Lights SS smoke solutions were far more inhibitory in the hESC system than in the mouse. The data for the inhibitory responses for each cigarette type tested are shown in Table VIII.C-2. With both cell lines and sidestream smoke, mESC and hESC, attachment of the cells to the gel substrate at a non-cytotoxic dose of 0.1 PE was inhibitory with the highest tar and nicotine

cigarette consistently yielding the smallest effect reducing attachment of cells. The Quest cigarette on the other hand consistently produced the greatest inhibition of attachment to the cells. Interestingly, the percentage of colonies that underwent shrinkage (reduced attachment) during exposure was very low in the groups treated with MS smoke, regardless of the brand tested.

The authors note that the attachment of cells to a substrate is essential in embryological development and prevents apoptosis of cultured hESC. The data support the conclusion that smoke and/or nicotine inhibits attachment of embryonic and differentiated cells to substrates. In their study, 0.1 PE of SS smoke from all brands inhibited attachment of hESC cells to the gels. Similar inhibition of hESC attachment occurred with 1.8–3.71 M of nicotine alone, and this inhibition was reversible by the nicotine antagonist tubocurarine, suggesting that action was through a nicotine receptor. However, the authors note that Quest smoke solutions did not contain nicotine, yet significantly inhibited attachment indicating that perhaps other factors in smoke must also impair attachment. Furthermore, of particular interest in the study was the finding that SS smoke from harm reduction brands was a more potent inhibitor of attachment than SS smoke from a conventional brand.

(f) *Effect of full flavor and denicotinized cigarettes exposure on the brain microvascular endothelium: a microarray-based gene expression study using a human immortalized BBB endothelial cell line, with the exception of Nicotine, both full flavor (3R4F) and denicotinized (ULN) cigarettes activated inflammatory and oxidative stress pathways.*

Tobacco smoke is capable of inducing oxidative stress and vascular inflammation triggering pathophysiological changes in peripheral vasculatures (Zuo et al. 2014 [pg36]; Messner and Bernhard 2014 [pg 35]). Oxidative stress and inflammation are involved in the pathogenesis

of diseases such as cancer, ischemic heart disease and chronic obstructive pulmonary disease, and neurological and neurovascular diseases leading to Alzheimer's disease, multiple sclerosis, and cerebral stroke (Hernán *et al*., 2001 [pg34]; Mannami *et al.* 2004[pg35]; Rusanen *et al.* 2011 [pg35]). Naik et al. (2015 [pg35]) in vestigated the role of oxidative stress and antioxidant mechanisms such as NF-E2 related factor and the Nrf2 signaling pathway activated at the blood brain barrier (BBB) endothelium, as one of the main protective mechanisms triggered to counteract oxidative insult from toxicants and tobacco smoke. In the study human BBB endothelial cells (hCMEC/D3 cell line) were chronically exposed to vehicle control, nicotine, or 3R4F, ultra low nicotine (ULN) cigarette (SPECTRUM) smoke extracts (CSE) over a period of 24 hours. Nrf2 expression and its downstream pathway targets were evaluated for oxidative and/or inflammatory potential in a side-by-side comparative analysis of antioxidant mechanisms triggered by nicotine, and CSE from regular full flavor cigarette (3R4F) and ultra-low nicotine (ULN) cigarettes. Exposure to CSE from both 3R4F and ULN cigarettes rapidly increased endothelial reactive oxygen species (ROS) as early as 3 hours post-exposure compared to control. Exposure to nicotine elicited only a mild oxidative response. Nrf2 is a redox-sensitive transcription factor and a master regulator of cellular redox homeostasis. It has been demonstrated that increased levels of ROS trigger the activation and subsequent nuclear translocation of Nrf2 which triggers the expression/activation of molecular changes involved in cellular cytoprotection against oxidative and inflammatory stress (Ma Q, 2013; Hayes, 2014). Nrf2 gene expression levels in response to CSE from 3R4F and ULN cigarettes were slightly elevated. However, using RT-PCR Nrf2 transcription was significant in BBB endothelial cells exposed to ULN-derived CSE compared to control but not in cultures exposed to CSE derived from 3R4F cigarettes.

