Title
Elucidating the Role Genotoxic Susceptibility Plays in Pulmonary Diseases of the Mouse and Human

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Publication Date
2013

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Elucidating the Role Genotoxic Susceptibility Plays in Pulmonary Diseases of the Mouse and Human

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Toxicology

by

Aaron Chapman

2013

ABSTRACT OF THE DISSERTATION

Elucidating the Role Genotoxic Susceptibility Plays in Pulmonary Diseases of the Mouse and Human

by

Aaron Chapman

Doctor of Philosophy in Molecular Toxicology

University of California, Los Angeles, 2013

Professor Robert H. Schiestl, Chair

Assessing the ability of diseases states and/or toxicants to cause genomic perturbations is an underpinning of science that should never be overlooked. In human and murine disease models genomic changes that occur many times exacerbate diseases and cause long term effects. Understanding how genomic perturbations are caused by various disease states independent of external exposure as well as toxicant induced genomic changes and how these perturbations can be used as predictive indicators of disease prognosis is a niche which science should investigate. Many diseases and toxicants can cause genomic changes, however I will present work elucidating i) the effects of side-stream tobacco smoke and smoke extract on glutathione-and oxidative DNA repair deficient mice and blood cells ii) the increased susceptibility to cigarette smoke induced genotoxicity in smoking and non-smoking lung cancer patients iii) the genotoxicity caused by asthma in the CC10-rtTA-IL13 transgenic (TG) over-expressing mouse model.

The dissertation of Aaron Chapman is approved by.

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2013

Thank you LORD.

In Loving Memory of: PawPaw Eugene, Mama Gert, Big Papa Eli, Mama Adele, Antie Elnora and Mrs. Dorothy Matthews

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Acknowledgements

I first will like to thank my Lord and Savior Jesus Christ for being my rock and my salvation even when times seemed bleak HE was my foundation and my continued source of strength.

I thank my advisor Dr. Robert Schiestl for his continued support and guidance through my time as a UCLA graduate student. I thank the members of my thesis committee Dr. Jesus Araujo, Dr. Oliver Hankinson, and Dr. Steve Dubinett for their help and guidance.

I thank the past and present members of the Schiestl lab team, especially those mentioned below. I thank Drs. Lynn Yamamoto, Zorica Scuric, and Aya Westbrook for being unselfish mentors who helped guide me down the correct path even when I had many probing scientific questions that seemed to never end . I thank Dr. Liuba Parfenova for constantly being kind and patient with me even when I waited to the last minute to place large orders. I want to thank Dr. Heather Tarleton for being both a spiritual and academic mentor. I thank the past and current members of "Team Get Aaron out of graduate school" consisting of Kristin Yamada, Dr.Sarah Kobylewski, Anthony Nyguen, Meng Deng, Sarkis Aroyan, Jared Liu, Ryan Bakhit, Danny Malkin, Jessica Camacho, Aleksandra Durisic, Ana Scuric, Nami Moradi, and ATS statistical consultants I definitely could not have made it without all of your tireless effort and support. I want to thank all the friends and family members who were encouraging throughout my time in graduate school especially Rod & Toya Bailey who are my "California parents". I Furthermore want to thank my Grandparents Calvin & Brenda Severin, Sarah Porter, Julia Chapman, and Eddie Henderson for being the constant spiritual pillars in my life that always encouraged me to stay focused and steadfast in both my studies of school and of the LORD. I want to thank my brother Andrew Chapman for being my spiritual and comical support system who helped me

pray and laugh my way throughout the toughest times in my life. I could always count on him to keep me grounded and to tell me not to take life nor myself so seriously. Last but definitely not least I would like to thank my parents Michael and Monica Chapman for their support, prayers, words of encouragement, and both spiritual and life direction. You have been the absolute best parents a man could ask for. Thank you for the late night encouragement when I didn't think I would make it and all the little and big things you have done for me throughout my life I definitely would not have made it without you guys.

Chapter 1 is a version of a paper that is in preparation for submission: Elucidating the genotoxic effects of interleukin-13 in asthma utilizing the CC10-rtTA-IL-13 over-expressing transgenic mouse model. Co-authors include Daniel Malkin, Jessica Camacho, (Molecular Toxicology, UCLA) and Robert H. Schiestl, Ph.D (Principle Investigator, Molecular Toxicology and Environmental Health Sciences faculty, UCLA). This work could not be completed without the gracious gift of IL-13 over-expressing mice from Dr. Talal Chatila (Harvard University),Dr. Brigitte Gomperts. I am also thankful for the aid of Dr. Nora Rozengurt, Petra Wise and Ko Kiehle for mouse pathological assessment, aid in initial experimental setup, and mouse IHC staining respectively. Chapter 2 is a version of a paper that has been submitted to Mutational Research and is under review. The original version of this chapter is printed in Chapter 3 of Lynn Yamamoto dissertation entitled "Gene –Environment Interactions: Roles of DNA Repair Proteins in Preventing Disease and Injury". I conducted all *ex vivo* experiments as well as aided in smoke exposures, benzo(a)pyrene exposures, organ collection, colony maintenance and writing of manuscript. Chapter 3 is in collaboration with Dr. Steve Dubinett who provided the necessary blood samples for assessment of biomarkers. I want to especially thank the members of Dr. Dubinett's group Dr. Brian Gardner and Dr. Gina Lee who aided in blood collection,

storage, and proper sample identification. Furthermore, I would like to thank Fran Rosen for giving valuable insight on the IRB process, and Dr. Andrew Dannenburg (Cornell University) who provided cigarette smoke extract. I would like to give a very special thanks to Dr. Joni Ricks, Dr. Andy Lin, and the rest of the ATS core for their aid in statistical consultations. I was responsible for experimental design and data analysis and will write any manuscript which results from this work. Finally I would like to thank the UCLA Affiliates, Eugene V. Cota Robles fellowship, Graduate research mentorship program, UCLA ACCESS program, and Molecular Toxicology IDP program for their financial contributions to me throughout my graduate school tenure.

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Articles in Submission

Mitsuko L. Yamamoto*, **Aaron M. Chapman***, and Robert H. Schiestl Effects of side-stream tobacco smoke and smoke extract on glutathione- and oxidative DNA damage repair-deficient mice and blood cells; *Under Revision, Mutational Research.*.

Articles in Progress

Aaron M. Chapman*, Daniel Malkin*, Jessica Comacho, and Robert H. Schiestl. Elucidating the genotoxic effects of interleukin-13 in asthma utilizing the CC10-rtTA-IL-13 over-expressing transgenic mouse model. *In Preparation.*

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Part I: Introduction to DNA Repair and Diseases

Maintaining genomic integrity is imperative to the life of the cell. The basic premise being that the survival of the entire organism is dependent on the accurate transmission of genetic material from one daughter cell to the next.[1] In order to secure proper survival it is imperative the cell employs processes that protect, as well as assist in maintaining proper genomic integrity and cellular function; this is accomplished through various proteins and pathways that are collectively grouped as DNA repair mechanisms. These repair mechanisms refer to a collection of processes, by which a cell identifies and corrects damage to the DNA molecules that encode its genome. [2] On a normal basis the genome of a typical mammalian cell accumulates many thousands of lesions caused by endogenous and exogenous perturbations during a 24-hour period. Endogenous insults to DNA can be caused in various ways including reactive oxygen species (ROS) induced oxidation that is generated from normal metabolic processes, internal alkylation agents such as S-adenosylmethionine, reactive carbonyl species induced by bulky adduct caused by attack formed during lipid peroxidation, inflammation related processes, hydrolytic depurination leading to the formation of abasic sites, or base deamination [3] Exogenous insults include various forms of radiation, chemicals, disease states, and toxic agents such as cigarette smoke. However, as a result of DNA repair, less than 1 in 1000 of these endogenous or exogenous insults causes a mutation. [2, 3] Therefore, DNA repair is central to maintaining normal cellular homeostasis, and defective DNA repair is one of the factors responsible for progression to carcinogenesis.[4] Repair genes can be grossly categorized into five well-characterized repair pathways that recognize and aid in the removal of specific DNA perturbations : base excision repair, nucleotide excision repair, mismatch repair, double-strand break repair (including homologous recombination and non-homologous end joining), and transcription-coupled repair[5] .Generally the cellular responses elicited by organisms to DNA

damage fall into three major categories: direct reversal of damage, excision of damage, and post-replication repair. [5] the Deficiencies in any number of these repair pathways decrease DNA repair capacity and are associated with a greater than two fold increase in the risk of many cancers. [5] In this work we will discuss few key proteins that fall within these pathways.

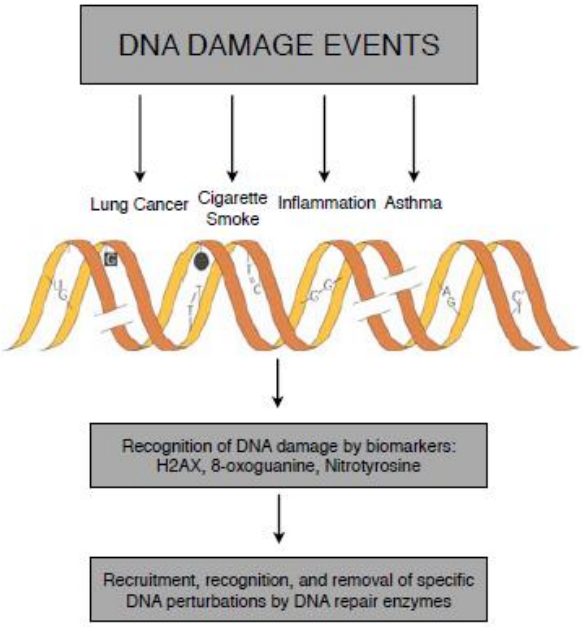

Fig.1 DNA damaging agents such as cigarette smoke or disease states such as asthma or lung cancer cause damage that can be recognized by biomarkers. DNA picture © 2001 Nature Publishing Group Hoeijmakers, J. H. J. Genome maintenance mechanisms for preventing cancer. *Nature* **411,** 366–374 (2001) doi:10.1038/35077232.

Base Excision Repair

Base Excision Repair (BER) is a process involving the removal of a single damaged DNA base by a DNA glycosylase-type enzyme that recognizes specific mutation and removes the affected base by cleavage of the N-glycosyl bond. [2, 6, 7] The base removal causes an intact sugar phosphate back bone with an apurinic or apyrimidinic also called an abasic site at the affected position in the DNA. [2, 6, 7] Depending on the redox status of the abasic site short patch base excision repair will proceed with the incorporation of one nucleotide or long patch base excision will be performed with the incorporation of 2-10 nucleotides. [6] The site is then repaired by additional steps including DNA backbone incision, gap filling, and ligation[7]

Nucleotide Excision Repair

Nucleotide excision repair (NER) is a collection of repair processes that recognize and repairs bulky adduct forming DNA lesions that cause distortions in the helical structure of DNA. [2] In this process a multisubunit enzyme simultaneously hydrolyzes two phosphodiester bonds on the legion containing DNA strand on either side of the distortion producing a large missing fragment of nucleotides. The gap is then filled and ligated.[2, 8]

Mismatch repair

Mismatch repair (MMR) is a highly conserved process in which unpaired and mismatched paired bases generated during DNA replication, spontaneous or induced base modifications, and during recombination that have escaped proof-reading are repaired. [9, 10] The unaffected DNA strand is used as the template and is hemimethylated in a short sequence by an adenine specific labeling enzyme for strand discrimination. [2, 9]Replication mismatches in close proximity of this hemimethylated sequence are repaired according to the sequence of the template strand. [2]

Homologous Recombination and Non-homologous end joining

Homologous recombination (HR) and non-homologous end joining (NHEJ) are DNA repair pathways that are important in recognition and removal of the most lethal type of DNA strand break that occurs from endogenous or exogenous factors the DNA double strand break. [11-14](HR) repairs DNA double strand breaks by exchanging genetic information between the damaged DNA strand and the undamaged sister strand.[12] This results in the precise repair of the DNA lesion but requires the usage of homologous sequences elsewhere in the genome such

as a sister chromatid or homologous chromosome.[13] Conversely (NHEJ) does not require the use of homologous sequences present in the genome. (NHEJ) can roughly be divided into stages that include end detection in which a protein complex binds the end of double strand break, recruits other proteins to the site of the break and tether the DNA in a synaptic complex, processing the ends of the DNA and filling of the gaps by ligation.[11]

Maintaining genomic integrity is imperative; therefore mutations, deficiencies, or ablations in genes or proteins associated with repair pathways decrease DNA repair capacity, and cause susceptibility to environmental toxins such as cigarette smoke. Furthermore, improper maintenance of genomic integrity can also lead to susceptibility of debilitating diseases such as asthma and lung cancer to cause DNA damage. In the subsequent chapters I will describe studies aimed at i) Elucidating the role interleukin-13 overexpression plays in the genotoxicity of asthma ii) Investigating the Effects of side-stream tobacco smoke and smoke extract on glutathione- and oxidative DNA damage repair-deficient mice and blood cells iii) Assessing biomarkers of DNA damage to determine susceptibility to develop lung cancer.

