UNIVERSITY OF CALIFORNIA
RIVERSIDE

# Clinical Investigations on Human Health Responses to Thirdhand Smoke and Electronic Cigarette Exposure

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

by

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March 2022

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# Acknowledgements

I would like to thank my family, friends, and classmates for all the continued support of the course of my graduate school career. Without their support I would not be the person I am today, and I am eternally grateful to have a wonderful community surrounding me.

I would like to thank Dr. Prue Talbot for being an excellent mentor who guided me to become an independent and critical thinking scientist. She also funded me for many years of research and provided me with brilliant support during my PhD studies.

Thank you to my dissertation committee, Dr. Eastmond and Dr. Haga-Yamanaka, for their valuable feedback during my qualifying exam and annual reports which made my projects stronger and challenged me to develop my skills as a scientist.

ABSTRACT OF THE DISSERATION

Clinical Investigations on Human Health Responses to Thirdhand Smoke and Electronic Cigarette Exposure
by

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Doctor of Philosophy, Graduate Program in Cell, Molecular,
and Developmental Biology
University of California, Riverside, March 2022
Dr. Prue Talbot, Chairperson

**Background:** Cigarette smoking causes diseases such as cancer, heart disease, and stroke. The diseases associated with electronic cigarettes (ECs) are not as well understood. Cigarette smoking leaves a residue, thirdhand smoke (THS), on indoor surfaces. The health effects of THS on humans is limited to one study. Our purposes were to: (1) identify urinary biomarkers of potential harm and exposure and correlate these to metal concentrations in urine from cigarette smokers and EC users; (2) determine the responses of humans dermally exposed to THS; and (3) identify molecular changes in keratinocytes exposed to THS extract.

**Methods:** In Chapter 1, urine from non-smokers, EC users, and cigarette smokers was evaluated for biomarkers of exposure, effect, and potential harm. In Chapter 2, human participants were exposed dermally to clothing impregnated with filtered

clean air or THS; their urine was analyzed for biomarkers of exposure and harm, and their plasma was analyzed using proteomics. In Chapter 3, molecular responses of keratinocytes to extracts from THS impregnated fabric were determined.

**Results:** In Chapter #1, urinary biomarkers of exposure, effect, and potential harm were elevated in EC users and cigarette smokers; increased EC usage was correlated with elevated metal concentrations, which correlated with oxidative DNA damage. In Chapter #2, THS-exposure significantly elevated urinary 8-OHdG, 8-isoprostane, and protein carbonyls and activated pathways associated with inflammation, oxidative stress, and skin disease initiation. In Chapter #3, THS in keratinocytes caused proinflammation, and oxidative stress, mitochondrial damage, and elevated keratin 5 levels.

**Conclusion:** Cigarette smoking and EC vaping elevated DNA damage by oxidization which was correlated with total metal concentrations in the urine of cigarette smokers and EC vapers, a change which may lead to disease. Dermal THS exposure significantly increased oxidative damage to DNA, lipids, and proteins in humans. Inflammation was elevated at 3 hours and persisted for 22 hours, which could in the long-term lead to disease. Keratinocytes exposed to THS extracts had elevated molecular risk factors for developing inflammatory skin diseases, including cancer. THS-induced responses in keratinocytes were similar

to those in cigarette smokers, implicating THS as an environmental hazard that should be monitored.

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# Introduction

1) History of Tobacco Products
2) Electronic Cigarettes
3) Thirdhand Smoke

## History of Tobacco Products

The earliest archaeological discovery of tobacco usage was in the first century BC, when the Mayans of Central America smoked tobacco leaves for religious and ceremonial purposes. Archeological evidence has shown they migrated to the south of America between 470 and 630 AD traveling down the modern day Mississippi River, from there tobacco usage spread to neighboring tribes and villages[1]. Native American shamans believed tobacco had medicinal use and used it "cure" asthma, earaches, bowel problems, fever, sore eyes, depression, insect bites, burns, and other medical ailments[1]. In the late 15$^{th}$ century when Christopher Columbus arrived in America, he was gifted tobacco by the Native Americans. He documented tobacco was being used in pipes, cigars, and snuff. When tobacco reached Europe, it instantly became popular, and its popularity was further spread by Portuguese and Spanish Sailors around the world[1].

Spanish sailors introduced tobacco to Europeans in 1528 and the tobacco trade grew exponentially due to its “medical powers”. The French ambassador, Jean Nicot, sent tobacco leaves and seeds to King Francis II and the king’s mother. The king’s recurring headaches were “cured” by the instructions sent by Nicot to use tobacco as snuff. By the 1560s French cultivation of the crop began. Soon Spanish physicians, such as doctor Nicolas Monardes, wrote a book claiming tobacco could cure 36 medical problems. This promising medicinal plant was gaining immense popularity in Europe.

By 1573, an English naval commander brought tobacco seeds to England. For approximately the next 25 years tobacco smoking was touted in England being published in a letter called “Tabaco” in 1595 for its health-giving properties. However, by 1603, King James I attempted to ban the import of tobacco and stated the plant’s health risk to the brain and lungs due to the death of England’s Queen Elizabeth which he thought was related to tobacco usage[2]. In 1604, an English heavy tariff was placed on tobacco imports. By this time English colonizers had left England to settle in the New America. By 1609, a colony in Jamestown Virginia was growing tobacco because it was such a lucrative crop due to England’s demand for it and its versatility as a commercial trading tool with local Native Americans. To help the Virginia colony’s economy, King Charles II banned all England’s cultivation of tobacco and gave sole importation rights to the colony. This allowed for tobacco farming to boom in the early American colonies with some people making fortunes from their tobacco farms.

The need for expanded lands for tobacco farms in America by the English colonizers called the “policy of conquest” led to the Powhattan Wars (1610-1643) with Native Americans. Ultimately, it ended with Native Americans being pushed out of their lands, but sometimes it ended politically as the case of an arranged marriage between De La Warr and Pocahontas[3], which expanded the lands for tobacco farming. The expansion of tobacco farms required more labor, since this crop was extremely laborious, and led to an increase of importing slaves from Africa and enslaving Native Americans[3]. Dutch sailors recognized the profits of importing slaves, which led to the start of the slave trade and slavery being institutionalized in Virginia by 1661.

Europe’s demand for tobacco grew by the early 1700s. The colonies of Maryland and North Carolina became the second and third? biggest exporters of tobacco, behind Virginia. Merchants in London began a practice of depressed tobacco prices, but they kept the loans of American tobacco farmers high. This resulted in substantial debt owed by the American tobacco farmers, which they could not pay off. Merchants in London decided the loans could be paid off with tobacco used as currency, which outraged American farmers. This situation contributed in part to the American Revolutionary War.

Tobacco in the early 19th century would still impact the history of the early United States. As the northern states became more industrialized, the need for slaves diminished, and many of these states abolished slavery. However, the southern states were still heavily reliant on slaves for cotton and tobacco farming.

Also, unequal trading and taxation of shipped goods from the southern states to northern states led to disagreements of equitable trade and eventually a war broke out. This led to the American Civil War, which concluded with the southern Confederate side losing in 1865 and slavery being abolished.

The culture of tobacco cultivation changed from slave labor to share cropping. Also as an important technological advancement, James Bonsack, created a cigarette rolling machine in 1881. The demand in Europe for tobacco products changed from snuff, pipes, and cigars to cigarettes. James Buchanan Duke purchased rights to the machines in that same year and formed an empire called the American Tobacco Company and became the largest distributor of cigarettes in the 19th and 20th century. His machines allowed his company to out-sell all competitors and eventually all went out of business as he controlled a monopoly. He then lowered compensation to tobacco farmers which resulted in the Tobacco Wars 1904-1909. The wars concluded with a loss for the American Tobacco Company as it was dismantled by the Federal government in 1911. However, cigarettes remained popular in the United States due to their cheap cost and ease of mechanized manufacturing.

From the early 1900s to the 1960s, cigarette consumption peaked. Tobacco company researchers created different blends of cigarettes making milder flue-cured tobacco blends, which made inhalation smoother and increased nicotine absorption in the bloodstream. Army surgeons also highlighted the wound-relaxation and pain easing properties of cigarette smoking[4]. However, in the early

1900s, health reform movements led to early anti-smoking movements. Also, researchers started to identify the health problems associated with cigarette smoking. In 1956, the Surgeon's General scientific group identified a link between cigarette usage and lung cancer[4]. In England in 1962, the Royal College of Physicians also stated a link between cigarettes and lung cancer. Since that time, the number of research articles identifying the dangers and mechanisms of harm of tobacco smoke has grown exponentially.

From its peak in 1965, the popularity and consumption of tobacco began a steady decline to the early 2000s. In 1969, the advertisement of cigarettes on television and radios was banned. The Federal government then began regulating smoking in specific government domains. This was furthered aided by the 1972 Surgeon's General Report, the first of a series of science-based reports, to identify environmental tobacco smoke (ETS) as a health risk to non-smokers. The Army and Navy in 1975 eliminated cigarette rations which previously had been given to servicemen. By 1979, smoking became restricted in government facilities and was banned in the White House in 1993. In the 1990s all commercial airlines banned smoking aboard their planes. The Environmental Protection Agency (EPA) classified ETS as a "Group A" carcinogen in 1992, grouping it with the most dangerous carcinogens. In 2000 the United States Supreme Court ruled that the US Food and Drug Administration lacked jurisdiction under the Federal Food, Drug, and Cosmetic Act to regulate tobacco products. This leads us to more recent times where anti-smoking advertisements and life-experiences are considered the

best methods of encouraging abstinence from cigarette smoking. However, cigarette companies are still targeting new populations of users and, according to the Center for Disease Control's 2019 study, there are still 34.1 million adult cigarette smokers, which account for 14.0% of the entire USA population. Cigarette smoking, along with secondhand and thirdhand smoke (THS). remain to this day an epidemic problem. Smoking companies have adapted, encouraging the rise of electronic cigarettes (EC), electronic devices that compliment traditional cigarette smoking and continue to deliver nicotine.

ECs are electronic devices that enable users to inhale an aerosol created from a liquid which typically contains nicotine, flavorings, and other additives. EC designs have varied over the past decade since their inception, ranging from the e-pipe, e-cigar, large-size tank devices, medium-size tank devices, disposable EC, and rechargeable EC[5]. The impact and creation of these devices arose, in part, because the public was becoming aware of the dangers of cigarettes and the link to health diseases. The implementation and advertisements of "harm-reduction" nicotine delivering devices started with cigarette companies. In the 1990s and later new modified tobacco cigarettes (e.g., Advance, Omni); cigarette-like products, also called cig-a-likes (e.g., Eclipse, Accord); and smokeless tobacco products (e.g., Ariva, Exalt, Revel, snus) were invented. Advertisement slogans such as "All of the taste … Less of the toxins" were created by Brown and Williamson, and Vector's slogan "Reduced carcinogens. Premium taste" were the ploy attempted by cigarette companies.

Studies have shown consumers are interested in trying “harm-reduction” tobacco products, even if there is not sufficient scientific evidence to back the harm-reduction claim[6]. There were also concerns by scientists and tobacco control boards that these new devices would reduce smoking cessation rates and would lead to a new generation of smoking devices impacting children[7]. However, cigarette companies were aware that there was a steady decline of cigarette consumption annually and the smoking population was willing to try a new tobacco product.

**Electronic Cigarettes**

The first patented version of an EC was developed in 1963 by Herbert A. Gilbert and was patented in August 1965 (U.S. Patent No. 3,200,819). The purpose of the device was to provide a “smokeless non-tobacco cigarette” which provided “a safe and harmless means for and method of smoking” by replacing burning tobacco and paper with heated, moist, flavored air using a battery powered device. The Favor cigarette, introduced in 1986, was another alternative, non-combustible nicotine-containing tobacco product. However, it was not until 2003 that in China the EC would become widely popular.

The design of all ECs is similar. There is a battery which powers the device which is connected to a power button and atomizer (which vaporizes the e-liquid

into an aerosol). The atomizer is located inside a cartridge containing the wick where the e-liquid is stored. This leads to the mouthpiece which allows the user to inhale the aerosol.

The first popular EC was developed and patented in 2003 by the Chinese pharmacist Hon Lik, a former deputy director of the Institute of Chinese Medicine3. With the help of investors, under the company name Ruyan, they released an electronic atomizing cigarette which quickly gained popularity among Chinese smokers as a potential smoking cessation device[8]. By the mid 2000's the EC entered the American market. In 2013, Ruyan gained a U.S. patent for its EC product stating it as an "electronic atomization cigarette that functions as substitutes (sic) for quitting smoking and cigarette substitutes." (U.S. Patent No. 8,490,628 B2, 2013). Imperial Tobacco Company purchased the intellectual rights to this patent in August 2013 for $75 million. As of 2014, it is estimated 90% of the world's EC technology and products come from China (Barboza 2014?).

To appeal to the next generation of EC users, tobacco companies used advertisements for ECs through television and social media[9] and there was a huge growth in EC usage amongst adults and adolescents. By 2015, 16% of all high school students or roughly 2.4 million high school students had used an e-cigarette at least once in the past 30 days[5]. As of 2020, ECs have become more popular, especially amongst young adults rising to 19.6% of high schoolers or roughly 3.02 million high school students reported current EC usage (vaped at least one time in the past 30 days)[10]. This alarming trend has led to substantial discussion and

research amongst public health communities, state and national public health agencies (such as the FDA, NIH, and CDC), and scientists world-wide to address the health concerns of ECs.

The diversity and modifications of ECs currently on the market are constantly changing and innovating making research studying the long-term health effects and biological mechanisms of harm a difficult task. Tobacco companies understood the appeal the tasty flavoring, especially the fruity or candy flavors to attract youth to their tobacco products and hide the harshness of tobacco[5]. However, the addition of flavoring chemicals in the EC liquid of which there are currently over 19,000 unique e-liquids and 250 unique flavor descriptions[11] can contribute to toxicity in different mechanisms of harm. For example, Behar et al. 2016 demonstrated that cinnamaldehyde-containing refill fluids and aerosols are cytotoxic and genotoxic at low concentrations to respiratory cells. However, One recent study demonstrated that 81.5% of current youth ECs users said they used ECs “because they come in flavors I like”[12].

The first generation of ECs traditionally mimicked the look of a conventional cigarette. They had a slim white and tan shape, with the same anatomical look of a cigarette. These ECs were appropriately named cigalikes and contained a cartridge into which the e-liquid could be poured. The e-liquid was absorbed onto a cotton wick which was connected to the heating element and atomizer, which created the vapor the user inhaled.

Second generation ECs were larger, pen-shaped devices often with a larger cylindrical “tank system” that housed a greater volume of e-liquid. Third and fourth generations broke away from the traditional cigarette look for more aesthetically pleasing designs. For example, the widely popular Juul, which resembled a rectangular flash drive and offered a diverse range of popular flavors. EC users of this era often customized their devices which were called “mods”. The differences in design and engineering in each unique mod resulted in different concentrations of aerosolized chemicals and nicotine delivery.

The modifications of ECs led to larger batteries which not only extended the duration of the atomizing process, but also the temperature of the heating reaction. This potentially could create larger aerosol clouds containing more nicotine and greater concentrations of particulate matter[13]. The metal particulates come from leaching of the EC’s metal components (e.g. the heating coil and e-liquid container) and trace metals inside e-liquids[14]. Also, after continuous use, the EC heats up to a dangerous temperature and if no temperature limit is set, the solvents ,propylene glycol and glycerin, can react to form formaldehyde, a carcinogen[15]. There are potential dangers that harbor within the e-liquid too[14,16].

Studies of EC users have reported negative health effects affecting the nose, mouth, throat, and airways[17,18]. An epidemiological study of 45,000 adolescents living in Hong Kong with repeated EC use for more than? 1 month developed a chronic cough or phlegm build up[19]. A cross-sectional study of nearly 36,000 high school students in South Korea found that the EC users were more

likely to develop asthma and miss sick days at school because of severe asthma symptoms[20]. Another cross-sectional study of 6,089 high school students in Hawaii found a correlation between EC use and developing asthma[21]. The Health eHeart Study of nearly 40,000 subjects found that EC usage was associated with both asthma and *chronic obstructive pulmonary disease (*COPD)[22].

In 2019, there were deaths associated with EC usage and several hundred cases of reported acute respiratory illness prompting investigations from the FDA and CDC[23]. The novel disease was termed e-cigarette or vaping use-associated lung injury (EVALI). The 53 patients first diagnosed with EVALI they developed gastrointestinal (81%), constitutional (100%), and respiratory (98%) symptoms, dyspnea (87%), and cough (83%). Most patients had arterial hypoxemia (69%), elevated blood neutrophil counts (94%), and elevated transaminases (55%). 48 patients were scanned by computer tomography and diagnosed with “abnormal lung parenchyma, typically characterized by ground glass opacities in both lungs, sometimes with subpleural sparing”[24]. By February 2020 there were nearly 2,700 cases of EVALI[23]. It was theorized that tainted cannabis e-liquid containing high levels of vitamin E acetate was the culprit, though no singular conclusive determinant was found. Due in part to this unique “poisoned” e-liquid epidemic, the negative health effects of habitual EC usage have become more extensively studied.

Adverse respiratory health and toxicity studies with ECs have demonstrated lung toxicity with one common lung function test being spirometry measurements

(which is the forced inhalation and exhalation and the monitoring of airflow over time). One study demonstrated in 30 ECs users compared to their controls, that 1 hour post vaping abstinence lowered forced expiratory volume in 1 second ($FEV_1$) reflecting acute bronchospasms in the lung[25]. To determine chronic, permanent structural lung damage, clinical studies need to monitor decades of exclusive EC users. However, it should be noted that spirometry tests do not always detect lung damage because patients with cystic fibrosis who develop lung disease over long periods of time did not have decreased $FEV_1$[26]. ECs have now been used for over 10 years and their long-term effects will likely become apparent in the near future.

Other studies have demonstrated the physiological and molecular changes of acute EC exposure. EC usage with or without nicotine induced lung inflammation and repair responses in mice exposed to EC aerosol for 30 days (2 hours per day, 5 days per week)[27]. Airway mucosa secretion as detected by MUC5AC-positive mucin was increased in former cigarette smokers who switched to exclusive EC use[28]. Increased mucin (MU5AC +) levels correlated with declined lung function in severe cystic fibrosis (CF) patients[29] and increased EC use may possibly lead to developing CF. Elevated mucin levels inversely correlated with declined $FEV_1$ in COPD patients indicative of lung damage, and the mucins may serve as good biomarkers of lung harm[30]. Proteomics of EC one user's sputum has detected pathways of inflammation similar to cigarette smoking, such as neutrophil activation, lung inflammation due to production of MMP9, increased levels of innate defense proteins DMBT1, trefoil factor 3, lactoferrin, LYSC, and

increased mucin production[31]. There are dangers associated with increased protease production such as elastases and matrix metalloproteases (MMPs) in EC users which can degrade lung basement membranes and lead to COPD[32]. A clinical study demonstrated that acute EC vaping of fourth generation products induced lung inflammation and a sustained decrement in transcutaneous oxygen tension, which is consistent with injury to the small airways[33].

Inflammation of the lung airways can lead to respiratory diseases due to inflammatory reactions against inhaled antigens, such as hypersensitivity pneumonia. A clinical study looked at healthy participants with no history of cigarette or EC usage and exposed them to a single 1 hour EC smoking session and found in their blood endothelial microparticulates indicative of alveolar capillaries that were activated or injured[34].

Air-liquid-interface cultures (where the basal lung epithelial cells are submerged in media and the apical cells are exposed to EC vapor) showed epithelial remodeling and mucociliary dysfunction[35]. Impaired mucociliary clearance is a predisposition for developing pneumonia[33]. A study of 10 healthy non-smokers who vaped 20 puffs had alveolar macrophages harvested from a bronchoalveolar lavage, the macrophages were analyzed by RNA-seq and elevated pathways were related to pulmonary inflammation and migration[36]. Neutrophils were activated in chronic EC vapors too, as they had a propensity for neutrophil extracellular trap (NET) formation where the neutrophils eject their DNA to encapsulate foreign particles, often bacteria[31]. *In-vitro* aerosol exposure studies

to neutrophils altered the expression of proinflammatory surface markers CD11b and CD66b, and also increased production of inflammatory cytokines and proteases[37]. The secretion of these inflammatory proteins has been detected in the sputum of EC users too[31].

Acute lung epithelial EC exposure studies have shown inhibition of mitochondrial function such as suppressed bioenergetic activity, reduced ATP levels, and impaired cilia motility[38], and altered gene expression involved in oxidative and xenobiotic stress[39]. The question researchers have pondered is what EC chemical(s) cause toxicity? The two main constituents of e-liquids are propylene glycol and glycerin. These two solvents are regarded as safe for food consumption, however before e-liquid production they had rarely been tested for inhalation toxicity. Propylene glycol activates TRPV1 and TRPA1 channels, which are two irritant receptors expressed in sensory nerves innervating the airways[40]. Although it seems in normal concentrations, propylene glycol acts only as a mild to no symptom respiratory or ocular irritant[41]. However, that conclusion was based upon an acute 30 min to 4-hour exposure study. Perhaps, chronic exposure can cause more serious symptoms because TRPV1 and TRPA1 are promoters of asthmatic inflammation and airway hyper-reactivity[42]. Propylene glycol and vegetable glycerin can also increase mucin production[31], and as stated earlier this is inversely correlated with decreased lung function and susceptibility to developing COPD.

Nicotine, a naturally occurring compound in tobacco plants, binds nicotinic acetylcholine receptors (nAChR), which are ligand-gated ion channels highly expressed in the brain and lungs[43]. Activation of the nAChR has been extensively studied and has many biological altering properties. Nicotine can increase cell proliferation and inhibit apoptosis[44]. The role of nicotine in increased cancer risk is known because α3, α5, and β4 nAChR were associated with lung cancer and there was differential expression of nAChR between non-smokers and smokers with non-small cell lung cancer[45]. Nicotine can also cause smooth muscle cell proliferation, which can cause vascular dysfunction by increasing arterial stiffness[46]. Nicotine also increases neutrophil elastase production and IL-8 release, which can degrade the pulmonary extracellular matrix and plays a crucial role in lung destruction[47]. In summary, ECs that contain nicotine may increase the risk of lung inflammation and the risk of cancer.

E-liquids contain flavorings to enhance the taste of the inhaled vapor and there are currently over 7,700 unique e-liquid flavorings being sold[48]. However, certain flavors such as menthol and cinnamaldehyde are toxic when tested in-vitro with human embryonic stem cells (hESCs) and human pulmonary fibroblasts (hPFs)[38,49], and menthol e-liquid caused apoptosis, autophagy, inflammatory response, and mucin production in human middle ear epithelial cells (HMEECs)[50]. Human lung tissue exposed to menthol in the air-liquid-interphase (ALI) demonstrated increased oxidative stress and inflammatory responses[51]. The buttery flavor diacetyl can cause pulmonary toxicity and propensity for causing

bronchiolitis obliterans[52]. Also, aldehyde flavorings when mixed with propylene glycol (PG) and vegetable glycerin (VG) undergo chemical reactions, which form acetal compounds that act as irritant receptors[53].

A study released from our lab discovered ECs contain high amounts of trace metals, which can cause toxicity[54]. Metal/metalloid levels which include aluminum, antimony, arsenic, cadmium, cobalt, chromium, copper, iron, lead, manganese, nickel, selenium, tin, and zinc, were present in e-cigarette liquids[55]. The health effects of metal exposure is well documented and can cause cancer[56], cardiovascular disease[57], renal damage[58], neurotoxicity[59], and oxidative stress[60]. Metal leaching can occur from the atomizing unit, soldering joints, wires, wicks, sheaths, metal housing container, and even from e-liquids themselves[54]. A review study that measured metalloid levels in aerosol samples generally found higher concentrations of metals in tank-style models compared to cig-a-like devices[55]. The metals Al, Fe, Ni, and Zn were found in e-liquids and aerosols, whereas Cr, Cu, and Pb were found in aerosols[55]. Another study found only Zn in the e-liquid, but found Al, Cu, Fe, Mn, Ni, Pb, and Zn in aerosols[61]. A similar study found higher Al, As, Ni, and Zn in aerosols compared to their liquids[62]. These studies show that e-liquid can contribute to metal exposure. There is also increased metal contamination attributed to the metal components of ECs and the aerosolization process.

Several human studies found higher levels of metals present in EC aerosols in exclusively EC users compared to non-users. A study in Romania discovered

EC users had higher blood levels of Ag, Se, and V compared to non-smokers and cigarette smokers[63]. A study of 64 EC users found higher concentrations of Cr and Ni in urine, saliva, and exhaled breath condensate (EBC) (micrograms per liter) compared to non-smokers[64]. Collectively, these studies show EC usage is correlated with elevated metal exposure.

Once inside the body, inhaled metals are rapidly absorbed through the respiratory tract[65], and those identified in EC aerosols can have adverse health effects. Inhaled nickel (NI) exposure can cause lung inflammation and diseases such as rhinitis, sinusitis, asthma, and in general decreased lung function[66]. EC Ni exposure was suspected to have caused allergic dermatitis in a 52-year-old EC user because when she used a nickel-free EC device their skin allergy cleared up[67]. Ni is an established carcinogen and exceeds the minimum risk levels for Ni inhalation in 89-100% of aerosol samples generated from tank devices (n=54)[61]. Pb even in low concentrations is associated with increased risk of cardiovascular and kidney disease, and neurotoxicity[68]. Chronic Cu exposure has caused vomiting, gastrointestinal pain, hepatotoxicity, respiratory irritation, coughing, sneezing, and chest pain[69]. A highly toxic metal, As, is associated with cancer and cardiovascular diseases[70]. Excess Zn is associated with increased inflammation, chest pain, dyspnea, metal fume fever, sore throat, and shortness of breath[71].

In summary, there is overwhelming evidence EC usage increases the exposure to metals which are associated with adverse health effects. There is substantial heterogeneity in EC devices, e-liquids, customizable parts, heating

temperatures, and length of vaping time that contribute to differences in metal exposure. Most metal concentration studies have found there is a substantial increase in the concentration of metals found in the aerosols compared to the unused e-liquid, indicating that the metal components in the aerosolization process contribute to metal exposure. Chronic exposure may eventually lead to disease progression and more studies are needed to inform EC users of possible metal exposure through vaping.

The first concept of an EC was patented in 1963, and in 2003 use of ECs became widely popular. There are an estimated 8 million+ EC users in the US[72], and this will continue to increase unless the public is informed about the health hazards of EC usage. There are multiple toxic compounds in EC aerosols such as volatile carbonyls, carcinogens, reactive oxygen species, furans, metals, and flavorings that contribute to cytotoxicity via different mechanisms. The correlation between short- to intermediate-term (months to a year-long) EC usage and disease initiation is very apparent, and as more time progresses, researchers will discover the chronic (years to decades) effects of EC vaping, which may include cancer and cardiovascular disease. Research is important to determine vaping is harmful to human health so that regulatory agencies can limit or prevent EC usage and to inform the public about the toxicity of vaping.

## Thirdhand Smoke

Numerous studies have shown that secondhand smoke (SHS) exposure or environmental tobacco smoke (ETS) can have many adverse health consequences[73]. As THS was a relatively new concept, the general public was still unaware of its potential health dangers[74]. Scientific literature began introducing the topic of aging SHS and ETS, and to uniquely differentiate between SHS and THS[73]. Although the most commonly accepted term is THS, it is also known as *Residual tobacco smoke* or *Aged tobacco smoke*[75]. Since its identification experts in the field have come to a consensus statement to define THS as follows: *"THS consists of residual tobacco smoke pollutants that remain on surfaces and in dust after tobacco has been smoked; are re-emitted into the gas phase; or react with oxidants and other compounds in the environment to yield secondary pollutants"*[75]. The phrase “the four Rs” provide a description of THS which occur when tobacco chemicals “remain, react, re-emit, and/or are resuspended” long after active smoking has ceased. THS is comprised of thousands of chemicals and ultrafine particles that are capable of penetrating into materialistic items, such as furniture and clothing where they remain embedded for prolonged periods of time. During this time residual tobacco chemicals may react with atmospheric species creating other pollutants not present in the original fresh smoke, where they are re-emitted back into the indoor environment or stick to indoor surfaces[75,76]. Nicotine can react with common indoor atmospheric species to produce tobacco-specific nitrosamines (TSNAs). Chemical reactions

take place from the moment tobacco smoke is produced and may continue for weeks[77]. THS, in-contrast to SHS, can remain for pro-longed periods of time up to months or years.