Nrf2 nuclear translocation was statistically significant in BBB endothelial cells exposed to either ULN and 3R4F cigarette extracts but nicotine exposure did not affect Nrf2 gene expression, transcription or translocation. The NQO1 gene, a major enzyme of Phase I detoxification, had significant induction from both 3R4F and ULN CSE compared to control. Nicotine had no significant effect. Up-regulation of CYP enzymes CYP2S1 compared to control, for 3R4F and ULN was determined. Cytochrome P450 (CYP450) enzymes provide additional anti-oxidant response through metabolism-based detoxification of toxic chemicals. The authors reported up-regulation in the gene expression of the CYP enzymes CYP2S1 for 3R4F and ULN compared to control. CYP51A1 gene expression was also up-regulated however; the measured changes were statistically significant only for BBB endothelial cultures exposed to ULN but not for 3R4F-derived CSE. Nicotine alone did not elicit an effect. CSE from ULN cigarettes increased the gene expression of P-gp compared to control but not that of MRP4, while nicotine alone caused a modest reduction in P-gp transcription when compared to controls. Blood brain barrier drug efflux transporters such as P-glycoprotein that prevent the brain penetration and accumulation of toxic substances including xenobiotics were up-regulated by 3R4F CSE exposure.

CSE potentiates synthesis and activity of various antioxidants in blood brain barrier endothelial cells such as the major anti-oxidant glutathione (GSH), glutathione cysteine ligase and SLC7A11, involved in GSH synthesis. Exposure to 3R4F and ULN smoke extracts produced up-regulation of SLC7A11 and GCLM transcription. Nicotine did not produce any significant alterations in GCLM and SLC7A11 gene expression. This clearly suggests a depletion of cellular antioxidant protection against incumbent oxidative stress load caused by the exposure to smoke extracts and nicotine to a lesser extent.

The authors assessed the effects of nicotine, 3R4F and ULN exposure on gene expression, transcription and translation of heme oxygenase (HMOX-1), another component of the cellular antioxidant cytoprotective mechanisms. Using transcriptome analysis, they found a significant increase in the gene expression levels of HMOX-1 and other antioxidant molecules in response to 3R4F and ULN exposure. They also investigated whether CSE increases NFκβ expression and nuclear translocation, by testing the impact of tobacco smoke and nicotine on the p65 subunit of NFκB (NFκB-p65) gene expression, a transcriptional factor and potential activator of oxidative and inflammatory stress pathways the mRNA expression of NFκB-p65 was markedly elevated by CSE exposure of both products with ULN producing a stronger response than 3R4F, and also observed a significant increase in the activation and nuclear translocation of NFκB-p65 following exposure to both 3R4F or ULN smoke extracts. Nicotine exposure alone altered neither the mRNA expression nor the nuclear translocation of NfκB-p65.

Secretion of the pro-inflammatory cytokine interleukin 8 (IL-8) and the chemokine Monocyte Chemoattractant Protein-1 (MCP-1) are in agreement with NFκB-p65 activation. Both CSE from 3R4F and ULN cigarette products amplified the IL-8 secretory response by tenfold compared to control cultures. Nicotine (100 ng/mL) was also found to significantly elevate the release of IL-8 from BBB endothelial cells by more than 2-fold, although the magnitude of nicotine's effects was very low when compared to CSEs. In addition, release of vascular endothelial growth factor (VEGF), a known modulator of vascular angiogenesis, was also significantly increased in response to the exposure to 3R4F and ULN-derived CSE. Nicotine treatment alone was also found to induce the endothelial secretion of MCP-1 and VEGF by a marginal 1.5-fold increase over control.

Overall, reactive oxygen species (ROS) were increased after exposure of cells to CSE from either the ULN cigarettes or 3R4F cigarettes. Nicotine alone had a much lower effect. CSE from both types of cigarettes increased expression of Nrf2, a transcription factor involved in activation of molecular networks involved in cellular protection against oxidative and inflammatory stress. Nicotine alone did not affect Nrf2 expression. Expression of NFκβ and other genes associated with detoxification and anti-oxidant responses were also affected in a similar manner by CSE from ULN cigarettes and 3R4F reference cigarettes.

ii. Animal Studies

(a) Tobacco extract but not nicotine impairs the mechanical strength of fracture healing in rats.