Asthma is a chronic obstructive lung disease characterized by chronic inflammation of the airways and recurrent bronchospasms ranging from mild to debilitating.[15] It is well established that Interleukin-13 (IL-13) serves as a major mediator of the asthmatic process. [16]The pathophysiological characteristics of asthma predominate in the pulmonary airways and include inflammation of the bronchioles and bronchiole hyper responsiveness that can cause decreased airflow.[17] The large amounts of inflammation associated with asthma can cause DNA damage to surrounding cells of the lung.[18] We utilized mice overexpressing IL-13 to assess IL-13 role in genotoxicity concomitantly with the inflammatory asthmatic process.

In chapter one we will elucidate if over expression of a key asthma cytokine interleukin-13 in the lung causes systemic genotoxicity. Our study also demonstrates for the first time that this genotoxicity extends beyond the primary site of the lung to circulating leukocytes and erythroblasts in the bone marrow eliciting systemic effect driven by IL-13 over-expression. Furthermore, we showed that IL-13 mice exhibited sub-epithelial eosinophilic infiltration, peribronchiolar, and perivascular lymphoid infiltration. Airways of the transgenic mice also contained free floating eosinophilic crystals, many surrounded by aggregates of macrophages, giant cells and neutrophils PMNs all were phenotypes not present in wildtype (WT) mice.

In chapter two we investigated the effects of side-stream tobacco smoke and smoke extract on glutathione- and oxidative DNA damage repair-deficient mice and blood cells. Cigarette smoke causes direct oxidative DNA damage as well as indirect damage through inflammation.[19] Epidemiological studies show a strong relationship between secondhand smoke and cancer; however, the mechanisms of secondhand smoke-induced cancer are not well understood.[20] Animal models with either i) deficient oxidative DNA damage repair, or ii) a decreased capacity to combat oxidative stress may help determine the pathways important in mitigating damage caused by smoke.

In this study, we used mice lacking *Ogg1* and *Myh*, both of which are involved in base excision repair by removing oxidatively damaged DNA bases. Gclm-deficient mice, which have decreased levels of glutathione (GSH), were used to look at the role of smoke-induced oxidative damage. *Ex vivo* experiments show significantly elevated levels of DNA single-strand breaks and chromosomal aberrations in peripheral blood lymphocytes from $Ogg1^{-/-}Myh^{-/-}$ double knockout mice compared to wild type (WT) mice after 24 hours of exposure to cigarette smoke extract (CSE). The average γH2AX foci per cell was significantly elevated 3 hours after exposure to

CSE in cells from *Ogg1* $^{-/-}$*Myh* $^{-/-}$ double knockout mice compared to wildtype mice. *In vivo* we found that all mice had increased markers of DNA damage after exposure to side-stream tobacco smoke (SSTS). *Ogg1*$^{-/-}$ *Myh*$^{-/-}$ and *Gclm*$^{-/-}$ mice had altered levels of peripheral blood glutathione after SSTS exposure whereas wild type mice did not. This may be due to differential regulation of glutathione synthesis in the lung. We also found that *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice had a decreased lifespan after oral gavage with benzo[*a*]pyrene compared to wildtype mice and sham-exposed *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice. Our results are important in investigating the roles of oxidative stress and oxidative DNA damage repair in cigarette smoke-induced cancers and characterizing the role of genetic polymorphisms in smoke-related disease susceptibility.

In chapter 3 we assessed biomarkers of DNA damage to determine susceptibility to develop lung cancer. Lung cancer is the leading cause of death from cancer in both men and women in the United States. [21, 22] Lung cancer causes more deaths than the next three most common cancers combined (colon, breast and prostate), [23, 24] nearly 90 percent of individuals who develop lung cancer are smokers, yet only 10-15 percent of lifetime smokers will develop the disease. [22, 25] What is further perplexing is the notion that it is not well understood why some individuals are more susceptible to smoking induced lung cancer. This suggests that other factors concomitant with history of smoking may be involved in susceptibility to develop carcinogenesis in these select individuals. In this work we utilized markers of DNA damage and repair in peripheral leukocytes in the presence of cigarette smoke extract as an indication of increased susceptibility in individuals with known lung cancer. To further strengthen our analysis we combined our data with the known characteristics of confounding diseases, age, race, sex, family history of cancer, and other environmental exposures in each individual. In spite of our very small sample size we found statistically significant results correlating age and race to

increased genotoxicity as well as positive yet non-significant trends in gender, family history of cancer, pack years of cigarettes smoked, lung cancer difference to non-lung cancer, and individuals with history of other cancers.

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Chapter 1:
Assessing the Role of Interluekin-13 in the Genotoxicity of Asthma

Abstract

Asthma is a common heterogeneous disease with both genetic and environmental factors that affects millions of individuals worldwide. Thymus helper 2 (Th2) cells are thought to induce asthma through the secretion of many cytokines that activate inflammatory and residential effector pathways both directly and indirectly. Activated type 2 helper T cells secrete a panel of cytokines, including IL-13, a central immune regulator of many of the hallmark type 2 disease characteristics found in asthma including IgE synthesis, mucus hypersecretion, airway hyperreactivity, and tissue fibrosis. Furthermore IL-13 has been directly implicated as a potent stimulator of eosinophil, lymphocyte, mucus metaplasia, tissue fibrosis, and parenchymal remodeling. In our study we demonstrated that inflammation induced genotoxicity found in asthma extends beyond the primary site of the lung to circulating leukocytes and erythroblasts in the bone marrow eliciting systemic effects driven by IL-13 over-expression. Furthermore, we showed that IL-13 mice exhibited sub-epithelial eosinophilic infiltration, peribronchiolar, and perivascular lymphoid infiltration. Airways of the transgenic mice also contained free floating eosinophilic crystals, many surrounded by aggregates of macrophages, giant cells and neutrophils PMNs all were phenotypes not present in wildtype (WT) mice.

Introduction

Asthma is a common heterogeneous disease with both genetic and environmental factors that affects nearly 155 million individuals' world wide. In the United States, over 25 million individuals have asthma, about 7 million of which are children.[26] Asthma is clinically diagnosed by a barrage of symptoms which include wheezing, coughing, and shortness of breath.[27] Asthma can be subcategorized into two classes: allergic, and non-allergic asthma.

Patients with non-allergic asthma represent 10-30% of individuals with asthma. The clinical, difference between allergic and non-allergic asthma may be determined by a positive or negative reaction to aeroallergens skin prick tests. There are almost no observable differences in the types of physiological changes that occur between the two subcategories, however non-allergic asthmatics incur more severe and more frequent symptoms.[28]

The pathophysiological characteristics of asthma predominate in the pulmonary airways and include inflammation of the bronchioles and bronchiole hyper responsiveness that can cause decreased airflow.[17] Airways of asthmatic individuals are distinguished through structural modifications, collectively called airway remodeling. These airways comprise of multifaceted changes in composition, content, and organization of the various cellular and molecular constituents of both the small and large airways.[29] The most striking pulmonary abnormalities occurring in asthmatics include epithelial sloughing, goblet cell metaplasia, multiplied mucus glands, thickening of the lamina reticularis, increased airway smooth muscle mass, angiogenesis, and alterations in the extracellular matrix components.[30] Molecularly asthma increases migrating cell types including of B lymphocytes, T lymphocytes, eosinophils, neutrophils, macrophages, have all been associated with the classification of the disease.[31] In a failed attempt of the body to compensate with asthma mucus production increases in the lung to sequester foreign matter before it reaches the walls of the airway, airway restriction limits constrains intake of the foreign matter, as well as inflammation which promotes antibody contact with the foreign matter for its removal.[32] The increase in B lymphocytes, induces antibody release and involves the overproduction of immunoglobulin E, triggering the discharge of chemical mediators such as leukotrienes, prostaglandins, and histamines, leading to airway inflammation.[33]

The molecular mechanism through which airway remodeling occurs, involves a central role type 2 helper T cells.[34] Dendritic cells in the airway lumen collect allergens and enter tissues through disrupted epithelium. Activated dendritic cells advance to regional lymph nodes or to the local mucosa, where they present processed peptides derivatives from foreign matter upon the major histocompatibility complex (MHC) class II molecules to naive T cells. The assembly of the T-cell receptor with the MHCII complex initiates T cell activation. Secondary signals generated through the ligation of CD80 and CD86 on dendritic cells with their respective receptors on T cells, CTLA4 and CD28, allows T-cell activation to proceed.[34] It is well established that type 1 helper T cells regulate cellular immunity while type 2 helper T cells regular humoral immunity.[34] There is evidence that in asthmatic individuals, antigen presentation results in the preferred divergence of T-cell differentiation towards type 2 helper T cells, whereas non-asthmatic individuals display an inclination to produce type 1 helper T cells.[35]

T helper 2 (Th2) cells are thought to induce asthma through the secretion of many cytokines that activate inflammatory and residential effector pathways both directly and indirectly. [36] Activated type 2 helper T cells secrete a panel of cytokines, including IL-4 and IL-13, both of which serve as major participants in the progression of asthma.[16] IL-4 induces differentiation of naive helper T cells into type 2 helper T cells. This is a positive feedback mechanism where upon activation by IL-4, type 2 helper T cells subsequently produce additional IL-4 further promoting type 2 T cell differentiation.[16, 37] The IL-4 cytokine is structurally similar to and has functions similar to the IL-13 cytokine. IL-4 or IL-13 may activate a receptor complex that is composed of the IL-4Rα and IL-13R1α1 subunits on many cell types in the airway wall and these cytokines are thought to mediate many processes that are relevant to

asthma pathology as a result of activation of this receptor complex.[38, 39] IL-4 and IL-13 have an overlapping and pivotal role in asthma pathogenesis; overexpression of IL-13 is necessary and sufficient to induce asthma while IL-4 is not.[16, 40, 41] The IL-4Rα/IL-13R1α1 receptor complex is present on eosinophils, mast cells, B lymphocytes, fibroblasts, and airway smooth muscle cells.[41] The activation of the receptor complex, initiated via the cytokines IL-4 and IL-13 result in the phosphorylation of STAT6.[42] Following its phosphorylation, STAT6 dimerizes and translocates into the nucleus where it regulates gene transcription.[43] In Hematopoietic cells, STAT6 activation results in type 2 helper T cell differentiation and IgE release in B cells. In non-hematopoietic cells, STAT6 activation leads to cellular remodeling that includes epithelial cell mucus metaplasia and smooth muscle contraction in the airways. Activation of STAT6 in mast cells, eosinophils, and lymphocytes, causes these cells to migrate into the bronchial mucosa in asthmatic airways to contribute to airway inflammation and remodeling.[42] The effects of STAT6 activation through binding of IL-13 cause the epithelial cell changes evident in asthmatic airways. Overproduction of mucus, inflammation of the airways, and tissue fibrosis all result from IL-13 triggering STAT6.[37, 42, 44, 45] IL-13 also fuels the release of leukotrienes and chitinases in airway epithelial cells. The release of these mediators results in smooth muscle contraction. [37, 44, 45] IL-13 may also bind to the IL-13Rα2 receptor with a very high affinity. The consequence of IL-13Rα2 receptor activation is unknown and currently the molecule is thought to operate as a decoy receptor.[46] Although IL-13 is known to play a major role in the development and persistence of asthma, the complex combination of environmental and genetic origin of the disease obfuscate the solitary role of IL-13 in the disease. We therefore, used a genetically modified mouse model which conditionally

overexpresses IL-13 in the lungs to study the independent role of IL-13 in the progression of asthma.[47]

IL-13 is a major cytokine produced during asthma. The role Interleukin-13 plays in many down-stream immunological processes of the lung make it a provocative tool of further research. IL-13 has been directly implicated as a potent stimulator of eosinophil, lymphocyte, mucus metaplasia, tissue fibrosis, and parenchymal remodeling. [38] Furthermore, IL-13 is a central immune regulator of many of the hallmark type 2 disease characteristics, including IgE synthesis, mucus hypersecretion, airway hyperreactivity, and tissue fibrosis.[40] In this study we sought to elucidate the genotoxicity attributed to IL-13 induced asthma by assessing both tissue specific and systemic genotoxicity in IL-13 over-expressing mice. We therefore, used a genetically modified mouse model originally developed by the Elias lab [48] with two constructs which conditionally overexpresses IL-13 in the lungs. The first construct in these mice, CCL10-rtTA-hGH, contains the CCL10 promoter. The second construct, *tet*-O-IL-13-hGH, contains a *tet-O* and minimal CMV promoter, murine IL-13 cDNA, and a necessary stop signal. In the presence of doxycycline, rtTA is able to bind in trans to the *tet-O*peron and activate IL-13 gene transcription. These mice overexpress IL-13 specifically in the clara cells of the lung only when exposed to doxycycline.