THS and SHS are conceptually distinct, in SHS the aerosol (particulates and gas phase constituents released from the combusting tobacco cigarette) is only present while smoking is physically occurring. Therefore, exposure to SHS is associated with freshly emitted smoke only. The primary route of SHS exposure is by inhalation that occurs in acute periods often minutes (but sometimes hours in a larger gathering of smokers). In contrast, for THS there are multiple routes of exposure which include inhalation of contaminated dust particles, dermal absorption from contact with contaminated surfaces or clothing, and ingestion of THS from hand-to-mouth contact or food. For example, toddlers frequently mouth objects in their environment, which is another route of oral exposure to THS. Recent studies have documented exposure of THS dust through the routes of exposure described above[78]. In humans, there is an increased potential cancer risk correlated to exposure to tobacco specific nitrosamines through non-dietary ingestion and dermal exposure[79]. Another study has shown that non-smokers exposed to THS have an increased mortality risk associated when living with a smoker[80]. These impactful studies have provided enough evidence to identify THS as a dangerous health risk for adults and children. The California Consortium on Thirdhand Smoke was launched in 2011 and renewed in 2014 to carry out its

research agenda. The goal of this Consortium was to identify the chemical composition of THS, determine potential routes of exposure, mechanisms of toxicity, overall health effects of THS, and potential behavioral, economic, and sociocultural considerations and consequences posed by it.

The generation of THS samples for toxicology studies has been described in several studies[81,82]. To create the THS samples, materials (fabrics or paper) are exposed to cigarette smoke, either sidestream, or a mixture of sidestream and mainstream. In a controlled flow chamber, cigarette smoke is directed by airflow into the exposure chamber where the fabric/paper is impregnated with cigarette smoke. To generate standardized THS samples, multiple factors such as smoke concentration, flow rate, time, substrate, and storage conditions are accounted for. Smoking machines generate either sidestream smoke or a mixture of sidestream and mainstream smoke and provide an autonomous consistent generation of cigarette smoke. The THS samples used for my study were generated by Dr. Schick[82]. To standardize THS samples across multiple batches, the cigarette smoke exposed clothing contained at least 3000 mg of smoke particles per $m^{-3}$ of fabric. The smoke is diluted to concentrations that are typically expected for secondhand smoke from a smoker. This process takes months in order for the cloths to amass the necessary number of smoke particles. Once the clothing has reached 3000 mg of particles, the clothing is vacuum sealed inside mylar bags and stored in a -20$^{o}$ freezer. For my *in-vitro* experiments, THS-impregnated fabric was used for chemical extraction. The protocol as briefly described is to remove the

THS fabric from storage and cut it up into small centimeter sized pieces. The chemical extract concentration is 0.01 g of fabric/mL, therefore 0.05 grams of fabric is placed into 5 mL of media in a sterile glass vial. These vials are placed on a gentle rocker for 1 hour and the THS-fabric media is then filtered using 0.2 μm membrane filters to remove fabric particulates. The THS-extract is aliquoted, flash frozen on dry ice, then stored in a -20$^{o}$C freezer until the experiment is conducted.

A study from our lab determined that the type of fabric plays an important factor in the absorption of THS by comparing adsorption to polyester and cotton 19 months after storage, and discovered cotton contained higher levels of extractable THS constituents[83]. A collaborator with our lab, showed that THS can deposit on cotton clothing within 60 mins, leaving deposits of polycyclic aromatic hydrocarbons (PAHs), nicotine, and tobacco-specific nitrosamines on surfaces[82]. There are chemical processes involving atmospheric reactive species that react with THS constituents (mainly nicotine was measured in this study). The reaction forms new oxidation products, such cotinine, myosmine, *N*-methyl formamide, and nicotine-1-oxide[84]. There are also indoor combustion sources, such nitrous acid (HONO), hydrogen peroxide ($H_2O_2$), and free radicals that may drive the formation of new THS chemical reactions[73]. Even direct light can generate OH and $NO_3$ radicals from air[85]. These oxygen- and nitrogen-containing radicals, oxidants, and nitrosating species can react with THS chemicals to create oxidation byproducts such as carbonyls, amides, *N*-oxides, carboxylic acids, and TSNA's[73]. Also, these secondary organic aerosol formations produced by nicotine ozonolysis

can generate more ROS which can contribute to further oxidation[86]. Probably the most well-known THS chemical transformation is the nitrosation of nicotine by HONO to produce tobacco TSNAs, such *N*′-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 4-(methylnitrosamino)-4-(3-pyridyl)butanal (NNA)[87].

Storage of THS samples is an important factor, and time at room temperature can cause loss of toxic chemicals[83], therefore storage at −20 °C is recommended. The duration of cigarette exposure from start to finish should be noted, as well as time in storage. Some of the compounds in THS are volatile (such as acrolein), labile, and/or sensitive to UV degradation, so samples should be stored in airtight light impermeable packing.

Since the implementation of the California Consortium on Thirdhand Smoke, there have been numerous studies contributing to the body of evidence regarding the health risks of THS. *In-vitro* studies demonstrated potentially harmful effects of THS, however realistic concentrations of THS exposure first needed to be quantified. Researchers tested indoor surfaces of cigarette smoker's homes and found levels of NNA (2.2–3500 ng/m$^2$), and NNK (0.31–500 ng/m$^{2)}$ depending on the indoor surface used for testing. These real-world concentrations could be converted for cell culture exposures and the authors determined the *in-vitro* exposure concentrations for 0.39–1.82 ng/mL of NNA and 0.51–7.2 ng/mL of NNK[87].

Studies from our lab demonstrated THS extracts caused stress-induced mitochondrial hyperfusion and altered mitochondrial gene expression in mouse neural stem cells and human embryonic stem cells[38,88]. Cytotoxicity of THS in the MTT assay was isolated to acrolein[89]. Gene expression of THS-exposed human fibroblasts to low levels of acrolein ($10^{-6}$ M) inhibited expression of TFDP1 (which controls transcriptional activity from G1 to S phase of mitosis), *Casp3* (which transitions cells from G2 into the M phase of the cell cycle), and *AnaPC2* (which would inhibit metaphase-anaphase transition). *Wee1* expression increased, which is an inhibitory protein preventing the transition of DNA replication to mitosis. This study demonstrated that THS exposure to the volatile organic compound (VOC) acrolein can inhibit the cell cycle and slow proliferation[89].

Another THS *in-vitro* study showed that 24-hour THS exposure to a human liver cell line (HepG2) caused DNA strand breaks, which can increase the risk of mutagenesis and developing cancer[90]. BEAS-2B cells exposed to NNA had significant double stranded breaks (DSBs) in DNA, which caused histone H2AX to become phosphorylated. NNA also reacts with guanosine forming DNA adducts[91] and H2AX can serve as a biomarker for analyzing DSBs[90]. There were also increased levels of 8-oxo-dG, a major DNA oxidation product that causes DNA transversions and could lead to disease progression such as cancer[91]. In a metabolomic study of human male reproductive cell lines, THS caused changes consistent with an antioxidant response and alterations in nucleic acid synthesis[92].

The effects of THS accumulation were also assessed using terry cloth exposed to cigarette smoke generated in a controlled exposure chamber. Human neural stem cells and pulmonary fibroblasts were exposed to THS extract from this fabric and assessed using a MTT assay, which measures mitochondrial reductase activity. Toxicity was observed in extracts from the terry cloth equivalent to ~ 54 cigarettes over an 8-month period[77], which was considered a low dose of THS exposure since a pack-a-day smoker would consume 4,800 cigarettes in an 8-month period. A THS 60-day aging experiment where samples were taken from a smoker's car found there was a 100% concentration increases of cotinine and NNA compared to a non-smoker's car which caused DNA damage in mouse NSCs and human DF[77]. Another study exposed rat HepG2 to THS paper extracts created from 1, 3, 5, 10, 15, or 20 cigarettes in an acrylic chamber and cotton samples collected from a smoker's home (60 cigarettes smoked over 3 days). There was reduced cellular proliferation and lysosomal activity when the cells were exposed to the cloth samples taken from the smoker's home[90].

In mouse studies, researchers exposed newborn mice to THS during their first 3 weeks of life and found greater eosinophil numbers in blood circulation in both genders, with higher basophils in male mice and increased neutrophils in female mice[93]. There was also a significant increase in the percentage of B-cells and T-suppressor cells, with a decreased percentage of myeloid cells in adult mice[93]. Another mouse study investigated the effects of THS on liver, lung, skin healing, and behavior. The authors that in the liver, THS led to increased lipid

levels and non-alcoholic fatty liver disease, a precursor to cirrhosis and cancer. In the lung, THS stimulated excess collagen production and high levels of inflammatory cytokines, a pre-disease state for fibrosis, COPD and asthma. In wounded skin, healing in THS-exposed mice was decreased and wounds remained open for longer. Behavioral tests showed that THS-exposed mice become hyperactive[94]. They also measured urinary NNAL levels in THS-exposed mice and found that its concentration was similar to those seen in a cohort study 50 infants/toddlers aged 0.5 to 4 years exposed to SHS. This demonstrated that the exposure conditions were relatively consistent with children's passive cigarette exposure in smoker's homes. A study of THS-exposed mouse wounds found higher levels of oxidative stress, reduced levels of antioxidants, and elevated DNA damage that contribute to tissue dysfunction[95]. They found elastase was elevated, suggesting that elastin is degraded and the plasticity of the wound tissue is decreased[95]. Collectively, these results show the potential health effects of THS in humans.

A human exposure study identified the persistence of THS constituents (nicotine) in the houses of former smokers even after the former tenants moved out and performed thorough cleaning[80]. The same author identified that THS chemicals (nicotine, NNK, NNAL) can last up to 6 months in the dust and surface of former smoker's homes[96]. A comparison of hotels with a smoking ban to hotels without smoking bans found higher levels of surface nicotine. In addition, urinary NNAL and cotinine were elevated in guests that had stayed in the 10 most THS

contaminated rooms in hotels without a smoking ban[97]. Casinos that implemented smoking bans had lower levels of nicotine air contamination, however in reservoirs that can hold THS such as the carpet, furniture, and building materials harmful levels of nicotine contamination were present. Rental cars without smoking bans had higher levels of surface and dust nicotine, especially in smoking cars that were older and had higher milage usage[98].

A study identified a lung specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-1butanone (NNK) in 33 of 37 smoker's homes (700±788 pg/100cm$^2$) taken from cotton swap samples[99]. A human exposure study found that a toddler mouthing a piece of THS-impregnated fabric equivalent to 133 cigarettes exposure contains 16-fold higher concentrations of TSNA's than if they were exposed by passive secondhand inhalation[90]. This is extremely concerning because of the potential carcinogenic risk of TSNA exposure. A human risk study evaluated the relationship between THS exposure in people and the risk of cancer. By estimating exposure to carcinogen N-nitrosamines and tobacco-specific nitrosamines (TSNA) measured in house dust samples, they showed an increased risk of cancer in the homes of smokers especially if exposure occurred at an earlier age[100]. Other risk assessment studies found a relationship between an increase of respiratory symptoms in children who lived with parental smokers suggesting they also have increased THS exposure too[101]. An exposure risk study analyzed nicotine exposure in infants who had been brought into the neonatal intensive care unit (ICU) from mother's who smoked, and found increased nicotine contamination in

the infant incubators, and hospital equipment, and as well as urinary nicotine metabolites[102]. Even low levels of prenatal exposure to nicotine have been reported to cause infant cognitive defects at 5 weeks of age after birth[103].

The collection of studies above demonstrates the risk associated with THS exposure in humans. However as of 2021, there was only one human harm study associated with THS exposure. This study analyzed gene expression in nasal epithelium following acute inhalation of THS emitted from a controlled chamber. Affected genes were associated with increased mitochondrial activity, oxidative stress, DNA repair, cell survival, and inhibition of cell death[104], showing that humans respond to inhaled THS chemicals. This one study demonstrates the effects of THS inhalation exposure, but no human dermal exposures have been performed.

**Thirdhand Smoke Effects On The Skin**

Unlike primary and secondary tobacco exposure where the main route of exposure is through inhalation, the main route of THS exposure is through dermal contact. Though there is a lack of research on the effects of THS on the human skin, one can hypothesize the health effects from data on the effects of cigarette smoke exposure on skin. Smoking negatively impacts wound healing[105], and thus smoking cessation is recommended before surgery[106]. The constriction of blood vessels is reversible 1-hour post-smoking cessation, however a return to a normal immune system takes approximately 4 weeks[73]. From mouse studies, wounds exposed to THS show heavy keratinization of the epithelium which causes the

epidermis to lose its elasticity and causes keratinocytes to not properly differentiate. There is an increase of MMP-1 and MMP-9 production, which degrades the extracellular matrix further weakening the connections of keratinocytes[107]. Inhibitor metalloproteinase 1 (TIMP1) is also decreased, causing increased levels of MMP-1 activity. The levels of fibrillar collagen are decreased and degraded. The collagen that is produced is not fibrillar, which means the extracellular matrix of the epidermis is weakened[108]. This causes the epidermis to be more prone to re-opening due to a decrease in the amount of fibrillar collagen and abnormal keratinization, leading to a significant weaking of the epidermis. My two research projects Chapters Two and Three help fill this knowledge gap by demonstrating the biological responses to dermal THS exposure.

Research on thirdhand smoke is still in its early stages. Currently, there is only one clinical study of the effects of THS on human health. Previous research has shown the formation of THS chemicals such as nitrosation of nicotine to form TSNAs and the formation of secondary organic reactions that produce carbonyls, amides, *N*-oxides, carboxylic acids and ROS. While some THS chemicals persist for months, others volatile chemicals, such as acrolein, furan, acrylonitrile, and 1,3-butadiene are released back into the air and can be relatively toxic. THS *in-vitro* studies caused DNA damage, mitochondrial dysfunction, metabolic disorders, and skin damage. In mouse models, multiple organ systems are harmed (especially the lung, liver, and skin), there is altered immune responses, deficient metabolic

effects, increased LDL, decreased HDL defects in insulin metabolism, delayed wound healing, and oxidative damage (with anti-oxidant depletion). Human exposure studies have demonstrated THS chemicals persist for months even after smoking cessation has ceased for months and the harmful levels of tobacco-products metabolites have been measured in health individuals exposed to THS. The only human THS-inhalation study demonstrated biological harm (oxidative damage, DNA repair, and cell survival pathway activation) after just a 3-hour exposure, demonstrating THS is harmful to human health. Future studies should aim to find novel distinguishing THS biomarkers and further develop THS-exposed human harm research. Eventually, the goal of the THS Consortium is to provide enough data for the regulation of public health policies to limit the exposure of THS in contaminated properties and will help physicians, researchers, and the general public to become knowledgeable about the health hazards of THS by addressing the harmful effects of THS to human health. My hypothesis is that firsthand cigarette smoke and electronic cigarette exposure will elevate biomarkers of effect and exposure that would correlate with biomarkers of harm to demonstrate potential linkage to disease initiation due to exposure. My hypothesis for Chapters two and three is that exposure to THS will cause similar detrimental health effects in the human skin as mimicked by cigarette smoke exposure.

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# Chapter One

# Correlation Between Biomarkers of Exposure, Effect, and Potential Harm in the Urine of Electronic Cigarette Users

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## Abstract

**Objectives:** To determine if urinary biomarkers of effect and potential harm are elevated in electronic cigarette users compared to non-smokers and if elevation correlates with increased concentrations of metals in urine.

**Study Design and Setting:** This was a cross-sectional study of biomarkers of exposure, effect, and potential harm in urine from non-smokers (n=20), electronic cigarette users (n=20), and cigarette smokers (n=13). Participant's screening and urine collection were performed at the Roswell Park Comprehensive Cancer Center and biomarker analysis and metal analysis was performed at the University of California, Riverside.

**Results:** Metallothionein was significantly elevated in the electronic cigarette group (3761 ± 3932 pg/mg) compared to the non-smokers (1129 ± 1294 pg/mg, p=0.05). 8-OHdG (8-hydroxy-2' -deoxyguanosine) was significantly elevated in electronic cigarette users (442.8 ± 300.7 ng/mg) vs non-smokers (221.6 ± 157.8 ng/mg, p=0.01). 8-isoprostane showed a significant increase in electronic cigarette users (750.8 ± 433 pg/mg) vs non-smokers (411.2 ± 287.4 pg/mg, p=0.03). Linear regression analysis in the electronic cigarette group showed a significant correlation between cotinine and total metal concentration; total metal concentration and metallothionein; cotinine and oxidative DNA damage; and total metal concentration and oxidative DNA damage. Zinc was significantly elevated in the electronic cigarette users (584.5 ± 826.6 µg/g) compared to non-smokers (413.6 ± 233.7 µg/g, p=0.03). Linear regression analysis showed a significant

correlation between urinary zinc concentration and 8-OHdG in the electronic cigarette users.

**Conclusions:** This study is the first to investigate biomarkers of potential harm and effect in electronic cigarette users and to show a linkage to metal exposure. The biomarker levels in electronic cigarette users were similar to (and not lower than) cigarette smokers. In electronic cigarette users, there was a link to elevated total metal exposure and oxidative DNA damage. Specifically, our results demonstrate that zinc concentration was correlated to oxidative DNA damage.

**What is the key question?**

- Is increased electronic cigarette usage associated with elevated metal exposure and if such exposure can cause biological harm?

**What is the bottom line:**

- Biomarkers of exposure (cotinine and metals), effect (metallothionein), and potential harm (8-isoprostane and 8-OHdG) were elevated in electronic cigarette users and were similar to concentrations in cigarette smokers; also increased electronic cigarette usage (as measured by cotinine) was correlated with elevated urinary metal concentrations, which were correlated with oxidative DNA damage.

**Why read on:**

- This is one of the first studies to demonstrate a correlation between biological harm and electronic cigarette usage, suggesting the metal

constituents (in particular zinc) in electronic cigarette aerosol can cause oxidative DNA damage. Given the recent deaths and pulmonary illnesses related to electronic cigarette usage, it is important for readers to know about the potential health effects related to electronic cigarette usage.

**Strengths and Limitations:**

- This was a cross-sectional study with gender and age-matched populations to compare urinary biomarker levels and metal concentrations in electronic cigarette users versus cigarette smokers and non-smokers.
- This is the first study to demonstrate electronic cigarette users are exposed to increased concentrations of potentially harmful levels of metals (specifically zinc) that were correlated to elevated oxidative DNA damage.
- This study is based on a relatively small population (n=53) and small number of biomarkers and should be expanded.
- In the electronic cigarette and cigarette smoker groups, participants were not all using the same products and had different numbers of puffs/day.

# Introduction

Cigarette smoking causes more than 480,000 deaths annually in the United States and is the leading cause of preventable death[1]. Electronic cigarettes, which grew in usage over 900% between 2011-2015, do not burn tobacco and may be a safer product[2]. However, there are limited scientific data to prove that electronic cigarettes are actually less harmful than combustible tobacco products, although they may be harmful in different ways. To the contrary, some previous research has demonstrated that electronic cigarette aerosols contain potentially harmful chemicals, such as acrolein; formaldehyde and benzene[3]; cytotoxic flavor chemicals, such as diacetyl and cinnamaldehyde[4,5]; metals and ultrafine particles including tin, chromium and nickel nanoparticles[6,7]; and free radicals[8]. Moreover, some electronic cigarette refill fluids and aerosols showed cytotoxicity when tested in vitro[9,10], an effect that has been linked to metals in the refill-fluid[6,]. An in vitro study demonstrated that isolated human alveolar macrophages exposed to electronic cigarette vapour induces inflammation and reduces phagocytosis leaving the patient more susceptible to pulmonary infections[11]. Moreover, recent case reports have attributed electronic cigarette use to several adverse health effects, such as respiratory diseases[12], increased risk for cardiovascular disease[13], and impaired wound healing after surgery[14]. Several previous studies on electronic cigarettes have evaluated biomarkers of exposure in blood, urine, and saliva[15,16,17], but none has yet examined and quantified biomarkers of effect and potential harm in relation to metals in electronic cigarette users.

This study compares urinary biomarkers of exposure, effect, and potential harm in non-smokers, conventional cigarette smokers, and electronic cigarette users and accounts for the effect of gender and age on biomarker expression. Based on the above studies, we hypothesized that there would be an increase in the level of biomarkers of effect and potential harm in electronic cigarette users compared to non-smokers and a decrease compared to cigarette smokers. The urinary biomarker of effect, metallothionein, is a protein that responds to and protects against metal toxicity and free radical stress. Urinary biomarkers of potential harm were two markers of oxidative stress: (1) 8-isoprostane, a prostaglandin formed by fatty acid peroxidation, and (2) 8-OHdG, a product of DNA oxidation. Urinary biomarkers of exposure were: (1) cotinine, a nicotine metabolite to measure smoking or vaping usage, and (2) total concentration of 11 urinary metals, which are present in electronic cigarette aerosol[6,7,18] and are known to associate with metallothionein[19,20]. Regression analyses were performed to identify relationships between biomarkers of exposure (cotinine and metals), effect (metallothionein), and potential harm (8-OHdG). To isolate the observed oxidative effects to a specific metal, regression analyses were performed between the urinary concentrations of individual metals and 8-OHdG.

## Materials and Methods

***Subjects***: The urine samples were from participants who were non-smokers, cigarette smokers, and electronic cigarette users. Participants were recruited through local media and flyers posted in various locations around the Buffalo, New York area. Potential participants were provided with a brief description of the study and had an opportunity to ask questions about the study procedures. All potential participants were screened over the phone for inclusion and exclusion criteria. The exclusion criteria included concurrent use of smokeless tobacco, pipes, or cigars; alcohol or illicit drug dependence within the past six months or current illicit drug use (including marijuana; self-reported); psychiatric illness; and use of Nicotine Replacement Therapy (NRT). Information about medication and vitamins/antioxidants/metal usage was not collected. All eligible subjects who had been asked to come to the clinic for screening were given an informed consent form to read and sign. Copies of the signed consent forms were given to the research subject and were also stored in a secure location, along with the participant's research chart. Informed written consent was obtained from each participant prior to their participation. Eligible participants were then asked to come to Roswell Park Comprehensive Cancer Center for a one-time visit, which lasted approximately 1 hour. Spot urine samples were collected during this on-site visit. The Roswell Park IRB had reviewed all procedures prior to implementation (protocol number I 247313).

A total of 53 participants were gender and age matched and selected for biomarker analysis. Because age may affect the basal expression level of biomarkers, the subjects were separated into those ≤40 years old and ≥41 years old, with the groups containing 23 and 30 samples, respectively. Out of these age-separated samples, participants were selected from the non-smoker, cigarette smoker, and electronic cigarette user groups. Each group had approximately equal male and female samples. Using a one-way ANOVA and a Tukey's multiple comparison test, there were no significant differences in the ages of the younger participants or in the ages of the older participants; however, the ages of the younger and older groups were significantly different from each other. There were negligible levels of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in the non-smokers (2.8 ± 6.3 pg/mg of creatinine) and electronic cigarette users (13.3 ± 18.6 pg/mg of creatinine) indicative of no tobacco use, in contrast to the cigarette smokers (105.7 ± 87.4 pg/mg of creatinine) who had significantly elevated NNAL (Supplementary Figure 1.1). In the non-smokers, no samples had levels of cotinine ≥1.0 ng/mg (Supplementary Figure 1.2), confirming smoking abstinence. The demographics of the 53 participants who provided urine samples were organized by age, gender, and smoking group (Table 1.1).

**Table 1.1.** Demographics of the 53 participants included in this study separated by smoking group, age, and gender. All smoking groups were gender and age matched.

Table 1 Demographics of the 53 participants included in this study separated by smoking group, age and gender

| Age (≤40 years old) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Non-smokers** | | | | **Cigarette users** | | | | **Electronic cigarette users** | | | | |
| Sample ID | Sex | Age | Average | Sample ID | Sex | Age | Average | Sample ID | Sex | Age | Electronic Cigarette type | Average |
| 33B | Male | 23 | | 02A | Male | 28 | 28±0 | 04C | Male | 19 | Tank | |
| 07B | Male | 25 | | 21A | Female | 24 | | 35C | Male | 28 | Tank | |
| 38B | Male | 29 | | 17A | Female | 33 | 28.5±6.4 | 17C | Male | 30 | Tank | |
| 21B | Male | 37 | | | | | | 41C | Male | 34 | Tank | |
| 16B | Male | 40 | 30.8±7.4 | | | | | 16C | Male | 40 | Tank | 30.2±7.8 |
| 06B | Female | 27 | | | | | | 06C | Female | 29 | Tank | |
| 09B | Female | 32 | | | | | | 23C | Female | 32 | Tank | |
| 42B | Female | 33 | | | | | | 21C | Female | 33 | Tank | |
| 45B | Female | 33 | | | | | | 28C | Female | 39 | Tank | |
| 44B | Female | 38 | 32.6±3.9 | | | | | 27C | Female | 40 | Tank | 34.6±4.7 |
| Age (≥41 years old) | | | | | | | | | | | | |
| **Non-smokers** | | | | **Cigarette users** | | | | **Electronic cigarette users** | | | | |
| Sample ID | Sex | Age | Average | Sample ID | Sex | Age | Average | Sample ID | Sex | Age | EC Type | Average |
| 13B | Male | 42 | | 08A | Male | 41 | | 31C | Male | 45 | Tank | |
| 27B | Male | 46 | | 23A | Male | 49 | | 37C | Male | 47 | Tank | |
| 26B | Male | 58 | | 03A | Male | 65 | | 05C | Male | 57 | Tank | |
| 34B | Male | 58 | | 28A | Male | 66 | | 32C | Male | 60 | Tank | |
| 43B | Male | 66 | 54±9.8 | 13A | Male | 75 | 59.2±13.8 | 03C | Male | 66 | Cartomiser | 55±8.9 |
| 41B | Female | 41 | | 14A | Female | 46 | | 08C | Female | 44 | Tank | |
| 04B | Female | 46 | | 06A | Female | 49 | | 13C | Female | 50 | Tank | |
| 28B | Female | 52 | | 18A | Female | 57 | | 09C | Female | 55 | Tank | |
| 29B | Female | 59 | | 33A | Female | 59 | | 88C | Female | 55 | Tank | |
| 35B | Female | 61 | 51.8±8.5 | 36A | Female | 69 | 56±9.1 | 12C | Female | 62 | Tank | 53.2±6.7 |

All smoking groups were gender and age matched.

***Biospecimen Collection:*** Spot urine samples were collected from participants in a previous study[16], and cotinine, NNAL, and creatinine concentrations were determined at the Center for Disease Control (CDC) and the Roswell Park Comprehensive Cancer Center (RPCCC), respectively. Aliquots of 45 ml of fresh urine samples were transferred to 50-ml Falcon tube then centrifuged and immediately frozen at -20°C and stored at the RPCCC laboratory. Prior to shipping, samples were thawed, and 1.5 ml aliquots were transferred to smaller tubes and

shipped frozen to University of California, Riverside (UCR) for biomarker analysis. The biomarker study was approved under IRB protocol HS-12-023 from UCR.

***Selection of Biomarkers*:** Biomarkers were selected by studying previous literature pertaining to urinary biomarkers in smokers[21,22,23,24,25]. The selection criteria for our panel of urinary biomarkers was based on our goal to analyze metal exposure and oxidative stress (Table 1.2). To evaluate exposure, cotinine and metals were measured in urine samples. Metallothionein, which increases when metal exposure is elevated, was used as a biomarker of effect. Conventional cigarettes and electronic cigarettes generate free radicals that cause cellular oxidative stress[8,26,27]. Therefore, oxidative damage was evaluated in the three study groups by measuring urinary 8-isoprostane (a biomarker of lipid peroxidation) and 8-OHdG (a biomarker of DNA oxidation). Cigarette smoke and electronic cigarette aerosols contain a mixture of metals[6,7,28] that could lead to an increased production of metallothionein (a metal exposure and ROS scavenging biomarker), which is a cysteine-rich protein that functions in metal binding[25]. All selected biomarkers described above have been shown to be specifically associated with clinically relevant outcomes and diseases (Table 1.2).