Skott et al. (2006 [pg36]) reported on bone wound healing after controlled fractures of femur bones followed by observation of the healing process in groups of rats treated with nicotine, tobacco extract, tobacco extract with added nicotine and a saline control vehicle. Using implanted osmotic pumps, rats were infused with dosing solutions to achieve serum nicotine levels of 40–50 ng/mL to simulate the serum nicotine equivalent dose from one to two packs of cigarettes per day for one week prior to fracture and three weeks post-fracture. Evaluations of the femur bone after 21 days of healing showed the ultimate torque of the fractures was decreased by about 20% only in the group given tobacco extract alone, compared with the nicotine group and the saline vehicle. Energy absorption, ultimate stiffness, and bone mineral content were not significantly different from the nicotine or other treatment groups. The authors concluded that nicotine does not seem to influence the mechanical strength, when administered in a dose inducing serum nicotine levels similar to those observed in daily smokers, a result similar to Abulencia *et al.* (1999 [pg 34]) who showed that rats treated with the same amount of nicotine

as used in Skott's experiment (3 mg/kg/day) had normal mechanical strength of healing fractures after 3 and 6 weeks. Lenz *et al.* (1992 [pg34]) reported that tobacco extract without nicotine hampers collagen synthesis *in vitro* to a greater extent than does nicotine alone, supporting the findings of the current study. Skott and co-workers note that the "study shows that tobacco extract, but not nicotine, at clinically relevant doses, decreases mechanical strength of healing rat femoral fractures, when administered for 1 week prior to and 3 weeks after the fracture." Nicotine free tobacco extract from Quest cigarettes alone decreased the mechanical strength of the healing bone. Overall, tobacco extract from the Quest cigarettes resulted in a significant decrease in the mechanical strength. There was no significant effect of nicotine alone. In the nicotine plus Quest tobacco extract group, the mean ultimate torque and torque at yield point were higher, but not significantly different, from the values for the tobacco extract only group.

Conclusion: The present study shows that nicotine free tobacco extract impairs the mechanical strength of the healing fractures in this model, whereas administration of nicotine does not seem to influence the mechanical strength.

(b) *Potentially Reduced Exposure Cigarettes Accelerate Atherosclerosis: Evidence for the Role of Nicotine*

Catanzaro *et al.* (2007 [pg34]) studied the effects of conventional and reduced nicotine cigarettes in apolipoprotein E-deficient (apoE +/–) mice exposed by inhalation for 8 or 12 weeks using Quest-1 and Quest-3, Eclipse, 2R4F cigarettes and an air control group. In both studies, mice exposed to smoke from high-nicotine, high-tar Quest 1, and 2R4F cigarettes developed greater areas of lipid-rich aortic plaque lesions than did non-smoking controls. Exposure to smoke from the lower-nicotine products, Eclipse, and Quest 3, was associated with smaller lesion areas,

but animals exposed to smoke from all of the tested types of cigarettes had larger lesions than did control groups not exposed to cigarette smoke. In the 12-week study, lesion areas were approximately double the area of lesions in controls in the aortic arch, infrarenal aorta, and all aortic segments combined (total) of animals exposed to high nicotine 2R4F and Quest 1 smoke.

In the 8-week study, lesion areas were generally smaller than in the 12-week study animals, possibly due to shorter smoke exposure. Mice exposed to high-nicotine 2R4F and Quest 1 smoke had lesions 0.5 to 3-fold larger than in control mice. Lesion areas in mice exposed to the lower nicotine Quest 3 and Eclipse smoke were smaller than those in mice exposed to 2R4F or Quest 1 smoke but were significantly larger than controls. Suprarenal and total aortic lesion areas in Quest 3 and Eclipse exposed mice were significantly smaller than in 2R4F exposed mice. Similarly, Quest 1 exposed mice had significantly smaller lesion areas in the total aorta than did 2R4F exposed mice.

In addition, isoprostane F2 alpha VI, a sensitive and specific marker of lipid peroxidation and oxidative stress *in vivo*, was measured in urine collected overnight commencing immediately after smoke exposure for each study. Isoprostane F2 alpha VI levels were elevated in each of the smoke-exposed groups except for Quest 3, which has the lowest nicotine yield of all products tested. There was a clear dose–response relationship between nicotine yield and urinary levels of isoprostane F2 alpha VI.