Materials and Methods

Mice. The CC10-rtTA-IL13 transgenic (TG) mice were generated in Dr. Talal Chatila lab David Geffen School of Medicine University of California Los Angeles, USA. Mice were bred in an institutional specific pathogen free animal facility under standard conditions with a 12 hr light/dark cycle according to Animal Research Committee regulations. Mice were fed a standard

diet and doxycycline water *ad libitum*. As described previously in[49] The Clara cell 10-kDa (CC10) gene promoter was used to conditionally express IL13 in the mouse lung in the presence of doxycycline. In these experiments we exposed mice to doxycycline for a 3 week time period. Animals in the control group consisted of wild type (WT) mice were exposed to doxycycline. The IL13 transgene exhibits baseline leakiness in the absence of doxycycline allowing transgenic mice to exhibit minor elevation of IL13 expression and minor allergic airway inflammation.[50]. For this reasoning doxycycline untreated transgenic mice were not used as controls in our experiments. After the 3 week exposure to doxycycline all mice were euthanized and pathological assessments were conducted on the lungs.

Immunofluorescence. 50ul of whole peripheral blood was put into erythrocyte lysis buffer, cells were laid over poly -D-lysine-coated coverslips and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) at room temperature as described previously [51]. Subsequently, cells were permeabilized with 0.5% Triton X-100 (Sigma), followed by 5 rinses in PBS. Blocking was done in aluminum-covered plates overnight at 4°C in 10% FBS. Coverslips were then incubated for 1 hour at room temperature with mouse anti-phospho-Histone H2A.X (Upstate, Temecula, CA) at a dilution of 1:400, or Mouse anti–8-oxoguanine clone 483.15 (Upstate, Temecula, CA) at 1:250 respectively. Coverslips were then rinsed with 0.1% Triton X-100. Following a second 10% FBS blocking, cells were stained with FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:150 for samples with γH2AX primary and (1:200) for samples with 8-oxoguanine primary, respectively for 1 hour at room temperature. Coverslips were mounted onto slides using VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA). For both 8-oxoguanine and γH2AX assay analysis were done on a Zeiss automated microscope. At least 100 cells were counted per sample and

cells with more than four distinct foci in the nucleus were considered positive for γH2AX.[52] and cells that exhibited elevated fluorescent intensity compared to background were considered positive for 8-oxoguanine respectively. Apoptotic cells, which have an approximate 10-fold increased in nuclear foci in damaged cells, were not included in analyses [52, 53]. Statistical analyses were done using Poisson distribution 8-oxoguanine (STATA statistical analysis software) for γH2AX and using ANOVA and tukey's post hoc test for 8-oxoguanine analysis (GraphPad Prism).

Micronucleus assay. 3µl of whole blood were spread on a microscope slide and stained with Wright-Giemsa solution (Sigma-Aldrich, St. Louis, MO). At least 4000 erythrocytes were counted according to published recommendations [54]. MN were counted and scored with an Olympus Ax70 (Tokyo, Japan) at 100X magnification. Statistical analysis was done using repeated measures ANOVA followed by Tukey's post-tests (GraphPad Prism).

DNA single strand breaks. Oxidative DNA damage and DNA strand breaks were measured in peripheral blood cells using the alkaline comet assay. Peripheral blood was collected before doxycycline administration (Day 0), and on days 3, 6, 9, 12, 15, 18 and 21 days of doxycycline treatment. Blood was diluted 1:1 with RPMI + 20% DMSO, slowly frozen and stored at -80C until the assay was performed. The comet assay was done as described previously [55]. Briefly, cells were mixed with low melting-point agarose, and placed in triplicate onto normal agarose layed over gelbond (Lonza Inc. Rockland, ME). The gel was immerse in lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton, and 10% DMSO), then alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA). After 20 minutes in the electrophoresis buffer at 4°C, the gel was run for 45 minutes at 300 mA, allowed to dry and then stained with SYBR Gold (Molecular Probes). Comet tail-moments were analyzed using CASP (Comet Assay

Software Project, http://casp.sourceforge.net/). To measure oxidative DNA damage, the comet assay was modified to include an incubation step with hOGG1 (New England Biolabs, Ipswich, MA). As described previously, embedded cells were incubated with hOGG1 (1:300 in NEBuffer1 and BSA) at 37°C for 30 minutes following the lysis step [56]. Tail-moments were normalized to a control to account for inter-experimental variability. Statistical analyses were done using ANOVA (GraphPad Prism).

Blood Collection. Peripheral blood was collected by facial vein puncture using 5 mm sterile lancets (Medipoint Inc. Mineola, Ny) from experimental mice on specified days throughout the duration of experiment and on sacrifice day via terminal right ventricle cardiac puncture using a heparin-coated syringe (American Pharmaceutical Partners, Inc. Schaumburg, IL). Blood from each mouse was collected into EDTA-coated tubes (Sarstedt Aktiengesellschaft & Co., Numbrecht).

ELISA analysis. Serum was separated from blood taken via submandibular punctures on all mice immediately before doxycycline administration (Day0) and on days 3, 5,7,10,13,16,18, and on terminal (Day21). After collection serum samples were aliquoted into micro-centrifuge tubes and kept at -20˚C until analysis. Sandwich ELISAs were conducted on samples that were diluted 1:10. Analysis of total Mouse IgE was done according to manufacturer's instructions (BD biosciences). Each sample was done in triplicate and analyzed using the relative standard curve method optical density vs concentration. Statistical analysis was done using linear mixed model with repeated measurements nested within each mouse. (STATA statistical analysis software)

Gene expression analysis. Lungs from WT and IL-13 mice at the end of the 3-week doxycycline exposure mice were perfused and lavaged before immersion into RNAlater

(Qiagen). Lungs were kept at 4˚C for 24 hours then transferred to -80˚C until RNA was isolated using the RNeasy Mini kit according to manufacturer's instructions (Qiagen). cDNA was synthesized using SuperscriptIII (Invitrogen) according to manufacturer's recommendations. Quantitative real-time PCR was performed on an ABI Prism 7500 gene expression system (Applied Biosystems) using Taqman gene expression assays for *H2AX*, *8-oxoguanine*, *IL-13*, *IL-4*, *IL-5*, *CCL-11/Eotaxin*, *TGF-β*, *Tnfα*, and *Gapdh* was used as an internal control. Each reaction was done in triplicate and analyzed using the relative standard curve method.

Doxycycline water administration. As described in [38] CC10-rtTA-IL-13 transgene(+) (Tg+) mice and their WT littermate controls were maintained on normal water until one month of age. After one month of age doxycycline was administered to the drinking water 1000mg/L in 4% sucrose and kept in aluminum foil covered bottles to prevent light-induced degradation of doxycycline.

Bronchoalveolar lavage (BAL). The amount of lung inflammation cause by increased infiltration of immunocirculating cells was assessed by BAL. Briefly mice were euthanized the trachea was isolated via blunt dissection and small caliber tubing was inserted and secured in the airway. A volume of1ml of 1X PBS was flushed and removed 3 times successively from the lungs of WT and IL-13 mice until 3mL of BAL fluid was collected. BAL fluid was centrifuged at 1600rpm at 4°C for 10minutes. After centrifugation cells were re-suspended in 200ul of 1X PBS. A 1:1 ratio of cells to trypan blue was put into a hemocytometer and the number of viable cells was counted. After cell viability was assessed 200ul of remaining cells were put into cytospin and spun at 400 RPM for 5 minutes. Slides were removed from cytospin and allowed to air dry. After drying the slides were stained using (Thermo Kwik Diff staining kit) using manufactures staining recommendation. Slides were allowed to air dry overnight and were

mounted with paramount and a coverslip and allowed to dry. Finally at least 200 cells were differentiated by light microscopy based on conventional morphological criteria for each animal.

Immunohistochemistry. γH2A.X, 8-Hydroxyguanosine, and Nitrotyrosine stains were done on lung tissue of (WT) and IL-13 mice. The slides were placed in xylene to remove paraffin, then a series of ethanol washes. After a wash in tap water, the slides were incubated in 3% Hydrogen peroxide / methanol solution for 10 minutes. The slides were then washed in distilled water, and incubated for 25 minutes in Citrate Buffer pH6 (Invitrogen Corporation) at 95 degrees Celsius using a vegetable steamer. Next, the slides were brought to room temperature, rinsed with PBST (Phosphate Buffered Saline containing 0.05% Tween-20), then incubated at room temperature for 1 hour with Anti-gamma H2A.X (phosphor S139) antibody (abcam, ab22551), 2 hours with anti-8-Hydroxyguanosine antibody (abcam, ab48508) ,or for 45 minutes with Nitrotyrosine (Millipore, 06-284) at the dilutions of 1: 50 for γH2A.X, and 8-Hydroxyguanosine antibodies and at1:200 for the Nitrotyrosine antibody, respectively. The slides were then rinsed with PBST, and were incubated with Dako EnVision+ System –HRP Labelled Polymer Anti-Mouse (Dako, K4001) at room temperature for 30 minutes. Subsequently after a rinse with PBST, the slides were incubated with DAB (3,3'-Diaminobenzidine) for visualization. Finally, the slides were washed in tap water, counterstained with Harris' Hematoxylin, dehydrated in ethanol, and mounted with media.

Results

Fig 1-1. **IL-13 Mice show elevated reactive oxygen species induced genotoxicity**. Because IL-13 is a major mediator of allergenic asthma and induces higher levels of inflammation in asthmatic mice [57] , we hypothesized that inflammation induced DNA damage

would be more prevalent in IL-13 compared to wild type (WT) mice. Increase of asthma induced genotoxicity was assessed in peripheral white blood cells as a systemic measurement of DNA damage.8-oxoguanine is a mutagenic lesion caused by the interaction of a reactive oxygen species to DNA that causes G:C to T:A transversion mutations during replication[58] .Percent positive 8-oxoguanine staining in peripheral white blood cells was assessed using fluorescent microscopy. Blood was taken on day 0 as an assessment of baseline levels of 8-oxoguanine induction between both WT and IL-13 groups. After 6 days of doxycycline presence in drinking water IL-13 mice exhibited a slight increase of 8-oxoguanine immuno-staining compared to wild type mice this induction persisted and became statistically significant at **,$p<0.001$ at day 15 and remained elevated throughout the 21 day exposure to doxycycline. (Fig.1-1)

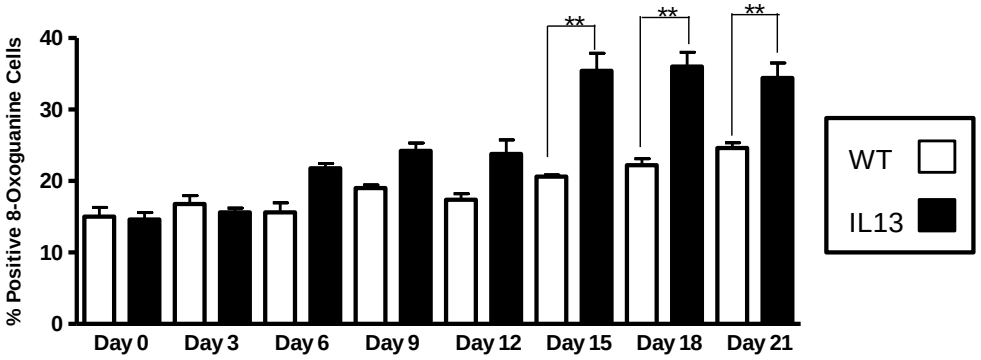


Fig. 1-1 Inflammation induced DNA damage measured via 8-oxoguanine induction. Percent positive cells for 8-oxoguanine induction in white blood cells. Presence of 8-oxoguanine was confirmed by immunofluorescence. Positive cells stain brightly green compared to no immunofluorescent staining for negative cells. White bars indicate Wild type (WT) animals and black bars indicate IL-13 animals. Data represent mean ± SEM. Statistical analyses were done using ANOVA testing and Tukey's post hoc analysis. n=5 in all groups. ** indicates $p<0.001$

Fig1-2. IL-13 Mice have persistent genotoxicity that induces micronucleus formation in erythrocytes.The in vivo micronucleus assay was conducted in mature normochromatic erythrocytes circulating in the peripheral blood to determine chromosomal damage. Micronuclei in erythrocytes/erythroblasts from the peripheral blood or bone marrow have been induced in the

presence of chromosome breaks, spindle abnormalities, or structurally abnormal chromosome.[54, 58] Mature micronucleated normochromatic erythrocytes represent the final developmental stage of erythroblasts containing micronuclei stemming in the bone marrow, and permit the simultaneous study of the generation and elimination of micronucleated erythrocytes. [58, 59] Blood was taken on day 0 as an assessment of baseline levels of 8-oxoguanine induction between both WT and IL-13 groups. After 6 days of doxycycline presence in drinking water IL-13 mice exhibited a significant increase of micronuclei formation in peripheral blood compared to WT animals at *, $p<0.05$. This statistically significant induction at *, $p<0.05$ of micronuclei persisted until terminal date Day 21.