**Table 1.2.** Clinical diseases associated with biomarkers measured in this study.

| Biomarker Type | Associated Diseases | References |
|---|---|---|
| **Exposure** | | |
| Selenium | Nausea, vomiting, "garlic breath", nail loss, hair loss, cardiovascular disease, cardiac arrest, cancer, | MacFarquhar 2010, See 2006, Rayman 2012 |
| Zinc | Nausea, vomiting, epigastric pain, fatigue, hypertension, hemotoxicity, bronchospasms, hepatotoxicity, neurotoxicity, cancer | Fosmire 1990, Nriagu 2007 |
| **Effect** | | |
| Metallothionein | Cancer, cardiomyopathy, oxidative stress, heavy metal toxicity | Eckschlager 2009, Zhou 2008, Ruttkay-Nedecky 2013, Klaassen 2009 |
| **Potential Harm** | | |
| 8-OHdG | Cancer, cardiovascular disease, neurodegenerative diseases | Kroese 2014, Valavanidis 2009, Kim 2015 |
| 8-Isoprostane | Coronary artery disease, atherosclerosis, interstitial lung disease, non-small cell lung cancer, breast cancer | Vassalle 2004, Morrow 2005, Montuschi 1998, Stathopoulos 2014, Rossner Jr 2006 |

***Urinary Creatinine Concentrations*:** Spot urine samples were used since biomarkers would not necessarily be stable in samples collected over 24 hours. Because spot urine samples were used, it was necessary to normalize the data to

creatinine, which is relatively stable in concentration over time. Creatinine concentrations in urine were analyzed at the RPCCC clinical laboratory in Buffalo. There were no significant differences in creatinine concentrations in relation to gender or age (Supplementary Figure 1.3).

***Biomarker of Exposure (Cotinine, NNAL and Metal Concentration) Analysis:*** Cotinine and NNAL were measured using previously published[29,30] and fully validated methods. Eleven elements/metals (antimony, cadmium, copper, indium, lead, nickel, rubidium, selenium, silver, titanium, and zinc) in urine samples were measured by inductively coupled mass spectrometry (ICP-MS) and used to calculate total urinary metal concentration. The 11 metals were selected for analysis because they have all been identified in electronic cigarette aerosols and are known to associate with metallothionein. There was no significant elevation of the total 11 metals in the smoking groups, though it is slightly elevated in the electronic cigarette group (Supplementary Figure 1.4). Details of metal analysis are given in the Supplementary Information.

***Biomarkers of Effect and Potential Harm Analysis Using ELISA:*** Each ELISA kit was quality tested for accuracy and reproducibility using urine samples collected in house. Samples were tested in duplicate on three different days, and the biomarker concentration was normalized to creatinine. A range of sample dilutions was tested to determine the optimal dilution for quantification of each biomarker

from the kits' standard curves. For all ELISA kits, the coefficient of variation for the three independent experiments was ≤15%, except for metallothionein, which was ≤20%. Any urine sample with a biomarker concentration outside the lowest or highest limit of quantification was excluded for statistical analysis. In all subsequent ELISA analyses, biomarkers were run in duplicate wells for each urine sample.

Following a 1:4 dilution in buffer, urine samples were analyzed to determine 8-isoprostane concentration using the Urinary 8-Isoprostane ELISA kit (Detroit R&D, MI, USA). The concentration of 8-OHdG was determined using a DNA Damage (8-OHdG) ELISA Kit (Stress Marq Biosciences, Victoria, Canada), following a 1:20 dilution. Urine samples were analyzed for metallothionein using a Human Metallothionein ELISA Kit (LifeSpan BioSciences, WA, USA), following a 1:20 or 1:40 dilution in sample diluent.

***Statistical Analysis*:** Two urine samples from the electronic cigarette group had abnormally high creatinine concentrations (≥3 mg/mL) as detected by a statistical outlier test and were removed from further analysis. For each urine sample, the biomarker concentration was normalized to its respective creatinine concentration. Because the normalized biomarker concentration data were not normally distributed, a Box-Cox transformation was performed after which a 3-way ANOVA was applied in MiniTab 17.0 (MiniTab Inc, PA, USA) using gender, age, and smoking group as factors. Outliers were removed if they had a large standardized residual (≥2.0 or ≤-2.0). In all the 3-way ANOVA models, the 2-way and 3-way

interactions were not significant, and our final model included age, gender, and smoking group. Post-hoc tests were used to compare different age groups, gender groups, and smoking groups. When the smoking group was analyzed independently (disregarding gender and age), a Dunnett's post-hoc test was used with the electronic cigarette group as the main comparison group, and the comparisons were electronic cigarette users vs. non-smokers and electronic cigarette users vs. cigarette smokers. All linear correlation analyses were performed using the Linear Regression Analysis ($R^2$ and p-value reported) in PRISM 7.0 (GraphPad, CA, USA). All graphs reported in this manuscript were made in PRISM 7.0.

***Patient and Public Involvement:*** No patients were involved in the research planning or design, nor were they involved in any aspect of the study besides urine collection. There are no plans to directly disseminate the results of the research to study participants. The dissemination of results will be achieved through publication or press release.

# Results

### *Biomarker of Effect*

Metallothionein, a biomarker of effect (due to metal and reactive oxygen species exposure), in the electronic cigarette group (3761 ± 3932 pg/mg) was significantly elevated when compared to the non-smokers group (1129 ± 1294 pg/mg, p=0.05), and these concentrations were similar to the cigarette smokers group (4096 ± 4320 pg/mg, p=0.95) (Figure 1.1A). There were no differences in age or gender.

### *Biomarkers of Potential Harm (Oxidative Stress)*

A significant elevation in urinary levels of the biomarker of DNA oxidation, 8-OHdG, occurred in electronic cigarette users (442.8 ± 300.7 ng/mg) vs. non-smokers (221.6 ± 157.8 ng/mg, p=0.01) (Figure 1.1B). There was no significant difference between electronic cigarette users (442.8 ± 300.7 ng/mg) and cigarette smokers (388 ± 235 ng/mg, p=0.75). Age affected 8-OHdG levels; those ≥41 years old (413.4 ± 256.4 ng/mg) had significantly elevated 8-OHdG compared to those ≤40 years (241.2 ± 214.1 ng/mg, p=0.02) (Figure 1.1C). There was no effect on gender.

The lipid peroxidation biomarker, 8-isoprostane, showed a significant increase in electronic cigarette users (750.8 ± 433 pg/mg) vs. non-smokers (411.2 ± 287.4 pg/mg, p=0.03) (Figure 1.1D). There was no significant difference between electronic cigarette users (750.8 ± 433 pg/mg) and cigarette smokers (784.2 ±

546.1 pg/mg, p = 0.96). Moreover, the ≥41-year-old population (777.6 ± 481.5 pg/mg) was significantly elevated in 8-isoprostane compared to those ≤40 years (392.6 ± 246.9 pg/mg, p=0.002) (Figure 1.1E). In addition, 8-isoprostane was significantly elevated in females (741.8 ± 489.3 pg/mg) vs. males (484.9 ± 345, p=0.04) (Figure 1.1F).

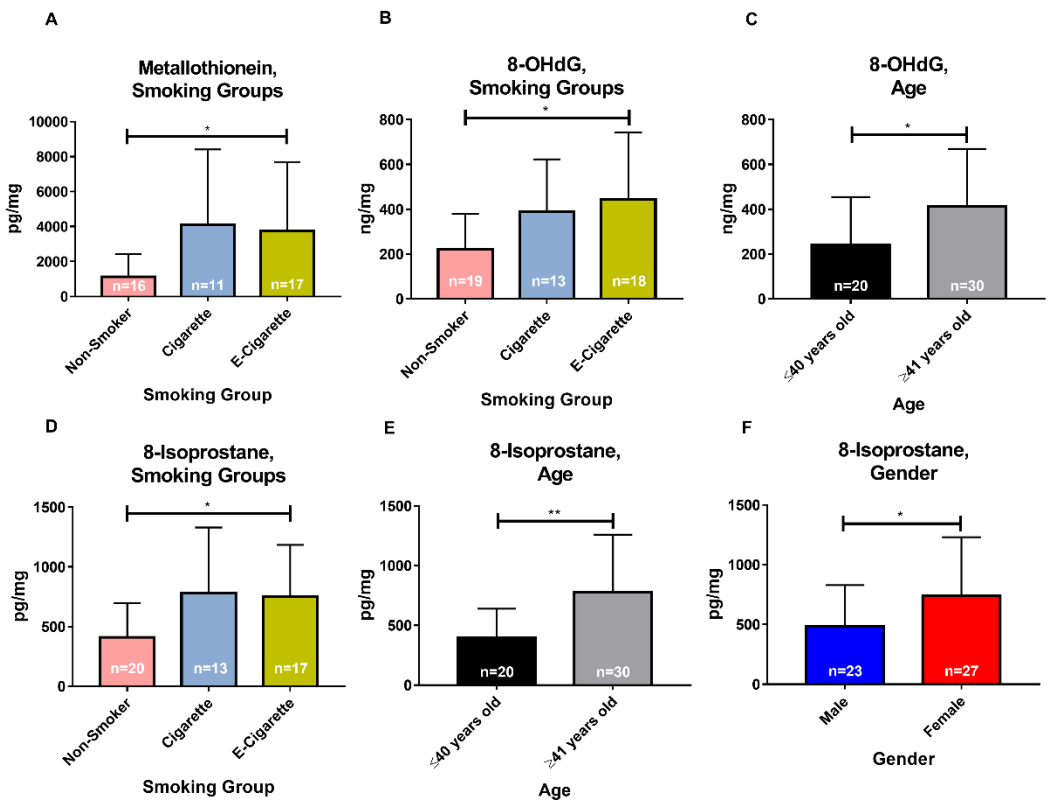


**Figure 1.1 Urinary metallothionein (pg/mg of creatinine), 8-OHdG (ng/mg of creatinine), 8-isoprostane (pg/mg of creatinine), are significantly elevated in e-cigarette users compared to non-smokers.** A. Metallothionein levels among the different smoking groups. B. 8-OHdG concentration in the different smoking groups. C. 8-OHdG concentration in the younger and older populations. D. 8-isoprostane levels among the different smoking groups. E. 8-isoprostane levels in the younger and older populations. F. 8-isoprostane levels in males and females. Bars are the means and standard deviations for each group. * p = < 0.05; ** p = <0.01.

***Biomarkers of Exposure are Correlated with Oxidative DNA Damage in E-Cigarette Users***

Results of linear regression analyses performed on the non-smokers, cigarette smokers, and electronic cigarette users are presented in Fig 2 for the following correlations: (1) cotinine and total metal concentration (Fig 2A-C), (2) total metal concentration and metallothionein (Fig 2D-F), (3) cotinine and 8-OHdG (Fig 2G-I), and (4) total metal concentration and 8-OHdG (Fig 2J-L). There were no significant correlations in the non-smokers (Fig 2A, D, G, and J). In the cigarette smokers group, only total metal concentration and 8-OHdG were significant (Fig 2K, $p=0.0003$). In the electronic cigarette users group, all linear regression analyses were significant: cotinine and total metal concentration (Fig 2C, $p=0.02$), total metal concentration and metallothionein (Fig 2F, $p=0.04$), cotinine and 8-OHdG (Fig 2I, $p = 0.02$), and total metal concentration and 8-OHdG (Fig 2L, $p = 0.007$).

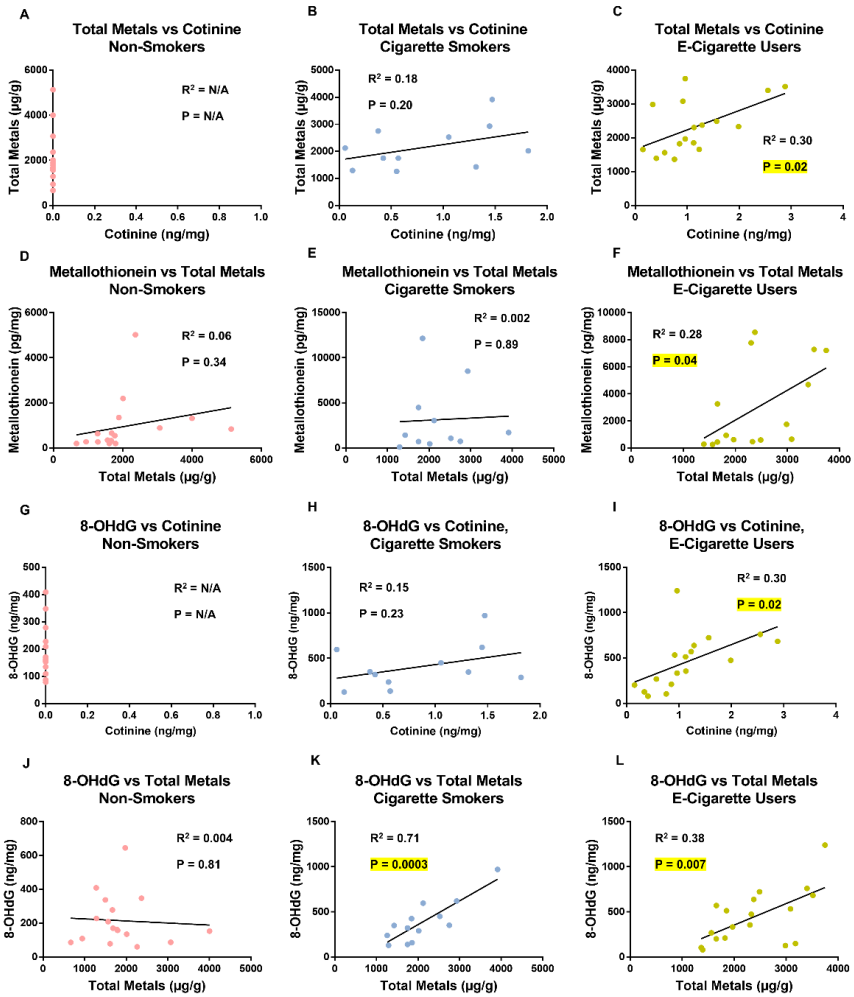


**Figure 1.2. Correlation between total metals and cotinine, metallothionein and total metals, 8-OHdG and cotinine, and 8-OHdG and total metals in urine.** A-C. Linear regression analysis comparing total metal (µg/g of creatinine) and cotinine concentration (ng/mg of creatinine) in urine of the non-smokers, cigarette smokers, and e-cigarette user groups. D-F. Linear regression analysis comparing metallothionein concentration (pg/mg of creatinine) and total metal concentration (µg/g of creatinine) in urine in the non-smokers, cigarette smokers, and e-cigarette users groups. G-I. Linear regression analysis comparing 8-OHdG (ng/mg of creatinine) and cotinine (ng/mg of creatinine) concentration in urine of the non-smokers, cigarette smokers, and electronic cigarette user groups. J-L. Linear regression analysis comparing 8-OHdG (ng/mg of creatinine) and total metal (µg/g of creatinine) concentration in urine of the non-smokers, cigarette smokers, and electronic cigarette user groups. N/A = not applicable since levels of cotinine in non-smokers was negligable.

### *Selenium and Zinc were Elevated in Electronic Cigarette Users*

Two of the 11 metals that were analyzed were significantly elevated in the electronic cigarette group. Selenium concentrations (Fig 3A) were significantly elevated in the electronic cigarette users (54 ± 20.6 µg/g) compared to non-smokers (41.8 ± 14.1 µg/g, p=0.04) and cigarette smokers (39.7 ± 17.3 µg/g, p=0.05). Zinc concentrations (Fig 3B) were significantly elevated in electronic cigarette users (584.5 ± 826.6 µg/g) compared to non-smokers (413.6 ± 233.7 µg/g, p=0.03). Zinc in the electronic cigarette users was not significantly elevated when compared to cigarette smokers (470.7 ± 223.6 µg/g, p=0.17).

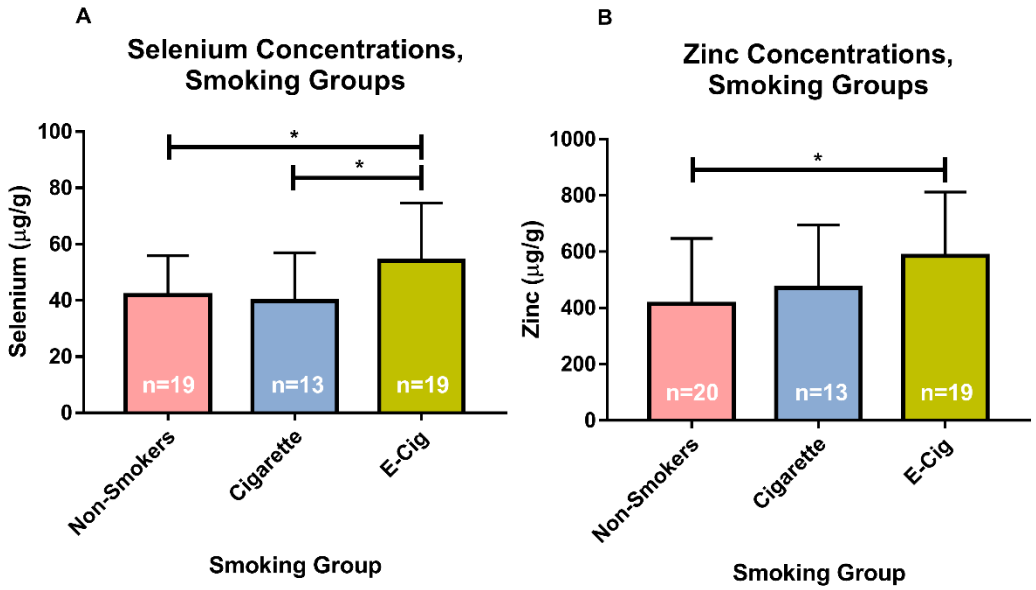


**Figure 1.3. Urinary selenium (µg/g of creatinine) and zinc (µg/g of creatinine) concentrations are significantly increased in the electronic cigarette users.** A. Selenium concentrations in the different smoking groups. B. Zinc concentrations in the different smoking groups. Bars are the means and standard deviations for each group. * p = < 0.05

***Zinc was Correlated with Oxidative DNA Damage in Electronic Cigarette Users***

Regression analysis were performed to compare urinary concentrations of selenium and zinc to 8-OHdG in the non-smokers, cigarette smokers, or electronic cigarette users (Fig 4). There were no significant correlations for selenium versus 8-OHdG (Fig 4A-C). In the electronic cigarette users only, zinc was significantly correlated to 8-OHdG (p=0.0066) (Fig 4F) In non-smokers and cigarette smokers, zinc was not correlated to 8-OHdG (Fig 4A, B).

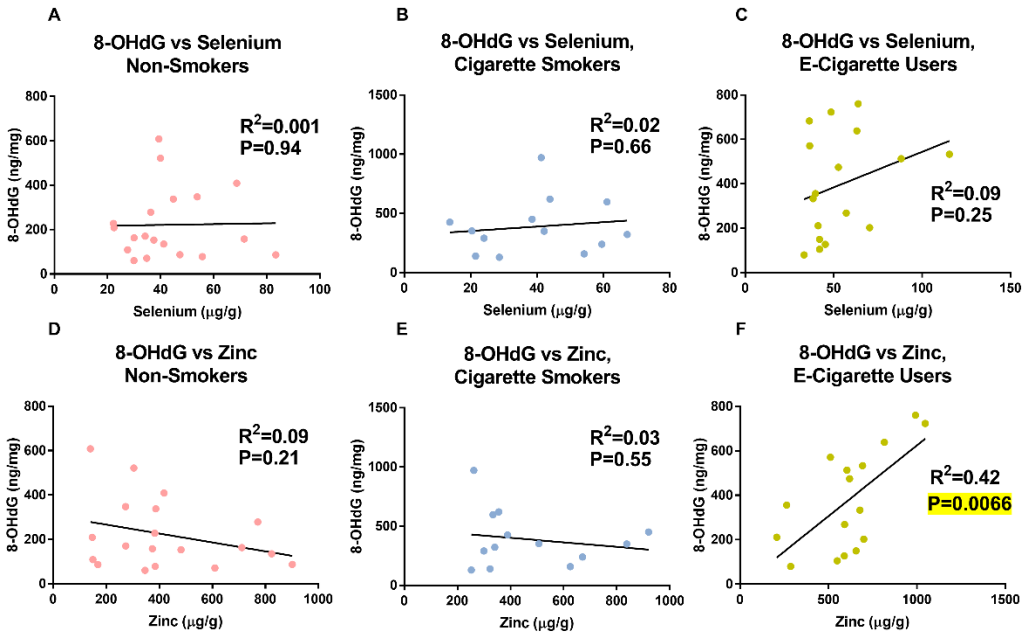


**Figure 1.4. Zinc concentrations (µg/g of creatinine) are significantly correlated to oxidative DNA damage in the electronic cigarette users.** A-C. Linear regression analysis comparing selenium (µg/g) of creatinine and 8-OHdG (ng/mg of creatinine) in urine of the non-smokers, cigarette smokers, and e-cigarette user groups. D-F. Linear regression analysis comparing zinc (µg/g of creatinine) and 8-OHdG (ng/mg of creatinine) in urine in the non-smokers, cigarette smokers, and e-cigarette users groups.

## Discussion

Consistent with our hypothesis, our study shows for the first time that biomarkers of effect and potential harm were elevated in the urine of the electronic cigarette users compared to non-smokers. Moreover, in electronic cigarette users, the levels of biomarkers of effect and potential harm were positively correlated with biomarkers of exposure to nicotine and metals. Importantly, electronic cigarette participants in our study did not report using other tobacco products and were not dual users of electronic cigarettes and conventional cigarettes. Before entering our study, all electronic cigarette users who were previous cigarette smokers had abstained from smoking cigarettes for a minimum of six months, and abstinence was confirmed by undetectable NNAL (Supplemental Figure 1.1). Previous literature has shown that abstinence from cigarette smoking was concurrently linked to a decrease in levels of 8-isoprostane and 8-OHdG, which returned to non-smokers levels[31]. Taken together, the above information supports the conclusion that the elevation of 8-isoprostane and 8-OHdG in urine was associated with electronic cigarette use specifically. Surprisingly, we did not find a significant reduction in biomarkers of effect and potential harm between electronic cigarette users and cigarette smokers. This observation may be explained by the fact that electronic and conventional cigarettes and their aerosols have anatomical, chemical, and particulate differences, which may contribute to physiological harm in separate ways.

Cigarette smoke and electronic cigarette aerosol contain a mixture of metals and free radicals[6,7,8,28,32] that could be contributing to the oxidative harm in our participants. The metals in electronic cigarette aerosols come mainly from the metal components in the atomizer and the e-fluid that is heated in the atomizer[7,33]. Metal concentration in urine was positively correlated with cotinine concentration, indicating that metals were elevated with increased aerosol exposure.

Metal increase in urine is further supported by the observed elevation in metallothionein, which acts as a heavy metal-binding protein and also protects cells from oxidative stress by scavenging ROS[25]. Metallothionein normally binds physiological metals, such as zinc and copper, but can also bind xenobiotic heavy metals such as cadmium, silver and arsenic[25,34] that are present in cigarette smoke[35] and electronic cigarette aerosols[7]. Metallothionein can also associate with at least 20 different elements/metals[19,20], and 11 of these have been found in cigarette smoke[28,36] or e-cigarette aerosol[6,7,18] and were present in the urine of our participants. The increase in metallothionein in the electronic cigarette user group was positively correlated with increasing metal concentration in their urine and was likely a response to metals inhaled by the electronic cigarette users. In cigarette smokers, metallothionein was not significantly correlated with increasing metal concentration, suggesting other factors such as ROS may be contributing to its activation. Also, cigarette smoke can have a different composition of metals than e-cigarette aerosol[6,7,18,28,36], which were not selected for in our 11 metal analysis,

and therefore the total metal concentration in smokers was not correlated to cotinine concentration.

Elevation of toxic metals can induce oxidative stress[37,38]. In the electronic cigarette group, there was a significant correlation between total metals and oxidative DNA damage. A similar correlation was observed for the cigarette smokers. Lipid oxidation was not significantly correlated with metal concentration in either the electronic cigarette or cigarette smokers groups. There are multiple isoprostanes and isoprostane metabolites formed in-vivo during oxidative conditions[39], and we measured only 8-isoprostane, which may account for the lack of correlation between lipid oxidation and metal concentration. In contrast, during DNA oxidation the guanine residue is highly oxidized compared to the other nucleic bases, leading to the formation of a single DNA oxidation product (8-OHdG), which makes correlation to oxidative stress straightforward.

Both zinc and selenium, which were significantly elevated in the electronic cigarette user group, are present in electronic cigarette aerosols, usually higher concentrations than most other elements[6,7]. However, only zinc concentration was correlated with oxidative DNA damage in the electronic cigarette group. While zinc is required for normal human health, its elevation above normal levels has been associated with oxidative stress[40]. Our data provide the first evidence that electronic cigarette usage increases the risk of zinc exposure, which in turn causes oxidative DNA damage in humans. Selenium is also a required trace element that can cause harm when elevated[41]. While its elevation in electronic cigarette users

was not linked to increased oxidative stress, future work may find that it has other adverse health effects.

Oxidative damage can lead to gradual harm of all organ systems[42] and if left unchecked can culminate in diseases such as atherosclerosis, coronary heart disease, pulmonary fibrosis, acute lymphoblastic leukemia, and lung cancer[43]. Of particular concern, increases in both 8-isoprostane and 8-OHdG were significantly greater in the older populations, suggesting that conventional cigarette users who give up smoking and switched to electronic cigarettes may be at greater risk for oxidative damage than young people who have not smoked previously. In the case of 8-isoprostane, females were more affected than males, suggesting that women should not be encouraged by physicians to use electronic cigarettes, especially when pregnant. There were no significant differences in the elevated concentrations of oxidative harm biomarkers between electronic cigarette users and cigarette smokers, suggesting their organ systems are exposed to similar levels of oxidative damage.

**Conclusions**:

Our data show for the first time that electronic cigarette use, which correlates with metal intake, leads to an elevation in metallothionein in the urine. The usage of e-cigarettes causes an increase in oxidative stress as measured by 8-OHdG and 8-isoprostane. E-cigarette users were exposed to elevated levels of selenium and zinc. The intake of metals (specifically zinc) is further correlated with

increased oxidative damage to DNA. These data indicate that electronic cigarette use is not harm free and that prolonged use with elevation of oxidative stress may lead to disease progression. Given these observations, physicians should use caution in recommending the use of electronic cigarettes to their patients and should be alert to possible adverse health outcomes associated with electronic cigarette use. The biomarkers used in this study may be valuable in clinical practice when evaluating the health of electronic cigarette users.

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## Footnotes

**Contributors:** SSC, CH, MG, PT were responsible for the study concept and design. SSC, MW and ANR performed the experiments for data collection. MG collected and shipped the urine samples to our lab. JL acted as a statistician. SMB helped design the use of the ICP-MS in TWL's lab and the analysis of the metal data. SSC, MW, CH, JL, MG, PT drafted the manuscript, and all authors read and provided comments on the manuscript. SSC, MW, and PT reviewed the data and take responsibility for the integrity and accuracy of the data. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted. SSC and PT are the guarantors.

**Funding:** This study was supported by the National Institute on Drug Abuse and the National Cancer Institute of the National Institutes of Health (awards R01DA037446 and P30 CA016056, respectively) and FDA Center for Tobacco

Products (CTP) and by an award from the Roswell Park Alliance Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

**Disclosures:** Dr. Goniewicz reports grants from Pfizer (2011 GRAND [Global Research Awards for Nicotine Dependence] recipient) and personal fees from Johnson & Johnson (as a member of the advisory board) outside the submitted work. Authors not named here have disclosed no conflicts of interest.