The authors conclude that a strong dose dependency exists between nicotine yield and lesion area in both studies, suggesting that nicotine plays an important role in stimulating lesion development, but also suggested that lesion area might also be related to tar yield since the small

study size and study design limited their ability to establish an association between tar content and lesion size. Plots of total lesion areas against the FTC nicotine and tar yields of the cigarettes tested revealed dose–response relationships between both nicotine and tar with total lesion area for both studies. They also opine that reduced nicotine exposure appears to have impacted the oxidative stress response in smoke-exposed mice since isoprostane F2 alpha VI was increased in mice exposed to smoke from all cigarettes tested except Quest 3, which delivers minimal nicotine. Histological and morphometric changes in plaque formation, foam cell and macrophages were similar for all smoke exposed groups with small increases in the number of elastic laminae present in the aortic arch of mice in the 2R4F, Quest 1, and Eclipse groups. Morphometry revealed that lumenal and medial cross-sectional areas were similar between controls and each treatment group.

Conclusion: Nicotine can affect the formation of lesions in the aorta in mice.

4. Bibliography

Abulencia, A.E, G.E. Friedlaender, N.W. Troiano, and T.C. Patel, Department of Orthopaedics and Rehabilitation, Yale School of Medicine. 1999. "The Influence of Nicotine on Fracture Repair in Rats." 45th Annual Meeting, Orthopaedic Research Society, Anaheim, California.

Benowitz, N. L., and S. G. Gourlay. 1997. "Cardiovascular Toxicity of Nicotine: Implications for Nicotine Replacement Therapy." *Journal of the American College of Cardiology* 29 (7): 1422–31.

Catanzaro, Daniel F., Ying Zhou, Rong Chen, Fangmin Yu, Sarah E. Catanzaro, Mariana S. De Lorenzo, Kotha Subbaramaiah, et al. 2007. "Potentially Reduced Exposure Cigarettes Accelerate Atherosclerosis: Evidence for the Role of Nicotine." *Cardiovascular Toxicology* 7 (3): 192–201. https://doi.org/10.1007/s12012-007-0027-z.

Chen, Jinguo, Richard Higby, Defa Tian, Duanjun Tan, Michael D. Johnson, Yingxian Xiao, Kenneth J. Kellar, Shibao Feng, and Peter G. Shields. 2008. "Toxicological Analysis of Low-Nicotine and Nicotine-Free Cigarettes." *Toxicology* 249 (2–3): 194–203. https://doi.org/10.1016/j.tox.2008.05.009.

Girdhar, Gaurav, Sulan Xu, Jolyon Jesty, and Danny Bluestein. 2008. "In Vitro Model of Platelet-Endothelial Activation Due to Cigarette Smoke under Cardiovascular Circulation Conditions." *Annals of Biomedical Engineering* 36 (7): 1142–51. https://doi.org/10.1007/s10439-008-9503-2.

Glantz, S. A., and W. W. Parmley. 1991. "Passive Smoking and Heart Disease. Epidemiology, Physiology, and Biochemistry." *Circulation* 83 (1): 1–12.

Hernán, M. A., M. J. Olek, and A. Ascherio. 2001. "Cigarette Smoking and Incidence of Multiple Sclerosis." *American Journal of Epidemiology* 154 (1): 69–74.

Lenz, L. G., W. K. Ramp, R. J. Galvin, and W. M. Pierce. 1992. "Inhibition of Cell Metabolism by a Smokeless Tobacco Extract: Tissue and Species Specificity." *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)* 199 (2): 211–17.

Lin, S., V. Tran, and P. Talbot. 2009. "Comparison of Toxicity of Smoke from Traditional and Harm-Reduction Cigarettes Using Mouse Embryonic Stem Cells as a Novel Model for Preimplantation Development." *Human Reproduction* 24 (2): 386–97. https://doi.org/10.1093/humrep/den419.

Lin, Sabrina, Shawn Fonteno, Jo-Hao Weng, and Prue Talbot. 2010. "Comparison of the Toxicity of Smoke from Conventional and Harm Reduction Cigarettes Using Human Embryonic Stem Cells." *Toxicological Science s: An Official Journal of the Society of Toxicology* 118 (1): 202–12. https://doi.org/10.1093/toxsci/kfq241.

Mannami, Toshifumi, Hiroyasu Iso, Shunroku Baba, Satoshi Sasaki, Katsutoshi Okada, Masamitsu Konishi, Shoichiro Tsugane, and Japan Public Health Center-Based Prospective Study on Cancer and Cardiovascular Disease Group. 2004. "Cigarette Smoking and Risk of Stroke and Its Subtypes among Middle-Aged Japanese Men and Women: The JPHC Study Cohort I." *Stroke* 35 (6): 1248–53. https://doi.org/10.1161/01.STR.0000128794.30660.e8.