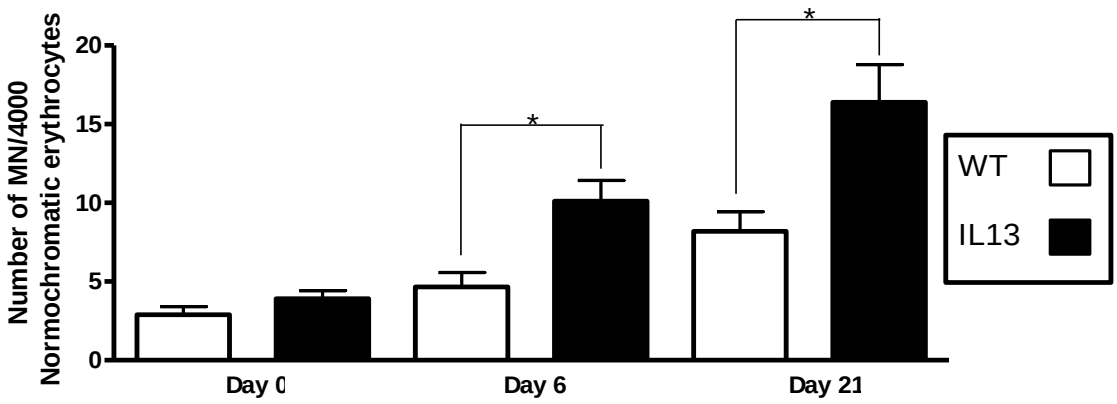


Fig.1-2 Persistent genotoxicity induced in micronuclei formed in peripheral blood erythrocytes. Number of micronuelated cells per 4000 noromorchromatic erythrocytes. Presence of micronuclei were confirmed by light microscope at 100X. White bars indicate Wild type (WT) animals and black bars indicate IL-13 animals. Data represent mean ± SEM. Statistical analyses were done using ANOVA testing and Tukey's post hoc analysis. n=9 for WT and n=10 for IL-13.* indicates $p<0.05$

Fig.1-3 IL-13 Mice have induction of stranded breaks. The alkaline comet assay is a gel electrophoresis assay that allows the detection of single and double strand breaks, and alkali labile sites at the single cell level.[52] Asthma is an inflammatory disease that produces large amounts of reactive oxygen species (ROS).[60, 61] Interaction of ROS with DNA may result in

mutagenic oxidative base modifications such as 8-hydroxydeoxyguanosine (8-oxo-dGuo) and induce DNA strand breaks[60] Transgenic animals exhibited an increase in the amount of single strand breaks occurring after 6 days of IL-13 over-expression compared to all other groups where no increase was observed. This induction of strand breaks was significant for indicated groups at *, $p<0.05$

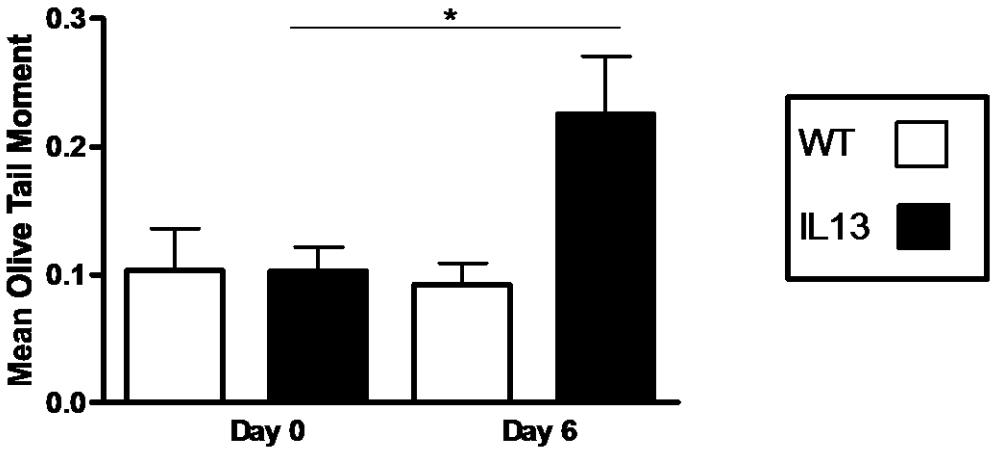


Fig.1-3 Single stranded breaks assessed via comet assay in peripheral blood after IL-13 over-expression. Assessment of single strand breaks were measured via comet assay before doxycycline administration at Day 0 and after doxycycline administration at days 6. At least 100 olive tail moments were counted via fluorescent microscopy and assessed using CASP software. White bars indicate Wild type (WT) animals and black bars indicate IL-13 animals. Data represent mean ± SEM. Statistical analyses were done using ANOVA testing and Tukey's post hoc analysis. * indicates $p<0.05$ n=5 for WT and IL-13 animals.

Fig1-4. IL-13 Mice have increase induction of double stranded breaks. As a measure of the amount of genotoxicity caused by an accumulation of DNA double strand breaks the γH2AX assay was assessed in the WT and IL-13 animals. H2AX is a member of the histone H2A protein family and becomes rapidly phosphorylated in presence of a DNA damaging event.[62] This rapid phosphorylation causes recruitment of DNA repair proteins to the site of the break and is detectable by specific antibodies to γH2AX. In Fig1-4 we assessed the amount of double strand breaks present in the WT and IL-13 animals. Transgenic animals exhibited an

increase in the amount of double strand breaks occurring at every time point after over-expression. This induction of γH2AX was significant on day 9 at **, p<0.002, at day 12 at *, p<0.02, and at day 18 day at **,p<0.001 respectively. A nearly significant induction of γH2AX at P=0.064 on day 21 was also observed in the IL-13 mice.

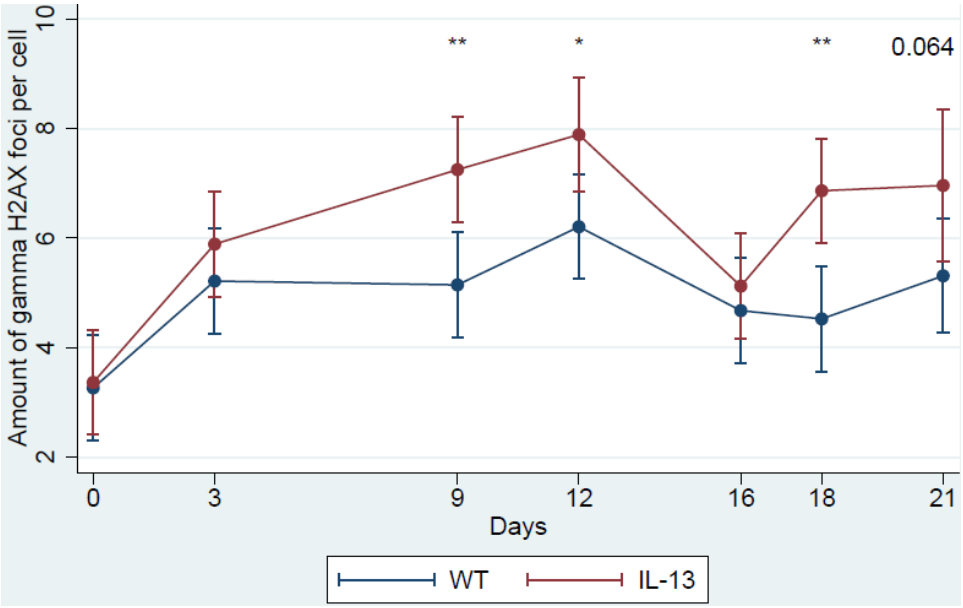


Fig.1-4 γH2AX genotoxic assessment in peripheral blood after IL-13 over-expression. Assessment of double strand breaks measured via γH2AX assay, were counted per cell using fluorescent microscopy before doxycycline administration at Day 0 and after doxycycline administration at days 3,9,12,16,18 and day 21 using a linear mixed model to determine genotoxic accumulation over time. * indicates p<0.02, ** indicates p<0.002 n=5 for WT and IL-13 animals.

Fig.1-5 IL-13 Mice exhibit increased inflammation of the lung.To understand the role IL-13 induced lung inflammation plays in the genotoxicity and progression of asthma we compared the lungs of WT and IL-13 animals. In (Fig.1-5A and 1-5 C) WT animals exhibit no increase of inflammation. In contrast IL-13 mice (Fig. 1-5B and 1-5 D) exhibit significant inflammation of the lung characterized in the airways bronchiolar lumen. IL-13 mice showed increase inflammatory cells migration in the bronchiolar epithelium, marked hyperplasia, goblet cell metaplasia, and eosinophilic intracytoplasmic

inclusion bodies present in clara cells. Thus IL-13 increases inflammation via recruitment of inflammatory cells migrating into airway spaces.

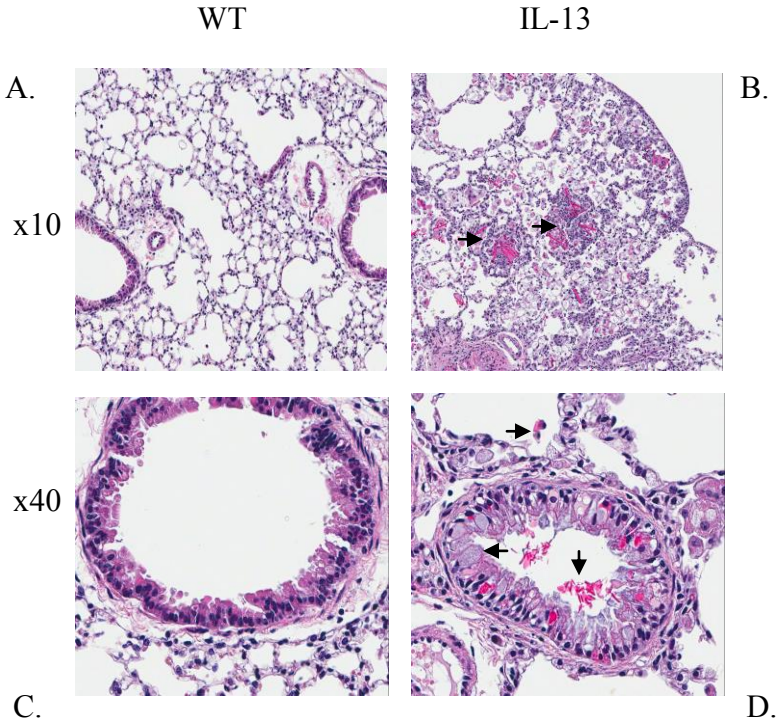


Fig.1-5. Effects of IL-13 Induction of asthma genotoxicity in IL-13 mice. Representative lung histology Hematoxylin & Eosin (H&E) staining at indicated magnifications. (A) 10x image of Wild type (WT) lung and Interleukin 13 over expressed mice (B), both at one month old. Arrows in (B) 10x image indicate formation of granuloma metafoci surrounding eosinophilic crystals. 40x image of WT (C) and (D) 40x image of IL-13 mice. Arrows in (D) indicate eosinophil migration, goblet cell metaplasia, and eosinophilic crystal formation in bronchial lumen. n=9 for WT and n=10 for IL-13

Fig.1-6. IL-13 mice exhibit persistent inflammation induced immune response and increased genotoxicity. To elucidate the role IL-13 plays in up-regulation of inflammation and genotoxicity in the lung we assessed key mediators of asthma Fig. 1-6A -1-6G, inflammatory disease Fig.1-6F, and genotoxicity Fig. via quantitative real-time PCR. As a measurement of further efficacy of our tissue specific asthma model we measured IL-13 levels in the lungs of both groups of experimental mice. The asthma mouse model exhibited an expected significant

increased gene expression of IL-13 at **, p<0.001 compared to WT animals. IL-4, TNF-α, IL-5, and γH2AX levels were significantly up-regulated in IL-13 mice at *,p<0.01 compared to WT animals. IL-13 mice also displayed a marked increase of TGF-β Fig.1-6E at **,p<0.001 compared to WT mice. Cc11 transcript levels in IL-13 mice were slightly higher but did not show a significant increase compared to WT mice Fig.1-6G These data are indicative of a chronically activated innate immune response present in IL-13 mice compared to WT mice.