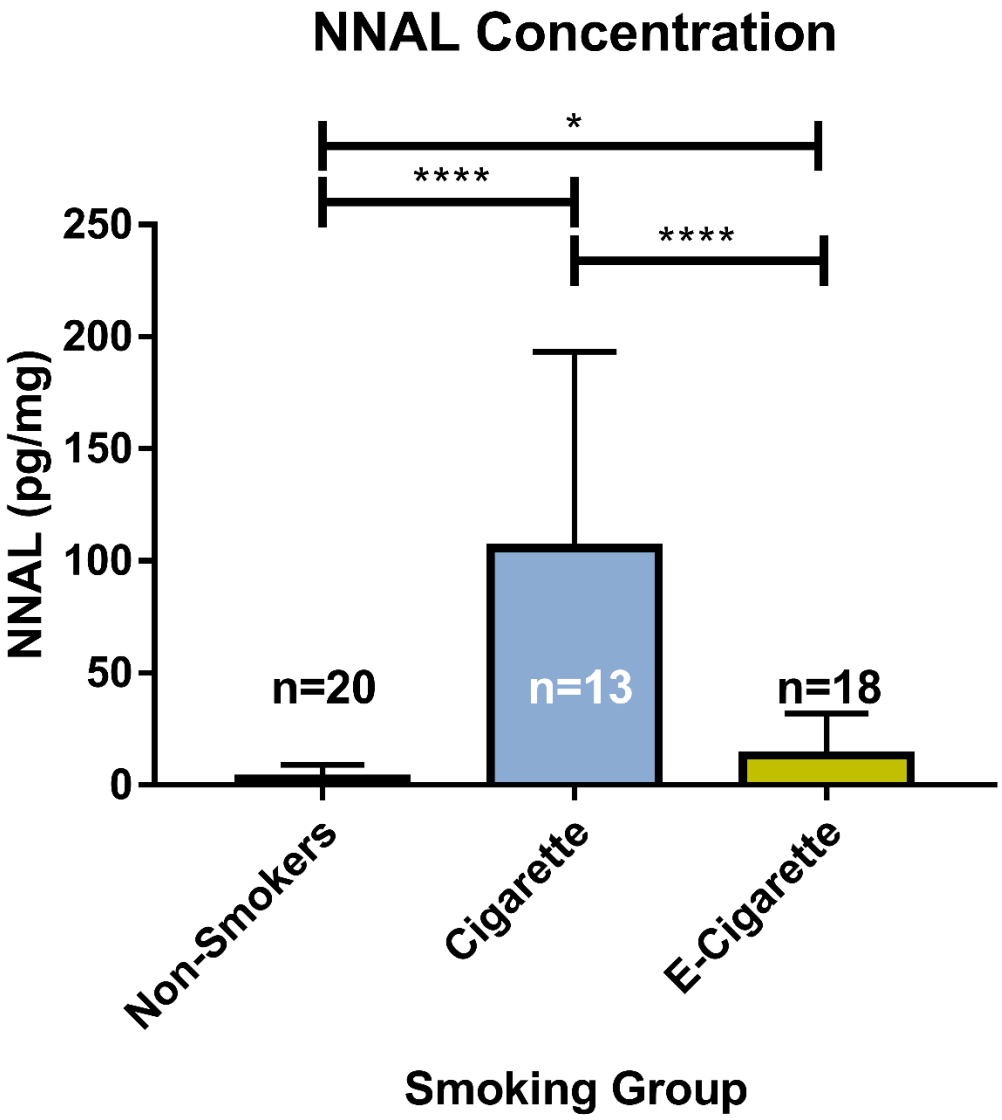
**Ethics Approval:** The study was approved by Roswell Park Comprehensive Cancer Center IRB (protocol number I 247313). The biomarker measurement study was approved under IRB protocol HS-12-023 from UCR.

**Data Sharing:** No additional data are available.

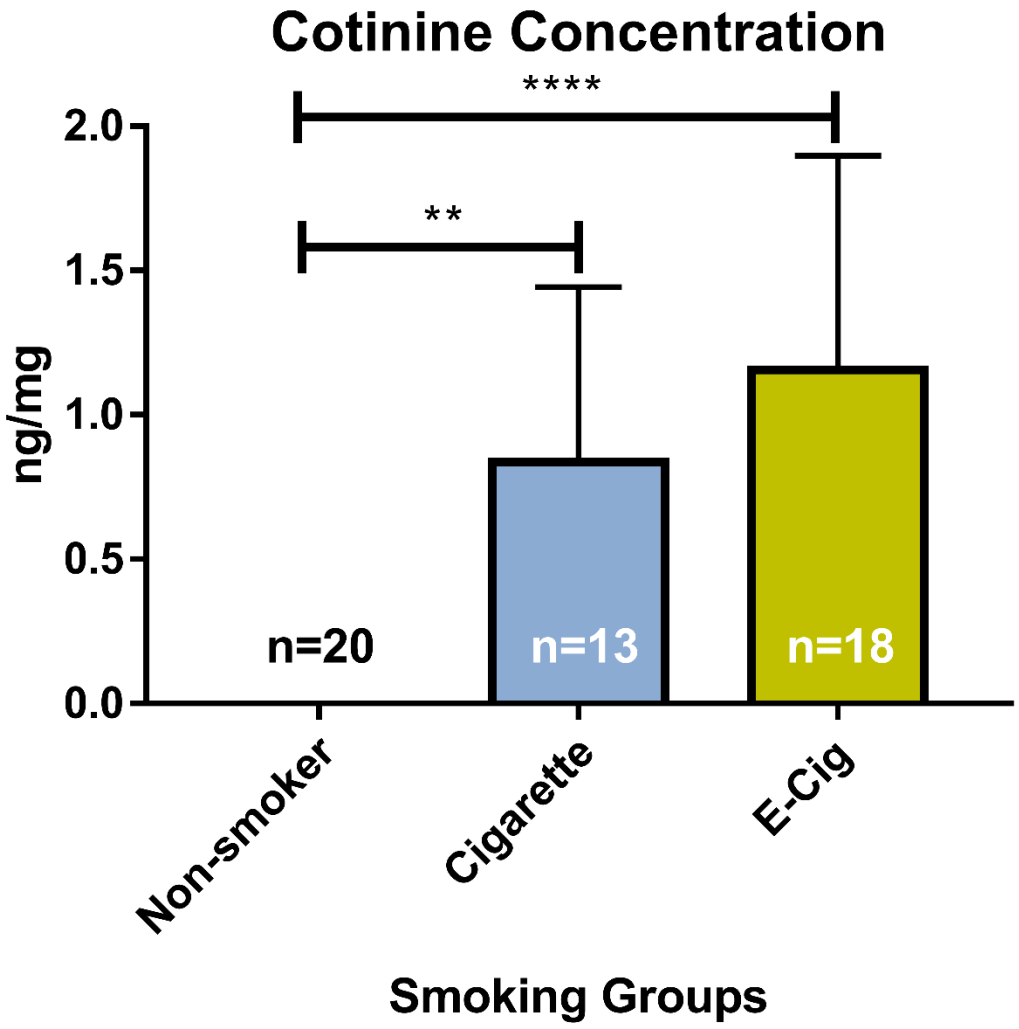
**Transparency:** The study guarantors affirm that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

Urinary Biomarker of Exposure (Metal Concentration) Analysis

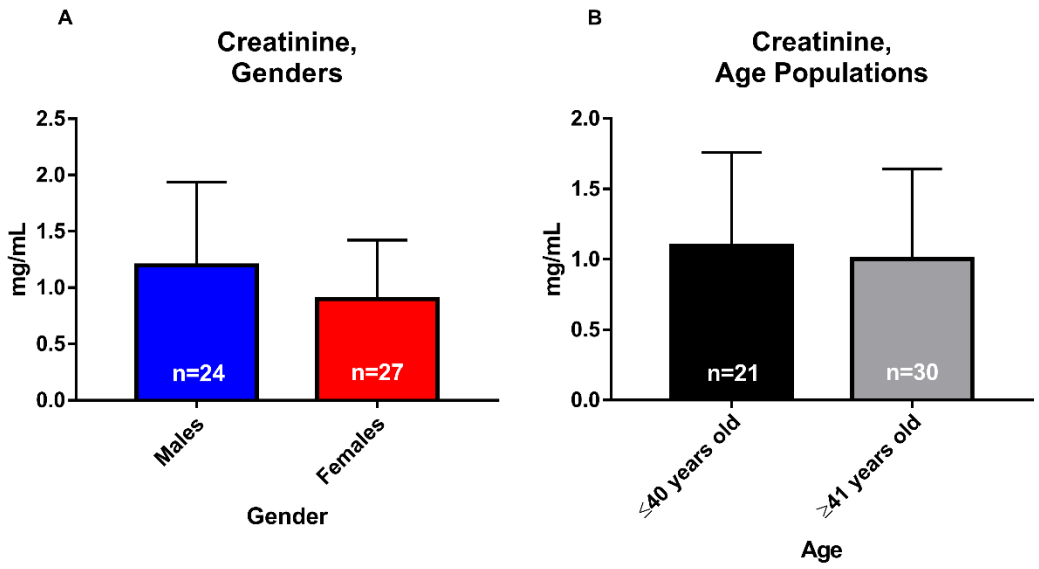
A 1:25 dilution was made for each urine sample by dissolving 400 µL into 7.6 mL of 2% nitric acid solution (GFS Chemicals, Columbus, OH). The 11 elements/metals were quantified using an Agilent 7900 ICP-MS (Agilent Technologies, Santa Clara, CA) with an ASX-500 series autosampler and an ISIS 3 Discrete Sample Uptake, spray chamber, and peristaltic pump. The ICP-MS was calibrated using a standard curve for all 11 metals of interest (Inorganic Ventures, Christiansburg, VA) in 0, 0.05, 0.10, 0.50, 0.75, and 1.0 ppm concentrations. Lithium, gallium, rhenium, scandium, and yttrium at 1 ppm were run in line with sample introduction into the nebulizer and used as internal standards. The blank contained 2% trace metal grade nitric acid by volume. Each sample was run in triplicate. To prevent interference, the cones were washed with nitric acid, and a detergent to bombard the cones. Quality control checks on calibration were then run using NIST standard reference materials 2668 Level I and Level II provided by the National Institute of Standards and Technology (Gaithersburg, MD). Machine calibrations and adjustments were controlled using Masshunter software.



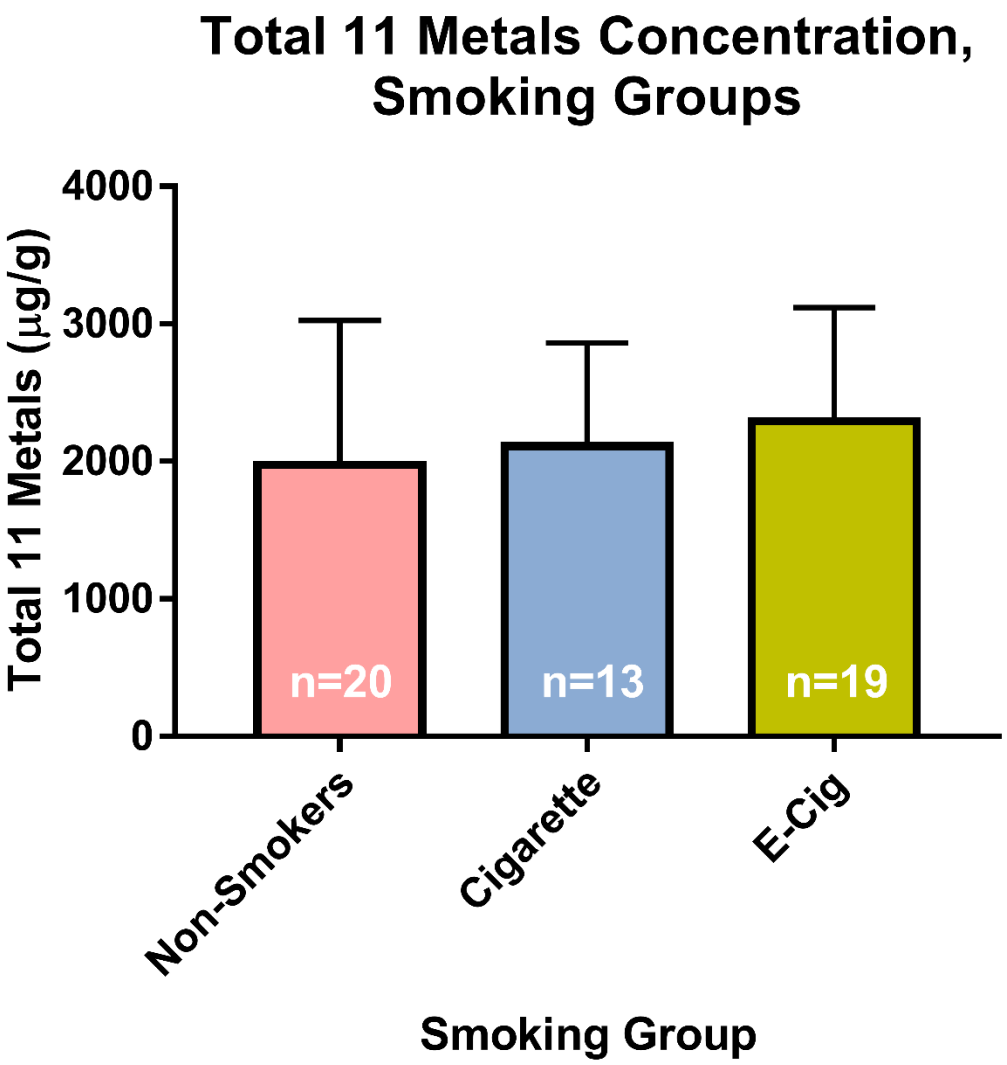

**Supplementary Figure S1.1. NNAL concentration (pg/mg of creatinine) among the different smoking groups.** Significant elevation of NNAL (a biomarker of tobacco exposure) was seen in the cigarette smokers. Bars are the means and standard deviations for each group. * p = < 0.05; **** p = < 0.0001.



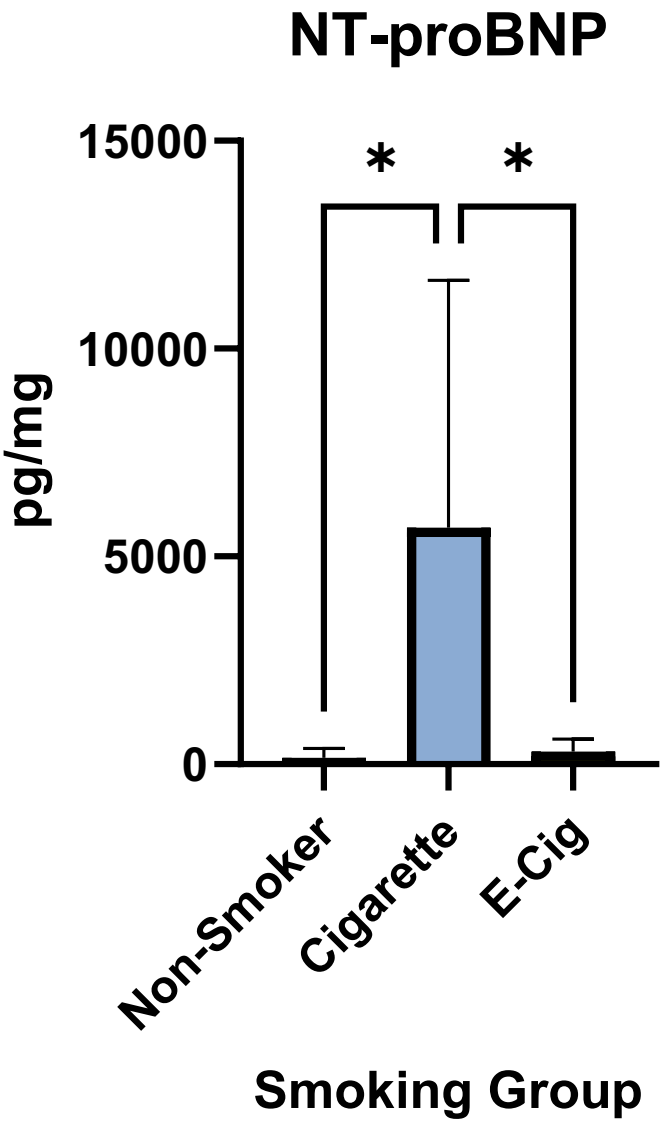

**Supplementary Figure S1.2. Cotinine concentration (ng/mg of creatinine) in the different smoking groups.** Cotinine concentration is elevated in the cigarette smokers and e-cigarette users compared to non-smokers. There is no difference between the cigarette smokers and e-cigarette users. Bars are the means and standard deviations for each group. ** p = < 0.01; **** p = < 0.0001.




**Supplementary Figure S1.3. Urinary creatinine concentration (mg/mL) in different genders and age populations.** A. Creatinine concentrations in males and females. B. Creatinine concentrations in the younger and older population. There were no significant differences between genders or age groups. Bars are the means and standard deviations for each group.




**Supplementary Figure S1.4. The total concentration of 11 metals (µg/g of creatinine) in each smoking group.** There were no significant differences in the total metals concentrations in any of the smoking groups. Bars are the means and standard deviations for each group.




**Supplementary Figure S1.5. NT-proBNP, a cardiac stress biomarker, was significantly elevated in the cigarette smokers compared to the non-smokers and e-cig users.** Bars are the means and standard deviations for each group.

# Chapter Two

# Dermal Thirdhand Smoke Exposure Induces Oxidative Damage, Initiates Inflammatory Markers in the Skin, and Adversely Alters the Human Plasma Proteome

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# Abstract

**Objective:** To evaluate the potential health effects of 3-hour dermal THS exposure from urine and plasma.

**Design:** A randomized, crossover, unblinded clinical trial.

**Setting:** This study was conducted in the Human Exposure Laboratory at the San Francisco General Hospital.

**Participants:** 10 healthy, non-smoking subjects.

**Intervention:** Dermal exposure for 3 hours exposed to clothing impregnated with filtered clean air or THS. Exposures to clean air or THS occurred 20-30 days apart.

**Main outcomes and measures:** Urine was analyzed for biomarkers of exposure and harm. Plasma was analyzed by global proteomics.

**Results:** In THS-exposed group, there was a significant elevation of urinary 8-OHdG, 8-isoprostane, protein carbonyls. Ingenuity Pathway Analysis (IPA) bio and disease function analysis of the THS 3-hour exposure identified pathways of inflammatory response ($p = 2.18 \times 10^{-8}$), adhesion of blood cells ($p = 2.23 \times 10^{-8}$), atherosclerosis ($p = 2.78 \times 10^{-9}$), and lichen planus ($p = 1.77 \times 10^{-8}$). Nine canonical pathways were significantly activated including leukocyte extravasation signaling (z-score = 3.0), and production of nitric oxide and reactive oxygen species in macrophages (z-score = 2.1). The THS 22-hour bio and disease functions revealed inflammation of organ ($p = 3.09 \times 10^{-8}$), keratinization of the epidermis ($p = 4.0 \times 10^{-7}$), plaque psoriasis ($p = 5.31 \times 10^{-7}$), and dermatitis ($p = 6.0 \times 10^{-7}$). Two

activated canonical pathways were production of nitric oxide and reactive oxygen species in macrophages (z-score = 2.646), and IL-8 signaling (z-score = 2.0).

**Conclusion:** Acute human dermal exposure to THS caused adverse health reactions similar to tobacco cigarette smoking and significantly increased oxidative damage to DNA, lipids, and proteins. The molecular biomarkers of inflammation-induced skin diseases were elevated at 3 hours and persisted 22 hours later. These findings provide insights into the molecular mechanisms involved in THS adverse effects, and may aid in establishing regulatory policy for THS.

**Strengths and Limitations:**

- This is the first clinical study to demonstrate that acute dermal exposure to THS mimics the harmful effects of cigarette smoking, alters the human plasma proteome, initiates mechanisms of skin inflammatory disease, and elevates urinary biomarkers of oxidative harm.
- This clinical trial involving participants acutely exposed to THS for 3 hours provides proof-of-concept data for our hypothesis that dermal THS exposure can produce adverse health effects in humans.
- These results will aid physicians in the diagnosis of patients exposed to THS and guide future research in the molecular mechanisms of THS toxicity.

- Future studies should examine larger populations of subjects that are exposed to THS for longer periods of time and determine if there are racial, gender, and age differences in response to THS.

## Introduction

Thirdhand smoke (THS) is the aged residue from secondhand smoke (SHS) that persists on indoor surfaces after smoking has stopped[1]. Some THS chemicals, including nicotine, react with environmental oxidants and produce secondary pollutants, such as tobacco-specific nitrosamines, that are harmful[2,3]. THS can remain on indoor surfaces indefinitely causing potentially harmful exposure to both smokers and non-smokers[4,5].

*In-vitro* studies have demonstrated numerous harmful effects of THS exposure, including stress-induced mitochondrial hyperfusion and altered mitochondrial gene expression in mouse neural stem cells and human embryonic stem cells[6]. Cytotoxicity of THS in the MTT assay was attributable to acrolein[7]. THS also produced DNA strand breaks in human liver (HepG2)[8] and caused metabolomic changes consistent with an antioxidant response in male reproductive cells[9]. Mice exposed to THS fabric in their cages had increased liver lipids and non-alcoholic fatty liver disease, excess collagen and increased inflammatory cytokines in their lungs, and decreased wound healing capacity[10]. THS-exposed mice showed increased platelet aggregation and hyperactivity, which increased the risk for thrombosis[11]. A 40-week THS-exposure in mice

increased the risk of lung cancer incidence[12], and another mouse metabolomics study showed liver damage attributed to oxidative stress[13].

Clinical research of harm caused by THS is limited to two studies. One study evaluated the relationship between THS exposure of people living in homes of smokers and the risk of cancer. They measured nitrosamines in house dust, estimated a lifetime exposure, and found a greater cancer risk if people were exposed at a young age[14]. A second study analyzed gene expression in nasal epithelium following acute inhalation of THS emitted from a controlled chamber. Affected genes were associated with increased mitochondrial activity, oxidative stress, DNA repair, cell survival, and inhibition of cell death[15], showing that humans respond to inhaled THS chemicals. However, no studies have been done on humans exposed dermally.

The three main routes of THS exposure are inhalation, ingestion, and dermal. Skin may receive the greatest exposure to THS due toits large surface area. The purpose of our crossover clinical trial was to assess the potential health effects of dermal exposure to THS by measuring biomarkers of oxidative stress in urine and plasma and changes in the plasma proteome. Ten healthy, adult nonsmokers wore clothing impregnated with THS for 3 hours with 15 minutes of exercise in each hour to induce perspiration. Control exposure participants wore clean clothing for the same time with the same exercise regime. Urine samples collected before exposure, immediately after exposure (3 hours), the next morning,

and 22 hours after exposure were analyzed for biomarkers of exposure and harm, and the plasma proteome was analyzed to identify effects on protein expression.

## Methods

### Trial Design and Oversight

The study CONSORT diagram describes the protocol, patient's exposure, and sample collection (Figure 2.1). The trial was approved with patient informed consent by the human research protection program institutional review board (IRB#15-17009) by the San Francisco General Hospital Panel. Patient demographics (See Supplementary 1 Table 2.1) and sample analysis workflow (See Supplementary 1) are provided.

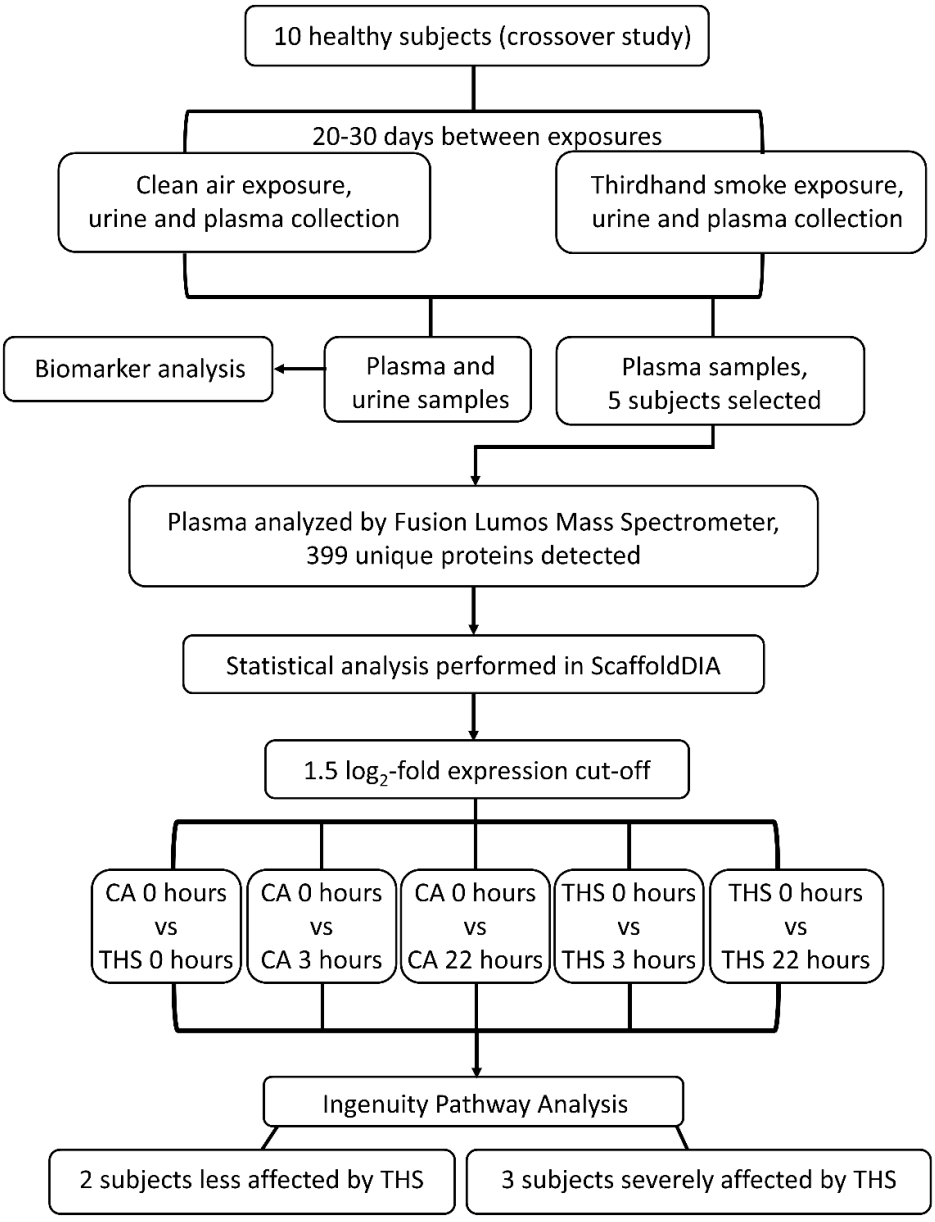



**Figure 2.1. CONSORT flow chart**. 10 healthy subjects were selected for a cross-over clean air or THS exposure for 3 hours (exposures were separated 20-30 days apart). Urine and plasma were collected at pre-determined timepoints up to 22 hours and analyzed for urinary biomarkers of harm and global plasma proteomics. Proteomics results from the exposures were analyzed for changes to canonical and disease pathways by IPA.

## Trial Interventions

Briefly, 10 healthy, non-smoking patients participated in a cross-over, randomized, unblinded study wearing clothing with or without THS for 3 hours in the Human Exposure Laboratory at the San Francisco General Hospital. To increase dermal uptake, the participants exercised on a treadmill for 15 minutes/hour to induce perspiration. The order of the exposures was randomized and separated by 20-30 days. Each participant received both exposures. The protocol for generating the clothing was described previously[16]. Urine and plasma were collected before exposure (0 hour) and at 3 and 8 hours after the start of exposure, upon waking the next morning, and at 22 hours after the start of exposure. Samples were centrifuged, aliquoted, stored at -80$^{o}$C, and shipped frozen to UCR for analysis.

Urine samples from 0, 3, 8, and 22 hours were analyzed for biomarkers and a subset of plasma samples from 5 participants, collected at 0, 3, and 22 hours, were analyzed by proteomic methods.

## Sample Size Calculation

Sample size was determined based on the number of people sufficient to detect increases in markers of inflammation in previous studies of secondhand cigarette smoke exposure. A total of sixteen participants were recruited.

## Data Collection and Statistical Analysis

### Urinary Biomarkers

Urinary biomarkers were analyzed by ELISA and detailed information is provided (See Supplementary 1).

### Plasma Proteomics

Five subjects with increased urinary biomarkers of oxidative harm were selected for plasma proteomics, performed by a Fusion Lumos Mass Spectrometer (Invitrogen). Proteins were referenced by the PanHuman database containing 399 total proteins. Significantly expressed proteins were identified using a threshold cut-off of ±1.5 $\log_2$ fold change and a 1% false detection rate. To determine significant protein expression between the clean air and THS groups at different timepoints, the subjects were compared as follows: baseline (clean air 0-hour vs THS 0 hour), clean air 3 hours (clean 0 hours vs clean air 3 hours), clean air 24 hours (clean air 0 hours vs clean air 24 hours), THS 3 hours (THS 0 hours vs THS 3 hours), and THS 24 hours (THS 0 hours vs THS 24 hours). The list of proteins analyzed in each group are provided (See Supplementary 1 Table 2.2). Detailed proteomics methods are described (See Supplementary 1).