Messner, Barbara, and David Bernhard. 2014. "Smoking and Cardiovascular Disease: Mechanisms of Endothelial Dysfunction and Early Atherogenesis." *Arteriosclerosis, Thrombosis, and Vascular Biology* 34 (3): 509–15. https://doi.org/10.1161/ATVBAHA.113.300156.

Mortelmans, K., and E. Zeiger. 2000. "The Ames Salmonella/Microsome Mutagenicity Assay." *Mutation Research* 455 (1–2): 29–60.

Naik, Pooja, Ravi K. Sajja, Shikha Prasad, and Luca Cucullo. 2015. "Effect of Full Flavor and Denicotinized Cigarettes Exposure on the Brain Microvascular Endothelium: A Microarray-Based Gene Expression Study Using a Human Immortalized BBB Endothelial Cell Line." *BMC Neuroscience* 16 (June): 38. https://doi.org/10.1186/s12868-015-0173-3.

Ramachandran, Jaimohan, David Rubenstein, Danny Bluestein, and Jolyon Jesty. 2004. "Activation of Platelets Exposed to Shear Stress in the Presence of Smoke Extracts of Low-Nicotine and Zero-Nicotine Cigarettes: The Protective Effect of Nicotine." *Nicotine & Tobacco Research* 6 (5): 835–41. https://doi.org/10.1080/1462220042000274284.

Roemer, E, H Schramke, H Weiler, A Buettner, S Kausche, S Weber, A Berges, et al. 2012. "Mainstream Smoke Chemistry and in Vitro and In Vivo Toxicity of the Reference Cigarettes 3R4F and 2R4F." *Beiträge Zur Tabakforschung International/Contributions to Tobacco Research* 25 (1). https://doi.org/10.2478/cttr-2013-0912.

Rubenstein, David, Jolyon Jesty, and Danny Bluestein. 2004. "Differences between Mainstream and Sidestream Cigarette Smoke Extracts and Nicotine in the Activation of Platelets under Static and Flow Conditions." *Circulation* 109 (1): 78–83. https://doi.org/10.1161/01.CIR.0000108395.12766.25.

Rusanen, Minna, Miia Kivipelto, Charles P. Quesenberry, Jufen Zhou, and Rachel A. Whitmer. 2011. "Heavy Smoking in Midlife and Long-Term Risk of Alzheimer Disease and Vascular Dementia." *Archives of Internal Medicine* 171 (4): 333–39. https://doi.org/10.1001/archinternmed.2010.393.

Shen, Y., V. Rattan, C. Sultana, and V. K. Kalra. 1996. "Cigarette Smoke Condensate-Induced Adhesion Molecule Expression and Transendothelial Migration of Monocytes." *The American Journal of Physiology* 270 (5 Pt 2): H1624-1633. https://doi.org/10.1152/ajpheart.1996.270.5.H1624.

Skott, Martin, Troels T. Andreassen, Michael Ulrich-Vinther, X. Chen, Dan E. Keyler, Mark G. LeSage, Paul R. Pentel, Joan E. Bechtold, and Kjeld Soballe. 2006. "Tobacco Extract but Not Nicotine Impairs the Mechanical Strength of Fracture Healing in Rats." *Journal of Orthopaedic Research* 24 (7): 1472–79. https://doi.org/10.1002/jor.20187.

Smith, C.J, and C Hansch. 2000. "The Relative Toxicity of Compounds in Mainstream Cigarette Smoke Condensate." *Food and Chemical Toxicology* 38 (7): 637–46. https://doi.org/10.1016/S0278-6915(00)00051-X.

Stone, P. C. W., A. C. Fisher, G. E. Rainger, and G. B. Nash. 2002. "Neutrophil Capture by Selectins on Endothelial Cells Exposed to Cigarette Smoke." *Biochemical and Biophysical Research Communications* 295 (5): 1150–55.

Zuo, Li, Feng He, Georgianna G. Sergakis, Majid S. Koozehchian, Julia N. Stimpfl, Yi Rong, Philip T. Diaz, and Thomas M. Best. 2014. "Interrelated Role of Cigarette Smoking, Oxidative Stress, and Immune Response in COPD and Corresponding Treatments." *American Journal of Physiology. Lung Cellular and Molecular Physiology* 307 (3): L205-218. https://doi.org/10.1152/ajplung.00330.2013.