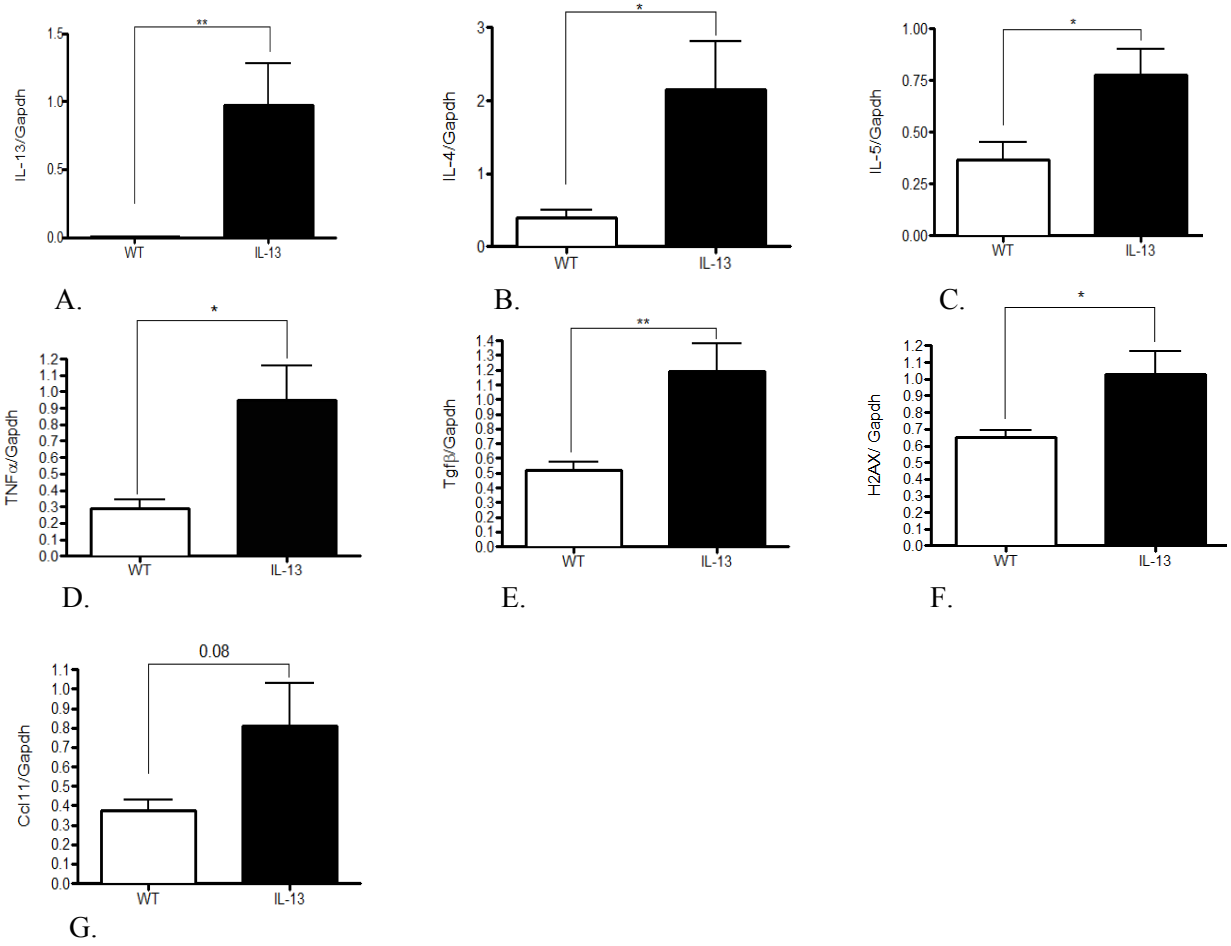


Fig.1-6 Assessment of cytokine panel in lung mRNA measured by quantitative real-time PCR. Mean expression divided by Gapdh, the internal control gene. * indicates p<0.01, ** indicates p<0.001, analysis were conducted using two tailed Student's unpaired T-test. n=9 for WT animals and n=10 for IL-13 animals.

Fig.1-7. IL-13 mice exhibit increase immune cell infiltration in BAL fluid.To delineate what immune cells may be implicated in the persistent inflammatory and genotoxic response present in asthma mouse model we determined the cellular composition of the BAL fluid in both the WT and IL-13 mice. IL-13 animals had a near 5-fold increase in eosinophil presence in BAL fluid compared to WT animals at $p<0.04$. IL-13 mice also exhibited significantly more circulation of neutrophils in the BAL fluid at ***,$p<0.0004$ compared to WT littermates. IL-13 mice also exhibited and increase induction of lymphocytes although this observation was not significantly different from WT animals.

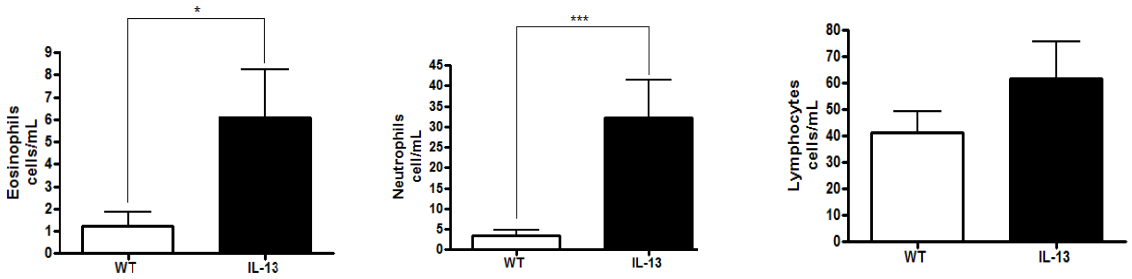


Fig.1-7. Inflammatory cell composition of bronchial alveolar lavage fluid (BALF).
Differential cell analysis were determined by light microscopic evaluation n=9 for WT, and n=10 for IL-13 animals ,* indicates $p<0.05$,*** indicates $p<0.0004$ respectively analysis were conducted using two tailed Student's unpaired T-test with Mann-Whitney determination.

Fig.1-8 Serum IgE levels are induced in IL-13 Mice. Increased level of IgE is a major factor in the etiology of asthma and is found abundantly in the serum of both human and murine models of asthma [63-65]. Therefore we assessed the levels of IgE in our transgenic IL-13 over-expression model. We observed that the levels of IgE were higher in the transgenic mice 12 days after IL-13 over-expression and were consistently higher throughout the experiment until terminal day 21. Although IL-13 mice exhibited higher IgE levels this induction was only significant at day 21 at **,$p<0.007$

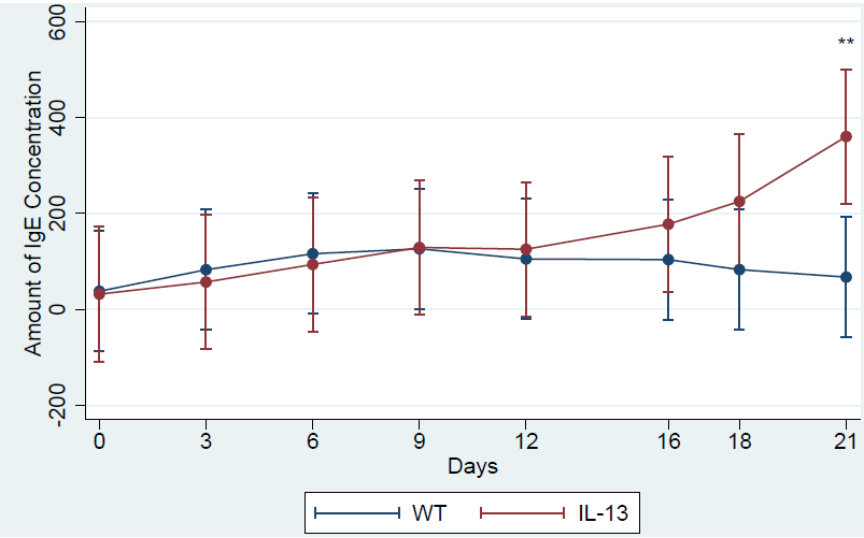


Fig.1-8. IgE concentration assessed via sandwhich ELISA. **indicates p<, 0.007 n= 5 in both IL-13 and WT animals.

Fig.1-9. IL-13 Mice have increase staining of markers of genotoxicity in lung tissue. To further assess asthma induced genotoxicity the lungs of WT and IL-13 mice were stained with γH2AX, anti-8-Hydroxyguanosine , and Nitrotyrosine antibodies. IL-13 mice exhibited increase staining with markers of genotoxicity in the lung in comparison to WT animals.

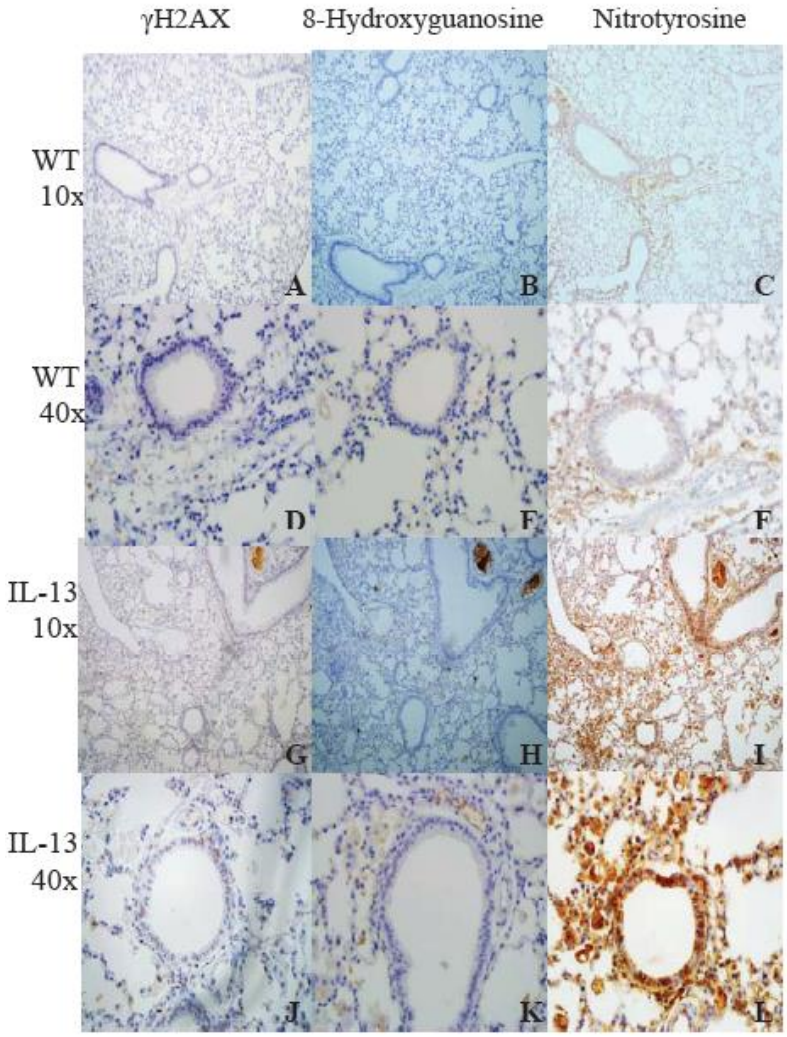


Fig.1-9. Staining of markers of genotoxicity in lung tissue as measured by immunohistochemistry. Markers of double stranded breaks (A-J) , reactive oxygen species (B-K), and inflammation (C-L) induced genotoxicity were stained in WT and IL-13 mice. Lung tissue in IL-13 mice (G-L) exhibited increased staining in all genotoxic parameters in comparison to WT mice (A-F). n=9 for WT animals and n=10 for IL-13 animals.