Biological processes, networks, and pathways were analyzed using Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA). In both GO and IPA, significant processes and pathways were identified ($p \leq 0.05$). A principal component analysis suggested that two (361 and 366) of the five subjects were

less responsive to THS exposure (See Supplementary 1 Figure 2.2). To gain a better understanding of the health of effects of THS, the three responsive subjects (234, 364, 370) were further analyzed. Similar variations in response have been reported in proteomic studies of cancer biomarkers in cigarette smoker's plasma[17]. All THS participants had had elevated urinary cotinine at 8 hours and 22 hours, indicative of nicotine exposure (See Supplementary 1 Figure 2.3).

### Patient and Public Involvement

No patients were involved in the design of the research or the outcome measures, nor were they involved in developmental plans for recruitment, implementation, or interpretation of the study. Patients were provided with informed consent prior to the start of the study.

## Results

### Patients

This study was completed in 2018 when the target of 16 participants was met. Ten (62.5%) of these participants were selected for urinary biomarker analysis to gender and age match the populations, while five (31.2%) participants were further analyzed for plasma proteomics (See Supplementary 1 Table 2.1).

## Primary Outcomes

### Urinary Biomarkers of Harm

#### 8-OHdG

Urinary 8-OHdG is a biomarker of DNA oxidation. At baseline, there was no significant difference between 8-OHdG concentrations in the THS group (100 ± 81.1 SD ng/mg) and clean air group (78.9 ± 48.0 SD ng/mg) ($p = 0.61$) (Figure 2.2A-E). At all subsequent times, 8-OHdG was higher in the THS group vs the clean air control, $p = 0.03$ for 3 hours, $p = 0.39$ for 8 hours, $p = 0.01$ first morning, and $p = 0.05$ for 22 hours.

#### 8-isoprostane

Urinary 8-isoprostane is a biomarker of lipid peroxidation. At baseline, there was no significant difference in 8-isoprostane concentration between the THS group (45.6 ± 33.4 pg/mg) and the clean air group (40.4 ± 45.5 pg/mg) ($p = 0.74$) (Figure 2.2F-J). There was a significant increase of 8-isoprostane concentration in the THS group compared to the clean air group at all times: 3 hours ($p = 0.04$), first morning ($p = 0.01$), and 22 hours ($p = 0.01$), except 8 hours ($p = 0.12$).

Protein Carbonyl

Urinary protein carbonyl is a biomarker of protein oxidation. At baseline, there was no significant difference in protein carbonyl concentration between the THS group (4.1 ± 1.2 nmol/mg) and the clean air group (3.2 ± 1.1 nmol/mg) ($p = 0.25$). In contrast, there was a significant increase in protein carbonyls in the THS group vs clean air group at all other timepoints: 3 hours ($p = 0.02$), 8 hours ($p = 0.05$), first morning ($p = 0.02$), and 22 hours ($p = 0.001$) (Figure 2.2K-P).

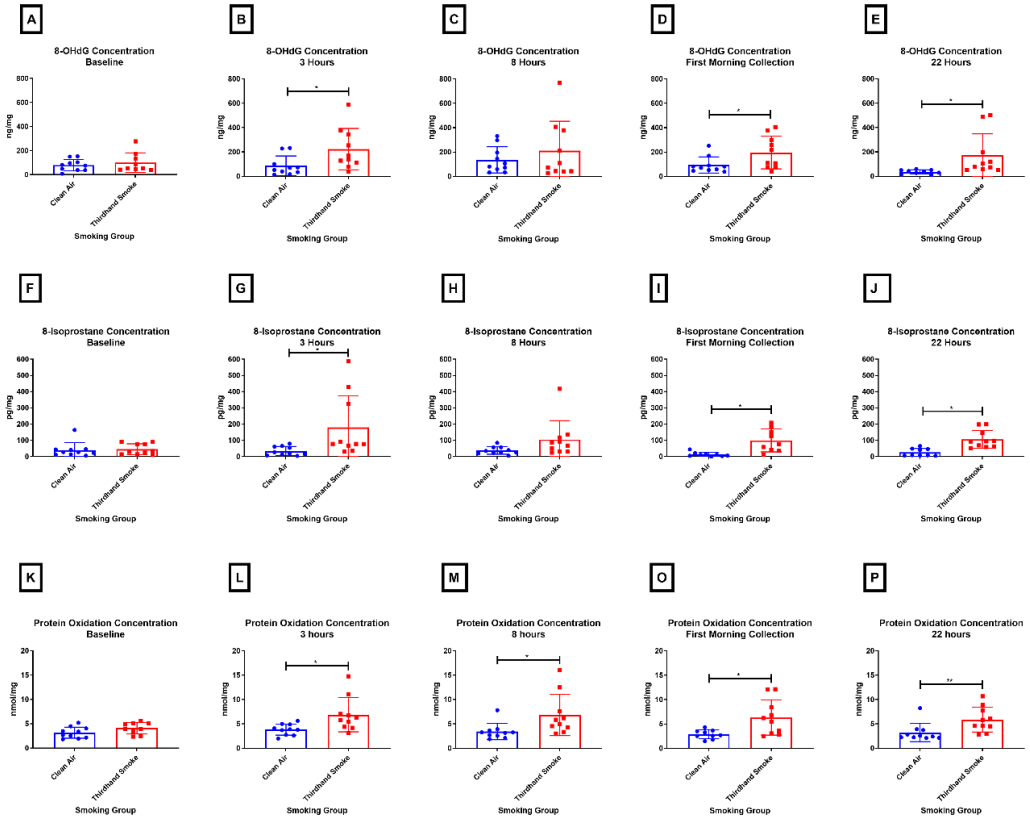



**Figure 2.2. The THS-exposed subjects had significantly elevated urinary 8-OHdG, 8-isoprostane, and protein carbonyls compared to their clean air exposure timepoints.** a-e. 8-OHdG concentrations (ng/mg of creatinine) at baseline, 3 hours, 8 hours, first morning collection, and 22 hours, respectively. f-J. 8-isoprostane concentrations (pg/mg of creatinine) at baseline, 3 hours, 8 hours, first morning collection, and 22 hours, respectively. k-p. Protein carbonyl concentrations (nmol/mg of creatinine) at baseline, 3 hours, 8 hours, first morning collection, and 22 hours, respectively.

## Gene Ontology

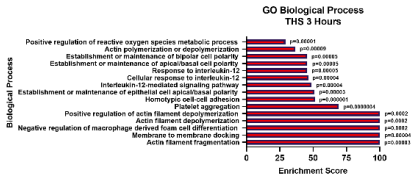
Proteins with ≥1.5-fold log change in expression from the THS 3 and 24-hour comparison were analyzed by GO which identified 13 biological processes in the 3-hour group and 15 biological processes in the 24-hour group that could be affected by THS exposure (Figure 2.3A, B). In the THS 3-hour group, significant biological processes included “actin polymerization or depolymerization”, “platelet aggregation”, “IL-12 mediated signaling”, “homotypic cell-cell adhesion”, and “positive regulation of ROS metabolic process” (Figure 2.3A). In the THS 24-hour exposure, biological processes included “endothelial cell development”, “opsonization”, epithelial cell maintenance, “protein localization to the endosome”, “membrane to membrane docking”, “leukocyte aggregation” and “leukocyte cell-cell adhesion” (Figure 2.3B).

## IPA Networks and Associated Diseases or Functions

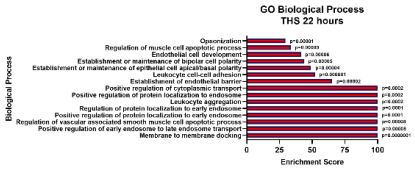
The top IPA-scored network from the THS 3-hour exposure was “cell-to-cell signaling and interaction, cellular function and maintenance, and infectious disease pathways” (Figure 2.3C). Extracellular signal-regulated kinase 1/2 (*ERK1*/*2*) was predicted to be central to these signaling pathways. Some diseases and functions associated with the THS 3-hour network were inflammatory response, engulfment and phagocytosis of myeloid cells and phagocytes,

engulfment of blood cells, and atherosclerosis formation. (Figure 2.3E). The top IPA scored network from the THS 24-hour exposure was related to organismal injury and abnormalities, inflammatory response, and infectious disease. Heat shock protein 90 (Hsp90) was central to signaling proteins in this network. Diseases associated with this network were “inflammation of organs”, “keratinization of epidermis”, “plaque psoriasis”, “dermatitis”, and “exanthem of skin” (Figure 2.3F).

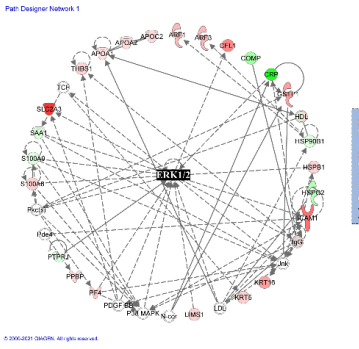
A



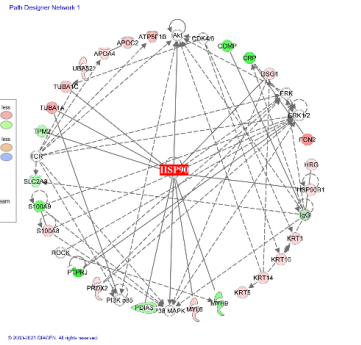

B




C THS 3 Hour Network #1




D THS 22 Hour Network #1




E

| THS 3 hours Network #1 Diseases or Functions Association | p-value | Molecules | # Molecules |
|---|---|---|---|
| Endocytosis | 2.51E-10 | APOA1,APOA2,APOC2,ARF1,CRP,GSTP1,HSP90B1,HSPG2,ICAM1,IgG,LDL,P38 MAPK,PF4,Pkc(s),S100A9,SAA1,THBS1 | 17 |
| Engulfment of phagocytes | 2.71E-10 | APOA1,APOA2,CRP,GSTP1,ICAM1,IgG,PF4,S100A9,SAA1,THBS1 | 10 |
| Severe COVID-19 | 3.15E-10 | APOA1,APOA2,CRP,PF4,PPBP,S100A8,S100A9,SAA1 | 8 |
| Engulfment of leukocytes | 4.92E-10 | APOA1,APOA2,CRP,GSTP1,ICAM1,IgG,PF4,Pkc(s),S100A9,SAA1,THBS1 | 11 |
| Phagocytosis of myeloid cells | 2.52E-09 | APOA1,APOA2,CRP,ICAM1,IgG,PF4,S100A9,SAA1,THBS1 | 9 |
| Phagocytosis of phagocytes | 2.71E-09 | APOA1,APOA2,CRP,ICAM1,IgG,PF4,S100A9,SAA1,THBS1 | 9 |
| Atherosclerosis | 2.78E-09 | APOA1,APOA2,APOC2,CRP,HDL,HSPG2,ICAM1,IgG,LDL,P38 MAPK,PDGF BB,PF4,S100A8,S100A9,SAA1,THBS1 | 16 |
| Engulfment of blood cells | 4.31E-09 | APOA1,APOA2,CRP,GSTP1,ICAM1,IgG,LDL,PF4,Pkc(s),S100A9,SAA1,THBS1 | 12 |
| Lichen planus | 1.77E-08 | CFL1,COMP,KRT16,KRT5,S100A8,S100A9 | 6 |
| Internalization of cells | 2.12E-08 | APOA1,APOA2,CRP,GSTP1,ICAM1,IgG,Jnk,LDL,P38 MAPK,PF4,S100A9,SAA1,THBS1 | 13 |
| Inflammatory response | 2.18E-08 | APOA1,CRP,ERK1/2,HDL,HSPB1,HSPG2,ICAM1,IgG,Jnk,KRT16,LDL,P38 MAPK,PF4,Pkc(s),PPBP,PTPRJ,S100A8,S100A9,SAA1,THBS1 | 20 |
| Adhesion of blood cells | 2.23E-08 | APOA1,CRP,ERK1/2,HSPG2,ICAM1,IgG,Jnk,LDL,P38 MAPK,PF4,Pkc(s),PPBP,S100A8,S100A9,SAA1,THBS1 | 16 |

F

| THS 22 hours Network #1 Diseases or Functions Association | p-value | Molecules | # Molecules |
|---|---|---|---|
| Severe COVID-19 | 1.65E-08 | CRP,FCN2,HRG,S100A8,S100A9,TUBA1A,TUBA1C | 7 |
| Inflammation of organ | 3.09E-08 | Akt,APOA4,CRP,DSG1,ERK,FCN2,HSP90B1,IgG,KRT1,KRT10,KRT14,KRT5,MYH9,P38 MAPK,PDIA3,PI3K, p85,PRDX2,S100A8,S100A9,TCR,TUBA1A,TUBA1C | 22 |
| Keratinization of epidermis | 4.00E-07 | DSG1,KRT1,KRT10,KRT14,KRT5 | 5 |
| Plaque psoriasis | 5.31E-07 | CRP,IgG,KRT1,KRT10,KRT14,S100A8,S100A9 | 7 |
| Dermatitis | 6.00E-07 | DSG1,IgG,KRT1,KRT10,KRT14,KRT5,S100A8,S100A9,TUBA1A,TUBA1C | 10 |
| Benign lesion | 6.00E-07 | CRP,DSG1,ERK1/2,KRT1,KRT10,KRT14,KRT5,MYH9,MYL6,PRDX2,ROCK,S100A8,S100A9,TUBA1A,TUBA1C | 15 |
| Exanthem of skin | 1.00E-06 | Akt,CRP,IgG,KRT1,KRT10,KRT14,S100A8,S100A9 | 8 |

**Figure 2.3. Gene ontology biological processes and IPA's predicted network activity due to THS 3- and 22-hour treatment**. a, b. Gene ontology biological processes ranked by the gene enrichment score at the THS 3 and 22 hours. c, d. IPA's top predicted network activity at the THS 3- and 22-hour timepoint. e, f. IPA's predicted diseases and function associated with their THS 3- and 22-hour network, respectively.

## IPA Regulator Effect Networks

IPA identified two regulator effect networks in the THS 3-hour data. The first was composed of three upstream regulators (TP63, BRD4, or VIPAS39) that predictively activated seven proteins (Figure 2.4A). This activation may increase adhesion of cells, cell movement of leukocytes, hemostasis, activation of cells, and homing of cells (Figure 2.4A). The other regulator effect network predicted SMARCA4 to activate five proteins that cause proliferation of immune cells (Figure 2.4B). There were no other regulator networks discovered for the other exposures.

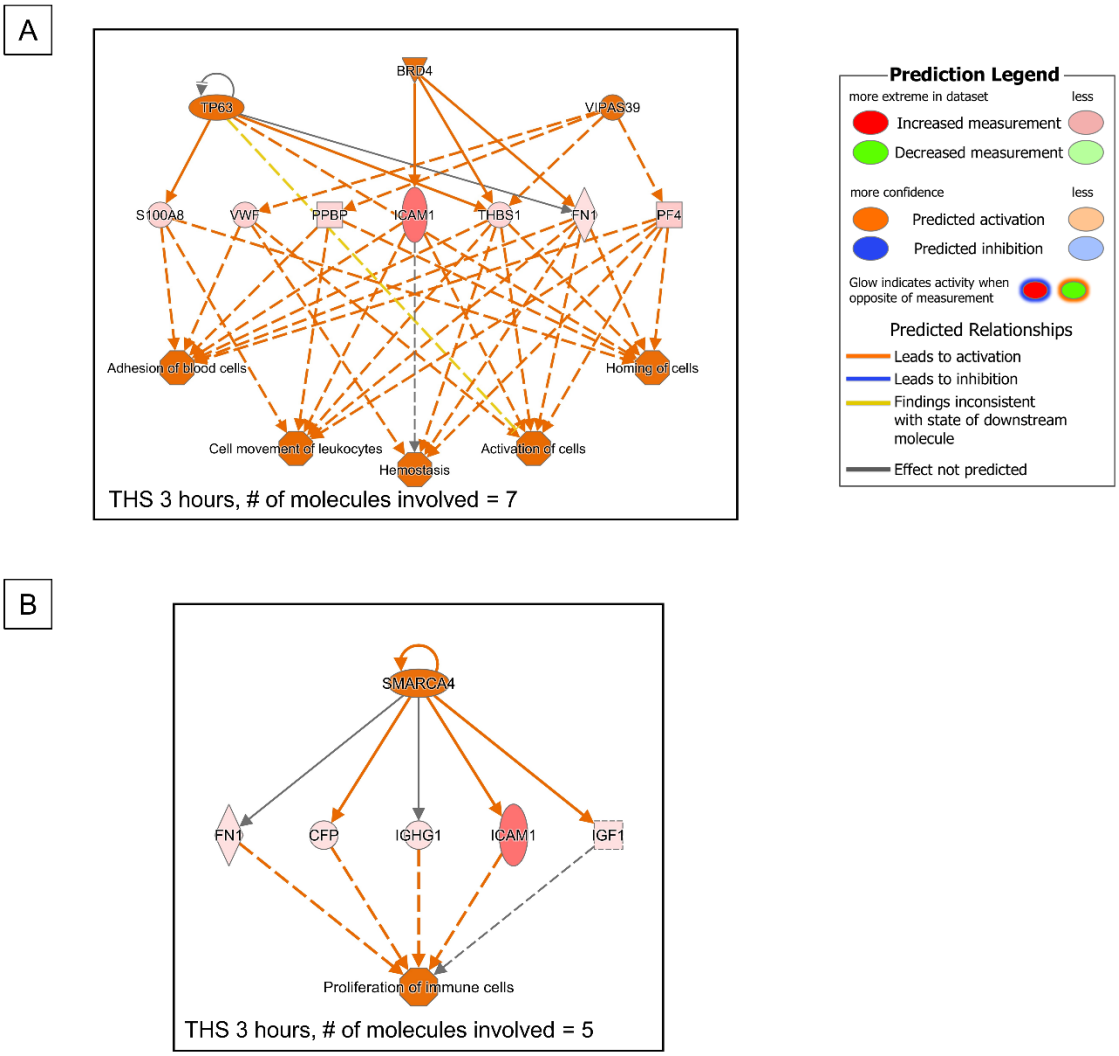



**Figure 2.4. Upstream regulator effects predicted in the THS 3-hour treatment by IPA**. a. Predicted activation of cells, adhesion of blood cells, cell movement of leukocytes, hemostasis, and homing of cells pathways. Proteins elevated in our subjects were S100A8, VWF, PPBP, ICAM1, THBS1, FN1, and PF4 that were predicted to be regulated by TP63, BRD4, or VIPAS39. b. Predicted proliferation of immune cells pathway. Proteins elevated in our subjects were FN1, CFP, IGHG1, ICAM1, and IGF1 that were predicted to be regulated by SMARCA4.

## Canonical Pathway Analysis Using IPA

### Baseline, Clean Air 3 and 22-hour Pathways

The baseline comparison was performed to identify protein baseline differences between the clean air and THS data, and only one significant canonical pathway was identified, indicating that the biological changes were caused by THS exposure (Table 2.1). The clean air exposures were performed as a control, but also to determine the potential effects of exercise. In the clean air 22-hour exposure "metabolism of reactive oxygen species" was elevated affirming exercise-induced ROS[18]; however, because no urinary biomarkers of oxidation in the control group were elevated, this suggests that there was a homeostatic redox rate. Any further ROS damage was due to THS exposure. Also, no plasma inflammatory biomarkers were found to be elevated in the clean air exposure (See Supplementary 1 Figure 2.4).

**Table 2.1. Predicted significant IPA canonical pathways for each comparison group.**

| Timepoint | Canonical Pathways | Z-score | Predicated Activation State |
|---|---|---|---|
| | | | |
| **Baseline (clean air 0 hours vs THS 0 hours)** | | | |
| | Huntington's Disease Signaling | -2 | Decreased |
| | | | |
| **Clean Air 3 hours (clean air 0 hours vs clean air 22 hours)** | | | |
| | RhoGDI Signaling | 2 | Increased |
| | Huntington's Disease Signaling | -2.236 | Decreased |
| | Integrin Signaling | -2.236 | Decreased |
| | Actin Cytoskeleton Signaling | -2.449 | Decreased |
| | | | |
| **Clean Air 22 hours (clean air 0 hours vs clean air 22 hours)** | | | |
| | Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes | -2 | Decreased |
| | Xenobiotic Metabolism PXR Signaling Pathway | -2 | Decreased |
| | Huntington's Disease Signaling | -2.236 | Decreased |
| | | | |
| **THS 3 hours (THS 0 hours vs THS 3 hours)** | | | |

| | | | |
|---|---|---|---|
| | Leukocyte Extravasation Signaling | 2.828 | Increased |
| | RhoA Signaling | 2.714 | Increased |
| | Actin Cytoskeleton Signaling | 2.673 | Increased |
| | Paxillin Signaling | 2.449 | Increased |
| | Signaling by Rho Family GTPases | 2.333 | Increased |
| | Coronavirus Replication Pathway | 2.236 | Increased |
| | Tumor Microenvironment Pathway | 2.236 | Increased |
| | Production of Nitric Oxide and Reactive Oxygen Species in Macrophages | 2.121 | Increased |
| | Cardiac Hypertrophy Signaling | 2 | Increased |
| | Phospholipase C Signaling | 2 | Increased |
| | | | |
| **THS 22 hours (THS 0 hours vs THS 22 hours)** | | | |
| | Production of Nitric Oxide and Reactive Oxygen Species in Macrophages | 2.646 | Increased |
| | Huntington's Disease Signaling | 2 | Increased |
| | MSP-RON Signaling In Cancer Cells Pathway | 2 | Increased |
| | IL-8 Signaling | 2 | Increased |

THS 3 and 22-hour Canonical Pathways

The canonical pathway analysis revealed some similarities in the changes caused by THS exposure at 3 and 22 hours. In the baseline to THS 3-hour comparison, IPA identified nine significant canonical pathways, which included “production of nitric oxide and ROS in macrophages”, “leukocyte extravasation signaling”, and “cardiac hypertrophy” (Table 2.1). The THS 22-hour group had four significant canonical pathways which correlated with our biomarker results and included production of “nitric oxide and ROS in macrophages”, “leukocyte extravasation signaling”, and “IL-8 signaling”.

THS 3 and 22-hour Disease and Bio Functions

The IPA’s “Disease and Bio Functions” analysis found similar elevated pathways in the THS 3- and 22-hour groups. Fourteen “Disease and Bio Functions” were activated in the THS 3-hour comparison, and these included “cell movement”, “migration of cells”, cell movement of leukocytes, lymphocytes, and phagocytes, “adhesion of blood cells”, and “hemostasis”. IPA predicted nine Bio Functions for the THS 22-hour group, which included “cell movement”, “migration of cells”, “cell spreading of tumor cell lines”, “cell movement of phagocytes”, “vasculogenesis”, and “angiogenesis” (Table 2.2).

**Table 2.2. Predicted significant IPA disease and biological functions for each comparison group.**

| Timepoint | Disease and Biological Functions | Z-score | Predicated Activation State |
|---|---|---|---|
| | | | |
| **Baseline (clear air 0 hours vs THS 0 hours)** | Binding of mononuclear leukocytes | 1.964 | Increased |
| | Lymphocyte migration | 1.962 | Increased |
| | Proliferation of cancer cells | -1.964 | Decreased |
| | Viral Infection | -2.025 | Decreased |
| | Vasculogenesis | -2.157 | Decreased |
| | Growth of connective tissue | -2.204 | Decreased |
| | | | |
| **Clean Air 3 hours (clean air 0 hours vs clean air 3 hours)** | | | |
| | Survival of vascular cells | -2 | Decreased |
| | Formation of actin stress fibers | -2.2 | Decreased |
| | Survival of sarcoma cell lines | -2.201 | Decreased |
| | | | |
| **Clean Air 22 hours (clean air 0 hours vs clean air 22 hours)** | | | |
| | Migration of granulocytes | 2.207 | Increased |
| | Metabolism of reactive oxygen species | 2.02 | Increased |
| | Apoptosis of endothelial cell lines | -1.98 | Decreased |
| | Formation of actin filaments | -2.207 | Decreased |
| | Formation of filaments | -2.207 | Decreased |
| | Chemotaxis of phagocytes | -2.23 | Decreased |

| | | | |
|---|---|---|---|
| **THS 3 hours (THS 0 hours vs THS 3 hours)** | | | |
| | Cell movement | 2.892 | Increased |
| | Activation of cells | 2.701 | Increased |
| | Cell movement of leukocytes | 2.684 | Increased |
| | Migration of cells | 2.631 | Increased |
| | Leukocyte migration | 2.618 | Increased |
| | Cell movement of mononuclear leukocytes | 2.615 | Increased |
| | Adhesion of blood cells | 2.471 | Increased |
| | Cell movement of lymphocytes | 2.233 | Increased |
| | Organization of cytoplasm | 2.231 | Increased |
| | Organization of cytoskeleton | 2.231 | Increased |
| | Hemostasis | 2.203 | Increased |
| | Homing of cells | 2.093 | Increased |
| | Proliferation of immune cells | 2.088 | Increased |
| | Proliferation of mononuclear leukocytes | 2.088 | Increased |
| | Cell spreading | 2.068 | Increased |
| | Chemotaxis | 2.059 | Increased |
| | Adhesion of immune cells | 2.001 | Increased |
| | | | |
| **THS 22 hours (THS 0 hours vs THS 22 hours)** | | | |
| | Vasculogenesis | 3.087 | Increased |
| | Cell spreading | 2.795 | Increased |
| | Cell movement | 2.759 | Increased |
| | Cell spreading of tumor cell lines | 2.646 | Increased |
| | Angiogenesis | 2.45 | Increased |
| | Migration of cells | 2.342 | Increased |
| | Proliferation of blood cells | 2.155 | Increased |
| | Cell movement of phagocytes | 2.135 | Increased |
| | Activation of cells | 2.046 | Increased |

**Western Blot for Integrin Linked Kinase (ILK) and Beta-3 Integrin**

To validate the proteomics data, Western blots were performed on plasma from the subjects used in the proteomics study. There were similar increases in ILK and beta-3 integrin expression at the 3- and 22-hour THS exposure and no change in the clean air exposure (See Supplementary 1 Figures 2.5,2.6).

# Discussion

This is the first human clinical trial to identify molecular pathways and potential health risks associated with dermal exposure to THS, which is likely the main route of uptake of THS chemicals from the environment. Acute THS exposure caused oxidative damage to DNA, lipids, and proteins recovered in the urine by 3 hours, and biomarkers of damage remained high after exposure stopped. Cotinine, a nicotine metabolite, was significantly elevated in the urine of THS subjects after 8 hours verifying systemic organ exposure to THS chemicals. Proteomics analysis detected differences between the THS and clean air exposures consistent with oxidative damage caused by THS toxicants, and implicated activation of the pro-inflammatory innate immune system, which initiated molecular risk factors for inflammatory skin disease.

Cigarette smoking increases inflammation[19], recruits leukocytes to injured tissue[20], and stimulates a pro-inflammatory immune response[21]. Smoking is also a risk factor for thrombosis-induced stroke because it elevates red blood cell

counts[22] and forms sticky fibrin clots[23]. Like cigarette smoking, THS exposure activated functional pathways characteristic of an innate immune response (e.g., “inflammatory response”, “inflammation of organ”, “leukocyte migration”, “proliferation of immune cells”, and “phagocytosis by myeloid cells”). IPA predicted two upstream regulatory pathways from the THS 3-hour exposure, which included “elevated hemostasis”, “adhesion of blood cells”, “cell activation”, “movement of leukocytes and homing of cells” and “increased proliferation of immune cells”. IL-6, a pro-inflammatory cytokine, was significantly higher after 3-hour exposure to THS and remained elevated at 22 hours. Similar increases in inflammatory cytokines occurred in a 3D model of human epidermis (EpiDerm) exposed to the residue from exhaled electronic cigarette aerosol[24]. Furthermore, the top scored biofunctions network were “inflammatory response” at 3 hours and “inflammation of organs” at 22 hours. Together, our results demonstrate that exposure to THS increases hemostasis (“adhesion of blood cells”) and initiates a pro-inflammatory response, which are risk factors for thrombosis.