Discussion

Asthma is a chronic obstructive lung disease characterized by chronic inflammation of the airways and recurrent bronchospasms ranging from mild to debilitating.[15] It is well established that Interleukin-13 serves as a major mediator of the asthmatic process. [16]This study is the first to assess IL-13 role in genotoxicity concomitantly with the inflammatory asthmatic process. Our study also demonstrates for the first time that this genotoxicity extends beyond the primary site of the lung to circulating leukocytes and erythroblasts in the bone

marrow eliciting systemic effect driven by IL-13 over-expression. We utilized the well characterized inducible over-expression CC10-rtTA-IL13 transgenic (TG) mouse to study the effect interleukin-13 may play in genotoxicity of asthma. The connection between inflammation and asthma is well documented. Our results showed that IL-13 mice exhibited sub-epithelial eosinophilic infiltration, peribronchiolar, and perivascular lymphoid infiltration. Airways of the transgenic mice contained free floating eosinophilic crystals, many surrounded by aggregates of macrophages, giant cells and neutrophils PMNs. Previous studies [16, 17, 66] described similar inflammatory processes. Furthermore migration and infiltration of these immune cells can induce cytokine production that help to perpetuate inflammation during asthma.[17] In this work we show that IL-13 over-expressing mice exhibit an induction of cytokines involved in inflammation and tissue morphology. Transgenic mice had increase gene expression of IL-13, IL-4,IL-5,TNFα, Tgfβ, γH2AX, and Ccl11 produced in the lung. IL-4 along with IL-13 is a key mediator of inflammation, has an overlapping biological function as IL-13 yet a distinct role in asthma progression.[40] IL-4 is best known for its role for defining the Th2 phenotype of lymphocytes in asthma, but it also exacerbates the asthmatic phenotype by increasing airway hyperresponisveness, eosinophil recruitment, and mucus over-production.[16, 66] Recent work has shown that IL-13 signaling is mediated by the type-2 IL-4 receptor, which consists of the IL-4R alpha and IL-13R alpha 1 chains [40, 46] Yet IL-13 alone is necessary and sufficient to render the major pathophysiological effects of asthma.[16] As further indication of IL-13 self sufficiency, it has been shown that IL-13 independent of IL-4 renders resistance to most gastrointestinal nematodes and specifically modulates resistance to intracellular organisms including Leishmania major, Leishmania mexicana, and Listeria monocytogenes. [67] We next investigated mRNA levels of tumor necrosis factor-alpha (TNF-alpha) a pro-inflammatory

cytokine that has been implicated in many aspects of the airway pathology in asthma including airway hyperresponsiveness [68]. We observed a significant increase in the lungs of IL-13 over-expressing mice. This observation is similar to what was found [38, 67] . TNFα induction was also seen in mice with multiple challenges, using toluene diisocyanate (TDI), a known cause of occupational asthma [69] . Conversely, Horiba et.al [70] found that after intranasal administration of various combinations of IL-1b, TNF-α and IL-13 Inflammatory cell influx was increased by all cytokines and combinations except IL-13 in OVA-sensitized mice. This unusual effect may be attributed to the different mechanism of the asthma inducers or to the pleiotropic nature of IL-13. Furthermore we studied the role of IL-5, another major cytokine that is implicated in the development of eosinophilc inflammation and airway hyperresponsiveness in asthma. [71] In our work transcript levels of IL-5 were significantly higher in animals over expressing IL-13 compared to WT animals. This significant increase in IL-5 levels may further confirm the influx of eosinophils in the bronchial alveolar lavage fluid in our experimental model. A recent study in human asthmatics show intriguing results that demonstrate that IL-5 priming enhances monoclonal antibody –mediated mitochondrial and ROS-dependent eosinophil apoptosis and eliminates caspase dependence. [72]. These findings suggest that IL-5 can serve as a therapeutic target in asthma for multiple reasons; for the attenuation of a sustained eosinophilic response, as well as, the induction of cytokine priming that may render eosinophils more susceptible to pro-apoptotic effects of monoclonal antibody targeting. We next explored the role of IL-13 over-expression plays in Tgfβ production in asthma. Our experiments show a significant increase in expression levels of Tgfβ in the lungs of IL-13 mice compared to wildtype littermates. The significantly increased Tgfβ levels coincide with the preceding data showing increase tissue remodeling present in the IL-13 mice. Our observation further corroborated with

previous studies that demonstrated the fibrotic effects of IL-13 are greatly mediated by Tgfβ. [57]. Another recent study by[47] demonstrate that IL-13 is a potent stimulator of MCPs and other CC chemokines and document the importance of MCP-CCR2 signaling in the pathogenesis of the IL-13-induced pulmonary phenotype in asthma including increased Tgfβ levels. Futhermore we observed a induction of CCL-11/eotaxin in the lungs of IL-13 mice in relation to their wildtype littermates although this increase was not statistically significant. Finally to determine if the genotoxicity that was found in the blood of the transgenic mice was also able to cause systemic genotoxicity in the lung we assessed if there was an increase amount of γH2AX and 8-oxoguanine. Phosphorylation of histone H2A to form γH2AX in the presence of a DNA damaging event is used as a biomarker of cellular response to DSB and has potential for monitoring DNA damage and repair in human and mice[73, 74]. 8-oxo-7,8- dihydroguanine (8oxoG) is a abundant ROS induced legion that when accumulated has been associated with numerous diseases[52, 75, 76] Our experiments show that there was a significant increase in amount of γH2AX in the lungs of the IL-13 overexpressing mice compared to wildtype animals. An induced yet non-significant increase in 8-oxoguanine was also observed. This observation of an induction of γH2AX mRNA levels further confirms increased genotoxicity in our IL-13 overexpression model and correlates with our hypothesis of systemic genotoxicity due to the presence of this marker of double strand breaks in the lung mRNA and in the peripheral blood.

To evaluate the inflammatory cell composition we determined the differential cell percentages in the bronchoalveolar lavage fluid of wildtype and IL-13 mice after doxycycline administration. We observed a significant influx of eosinophils and neutrophils, and increase yet not significant induction of lymphocytes of IL-13 that is not present in wildtype animals. Eosinophils have long been established as the major cellular hallmark during an asthmatic

response.[77] In inflamed tissues, eosinophils act as key mediators of terminal effector functions and innate immunity and in linking to adaptive immune responses[78] In mouse models of allergic respiratory inflammation, eosinophils appear to play a role during the secondary immune responses leading to the activation and proliferation of Ag-specific memory T cells and the subsequent recruitment of newly formed T effector cell populations to the lung [79]. It has been shown that eosinophilic recruitment to the airways is primarily regulated by IL-5 in combination with CCL11/eotaxin-[77, 80]. Furthermore it has been shown that eosinophil-rich infiltrations have an important mechanism for the manifestation of AHR via antagonism of the acetylcholine-inhibitory neuronal muscarinic M2 receptor by eosinophil major basic protein in asthma. [81] In this current work we observe a significant increase of eosinophils in the bronchoalveolar lavage fluid of IL-13 overexpressing mice and an induced yet non-significant increase in CCL-11/eotaxin transcript present in the lung mRNA. Conversely to this finding we observed a significant increase in IL-5 lung mRNA transcript. A possible explanation to the induced yet non-significant increase in CCL11/eotaxin transcript may be found in the work of [82]; where it is described that eotaxin is cleared or metabolized faster from the lung tissue than from the lumen creating a gradient of eotaxin between the tissue and the microvessel lumen. Inversely during the active chemokine secretion the eotaxin concentration in the fluid lining the airway will be lower than in the tissue and the direction of the gradient will inhibit migration into the airway lumen in an IL-5 independent manner. These data suggest that IL-5 increase independent of CCL11/eotaxin may correlate to the significant induction we found in migrating eosinophils in the bronchoalveolar lavage fluid but does not appear to be a stimulus for eotaxin production. Directly correlating with the previous study by [77]. Conversely we understand that not

exhibiting a significant induction of CCL-11 and IL-5 concomitantly may also explain the predominance of neutrophils in our experiments.

Although not significantly present in our study lymphocytes plan a prevalent role in the asthmatic response. Specifically T helper 2 (Th2) lymphocytes play a prevalent role in the development of asthma. Th2 lymphocytes induce the production of cytokines that are inflammatory mediators of the asthmatic response, induce morphological changes in the lung of asthmatic individuals, and help sustain bronchial airway hyperresponsiveness.[83, 84] Our experiments show an induction that is not clearly significant in lymphocytes in the bronchoalveolar lavage fluid but further experiments may delineate the role of lymphocytes in our model of asthma. We suggest that elucidating the role of specific lymphocytes such as CD4+ cell populations may prove to be more advantageous. [85]

Neutrophils are polymorphonuclear leukocytes that play a major role in the immune system acting as the first line of defense against bacterial and fungal infections.[86] Our study shows a highly significant accumulation of neutrophils in the bronchoalveolar lavage fluid. This observation was consistent with previous studies, which have shown that either IL-1b or TNF-a can effect the accumulation of neutrophils through the induction of chemokines in the airways. [70] The idea that neutrophil infiltration of the airway may play a major yet alternative role in asthma than eosinophils has become more readily acceptable since first introduced.[87-89].In a longitudinal patient study [90] shows a subgroup of asthma patients exhibiting mild to moderate asthma is non-eosinophilic. Furthermore recent data suggest a key role neutrophils play in late asthma response in both man and murine models respectively. [91] Another recent human study observed that the sputum of older chronic asthmatic individuals exhibit significant influx of neutrophil migration suggesting the predominance of neutrophilic airway inflammation leads to a

more severe asthmatic phenotype. [92] In our study the increase prevalence of neutrophils over that of eosinophils may also depict the presence of a more chronic asthmatic phenotype as shown by the increase neutrophil migration in the bronchoalveolar lavage fluid. An idea that is in parallel with [93]. Moreover this significant influx of both neutrophils and eosinophils may also loosely correlate to the enhanced systemic genotoxic response found in the blood.

To elucidate the role increased IgE plays in our model of asthma we assessed total serum IgE levels in the blood serum of wildtype and IL-13 mice. Immunoglobin E (IgE) is induced in response to environmental antigens and is a hallmark of bronchial asthma. [63] IgE is shown to increase eosinophilic migration in asthmatic mice yet is not completely required for all phenotypes of asthma.[16] Our data shows that there is a significant increase in IgE levels in IL-13 mice compared to wildtype mice observed only at day 21 suggesting that IgE alone is not necessary to render an asthmatic. A further possible explanation to this late IgE increase over time can be found in the strain specific immune modulation of the C57BL/6 murine background. Historically the transgenic mouse model used plays a key role in the various aspects of asthma phenotype and progression.[94] Typically C57BL/6 mice are low IgE and Th2-nonbiased mice making the induction of a fully robust Th2 mediate response difficult to render.[71, 94-96] For these reasons IgE levels only significantly increase after a persistent asthmatic response.These data show that IL-5, but not B cells or allergen-specific IgE, are required for eosinophil airway infiltration and the development of AHR following allergen/alum sensitization and repeated airway challenge with allergen. These results emphasize that the use of different sensitization and challenge protocols can influence the requirements for development of AHR.[71]

To assess the genotoxicity attributed to IL-13 induced asthma we observed the accumulation of the 8-oxoguanine mutagenic lesion present in the IL-13 over-expressing

asthmatic mice compared to the WT mice. Radical oxygen species (ROS) generate various modified DNA bases. Among them 8-oxo-7,8-dihydroguanine (8oxoG) is the most abundant and seems to play a major role in mutagenesis and in carcinogenesis.[76]. The induction of mutagenesis or carcinogenesis may explain the observed increase of this ROS mediated mutagenic lesion present in blood cells after conditionally over-expressing IL-13 in the lung of the transgenic mice compared to wildtype littermates. The slight increase of 8oxoG presence in the WT animals most likely can be attributed to the repeated blood draws that caused a moderate increase in the production of ROS- induced DNA damage. This observation is similar to what was described in [97]. In our IL-13 mouse model we detected a significant increase of single and double stranded breaks in the peripheral blood and lung, a significant induction of micronucleus formation in the normochromatic erythrocytes present in the peripheral blood leukocytes, as well as increased staining of markers of genotoxicity in the lungs . [58] *Westbrook, et. al* show that an accumulation of double-strand breaks can lead to chromosome breaks and micronucleus formation. Perturbations to erythroblasts in the bone marrow may be a humoral effect of inflammation-associated DNA damage, as with the peripheral leukocytes. We suggest that increased inflammation in our experimental mice causes a significant induction of migratory cells that preferentially release pro-inflammatory cytokines at sites of inflammation. This re-circulating pool of activated cells may recruit more effector cells, which come into contact with erythroblasts in the bone marrow causing the observed clastogenecity. The induction of systemic strand breaks which are prevalent in many types of cancer were found to be significantly increased in our IL-13 induced asthma model. Recent studies also point to the fact that asthmatic patients have higher cancer risk.[98-100] Recently IL-13 was shown to induce genes whose expression exhibited decreased methylation including those involved in tissue remodeling,

leukocyte influx, and a Th2 influx. [49] The process of hypomethylation at CpG gene promoters are prevalent in cancer cells and are observed during the early stages of tumorigenesis or in abnormal tissue such as hyperplasia .[101] This epigenetic change in tissue can attribute to the progression of cancer and serves as a point of interest in observing the increase prevelance of cancer in asthmatics. Moreover the induction of hypomethylation concomitantly with immunoregulatory ability by IL-13 makes it a provocative player due to the possible tie to the increased incidence of cancer in asthmatic patients.

In conclusion we proposed that the key asthmatic mediator interleukin-13, increases important elements of the inflammatory response including ROS derived oxidative stress causing an induction in genotoxicity that has wide reaching systemic genotoxic effects. We show IL-13 is an essential regulatory molecule present in asthma and is associated with a systemic induction of genotoxic parameters such as oxidative DNA damage, single and double DNA strand breaks, micronucleus formation, and protein nitration. We further suggest increased immune cell infiltration and inflammation byproducts as a possible culprit to this genotoxic induction. Previous studies in human asthmatics also identified increase strand breaks produced during the direct interaction of ROS with DNA or during the repair process of damaged DNA. [18, 60]. This study further implicates IL-13 as a potential therapeutic target for other pulmonary diseases involving carcinogenesis.

Chapter 2

Effects of Side-Stream Tobacco Smoke and Smoke Extract on Glutathione- and Oxidative DNA Damage Repair-Deficient Mice and Blood Cells

[1]

[1]Parts of this document were originally published in Chapter 3 of Lynn Yamamato's thesis dissertation entitled **Gene-environment interactions: Roles of DNA repair proteins in preventing disease and injury**. This chapter has been modified from the original version and this newly modified version is under review in Mutational Research Manuscript Number: MUT-D-12-00136R1.

Abstract

Cigarette smoke causes direct oxidative DNA damage as well as indirect damage through inflammation. Epidemiological studies show a strong relationship between secondhand smoke and cancer; however, the mechanisms of secondhand smoke-induced cancer are not well understood. Animal models with either i) deficient oxidative DNA damage repair, or ii) a decreased capacity to combat oxidative stress may help determine the pathways important in mitigating damage caused by smoke. In this study, we used mice lacking *Ogg1* and *Myh*, both of which are involved in base excision repair by removing oxidatively damaged DNA bases. Gclm-deficient mice, which have decreased levels of glutathione (GSH), were used to look at the role of smoke-induced oxidative damage. *Ex vivo* experiments show significantly elevated levels of DNA single-strand breaks and chromosomal aberrations in peripheral blood lymphocytes from *Ogg1*$^{-/-}$*Myh*$^{-/-}$ double knockout mice compared to wild type (WT) mice after 24 hours of exposure to cigarette smoke extract (CSE). The average γH2AX foci per cell was significantly elevated 3 hours after exposure to CSE in cells from *Ogg1* $^{-/-}$*Myh* $^{-/-}$ double knockout mice compared to wildtype mice. *In vivo* we found that all mice had increased markers of DNA damage after exposure to side-stream tobacco smoke (SSTS). *Ogg1*$^{-/-}$ *Myh*$^{-/-}$ and *Gclm*$^{-/-}$ mice had altered levels of peripheral blood glutathione after SSTS exposure whereas wild type mice did not. This may be due to differential regulation of glutathione synthesis in the lung. We also found that *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice had a decreased lifespan after oral gavage with benzo[*a*]pyrene compared to wildtype mice and sham-exposed *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice. Our results are important in investigating the roles of oxidative stress and oxidative DNA damage repair in cigarette smoke-induced cancers and characterizing the role of genetic polymorphisms in smoke-related disease susceptibility.

Introduction

In 1964 The Surgeon General's report classified tobacco smoke as a carcinogenic compound and in 1992, the EPA classified secondhand smoke as a Group A human carcinogen after evaluating human and animal data (Respiratory health effects of passive smoking: lung cancer and other disorders. 1992. EPA: Washington D.C.). Secondhand smoke contains over 4000 chemicals of which at least 250 are toxic or carcinogenic (Report on Carcinogens, Eleventh Edition, 2005, U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program). While the negative health effects of smoking are well known, 43.4 million adults continue to smoke, exposing approximately 126 million nonsmokers (Cancer Facts & Figures, 2009, American Cancer Society), making secondhand smoke a major public health concern.

Both *in vitro* and *in vivo* studies in laboratory animals as well as in non-smokers show genotoxic effects of second hand and side-stream tobacco smoke (SSTS) [102]. DNA adducts and oxidative DNA damage have been found in people exposed to SSTS [19, 102]. Compounds in smoke can react directly with DNA leading to DNA adducts as well as indirectly by producing reactive oxygen species (ROS). Benzo[a]pyrene (B[a]P) is one of the most well-known carcinogenic components of smoke and causes DNA adducts, often leading to GC→TA mutations [102, 103]. B[a]P has been shown to increase pulmonary DNA adducts *in vivo*, and causes altered mutational profiles in human lung tumors [104-106]. Although G to T transversions are most often attributed to DNA adducts caused by compounds in smoke such as polyaromatic hydrocarbons (PAHs), oxidative DNA damage also commonly leads to GC→TA mutations.

It is well established that second hand smoke increases the risk of diseases such as cancer [107] and heart disease [108], however, mechanisms of smoke-induced injury are still not fully

understood. The classic model is that smoke exposure causes DNA damage and mutations in tumor suppressor genes or oncogenes which lead to carcinogenesis. More recently, other factors have been suggested to increase susceptibility to smoke-induced lung cancer including inflammation, cell cycle signaling, and rates of activation and detoxification of smoke particles [109, 110]. Understanding how smoke exposure causes lung cancer will help to clarify why some smokers develop lung cancer while others do not. Approximately 85% of lung cancers are attributed to smoking, however, only 10-15% of smokers develop lung cancer [110], implying that a subset of smokers is more susceptible to smoke-induced injury. The association between secondhand smoke and lung cancer is weaker so that susceptibility may play a more prominent role.

Since oxidative stress is important in smoke-induced damage, we studied the roles of antioxidant and DNA damage repair capacity in combating the effects of smoke. We assessed the effects of cigarette smoke extract (CSE) and SSTS on DNA damage and oxidative stress in different mouse models. We used mice deficient in Ogg1 (8-oxodG glycosylase) and Myh (MutY homologue), as well as mice deficient in Gclm (Glutamate-cysteine ligase, modifier subunit). Ogg1 is considered to be the major enzyme for repair of 8-oxodG [75]. Myh primarily repairs 8-oxodG:A and 8-oxodG:G mismatches during DNA replication and its activity is directed towards the daughter strand [75]. Spontaneously, over 30% of double mutant *Ogg1*$^{-/-}$ *Myh*$^{-/-}$ mice develop lung tumors after 12 months [111]. Further characterization of these lung tumors show that 75% of sequenced K-ras alleles from tumors had a G to T transversion compared to no transversions in adjacent normal tissue [111]. This is consistent with mice deficient in 8-oxodG repair and the types of mutation which lead to tumorigenesis [111].

Subsequently, it was found that the number of 8-oxodG lesions in the liver, lungs, and small intestines of *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice were at least tripled compared to wildtype controls [112].

We used *Gclm*$^{-/-}$ mice to study the role of anti-oxidant defense in smoke-induced disease and injury. Gclm is important for glutathione synthesis. Glutathione is one of the most abundant reducing agents in the cell and has an important role in protection against reactive oxygen species (ROS), metabolism of nutrients and xenobiotics, and regulation of intracellular redox status [113]. *Gclm*$^{-/-}$ mice have 9-16% of the normal GSH levels in the liver, lung, pancreas, erythrocytes, and plasma compared to wildtype littermates [114]. *Gclm*$^{-/-}$ mice also exhibit reduced cysteine levels in the kidney, pancreas, and plasma [114].

To study the roles of DNA damage and oxidative stress in environmental tobacco smoke-induced cancer, we exposed *Ogg1*$^{-/-}$*Myh*$^{-/-}$, *Gclm*$^{+/-}$, and *Gclm*$^{-/-}$ and wildtype mice or cells to SSTS or CSE and measured markers of DNA damage and oxidative stress. We observed that CSE induced DNA double strand breaks in mononucleated white blood cells, single strand breaks in leukocytes, and micronucleus formation in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mutant but not in wildtype cells. We also found that γH2AX foci were increased in mice after SSTS exposure; however single-strand breaks and hOGG-1-induced DNA strand breaks were not significantly increased. SSTS increased GSH levels in *Ogg1*$^{-/-}$*Myh*$^{-/-}$, *Gclm*$^{+/-}$, and *Gclm*$^{-/-}$ mice but not wildtype mice. Gene expression of oxidative stress and GSH regulatory proteins were also altered in the lungs of SSTS-exposed animals. Finally, we show that DNA repair deficient animals had decreased survival proportions after oral gavage of benzo[*a*]pyrene compared to wildtype animals. These results show that mice deficient in base-excision repair have a mild but significantly increased susceptibility to smoke and smoke components and that GSH is an important molecule in mitigating these effects.

Materials and Methods

Mice Breeding and Care. Myh- and Ogg1-deficient mice have been described previously [111] and were backcrossed with C57BL/6J mice at least 4 times. Additionally, they have been backcrossed at least twice with C57BL/6J p^{un}/p^{un} mice. $Gclm^{+/-}$ mice have also been described previously and have been backcrossed at least 4 times with C57BL/6J p^{un}/p^{un} mice [115]. To obtain $Gclm^{-/-}$ mice, heterozygous females were crossed with heterozygous males. $Myh^{-/-}Ogg1^{-/-}$ mice were obtained by crossing $Ogg1^{+/-}Myh^{+/-}$ males and females and by crossing $Ogg1^{-/-}Myh^{+/-}$ mice with $Ogg1^{+/-}Myh^{-/-}$ or $Ogg1^{-/-}Myh^{+/-}$ and $Ogg1^{+/-}Myh^{-/-}$ with $Ogg1^{+/-}Myh^{+/-}$ mice. Genotyping was be done by PCR as described previously [111, 115]. Wild type mice were obtained from $Gclm^{+/-}$ crosses, $Ogg1^{+/-}Myh^{+/-}$ crosses, or from our wildtype C57BL/6J p^{un}/p^{un} colony, used in all backcrosses. 8-10 week old male and female mice were used for *in vivo* experiments and 12-13 month old male and female mice were used for *ex vivo* experiments. Mice were bred in an institutional specific pathogen free animal facility under standard conditions with a 12 hr light/dark cycle according to Animal Research Committee regulations. Mice were fed a standard diet and water *ad libitum*.

Blood Collection. For exposure to CSE, peripheral blood was collected from experimental mice via terminal right ventricle cardiac puncture using a heparin-coated syringe (American Pharmaceutical Partners, Inc. Schaumburg, IL). At least 1 mL of blood was collected from each animal, aliquoted into 2 tubes for exposure to CSE or PBS and incubated at 37°C. For *in vivo* experiments, blood was collected by facial vein puncture using 5 mm sterile lancets (Medipoint Inc. Mineola, Ny). Blood from each mouse was collected into EDTA-coated tubes (Sarstedt Aktiengesellschaft & Co., Numbrecht).

Whole blood CSE exposure. Frozen stocks of cigarette smoke extract were supplied by the lab of Andrew Dannenberg at Cornell University as described previously [116]. Concentrated 40.3 puffs/mL cigarette smoke extract was diluted to a working solution of 5 puffs/mL with PBS. 5 puffs/mL cigarette smoke extract (CSE) was administered directly into whole peripheral blood to a final concentration of 1 puff/mL CSE and allowed to incubate in a shaking 37°C incubator for 3,6, or 24 hours.

SSTS exposure. Mice were exposed to SSTS in a Teague Enterprises Model TE-10 cigarette-smoking machine (http://memebers.dcn.org/svteague/catalog.html). Smoke from a smoldering Kentucky reference cigarette 2R4F was collected in a mixing chamber, then flowed through a dilution chamber before entering the exposure chamber. Mice were exposed in open cages with access to food and water *ad libitum*. 8-10 week old mice were exposed to an average of 43 mg/m^3 TPM for 6 hours/day for 14 days. Daily exposures were separated with a 1 hour break between 3-hour exposures. TPM was measured by a particulate counter (DusTrak, TSI Inc., St. Paul, MN) and calibrated for tobacco smoke.

γ- H2AX Immunofluorescence. After erythrocyte lysis, cells were laid over poly -D-lysine-coated coverslips and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) at room temperature as described previously [51]. Subsequently, cells were permeabilized with 0.5% Triton X-100 (Sigma), followed by 5 rinses in PBS. For *ex vivo* experiments blocking was done in aluminum-covered plates overnight at 4°C in 10% FBS. For *in vivo* experiments blocking was done for 1 hour at room temperature in 10% FBS. Coverslips were then incubated for 1 hour at room temperature with mouse anti-phospho-Histone H2A.X (Upstate, Temecula, CA) at a dilution of 1:400, then rinsed with 0.1% Triton X-100. Following a second 10% FBS blocking, cells were stained with FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch,

West Grove, PA) at a dilution of 1:200 for 1 hour at room temperature. Coverslips were mounted onto slides using VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA). Foci were analyzed on a Zeiss automated microscope. At least 100 cells were counted per sample and cells with more than four distinct foci in the nucleus were considered positive for γH2AX.[52]. Apoptotic cells, which have an approximate 10-fold increased in nuclear foci in damaged cells, were not included in analyses [52, 53]. Statistical analysis was done using ANOVA and tukey's post hoc test in *ex vivo* experiments and two-way repeated measures ANOVA followed by Bonferroni post-tests for *in vivo* experiments (PRISM).