Upregulation of “RhoA signaling” and “signaling of Rho family GTPases” in the 3-hour THS exposure group provides further evidence for activation of the innate immune system. RhoA, a member of the RhoA GTPase family, regulates actin cytoskeletal organization[25], NF-κB transactivation[26], and IL-8 synthesis in endothelial cells[27]. Exposure of human epithelial cells to cigarette smoke extract for 3 hours activated a RhoA-dependent NF-κB signaling pathway that stimulated pro-inflammatory cytokine production[28]. Similarly, the 3-hour THS exposure

increased RhoA signaling, which activated “actin cytoskeleton signaling” and pro-inflammatory cytokine “IL-8 signaling” the next day. Macrophages produce large amounts of ROS from their “oxidative burst”[29] and similarly in both THS exposures “production of nitric oxide and reactive oxygen species in macrophages” were elevated. The pro-immune system is likely to contribute to the elevation of oxidative harm in the THS exposure. These results support the idea that THS exposure mimics the oxidative damage and immune activation previously observed in cigarette smokers[19,21,30].

Our THS exposures were brief, did not cause skin irritation, and were unlikely to induce skin disease, nevertheless markers associated with early-stage activation of contact dermatitis, psoriasis and other skin conditions were elevated. Allergic contact dermatitis is a rash caused by an immune reaction to materials that touch the skin[31], and psoriasis is characterized by cutaneous plaques caused by inflammatory infiltrates and epidermal hyperproliferation[32]. Psoriasis is linked to genetic susceptibility and can be triggered by environmental factors[33]. These skin diseases have been linked to cigarette usage[32,34] and markers of both were elevated in the plasma from subjects exposed to THS. At 22-hours after THS exposure, the proteomics data showed higher concentrations of markers associated with “keratinization of the epidermis”, “plaque psoriasis”, “dermatitis”, and “exanthem of the skin”. Concentrations of keratin 5 (KRT5) and keratin 14 (KRT14), the major cytokeratins of the epidermis, were elevated 22-hours after THS exposure. Repeated cigarette exposure to epithelial cells produced similar

increases in KRT5 and KRT14[35], which are biomarkers linked to inflammation-driven skin diseases, such as dermatitis and psoriasis[36,37]. While our subjects did not develop these conditions during their 3-hour exposure to THS, molecular changes characteristic of skin irritation and inflammation occurred, supporting the idea that dermal exposure to THS could lead to molecular initiation of inflammation-induced skin diseases.

Chronic exposure to THS may cause other diseases. Unrepaired DNA damage increases the risk of developing cancer[38]. "Positive regulation of reactive oxygen metabolic processes" was upregulated at THS 3-hour exposure. 8-isoprostane, indicative of lipid oxidation, is associated with atherosclerosis, cardiac failure, cancer, and immunological disorders[39]. Protein oxidation causes protein fragmentation and protein-protein cross-linking, which impair function, potentially leading to diseases such as chronic obstructive pulmonary disease (COPD)[40].

Cigarette smoke-induced atherosclerosis includes activation of a pro-inflammatory system and remodeled vasculature[41,42,43]. Macrophages phagocytose and clear oxidized lipids and become foam cells, preventing lipid clots[43]. In cigarette smokers, vascular smooth muscle cells (SMCs) increase matrix remodeling and inflammatory gene expression[44]. The activation of MMPs causes vascular SMC apoptosis leading to a loss of vascular structural integrity[45]. Our THS-exposed subjects showed activation of atherosclerosis and similar atherogenesis-related pathways. IPA predicted "negative regulation of macrophage derived foam cell differentiation" that would reduce clearance of

lipids, leading to accumulation of oxidized lipids in blood vessels. The “regulation of vascular associated SMC apoptotic process” and elevation of inflammation pathways mimic atherogenesis. The “adhesion of blood cells” pathway could lead to platelet aggregation. Our data suggest that prolonged THS exposure could produce arterial plaques and atherosclerosis, as seen in cigarette smokers.

We explored the predicted molecular proteins that regulated the biological pathways affected in our subjects. THS exposure activated biological functions associated with increased cell migratory and survival pathways, such as “cell movement”, “cell spreading of tumor lines”, “migration of cells”, “vasculogenesis”, and “angiogenesis”. The highest scored IPA THS 3-hour network was “cell-to-cell signaling and interaction, cellular function and maintenance, and infectious disease pathways”. ERK1/2, a protein-serine/threonine kinase, was predicted as a central signaling molecule in this network. ERK1/2 signals in pathways that regulate cell migration, cell survival, cell cycle progression, differentiation, and proliferation[46]. Nicotine binds to nAChR and β-adrenoceptors resulting in a downstream cascade of ERK/1/2/Stat3-signaling, which in turn activates NF-κB transcription of the cell-cycle regulator cyclin D1, a promoter of cell proliferation[47]. The THS 22-hour exposure’s highest scored IPA network was “organismal injury and abnormalities, inflammatory response, and infectious disease” pathways. HSP90, which acts as a chaperone for ERK1/2[48], was central to interacting with the majority of proteins in this network. We predict HSP90 was induced as a

survival response to help cells maintain protein homeostasis and DNA stability in an oxidative and chemically toxic environment.

## Conclusions

This is the first clinical trial to demonstrate that acute dermal exposure to THS increased oxidation of DNA, lipids, and proteins recovered in the urine and produced changes in the plasma proteome that are similar to changes observed in cigarette smokers. THS exposure activated biomarkers of the innate immune system, increased oxidative stress, and elevated biomarkers associated with skin diseases, such as contact dermatitis and psoriasis. Pathways associated with other cigarette-induced diseases not involving the skin, such as atherosclerosis and cancer, were also elevated in THS-exposed subjects. Though the subjects did not develop these diseases during a 3-hour exposure to THS, our results provide the first molecular evidence that the concentrations of proteins involved in disease pathways change in humans after acute dermal exposure to THS. Our data will be useful in devising regulatory policies to limit exposure to THS in contaminated properties and will enable health care workers to advise their patients on the risks associated with THS exposure. For future studies electronic cigarettes also deposit a residue that comes into contact with the skin[24,49,50] and this could be evaluated.

**Ethics and Dissemination**: Prior to the experiment patients were provided with informed consent. Patient information and trial protocol can be made available by contacting Dr. Schick by email.

**Ethics approval**: This study involved human participants and was given IRB approval (#15-17009) by the San Francisco General Hospital panel.

**Additional Article Information**

**Author Contributions:** Mr Sakamaki-Ching and Dr Talbot had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

*Concept and design:* All authors.

*Acquisition, analysis, or interpretation of data:* All authors.

*Drafting of the manuscript:* Sakamaki-Ching, Talbot.

*Critical revision of the manuscript for important intellectual content:* All authors.

*Statistical analysis:* Sakamaki-Ching

*Obtained funding:* Schick, Talbot.

*Administrative, technical, or material support:* Sakamaki-Ching, Schick, Grigorean, Talbot.

*Supervision:* Schick, Talbot.

**Conflict of Interest Disclosures:** All authors declare no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

**Funding/Support:** Research performed at the University of California, Riverside, was funded by a grant from the Tobacco Related Disease Research Program (TRDRP) 24RT-0037 to Dr Talbot. Mr Sakamaki-Ching was supported in part by a Deans Fellowship from the University of California, Riverside, Graduate Division. The indoor exposure experiment at the University of California, San Francisco, was supported by grants from TRDRP 24RT-0039 and 28PT-0081 to Dr Schick.

**Data Sharing Statement:** Patient information and trial protocol can be made available by contacting Dr. Schick by email.

**Acknowledgements:** Brett Phinney, PhD made contributions to the study. He is employed by the University of California, Davis Proteomics Core Facility.

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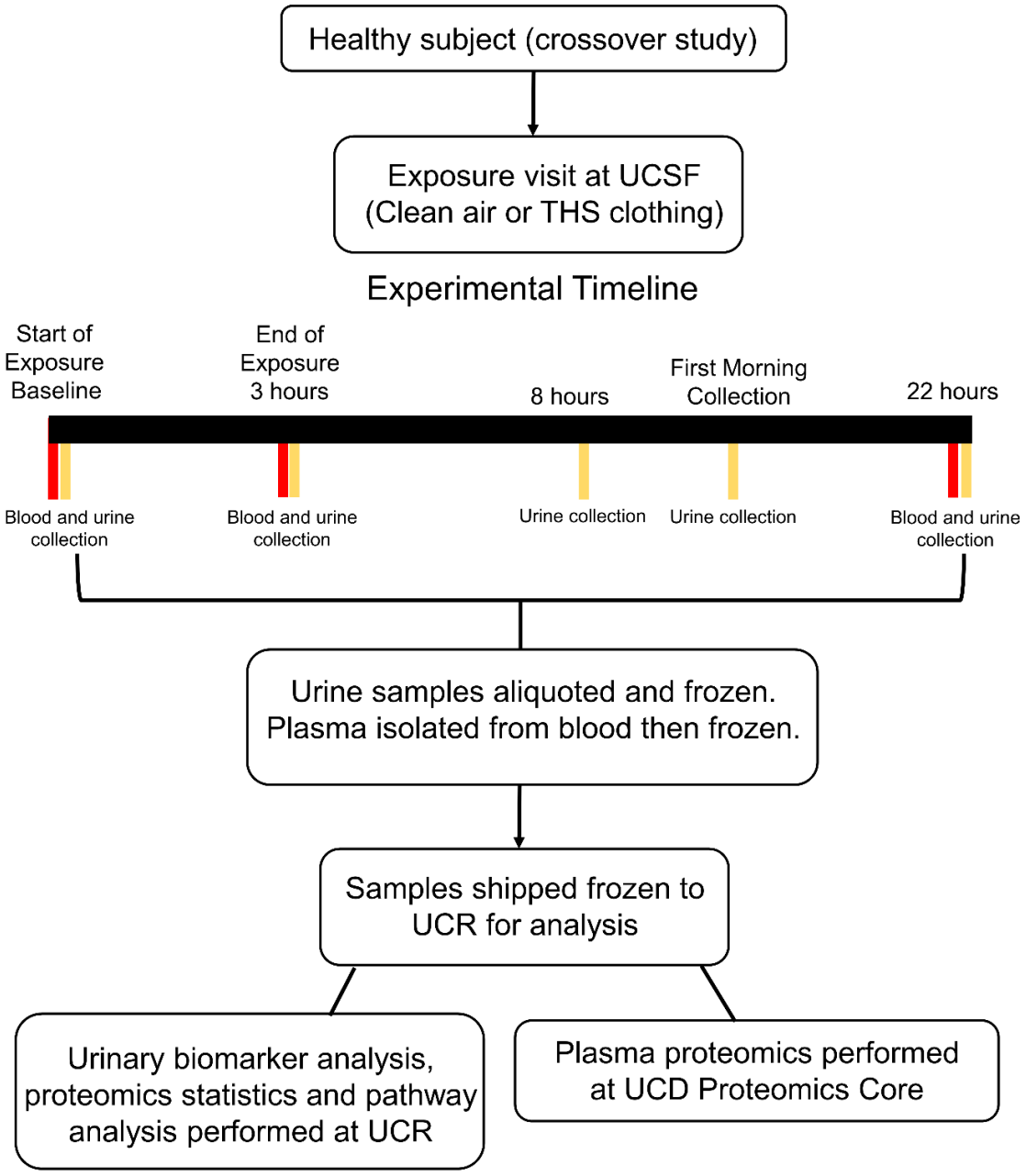
**Supplementary Table S 2.1. Clinical characteristics of the subjects.** All subjects were healthy, non-smokers. This was a cross-over study, the same subjects were exposed to either exclusively clean air clothing or THS clothing 20-30 days apart. Both genders had the same average age, while the females were within the healthy BMI (body mass index) range, the males are considered slightly overweight. Height is in inches (in) and weight is in pounds (lbs). Subject selected for "B"=urinary biomarker analysis and/or "P"=plasma proteomics analysis

| Subject ID | Gender | Age | Height (in.) | Weight (lbs) | BMI | Ethnicity | Drug usage in past 30 days: | CA Visit | THS Visit |
|---|---|---|---|---|---|---|---|---|---|
| 234 [B,P] | Female | 35 | 65 | 132 | 21.96 | Caucasian | | None | None |
| 299 [B] | Male | 49 | 69 | 176 | 25.99 | Caucasian | | None | None |
| 340 [B] | Female | 34 | 63 | 162 | 28.69 | African Am | | Alcohol | Alcohol |
| 358 [B] | Male | 21 | 71 | 181 | 25.24 | Caucasian | | Alcohol | Alcohol |
| 361 [B,P] | Male | 22 | 69 | 206 | 30.42 | Asian | | Alcohol | Alcohol |
| 362 [B] | Female | 27 | 62 | 132 | 24.14 | Asian | | Alcohol | Alcohol |
| 364 [B,P] | Male | 26 | 71 | 136 | 18.97 | Caucasian | prescription drug | Alcohol, Bupropion | Alcohol |
| 366 [B,P] | Female | 24 | 65 | 144 | 23.96 | Asian | | Alcohol | Alcohol |
| 368 [B] | Female | 34 | 66 | 128 | 20.66 | Caucasian | | Alcohol | Alcohol |
| 370 [B,P] | Female | 23 | 65 | 151 | 25.12 | Asian | | Alcohol | Alcohol |

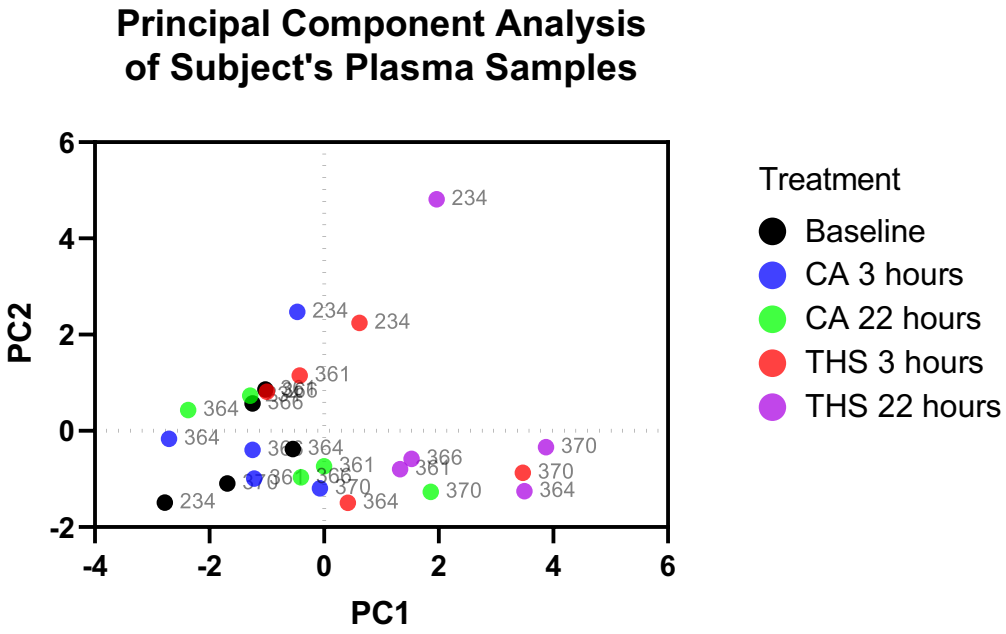
| Statistics | Gender (n) | Age | Height (in.) | Weight (lbs) | BMI |
|---|---|---|---|---|---|
| **Male** | 4 | 29.5 ± 13.2 | 70.0 ± 1.2 | 174.8 ± 29.0 | 25.2 ± 4.7 |
| **Female** | 6 | 29.5 ± 5.5 | 64.3 ± 1.5 | 141.5 ± 13.3 | 24.1 ± 2.8 |




**Supplementary Figure S2.1. Workflow of subject's sample collection for biomarker and proteomic analysis.**

**Uploaded to public database**

**Supplementary Table S2.2. List of protein ($log_2$ fold-change) concentrations analyzed from global proteomics.**




**Supplementary Figure S2.2. The top 12 upregulated proteins (≥1.5 log2-change) from the THS 22 hours timepoint were selected for a principal component analysis.** The clustering demonstrates that subject's 361 and 366 after 3 hours of THS exposure had a similar protein profile as the baseline and clean air exposure. Due to these expression similarities subjects 361 and 366 were removed from further pathway analysis to better determine the effects of THS exposure.

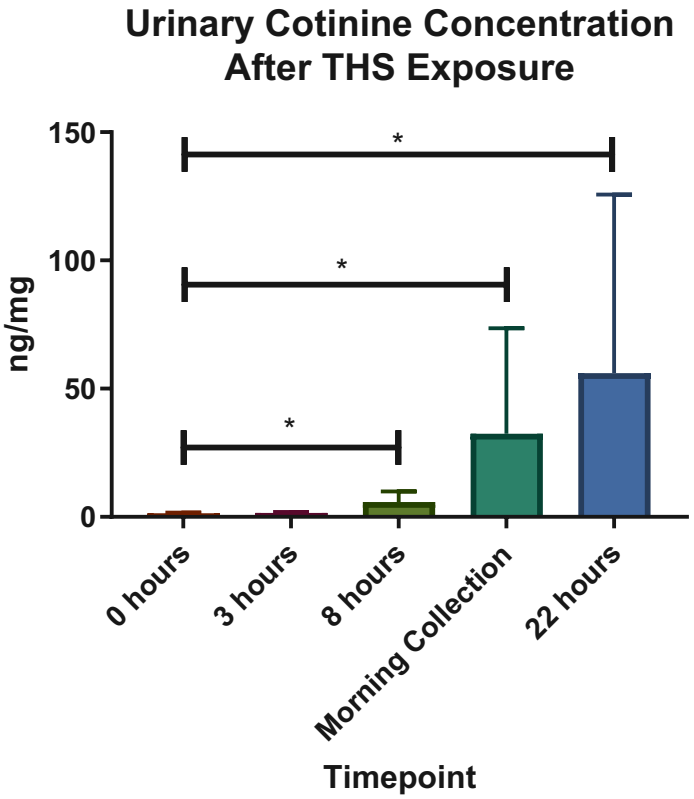



**Figure S2.3. Urinary cotinine (ng/mg of creatinine), a nicotine metabolite, in THS exposed subjects was significantly increased at 8 hours, first morning collection, and 22 hours post-THS exposure.**

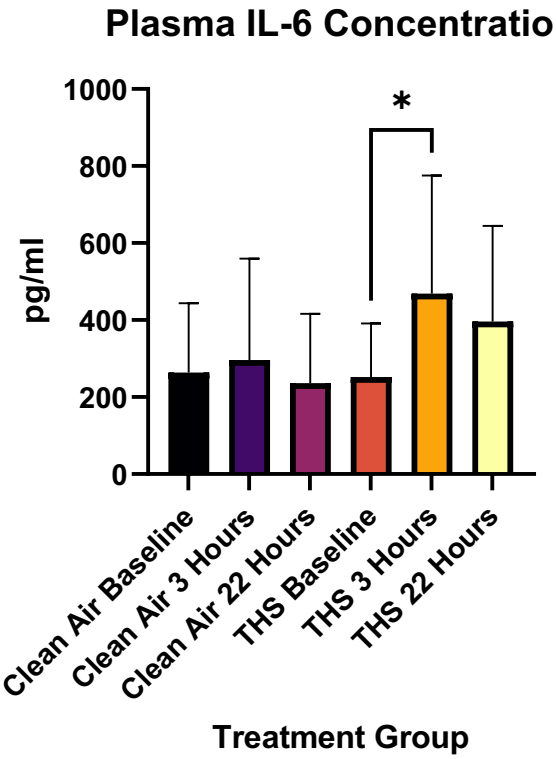



**Figure 2.4. Plasma IL-6, a pro-inflammatory cytokine, in THS exposed subjects was significantly increased at 3 hours.**

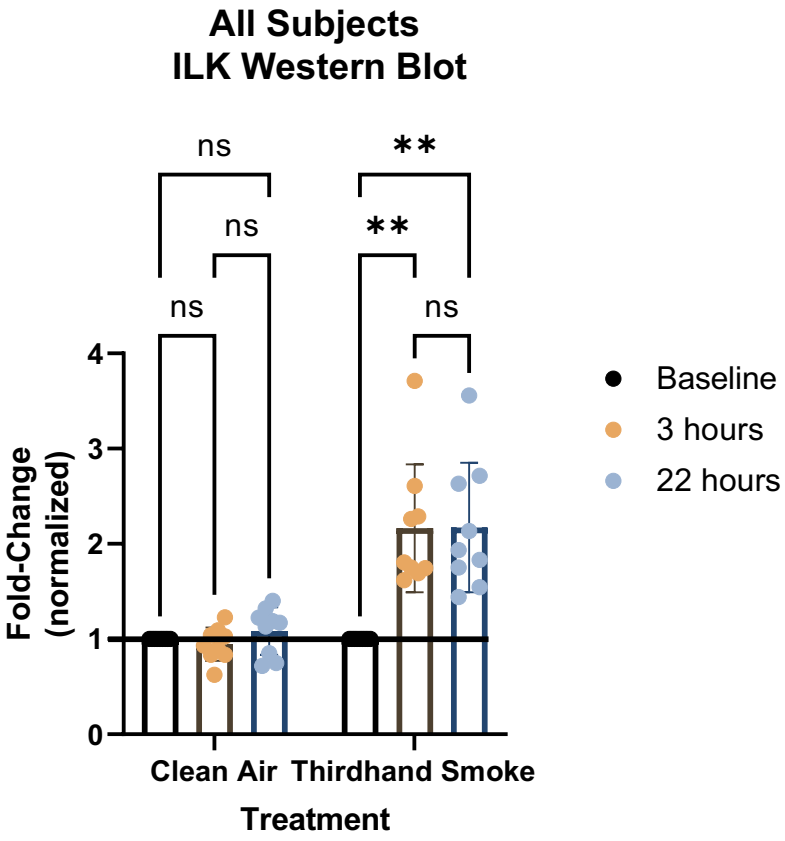



**Figure S2.5. Western blot validation for plasma protein integrin linked kinase.** ILK was significantly elevated in the THS 3- and 22-hour timepoint in both the western blot and proteomics results, while the clean air timepoints were insignificant. These Western blot results validate the proteomics results. This experiment was performed in triplicate from subject's 234, 361, and 370.

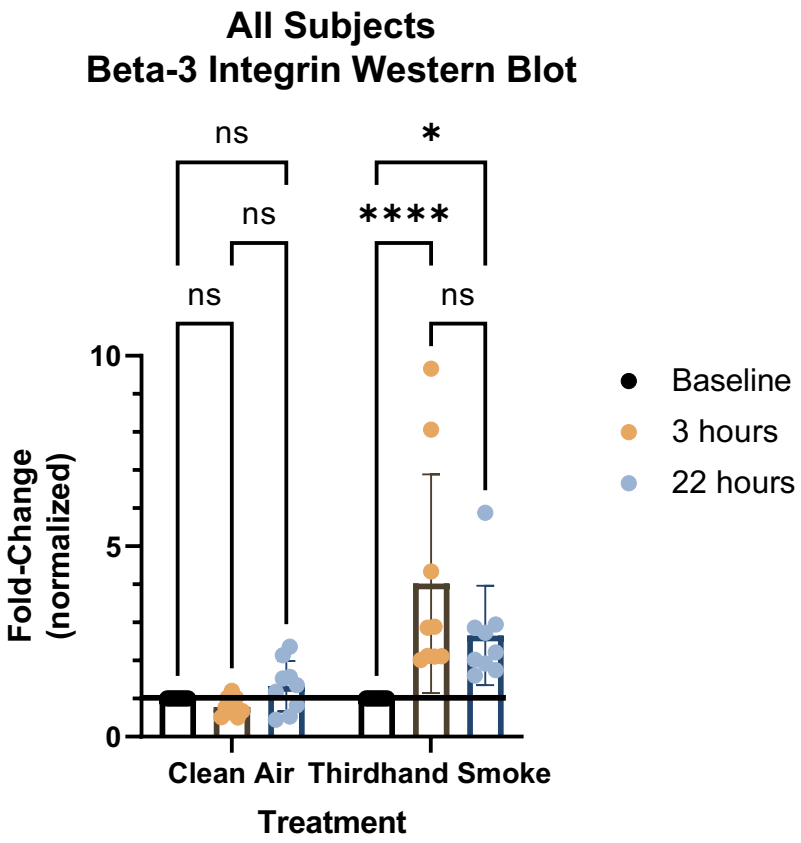



**Figure S2.6. Western blot validation for plasma beta-3 integrin.** ITGB3 was significantly elevated in the THS 3- and 22-hour timepoint in both the western blot and proteomics results, while the clean air timepoints were insignificant. These Western blot results validate the proteomics results. This experiment was performed in triplicate from subject's 234, 361, and 370.

Urinary Biomarkers

Urinary biomarkers were normalized to creatinine. 8-OHdG was measured using a DNA Damage ELISA Kit (Stress Marq Biosciences), following a 1:20 dilution. 8-isoprostane was measured following a 1:4 dilution using a Urinary 8-Isoprostane ELISA kit (Detroit R&D). Protein was quantified in 1:10 or 1:20 dilutions using the Pierce BCA Protein Assay Kit (Thermofisher Scientific). Protein carbonyls were analyzed using a Protein Carbonyl Assay Kit (Abcam) protocol with reagents that were ordered separately. Total oxidized protein (nmol) was normalized to the total protein concentration (mg). All biomarker graphs were made in Prism 9.0 (GraphPad). Statistical significance was determined by a paired t-test. * $p \leq 0.05$, ** $p \leq 0.01$. There was no effect on gender or age.

Plasma Proteomics Sample Preparation

The sera samples were solubilized in 50 µL Solubilisation Buffer, and subjected to reduction / alkylation / tryptic proteolysis by using suspension-trap (ProtiFi) devices. S-Trap is a powerful Filter-Aided Sample Preparation (FASP) method that consists in trapping acid aggregated proteins in a quartz filter prior enzymatic proteolysis. Disulfide bonds were reduced with dithiothreitol and alkylated (in the dark) with iodoacetamide in 50mM TEAB buffer. Digestion constituted of a first addition of trypsin 1:100 enzyme: protein (wt/wt) for 4 hours at 37 °C, followed by a boost addition of trypsin using same wt/wt ratios for overnight digestion at 37 °C. Peptides, still in S-Trap, were eluted using sequential elution buffers of 100mM

TEAB, 0.5% formic acid, and 50% acetonitrile 0.1% formic acid. The eluted tryptic peptides were dried in a vacuum centrifuge and re-constituted in 0.1 trifluoroacetic acid. The peptide extracts are reduced in volume by vacuum centrifugation and a small portion of the extract is used for fluorometric peptide quantification (Thermo scientific Pierce). One microgram of sample based on the fluorometric peptide assay was loaded for each LC-MS analysis.

Liquid Chromatography Tandem Mass Spectrometry

Peptides were desalted and trapped on a Thermo PepMap trap and separated on an Easy-spray 100 µm x 25 cm C18 column using a Dionex Ultimate 3000 RSLC at 200 nL/min. Solvent A= 0.1% formic acid, Solvent B = 100% Acetonitrile 0.1% formic acid. Gradient conditions = 2% B to 50% B over 60 minutes, followed by a 50%-99% B in 6 minutes and then held for 3 minutes than 99% B to 2%B in 2 minutes and total run time of 90 minutes using Thermo Scientific Fusion Lumos mass spectrometer. Two data acquisition modes were employed: data-dependent analysis DDA and data-independent analysis, DIA. The DDA mode was used to build spectral libraries which were used to perform deep searched on the samples, run in DIA mode. To obtain the DDA runs, we pooled peptides from all our thirdhand. Then this pooled sample was divided and eight DDA injections were performed. This is called gas-phase fractionation, because each injection covers 1/8th portion of the 400-1200 m/z range. MS data for samples run in DIA mode

covers entire range of m/z 400-1200. Both DDA and DIA were acquired using a collision energy of 35, resolution of 30 K, maximum inject time of 54 ms and a AGC target of 50K, using staggered isolation windows of 12 Da.

Data Analysis

DIA data was analyzed using Spectronaut (v.13, Biognosis) using the classic analysis DIA workflow with default settings. Prior to library-generation from the DIA LCMS runs and then to library-based analysis of the DIA LCMS runs, all raw files were converted into htrms files using the Htrms converter (Biognosys). MS1 and MS2 data were centroided during conversion, and the other parameters were set to default. A deep DDA spectral library was first generated by submitting the htrms files of the DDA gas-phase separation LCMS runs to a library generating step in Spectronaut. This uses the Pulsar search engine and all DDA runs are searched against the Homo sapiens sequence database -Uniprot UP00005640. The DIA htrms files were submitted to identification/quantitative analysis to Spectronaut, using the previously generated deep DDA spectral library (see above) and the reviewed Uniprot FASTA for Homo Sapiens, UP00005640. Default settings were used to perform this quantitative data analysis with the two libraries. Briefly, calibration was set to non-linear iRT calibration with precision iRT selected. DIA data was matched against the above-described spectral library supplemented with decoys (library size fraction of 0.1), using dynamic mass tolerances.

Quantitative and statistical analysis was performed processing protein peak areas determined by the Spectronaut software. The DIA data was processed for relative quantification comparing peptide peak areas from different conditions. For the DIA MS2 data sets, quantification was based on XICs of 3-6 MS/MS fragment ions, typically y- and b-ions, matching to specific peptides present in the spectral library. Interference correction was enabled on MS1 and MS2 levels. Precursor and protein identifications were filtered to 1% FDR, estimated using the mProphet algorithm,74 and iRT profiling was enabled. Quantification was normalized to local total ion chromatogram. Statistical comparison of relative protein changes was performed with paired t-tests. Finally, proteins identified with less than two unique peptides were excluded from the assay. The significance level was q-value less than 0.05%, and log2 ratio more than 0.58. Both DIA analysis results were filtered within Spectronaut by a 1% false discovery rate on peptide and protein level using a target-decoy approach, which corresponds to a Q value <0.01.

# Chapter Three

# The Effects of Acute Thirdhand Smoke Exposure on Human Keratinocytes

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## Abstract

**Background:** Our previous study of humans exposed dermally to THS fabric for three hours demonstrated activation of pro-inflammatory pathways, increased oxidative damage, and elevation of proteins associated with skin diseases in sensitive individuals. This study builds upon our previous results and analyzes the responses of keratinocytes exposed to thirdhand smoke extract *in vitro*.

**Methods:** Human keratinocytes (CCD 1106 KERTr) were treated with clean air or THS fabric extracts for 24-48 hours and analyzed for potential changes to cell migration, oxidative damage, antioxidant response, apoptosis or cell death, mitochondrial dysfunction, inflammatory mediators release, and keratin production.

**Results:** The concentrations of THS extract used were non-lethal to the keratinocytes, but adverse metabolic and biological responses were reported. Altered biological processes that resulted from THS exposure were a proinflammatory response, increased oxidative stress, mitochondrial damage, and keratin 5 elevation.

**Conclusion:** This is the first study to analyze the effects of THS on keratinocytes. Our results demonstrated an elevation of molecular risk factors associated with inflammatory skin diseases or skin cancer. The biological pathways activated were

similar to the skin responses from cigarette smoking, implicating THS as an environmental hazard that should be closely monitored as a potential factor contributing to skin harm in future studies.

## Introduction

Cigarette smoke settles on indoor surfaces during smoking and after smoking has stopped, forming residual chemical contamination called thirdhand smoke (THS), which can remain for months or even years[1,2].THS is a complex mixture of chemicals that can react with each other or with chemicals in the environment to form products that include reactive oxygen species (ROS), free radicals, and tobacco-specific nitrosamines (TSNAs), such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*′-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-4-(3-pyridyl)butanal (NNA)) [3]. The buildup of THS on indoor surfaces is now being recognized as a potential public health problem[2].

Mice exposed to THS using conditions that mimicked human exposure displayed delayed wound closure, impaired collagen deposition, altered inflammatory response, increased elastase, elevated oxidative stress, and lowered levels of anti-oxidant activity[4]. In a related study, THS-exposed mice showed delayed dermal wound healing due to increased keratin production, resulting in rigid, stiff skin prone to tearing[5]. Human pulmonary fibroblasts and neural stem cells (NSCs) were exposed in vitro to THS extract and assessed using the MTT assay, which measures mitochondrial reductase activity, and cytotoxicity was

attributed to acrolein, a volatile organic compound (VOC) in freshly made THS[6]. In mouse NSCs, THS extracts that no longer contained acrolein caused stress-induced mitochondrial hyperfusion (SIMH), causing punctate mitochondria to fuse into elongated networks. Decreased expression of genes involved in mitochondrial fusion (Fis 1, mitofusin-1) apparently produced the punctate phenotype[7]. In a later study, SIMH was linked to nicotine, the main chemical in THS, which by itself produced the networked phenotype[8]. Exposure of human HepG2 cells (a liver cell line) for 24 hours to a THS extract resulted in DNA strand breaks, demonstrating THS is also genotoxic[9]. Evidence of DNA damage was also observed in mouse NSCs and human dermal fibroblasts[10].

Nicotine has been detected in fabrics exposed using controlled laboratory conditions to THS[10,11,12], in the dust and on indoor surfaces in the homes of smokers who had implemented smoking bans[13], and in non-smoker's homes previously occupied by smokers[14]. Nicotine, a main chemical constituent of THS, can react with the indoor pollutant nitrous acid (HONO) to generate the carcinogen NNK[3]. Nicotine is more easily extracted from fabrics in humid atmospheres, suggesting that human exposure to THS chemicals may vary with climatic conditions[11]. A study from our lab estimated that THS contaminated terry cloth contained nicotine and TSNAs that are in higher magnitudes of concentration if dermal and ingestion exposure occurred rather than passively inhaled in toddlers[10]. A lifetime exposure to carcinogenic N-nitrosamines and TSNAs measured in house dust samples could increase the risk of cancer, particularly if

exposure occurred at an early age[15]. These studies demonstrate that THS remains a persistent contaminant with the potential to be harmful to human health.

Humans can be exposed to THS contamination on indoor surfaces through multiple routes, including inhalation, ingestion, and dermal contact. The first human clinical trial involving THS analyzed gene expression in nasal epithelium following acute inhalation of THS in a controlled laboratory exposure chamber[16]. Affected genes were associated with increased mitochondrial activity, oxidative stress, DNA repair, cell survival, and inhibition of cell death, showing that humans respond to inhaled THS chemicals. A second clinical study demonstrated that acute dermal exposure to THS can alter the human plasma proteome and mimics the effects of cigarette smoking, initiating the mechanisms of skin inflammatory disease and elevating urinary biomarkers of oxidative stress[17]. These two clinical studies demonstrate that human subjects respond to THS, which elevates biomarkers of harm and can potentially be a disease initiator. However, the molecular mechanisms of human keratinocyte responses to THS are largely unknown.

Current data show that THS may present a health hazard to humans with the potential to cause biological dysfunction and possibly disease. Clinically, there is correlative evidence between THS exposure by inhalation and dermal contact with adverse responses that could lead to disease. The purpose of this study was to determine if human keratinocytes exposed to THS show biological responses consistent with the molecular processes seen in inflammatory skin diseases and

to correlate these data to results of our earlier proteomics experiments on humans dermally exposed to THS.

## Methods

### THS Fabric Generation

THS impregnated fabric was created using a previously published protocol[18] and extracted into culture medium for cytotoxicity experiments[11]. To extract the THS chemicals, fabric was placed in glass vials at a concentration of 0.1 g fabric/mL medium and set on a shaker for 1 hour. The THS medium was then filtered using a 30 mL Norm-Ject syringe (Millipore Sigma, MA, USA) into a 50 mL tube on ice to remove the fabric. In sterile conditions, the THS medium was filtered using Acrodisc 25mm syringe filters with 0.2 µm Supor membrane (Pall Corporation, NY, USA). The THS medium was aliquoted into 5 mL Eppendorf tubes, flash frozen on dry ice, and stored in -80 $C^{o}$ freezer.

### Keratinocyte Culture Conditions

Human keratinocytes (human papillomavirus 16 (HPV-16) E6/E7 transformed) (ATCC® CRL-2309) were cultured, as described previously[19], in Keratinocyte-Serum Free Medium (Gibco 17005-042) with Keratinocyte Supplements (Gibco 37000-015) and 30 ng/mL of human recombinant epidermal growth factor (EGF;

BD cat# 354052). Medium was changed every 4 days, and cells were passaged in T-75 flasks upon reaching 90% confluency.

**MTT Assay**

Cytotoxicity of the THS extract was tested on the keratinocytes using the MTT assay which measures mitochondrial reductase activity. Keratinocytes were cultured to 90% confluency and treated with 0%, 25%, 50%, or 100% clean air or THS extract (diluted in keratinocyte medium) for 3 or 24 hours. After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, MO, USA) was added to wells and incubated for 2 hours at 37 °C. Solutions were removed from wells, and 100 µL of dimethyl sulfoxide (DMSO) were added to each well to solubilize the formazan crystals. Absorbance readings were taken against a DMSO blank at 570 nm using an Epoch microplate reader (Biotek, VT, USA). The MTT assay quantifies the conversion of a yellow tetrazolium salt (MTT) to purple formazan by active mitochondria. For each concentration tested, three independent experiments were performed.

**Cell Migration Assay**

Keratinocytes were cultured in µ-Dish$^{35mm}$ inserts (Ibidi, cat. no. 81176) placed in 24-well plates and grown to 90% confluency. Keratinocytes were stained with NucBlue (ThermoFisher Scientific, MA, USA). On the clean air or THS extract exposure day, the culture inserts were removed to allow cells to migrate into the separation zone. Keratinocytes were treated with clean air or THS extract with

culture medium containing 0%, 50%, 75%, or 100% extract concentrations. The plates were placed inside a BioStation CT (Nikon, NY, USA) for time-lapse phase contrast and fluorescent imaging that occurred every 15 mins for 48 hours. CLQuant software (Nikon, NY, USA) was utilized to monitor and track the cells to measure total cell area and count, as described in detail previously[20]. For each concentration tested, three independent experiments were performed.

**MitoSox Mitochondrial SuperOxide Measurement Assay**

Keratinocytes were cultured in Ibidi 8-well chambers (Ibidi, WI, USA) and treated with extracts of clean air or THS 0%, 50%, 75%, and 100% dilutions for 24 hours and then stained with MitoSox (ThermoFisher Scientific, MA, USA) for 15 mins. The staining medium was removed and washed with PBS(-) (ThermoFisher Scientific, MA, USA) three times and replaced with fresh medium. Live cells were immediately imaged using a TI inverted Nikon Eclipse microscope (Nikon, NY, USA) equipped with a LiveCell temperature and $CO_2$-regulating, heated stage (Pathology Devices Inc, SD, CA). Background filtering and fluorescent intensity measurements were performed using CLQuant (Nikon, NY, USA) and ImageJ (National institute of Health, MA, USA), as described previously[8].

**Mitochondrial Phenotype Assay**

Keratinocytes cultured in Ibidi 8-well chambers (Ibidi, WI, USA) were transfected with MitoTimer pMitoTimer #52659, AddGene) using Lipofectamine 3000

(Invitrogen, MA, USA), allowed to recover for 72 hours, then treated with extracts of clean air or THS 0%, 50%, 75%, and 100% dilutions for 24 hours. The transfection method followed the company's protocol with a plasmid concentration of 1.6 µg. Images were taken with a TI inverted Nikon Eclipse microscope (Nikon, NY, USA) equipped with a LiveCell temperature and $CO_2$-regulating, heated stage (Pathology Devices Inc, SD, CA). Mitochondrial phenotype analysis was performed by MitoMo software as previously published[8].

**Image Processing and Segmentation Using MitoMo**

Original single cell mitochondrial images were first processed using ImageJ (NIH). The images were duplicated and cropped to include a single cell per image, then saved as three-frame, uncompressed AVI files. These single-cell AVI's were then segmented using MitoMo (version 1.5.0) with one of two segmentation setting combinations[8].

Histogram matching was done to normalize the intensity levels of the video of interest. MitoMo provided additional intensity adjustment to ensure a normalized video. An Otsu thresholding method was performed to segment the video. Through this process, characteristics regarding the relative size and intensity of each segmented mitochondrion in each image were extracted. After initial segmentation, the watershed algorithm in MitoMo then differentiated between individual mitochondria in larger clumps by relative intensity comparison. The two setting combinations that were used to segment videos with varying morphologies

differed in the degree that the mitochondria were de-clumped. For the cells containing mostly network morphology, the settings aimed to de-clump to reflect the true connectivity of the mitochondria. For the cells with mostly punctate or swollen morphologies, the settings aimed to reflect the disconnectivity of the mitochondria.

**Classification of Mitochondrial Morphology**

For each segmented video, the mitochondria were manually classified into three categories: network, punctate, or swollen. The data extracted from the manual classification were compiled into a comprehensive library to train a learning algorithm. This library of data was loaded into two learning algorithms, k-nearest neighbor (KNN) and naive Bayes (NB), which were processed through the Matlab platform. The NB learning algorithm had the highest classification accuracy with a small standard deviation. To obtain data on mitochondrial morphology, the videos were processed using the most fitting setting combination, and then classified using the NB algorithm.

**8-OHdG ELISA**

Keratinocytes were cultured in 24-well plates and treated with extracts of clean air or THS 0%, 50%, 75%, and 100% dilutions for 24 hours. Supernatant was collected and the concentration of 8-OHdG was determined using a DNA Damage (8-OHdG) ELISA Kit (Stress Marq Biosciences, Victoria, Canada) following a 1:5

dilution as previously described[17]. For each concentration tested, three independent experiments were performed.

**Glutathione Assay**

Keratinocytes were cultured in 24-well plates and treated with extracts of clean air or THS 0%, 50%, 75%, and 100% dilutions for 24 hours. Cell lysate was harvested using RIPA Lysis and Extraction Buffer (ThermoFisher Scientific, MA, USA), and protein concentration was determined by Pierce BCA Protein Assay Kit (ThermoFisher Scientific, MA, USA) following the company's protocol. To measure total intracellular glutathione, a Glutathione Assay Kit (Fluorometric) (Abcam, Cambridge UK) was used. For each variable tested, three independent experiments were performed.

**Flow Cytometric Analysis of Cellular Apoptosis and Death**

Keratinocytes were cultured in 24-well plates and treated with extracts of clean air or THS 0%, 50%, 75%, and 100% dilutions for 24 hours. The cells were harvested using trypsin-EDTA 0.05% (Millipore Sigma, MA, USA), then washed in keratinocyte media and pipetted into single cell suspension, and the cell number was adjusted to a concentration of 1-5x$10^6$ cells/ml in ice cold FACS Buffer (PBS, 0.5-1% BSA or 5-10% FBS, 0.1% NaN3 sodium azide). Cells were transferred to polystyrene round-bottom 12 x 75 mm BD Falcon tubes (cat# 352052, ThermoFisher Scientific, MA, USA) in 100 µl of FACS buffer. 100 µl of Fc block

was added to each sample (Fc block was diluted in FACS buffer at 1:50 ratio), incubated on ice for 20 min, then centrifuged at 1500 rpm for 5 min at 4°C. The supernatant was discarded, and the pellet resuspended in 100 µl of cold FACS buffer. Cells were stained with 5 µl of PE Annexin V Apoptosis Detection Kit (BD Biosciences, NJ, USA) and Propidium Iodide Staining Solution (BD Biosciences, NJ, USA) and incubated for 30 minutes at room temperature. The cells were washed three times by centrifugation at 1500 rpm for 5 minutes and resuspended into 300 µl of ice cold FACS buffer with 4% formaldehyde solution for fixation for 10 minutes. Cells were washed with FACS buffer and resuspended into 300 µl FACS buffer. Cell populations were analyzed by the FACS Aria (BD Biosciences, NJ, USA). For each variable tested, three independent experiments were performed.

**IL-1α, IL-6, MMP-9 ELISA**

Keratinocytes were cultured in 24-well plates and treated with extracts of clean air or THS 0%, 50%, 75%, and 100% dilutions for 24 hours. The supernatant was collected and analyzed by a Human IL-1α Quantikine ELISA Kit (R&D, MN, CA), an ELISA MAX Deluxe Set Human IL-6 (BioLegend, SD, CA), or a Human MMP-9 Quantikine ELISA Kit (R&D Systems, MN, USA) following a 1:10 dilution. For each concentration tested, three independent experiments were performed.

**Keratin 5 Immunocytochemistry and Western Blot**

For immunocytochemistry analysis of keratin 5, keratinocytes were cultured in Ibidi 8-well chambers (Ibidi, WI, USA) and treated with extracts of clean air or THS 0%, 50%, 75%, and 100% dilutions for 24 hours. Cells were fixed with 4% formaldehyde solution for 10 minutes. The fixation solution was removed and cells were washed three times with PBS(-). For cellular permeabilization, a 0.1% Triton X-100 (in PBS) solution was used for 15 mins. The permeabilization solution was removed, and cells were washed three times with PBS(-). Cells were stained with primary rabbit anti-human cytokeratin 5 antibody (Santa Cruz Biotechnology, TX, USA) and primary goat anti-human acetylated α-tubulin antibody (Santa Cruz Biotechnology, TX, USA) overnight in a 4$^{o}$C refrigerator. The primary antibodies were removed and replaced with secondary mouse anti-rabbit IgG-FITC (Santa Cruz Biotechnology, TX, USA) and mouse anti-goat IgG PerCP (Santa Cruz Biotechnology, TX, USA) for 2 hours at room temperature. Cells were washed three times with PBS and protected with Mounting Medium with DAPI (Abcam, Cambridge UK). Fluorescent images were taken with a TI inverted Nikon Eclipse microscope (Nikon, NY, USA).

For western blot analysis of keratin 5, keratinocytes were cultured in 6-well plates and treated with with extracts of clean air or THS 0%, 50%, 75%, and 100% dilutions for 24 hours. Western blot analysis was performed as previously published[21]. To summarize, cell lysate was harvested using RIPA Lysis and Extraction Buffer (ThermoFisher Scientific, MA, USA), and protein concentration

was determined by Pierce BCA Protein Assay Kit (ThermoFisher Scientific, MA, USA) following the company's protocol. Protein concentration was equalized to 50 μg for all treatments for each well. The primary antibody was rabbit anti-human cytokeratin 5 antibody (Santa Cruz Biotechnology, TX, USA) and the secondary antibody was mouse anti-rabbit IgG-HRP (Santa Cruz Biotechnology, TX, USA). Blot images were collected using a BioRad Chemidoc XRS+ System (BioRad, CA, USA). Band intensities were normalized against the loading control to determine significant interactions.

**Statistics and Graphs**

All statistics and graphs were performed using Prism 9.0 (GraphPad, CA, USA). For experiments with only one factor (MTT assay, ELISA's, and glutathione assay), a one-way ANOVA with a Bonferroni's post-hoc test was utilized in which data in all groups were compared with one another. For experiments with two factors (live cell imaging, mitochondrial morphology, flow cytometry, and western blot), a two-way ANOVA with a Bonferroni or Dunnett's post-hoc test was used. Data were checked to determine if they satisfied the assumptions of ANOVA (homogeneity of variances and normal distribution). Means and standard deviations are reported. Statistical significance was represented as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

# Results

### Mitochondrial Reductase Activity in THS Treated Keratinocytes (MTT Assay)

To evaluate the effect of THS on mitochondrial activity, keratinocytes were treated with clean air or THS extracts at dilutions of 0%, 25%, 50%, or 100% for 3 and 24 hours. The clean air 100% 24-hour vs THS 100% 24-hour comparison was significant ($p<0.03$) (Figure 3.1A).

### Effect of THS on Keratinocyte Growth (Live Cell Imaging Analysis)

To examine the effect of THS on growth, keratinocytes were exposed to clean air or THS extracts of 50% or 100% for 48 hours in a BioStation CT, which collected images of live cells every 15 minutes. Video bioinformatics software was used to analyze keratinocyte area (measured in pixels) for each timepoint and treatment. Between 9-hours and 48-hours, the pixel area of cells in the THS 100% was significantly decreased compared to the clean air 100% ($p<0.05$) (Figure 3.1B-C). At 48-hours, the control/clean air 50% vs THS 50% was significant ($p<0.01$), as was the control/clean air vs THS 100% ($p<0.0001$) (Figure 3.1D). At 48-hours, the normalized number of cells/field was significant in the control/clean air 50% vs THS 50% ($p<0.01$, $p<0.05$) and control/clean air 100% vs THS 100% ($p<0.001$, $p<0.01$) comparisons (Figure 3.1E).

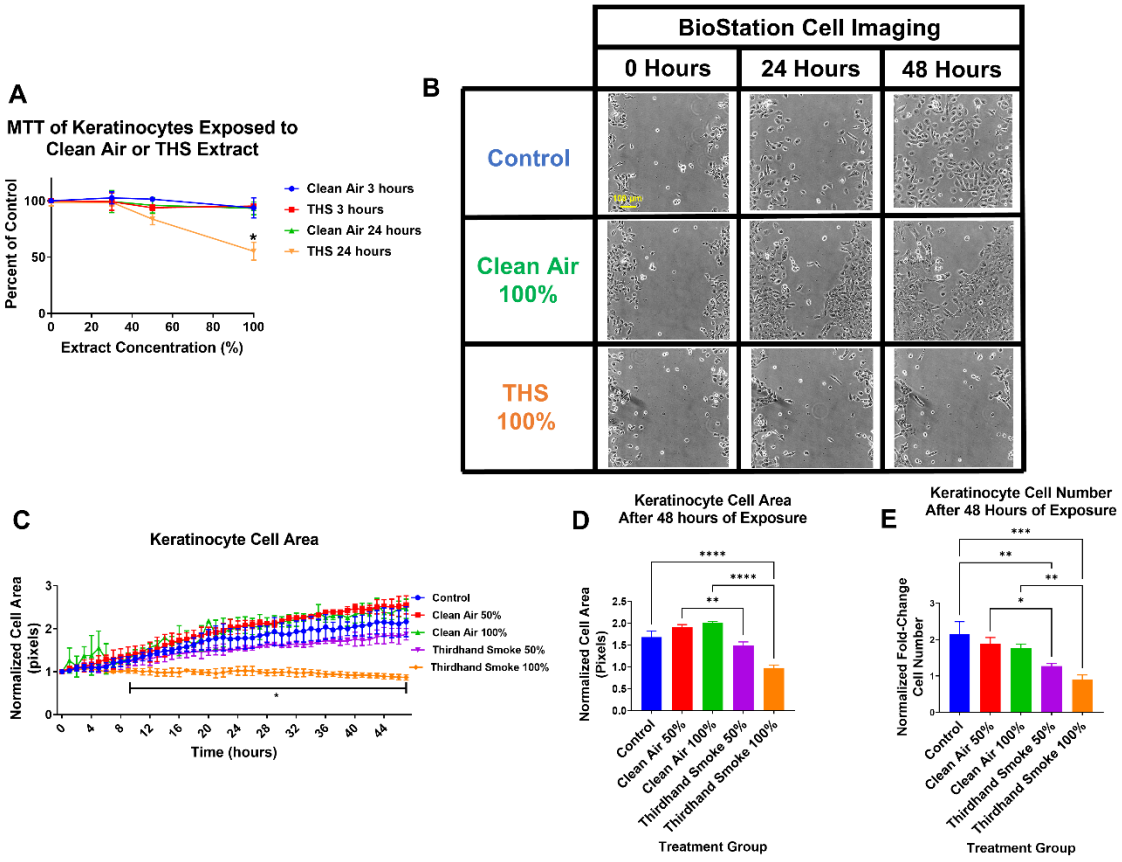


**Figure 3.1. Keratinocytes exposed to THS had reduced cellular metabolism, cell movement, and total cells after 24-48 hours. A**. MTT assay of keratinocytes exposed to THS extract at 25%, 50% and 100% dilutions for 3 and 24 hours. **B**. Keratinocytes were treated with THS extract for 24 hours and monitored in the BioStation CT for 48 hours with time-lapse and fluorescent images taken every 15 mins. **C**. Normalized fold-change of cell area (measured in pixels) per field of view was analyzed by CLQuant of keratinocytes treated with clean air or THS extract over 48 hours. **D**. Normalized percentage cell area (as measured in pixels) per field of view was analyzed by CLQuant of keratinocytes treated with clean air or THS extract after 48 hours. **E**. Normalized fold-change of total cell count as measured by DAPI fluorescent imaging per field of view of keratinocytes treated with clean air or THS extract after 48 hours. Each result is the mean ± standard deviation of three independent experiments. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001.

**Effect of THS on Mitochondrial Phenotype**

Healthy keratinocytes normally have networked mitochondria (Figure 3.2A/E), which can undergo fission forming the punctate phenotype. In the 50%, 75%, and 100% THS treatments, mitochondria fragmented and become swollen (Figure 3.2A-H). The percentage of punctate mitochondria increased significantly in the THS 75% compared to the clean air 75% ($p<0.02$), while the percentage of networked mitochondria significantly decreased in the THS 50% ($p<0.02$), THS 75% ($p<0.008$), and THS 100% ($p<0.0001$) compared to their respective clean air dilutions (Figure 3.2I). The average mitochondrial network size (measured in pixels) decreased in all the THS treated groups compared to their control (Figure 3.2J). The clean air treatment had no effect on mitochondrial phenotypes.

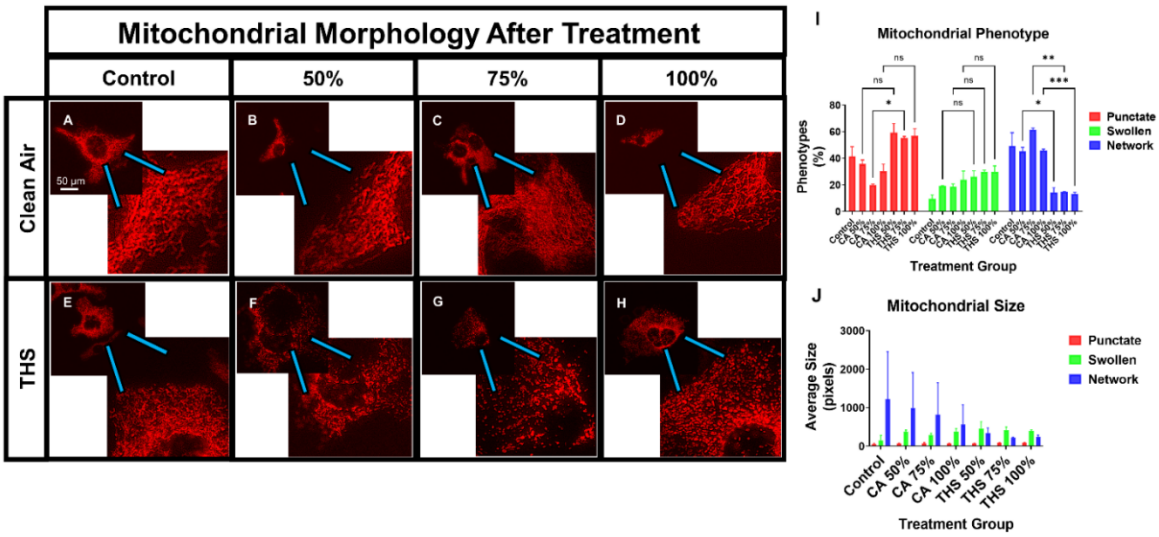


**Figure 3.2. Keratinocytes demonstrated mitochondrial fragmentation and punctate phenotype after 24 hours of THS treatment**. **A**. Keratinocytes in control media after 24 hours. **B-D**. Keratinocytes exposed to clean air extract concentrations of 50%, 75%, and 100% treatment, respectively. **E**. Keratinocytes in control media after 24 hours. **F-H**. Keratinocytes exposed to THS extract concentrations of 50%, 75%, and 100% treatment, respectively. **I**. The percentage of mitochondrial phenotypes (punctate, swollen, or networked) after 24 hours of clean air or THS extract treatment. The percentage of swollen mitochondria increased, while the percentage of networked mitochondria decreased in the THS treated groups. **J**. The average mitochondrial size (pixels) of the three mitochondrial phenotypes (punctate, swollen, or networked) were analyzed in MitoTimer-labeled cells after 24 hours of clean air or THS extract treatment. The networked size of mitochondria decreased due to fragmentation in the THS treated groups. Each result represents the mean ± standard deviation of three independent experiments. * p≤0.05, ** p≤0.01, *** p≤0.001.

**THS Increased Superoxide**

Keratinocytes were loaded with MitoSox (which measures superoxide oxidation) to determine if the THS 24-hour treatment increased oxidative stress. In the THS 100% treatment, MitoSox fluorescence increased in the mitochondria and nuclei (Figure 3.3A). In the THS 100% treatment, MitoSox fluorescence was significantly elevated in two of the separate cellular phenotypes: THS 100% cells with the nuclear oxidation but the nucleus was removed from the analysis ($p<0.002$), and cells with mitochondrial and nuclear oxidation ($p<0.0001$) compared to the clean air 100% treatment (Figure 3.3B). Mitochondrial oxidation was not increased in the clean air treatment.

**DNA (8-OHdG) Oxidation was Increased in THS Treated Keratinocytes**

Keratinocyte DNA was isolated from cells and analyzed by an 8-OHdG ELISA to determine if DNA oxidation was elevated in the THS 24-hour treatment. 8-OHdG concentrations was significantly elevated in the THS 50% ($p<0.0001$) compared to the clean air 50%, THS 75% ($p<0.0001$) compared to the THS 75% treatment, and THS 100% ($p<0.0001$) compared to the clean air 100% (p,0.0001) (Figure 3.3C). There were no differences in the clean air treatment.

**Total Glutathione Decreased in THS Treated Keratinocytes**

Protein lysates were isolated from treated keratinocytes, and total glutathione activity was measured using a glutathione detection kit. Total glutathione was normalized to BCA concentrations. A significant reduction of total glutathione (µg/mg) was observed in the control compared to the THS 75% vs clean air 75% ($p<0.006$), and THS 100% vs clean air 100% ($p<0.0001$) treatments (Figure 3.3D).

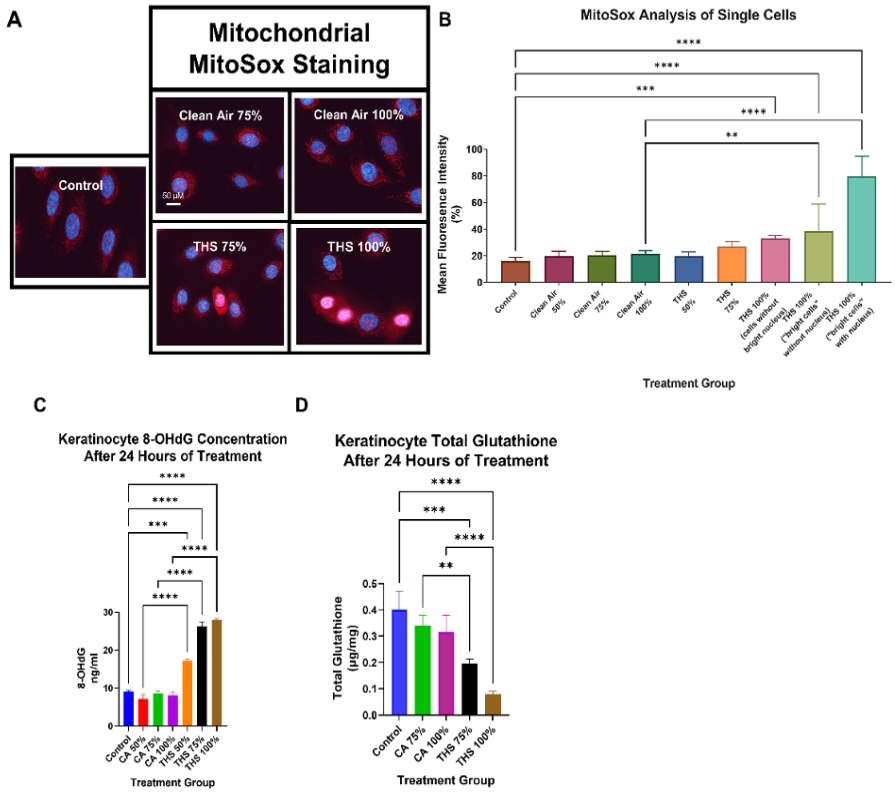


**Figure 3.3. Keratinocytes exposed to THS extract for 24 hours had increased mitochondrial and nuclear oxidation, increased 8-OHdG, and a depletion of the anti-oxidant glutathione**. **A**. Fluorescent images of THS or clean air extract treated keratinocytes after 24 hours then labeled with MitoSox. **B**. Measurement of the mean fluorescent intensity of MitoSox labeled single cells. **C**. 8-OHdG (ng/mL) concentration in keratinocytes treated with clean air or THS extract for 24 hours. **D**. Total glutathione (µg/mg) normalized to total protein (mg) in keratinocytes treated with clean air or THS extract for 24 hours. Each result represents the mean ± standard deviation of three independent experiments. ** p≤0.01, *** p≤0.001, **** p≤0.0001.

**Cell Death Analysis Using Flow Cytometry Analysis**

Keratinocytes treated with clean air or THS extracts for 24 hours were labeled with annexin-5 and propidium iodide to determine if cell death and apoptosis increased after treatment. The total number of double positive keratinocytes increased slightly in the THS 75% and 100% treatments, but most cells remained negative in all treatments (Figure 3.4A). The number of negatively stained cells was not significantly changed across all treatments. As a consensus the THS concentrations are relatively non-lethal to the cells, but rather affect metabolism or other survival pathways.

**THS Increased Release of IL-1α, IL-6, and MMP-9**

Keratinocytes contain IL-1α in their cytoplasm and upon chemical irritancy, extracellularly release it to recruit immune cells and to proceed with the wound healing process[22,23]. Keratinocytes released IL-1α into the culture medium after 24 hours of 100% THS treatment compared to the clean air 100% ($p<0.0001$) (Figure 3.4C). IL-6 is a pro-inflammatory cytokine; its release is stimulated by IL-1α receptor activation[24]. Keratinocytes released significant amounts of IL-6 after 24 hours of THS treatment in the 50% ($p<0.0001$), 75% ($p<0.0001$), and 100% ($p<0.0001$) treatments compared to their respective clean air treatments (Figure 3.4D). MMP-9 is released by keratinocytes during wound healing[25] and degrades extracellular collagen type IV to allow the transmigration of immune cells during inflammation[26]. MMP-9 release was significant after 24 hours of exposure to THS

50% ($p<0.0001$), 75% ($p<0.0001$), and 100% ($p<0.0001$) treatments compared to their respective clean air controls. (Figure 3.4E).

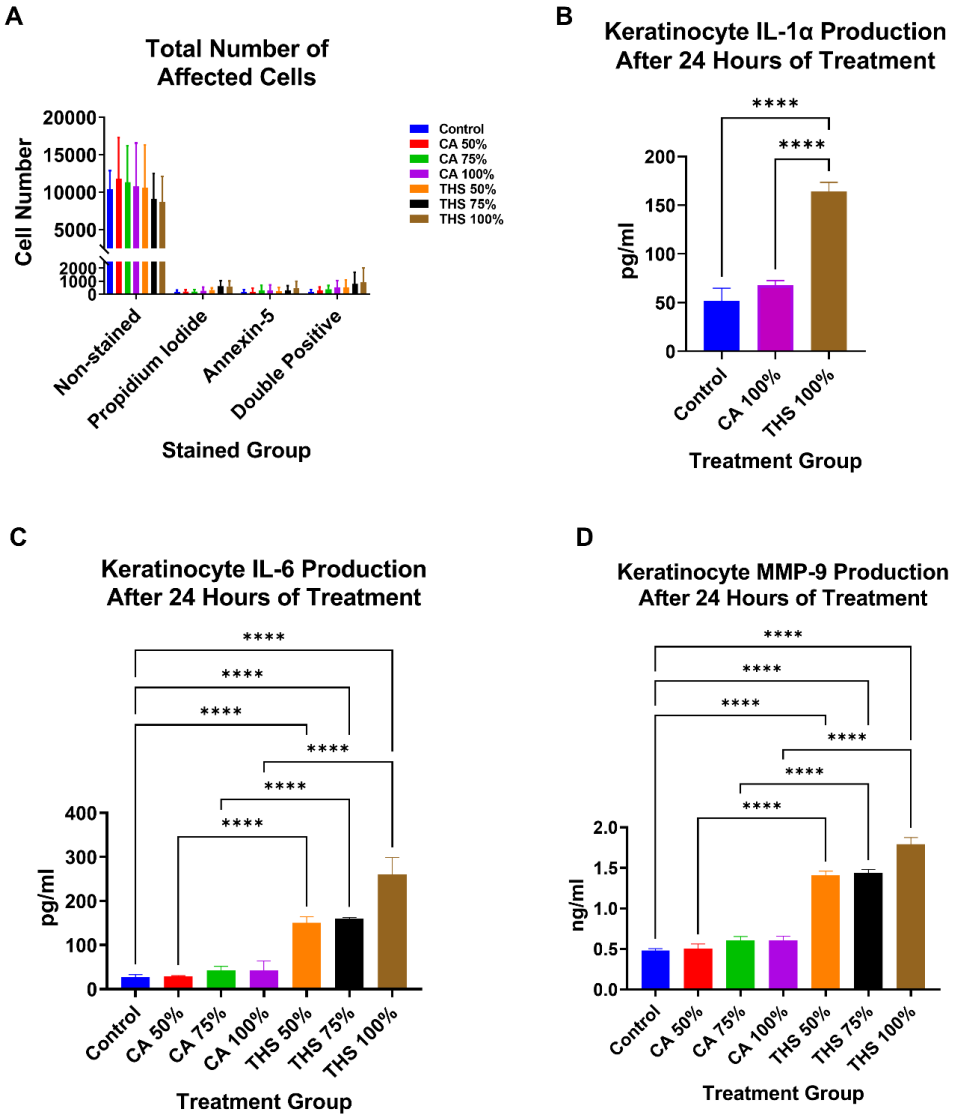


**Figure 3.4. Keratinocytes treated with clean air or THS extract for 24 hours did not have increased cellular apoptosis and death, but THS exposure was harmful enough to release inflammatory mediators IL-1α, IL-6, and MMP-9. A**. There was no significant increase in the total number of apoptotic or dead cells in the THS treated groups. **B**. IL-1α was significantly increased 100% dilution THS treatment. **C**. IL-6 was significantly increased of keratinocytes exposed to THS 50%, 75%, and 100% concentrations. **D**. MMP-9 production was significantly released in the THS treated keratinocytes in a dose-dependent manner. Each result represents the mean ± standard deviation of three independent experiments. **** p≤0.0001.

**Keratin 5 Assays**

Keratin 5 plays a role in regulating keratinocyte growth and differentiation. It is also elevated in some inflammatory-skin diseases, such as psoriasis[27] and atopic dermatitis[28]. After 24-hours, THS treatment immunohistochemistry staining showed increased keratin 5 fluorescence (Figure 3.5A). Western blotting was performed to quantify these results. Keratinocyte protein lysates were collected after 24-hours of treatment, and keratin 5 was normalized to GAPDH concentration. The fold-change for every treatment was normalized to its control. The THS 100% treatment significantly elevated keratin 5 expression ($p<0.001$) when compared to the clean air 100% treatment (Figure 3.5B). In atopic dermatitis patient's skin biopsies, there was an overexpression of keratin 5 (K5)[28] which can cause dysregulated skin growth[29].

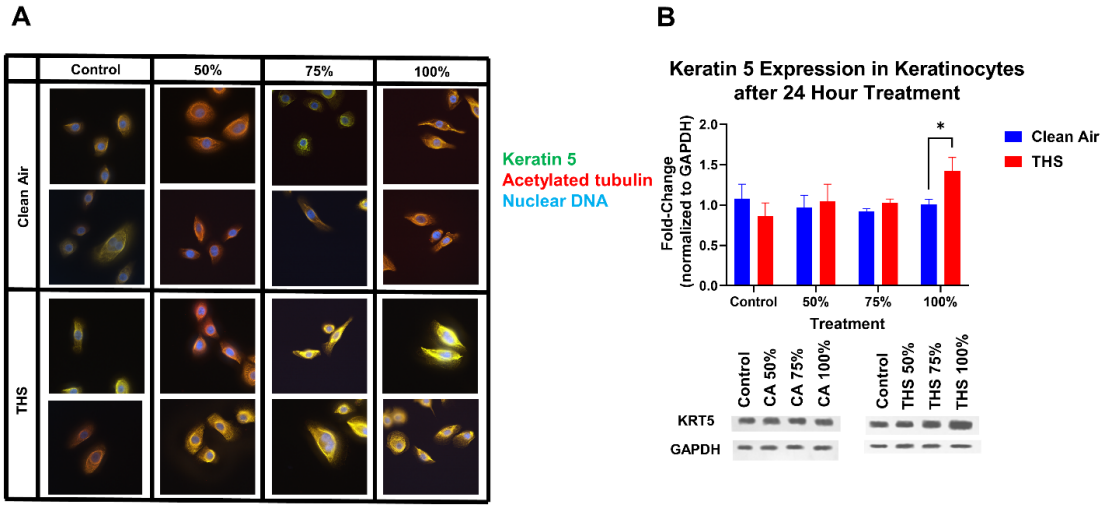


**Figure 3.5. Keratin 5 expression was significantly increased in keratinocytes by the 100% THS extract.** **A**. Immunohistochemistry of keratinocytes stained for keratin 5, acetylated tubulin, and DAPI after treatment with clean air or THS extract for 24 hours. **B**. Western blot analysis for keratin 5 normalized to GAPDH for keratinocytes treated with clean air or THS extract for 24 hours. THS treatment increased keratin 5 in a dose-dependent manner. Each result represents the mean ± standard deviation of three independent experiments. * p≤0.05.

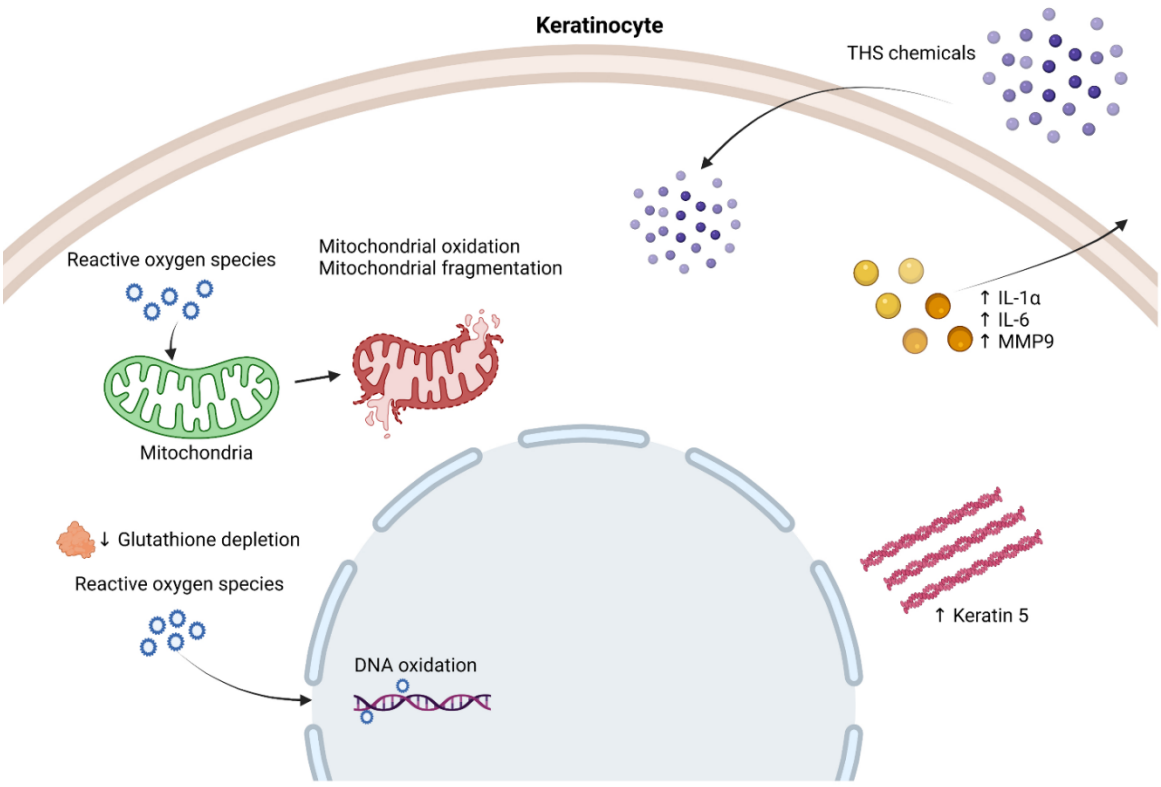


**Figure 3.6. Keratinocyte exposure to THS extract increased mitochondrial and nuclear oxidation, glutathione depletion, mitochondrial fission, release of inflammatory mediators, and elevated keratin 5.** These are molecular risk factors for inducing skin diseases such as atopic dermatitis, psoriasis, and skin cancer.

## Discussion

This study provides insight to the mechanisms of keratinocyte responses to THS. Acute 24-hour THS exposure reduced keratinocyte migration, elevated oxidative damage, depleted anti-oxidant concentrations, caused mitochondrial dysfunction (fragmentation), released inflammatory mediators, and elevated keratin 5. These responses are similar to what was seen in THS-exposed mice[4] and pathways modified in inflammatory-skin diseases[30]. The results of this study demonstrate the potential for THS to initiate skin disease.

THS-exposed keratinocytes had reduced migration and cell counts after 48 hours, similarly keratinocyte migration was reduced in the wound healing process which was attributed to nicotine and tobacco exposure[31]. Mitochondria play a critical role in keratinocyte regulation[32] and our results from the mitochondrial reductase activity assay demonstrated keratinocytes had reduced mitochondrial metabolism when exposed to the highest concentrations of THS. Furthermore, THS-exposed keratinocytes displayed fragmented mitochondria after 24 hours. Young, healthy keratinocytes possess networked mitochondria, but display fragmented mitochondria in damaged and stressed skin[33]. Cigarette smoke exposure can induce mitochondrial fragmentation in bronchial epithelial cells, a process regulated by mitochondrial fission proteins Drp1 and Fis1[34]. Mitochondrial fission occurs in highly damaged mitochondria a process which can be attributed to oxidative stress[35].

Our results showed increased mitochondrial and nuclear superoxide oxidation in THS-exposed keratinocytes. Keratinocyte mitochondrial impairment can be attributed to elevated levels of superoxide[36]. We saw elevated levels of 8-OHdG, a biomarker of DNA oxidation, and lowered the concentration of anti-oxidant glutathione demonstrating THS causes keratinocytes to become vulnerable to oxidative damage. The skin of THS-exposed mice had elevated levels of oxidative damage and a depletion of glutathione[4], confirming the results from our study. Clinical evidence has shown cigarette smoking causes depletion of antioxidants[37] and psoriasis patients skin biopsies showed reduced antioxidant levels compared to healthy patients[38]. Mechanistically ROS-induced mitochondrial damage releases cytochrome C from their intermembrane space triggering an enzymatic cascade leading to cellular apoptosis[39].

Increased apoptosis and cell death was observed in the THS-treated keratinocytes, although the total number of dead cells was relatively low. Another study demonstrated cells treated with THS lost their mitochondrial membrane potential and began the apoptosis process[7]. This result is significant because epidermal apoptosis has been documented in psoriatic patients[40] and because mitochondrial damage plays a critical role in the development of skin disease[41]. Keratinocyte apoptosis generates an inflammatory response which is connected to multiple skin diseases[42].

Inflammatory mediators IL-1α, IL-6, and MMP-9 were increased in the supernatant of THS-exposed keratinocytes. Keratinocytes store alarmin IL-1α in

their cytosol, but when their membrane becomes ruptured, release the cytokine into the surrounding area[43]. The release of IL-1α also stimulates IL-6 production in epithelial cells[24], and this indirectly recruits innate immune cell infiltration, collagen deposition, angiogenesis, and keratinocyte proliferation *in-vivo*[44]. The release of MMP-9 causes extra-cellular degradation which allows the transmigration of immune cells in the epidermis[26]. MMP-9 degrades collagen, a structural extracellular matrix protein, in the skin which reduces epidermal tissue tensile strength[45]. Collectively, the release of these inflammatory mediators indicates that THS exposure can cause skin inflammation.

Cigarette smoking increases the risk of developing skin diseases[46] such as psoriasis[47] and atopic dermatitis[48], and while THS contains some of the dangerous chemicals from cigarette smoke[2], we show further proof THS is a dermatological hazard. Our study demonstrated an increase of keratin 5 (K5) in THS-exposed keratinocytes. In inflammatory skin diseases such as atopic dermatitis there is a marked increase of K5 in the diseased skin[28]. K5 and keratin 14 (K14) are molecular regulators of keratinocyte proliferation and differentiation that signal through a phosphatidylinositol 3-kinase (PI3K)/Akt mediated pathway[29]. Normally, the K5/K14 co-pair is expressed in the basal epithelia and gradually decreases as keratinocytes proliferate and differentiate[29]. However, the dysregulation of these keratins contributes to the pathomechanisms of inflammatory skin diseases[49].

## Conclusion

To the best of our knowledge, this is the first *in vitro* human keratinocyte THS exposure study to demonstrate molecular pathways affected by THS. Acute exposure to THS extracts produced adverse effects in human keratinocytes that resemble those reported previously for mainstream cigarette smoke. We exposed keratinocytes to THS extracted from cotton fabric that mimicked one month's exposure from a pack a day cigarette smoker which would represent a realistic scenario of a person visiting a THS contaminated building, such as a casino or a smoker's home. Effective concentrations of THS did not kill cells but altered biological processes that resulted in a proinflammatory response, increased oxidative stress, mitochondrial damage, and K5 elevation (Figure 3.6). These types of responses, if chronic, could lead to skin diseases, such as atopic dermatitis, psoriasis, and skin cancer, which have been reported to be elevated in cigarette smokers. Our data may aid in the development of regulatory policies dealing with remediation of indoor environments contaminated with THS and may allow expansion of policies that limit indoor use of tobacco products.

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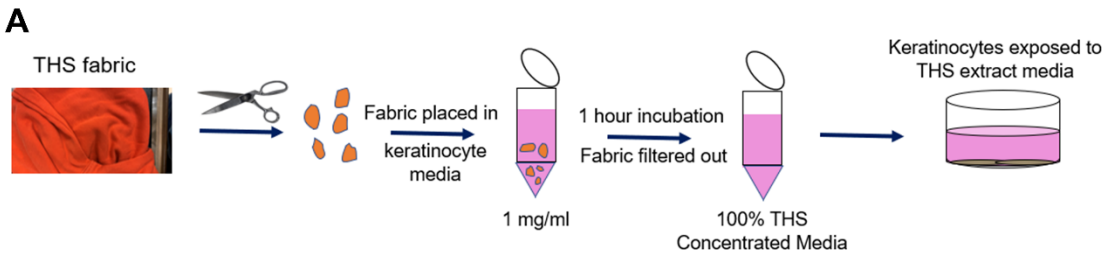
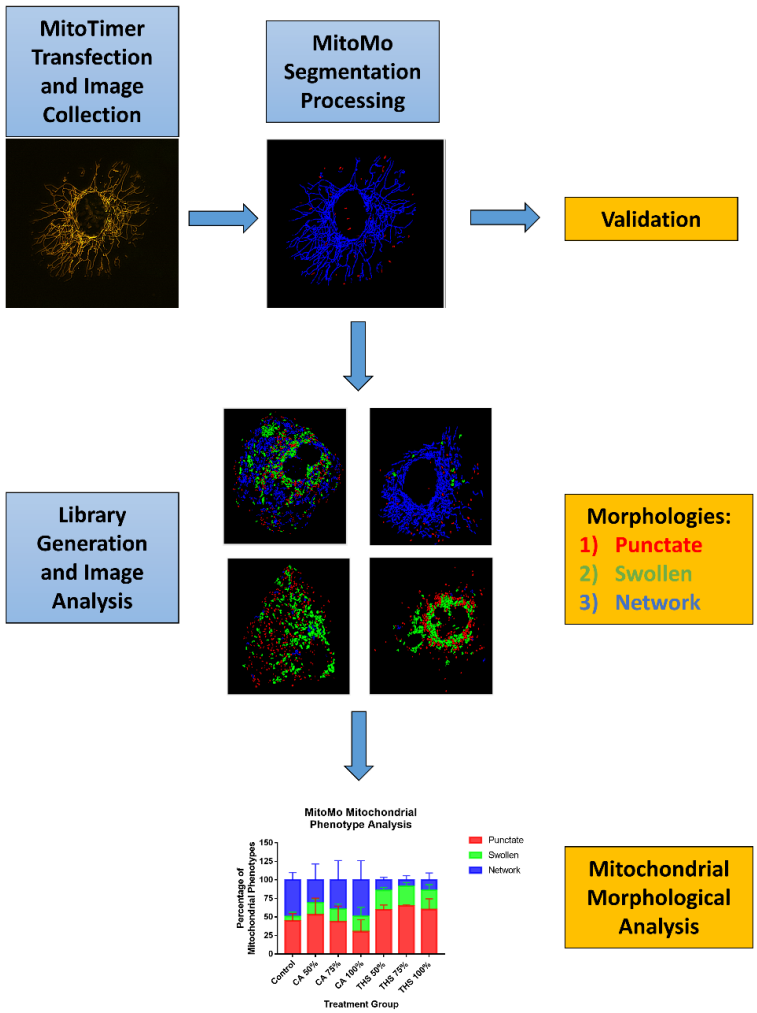
## Supplementary Information




**Supplementary Figure S3.1. Flow diagram showing the preparation of the clean air or THS extract media, and treatment of keratinocytes with the clean air or THS extract media and endpoint assays. A.** The clean air or THS fabric was extracted at a concentration of 1 mg of fabric/ 1 mL of keratinocyte medium creating a 100% concentration of extract medium, which was filtered, aliquoted, and diluted to the concentrations used for experiments. **B.** Keratinocytes were treated with the clean air or THS extract media for 48 hours for the BioStation live imaging experiment or treated for 24 hours for all other assays.




**Supplementary Figure S3.2. Flow diagram of the mitochondrial phenotype experiment.** Keratinocytes were transfected with MitoTimer plasmids to visualize the mitochondria. Then, they were treated with clean air or THS extract for 24 hours, and fluorescent images were taken. MitoMo software was utilized for segmentation and tracking of mitochondrial morphologies, which were punctate, swollen, or network phenotypes. The MitoMo software was trained to automatically detect the three mitochondrial morphologies and a collection of images were re-verified manually to ensure accurate results. Mitochondrial morphological analysis was performed from the data generated from using MitoMo.

## Conclusion

Our data show for the first time that EC and tobacco cigarette use elevate oxidative stress as measured by the biomarkers 8-OHdG (DNA oxidation) and 8-isoprostane (lipid oxidation). The biomarker of metal exposure, metallothionein, was elevated in both smoking groups. In both EC users and cigarette smokers, total metal concentrations correlated with increased oxidative DNA damage. EC users were exposed to elevated levels of selenium and zinc, and zinc concentrations correlated with oxidative DNA damage. In the cigarette smoking group, the cardiac biomarker of harm, NT-proBNP, was elevated. These data demonstrate that both EC use and cigarette smoking cause oxidative damage and metal exposure, while increased zinc exposure correlated with 8-OHdG concentrations in EC users, and cigarette smoking elevated a biomarker of cardiac harm.

We previously demonstrated that firsthand smoking elevated oxidative and cardiac harm. We now also show that acute dermal exposure to THS increased oxidation of DNA, lipids, and proteins. Plasma proteomics pathway analysis revealed that THS exposure activated a pro-inflammatory immune response,

increased oxidative stress, and elevated biomarkers associated with skin diseases, such as contact dermatitis and psoriasis. Pathways associated with other cigarette-induced diseases not involving the skin, such as atherosclerosis and cancer, were also elevated in THS-exposed subjects. Our results provide the first molecular evidence that the concentrations of proteins involved in disease pathways change in humans after acute dermal exposure to THS and with prolonged exposure could lead to disease progression.

Based on our previous discoveries, we performed the first research to characterize the response of human keratinocytes to THS *in-vitro*. We discovered that human keratinocytes exposed to THS extract for 24 hours activated the pathways associated with inflammatory skin diseases. THS treatment did not kill cells but altered biological processes that resulted in a proinflammatory response, increased oxidative stress, mitochondrial damage, and Keratin-5 elevation. This study demonstrates that human keratinocytes respond to THS in a manner that may lead to skin diseases, such as atopic dermatitis, psoriasis, or skin cancer.

Collectively, our studies aid in understanding the molecular effects of THS on the skin and the biomarkers of exposure and harm in EC users and cigarette smokers. Also, our data demonstrate that EC vaping and cigarette smoking are not harm free and support the conclusion that prolonged may elevate oxidative stress and lead to disease progression. Cigarette smoking caused an elevation of a cardiac stress marker. Our data may aid in the development of regulatory policies

dealing with remediation of indoor environments contaminated with THS and allow expansion of policies that limit indoor use of tobacco products.