Micronucleus assay. 3µl of whole blood were spread on a microscope slide and stained with Wright-Giemsa solution (Sigma-Aldrich, St. Louis, MO). At least 4000 erythrocytes were counted according to published recommendations [54]. MN were counted and scored with an Olympus Ax70 (Tokyo, Japan) at 100X magnification. Statistical analysis was done using repeated measures ANOVA followed by a Bonferroni or Kruskal-Wallis post-tests , or a t-test to compare 2 groups.(GraphPad Prism)

DNA single strand breaks. Oxidative DNA damage and DNA strand breaks were measured in peripheral blood cells using the alkaline comet assay. In *ex vivo* experiments peripheral blood was collected before CSE incubation, and immediately after 3, 6, or 24 hours of incubation with CSE. For *in vivo* experiments blood was collected before, and after 1 and 2 weeks of SSTS exposure. Blood was diluted 1:1 with RPMI + 20% DMSO, slowly frozen and stored at -80C until the assay was performed. The comet assay was done as described previously [55]. Briefly, cells were mixed with low melting-point agarose, and placed in triplicate onto normal agarose layed over gelbond (Lonza Inc. Rockland, ME). The gel was immerse in lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton, and 10% DMSO), then alkaline

electrophoresis buffer (0.3 M NaOH, 1 mM EDTA). After 20 minutes in the electrophoresis buffer at 4°C, the gel was run for 45 minutes at 300 mA, allowed to dry and then stained with SYBR Gold (Molecular Probes). Comet tail-moments were analyzed using CASP (Comet Assay Software Project, http://casp.sourceforge.net/). To measure oxidative DNA damage, the comet assay was modified to include an incubation step with hOGG1 (New England Biolabs, Ipswich, MA). As described previously, embedded cells were incubated with hOGG1 (1:300 in NEBuffer1 and BSA) at 37°C for 30 minutes following the lysis step [56]. Tail-moments were normalized to a control to account for inter-experimental variability. Statistical analyses were done using ANOVA (GraphPad Prism).

GSH measurements. Glutathione levels were determined in blood samples taken before, and after 1 and 2 weeks of SSTS exposure and stored at -80˚C until analysis. GSH was extracted using 5% metaphosphoric acid and analyzed using the *Oxis*Research Bioxytech GSH/GSSG-412 kit (Portland, OR) which is based on the Tietze method [117]. The assay was modified to fit into a 96-well plate. Statistical analyses were done using ANOVA.

Gene expression analysis. Lungs from mice at the end of the 2-week SSTS exposure or unexposed control mice were perfused and lavaged before immersion into RNAlater (Qiagen). Lungs were kept at 4˚C for 24 hours then transferred to -80˚C until RNA was isolated using the RNeasy Mini kit according to manufacturer's instructions (Qiagen). cDNA was synthesized using SuperscriptIII (Invitrogen) according to manufacturer's recommendations. Quantitative real-time PCR was performed on an ABI Prism 7500 gene expression system (Applied Biosystems) using Taqman gene expression assays for *Nrf2*, *Hmox*, *Gclc*, *Gclm*, *Gsr*, *Gstα4*, *Mcp-1*, *IL-1β*, *Tnfα*, and *Tgfβ*. *Gapdh* was used as an internal control. Each reaction was done in triplicate and analyzed using the relative standard curve method.

B[*a*]P survival proportions. Wildtype and *Ogg1*$^{-/-}$ *Myh*$^{-/-}$ double knockout mice were dosed twice a week for 1 month by oral gavage with Benzo[*a*] pyrene (Sigma, lot no.37F-0555). B[*a*]P was suspended in corn oil and mice were dosed at 100mg/kg body weight. Control mice were gavaged with corn oil only.

Results

Fig.2-1CSE exposure increased DNA double-strand breaks in mononucleated white blood cells from base excision repair deficient mice. The frequency of DNA double stranded breaks in white blood cells (WBCs) from wildtype and *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice was measured using the γH2AX assay. After 3 hours of exposure to 1 puff/mL CSE *ex vivo*, the average number of γH2AX foci per *Ogg1*$^{-/-}$ *Myh*$^{-/-}$ cell was four times higher than mononucleated WBCs in the non-exposed group (Fig. 2-1, $p<0.05$). No significant differences were found in wildtype WBCs exposed to 1 puff/mL CSE for 3 hours compared to non-exposed wildtype mononucleated WBCs. No differences were found between genotypes at any time point. (Fig. 2-1).

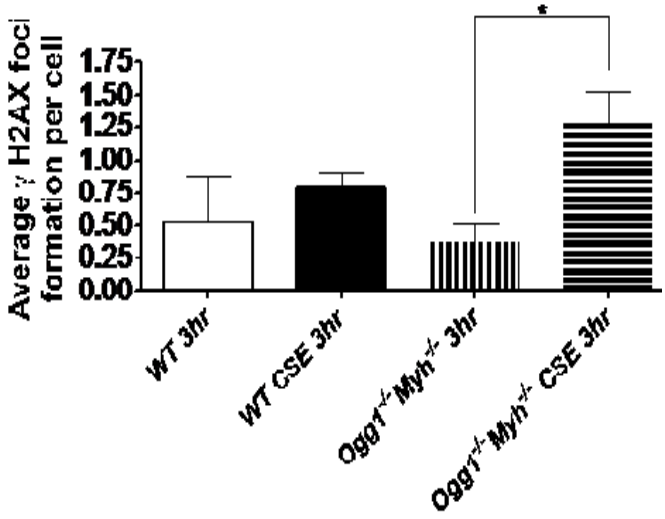


Fig 2-1 Average number of γH2AX foci formed in peripheral leukocytes. Presence of double strand breaks was confirmed by immunofluorescence of γH2AX. At least 100 cells were analyzed per sample. Data represent mean ± SEM. Statistical analyses were done using ANOVA testing and Tukey's post hoc analysis. n=5 in all groups. * indicates significant $p<0.01$ between no CSE exposed and CSE exposed lymphocytes at 3hr time point.

Fig. 2-2 CSE induced micronucleus formation in erythrocytes from base excision repair deficient mice after 24 hours. We found a significant induction of micronuclei in the peripheral blood from *Ogg1*$^{-/-}$ *Myh*$^{-/-}$ mice after incubation with CSE for 24 hours (Fig2-2, $p<0.05$). Micronuclei were slightly elevated in the peripheral blood from wildtype animals exposed to CSE for 24 hours, however, this difference was not significant. No differences were found between genotypes. These results suggest that cells from *Ogg1*$^{-/-}$ *Myh*$^{-/-}$ double knockout mice exhibit increased susceptibility to components of cigarette smoke extract.

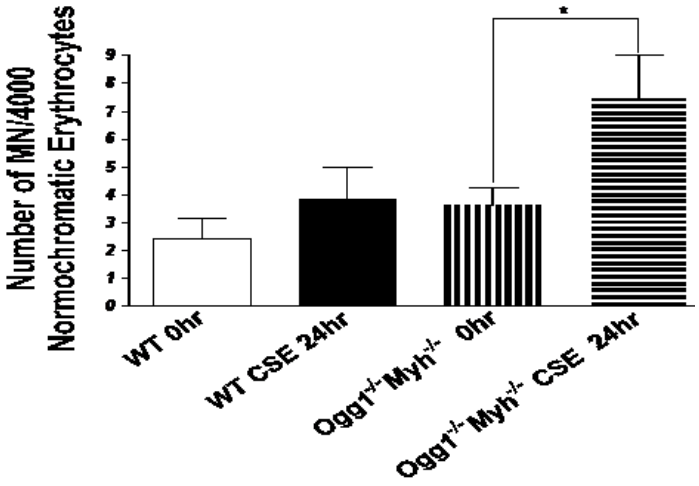


Fig 2-2 At least 4000 normochromatic erythrocytes were counted and scored for the presence of micronuclei. Data, statistical analyses, and error bars represent mean ± SEM of micronucleated normochromatic erythrocytes (MN-NCE) per 1000 NCEs. * indicate significant $p<0.05$ between no CSE exposed lymphocytes at 0hr and CSE exposed lymphocytes at 24hr. n=5 in all groups. Statistical analyses were done using two tailed student t-Test between relevant groups

Fig.2-3 CSE induced DNA strand breaks in peripheral leukocytes of wildtype and base excision repair deficient mice. Single- and double-strand breaks as well as alkali-labile sites were assessed by mean olive tail moment using the alkaline comet assay (Fig2-3). The mean olive tail moment from wildtype and *Ogg1*$^{-/-}$ *Myh*$^{-/-}$ double knockout mice remained low after 3 and 6-hour incubations with CSE. Mean olive tail moments were significantly higher after 24 hours of CSE incubation in both wildtype and *Ogg1*$^{-/-}$ *Myh*$^{-/-}$ double knockout mice compared to incubation with PBS only ($p<0.05$ and $p<0.001$, respectively) and compared to shorter incubation times. No differences were found between genotypes at any time point. (Fig.2-3).

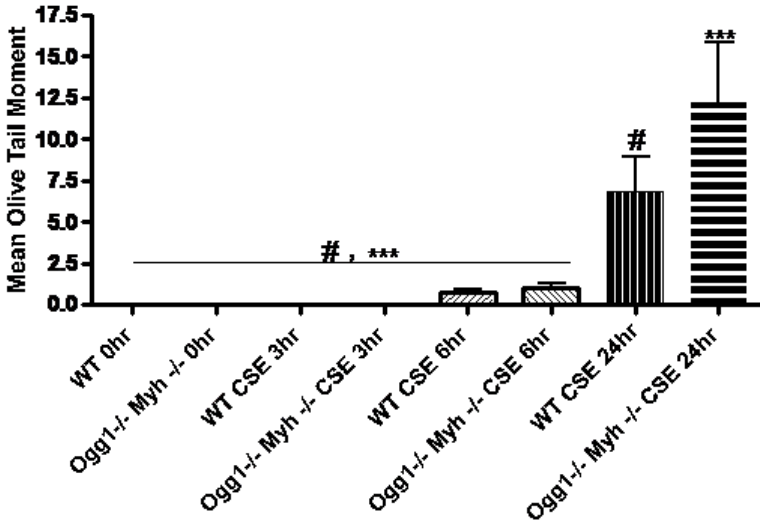
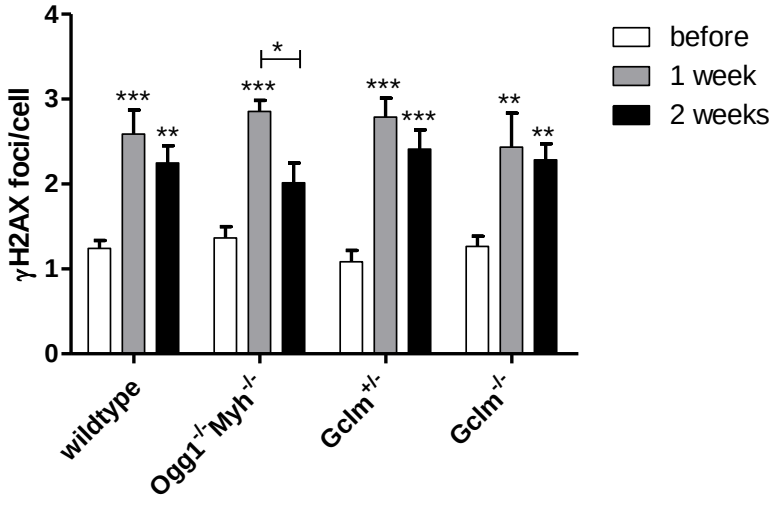


Fig.2-3 At least 100 "comets" were scored per mouse. To measure the amount of single strand breaks the comet assay was conducted. Data were log transformed before statistical analyses and error bars represent mean ± SEM. Statistical analyses were done using ANOVA testing and Tukey's post hoc analysis. # indicates $p< 0.05$ between WT CSE exposed. *** indicates $p \leq 0.001$. n=5 in all groups.

Fig.2-4 γH2AX foci and micronuclei are increased in mice after exposure to SSTS.

After 1 week of exposure to SSTS, γH2AX foci increased in all mice (Fig. 2-4A, $p<0.05$). After 2 weeks of exposure, average γH2AX foci per cell were still above spontaneous levels, but did not increase above week 1 levels. No differences were found between genotypes at any time point. In *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice, levels of γH2AX foci decreased significantly after 2 weeks of exposure compared to after 1 week. Since blood was taken within 15 minutes following exposure to SSTS, the γH2AX foci represent ongoing DNA damage and repair in these mice. Although γH2AX foci are increased after exposure to SSTS, micronuclei, which represent permanent damage following a double-strand break, are only significantly increased in wildtype and *Gclm*$^{+/-}$ mice after 2 weeks of exposure to SSTS (Fig.2-4B). Micronuclei are increased in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ and *Gclm*$^{-/-}$ mice, however the trend does not reach significance (Fig.2-4B). Importantly, however, spontaneous levels of micronuclei are lower in wildtype mice than *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice ($p<0.05$ using the t test). DNA single-strand breaks as measured by the comet assay were not increased significantly after exposure to SSTS. Oxidized DNA damage, measured by the hOGG1-modified comet assay, which induces breaks at oxidized bases, was also increased but not significantly in response to SSTS (data not shown). A lack of significant DNA damage seen by the comet assay could be due to the sensitivity of the assays or number of mice since our data show slight but not significant increases after SSTS exposure.

A.



B.

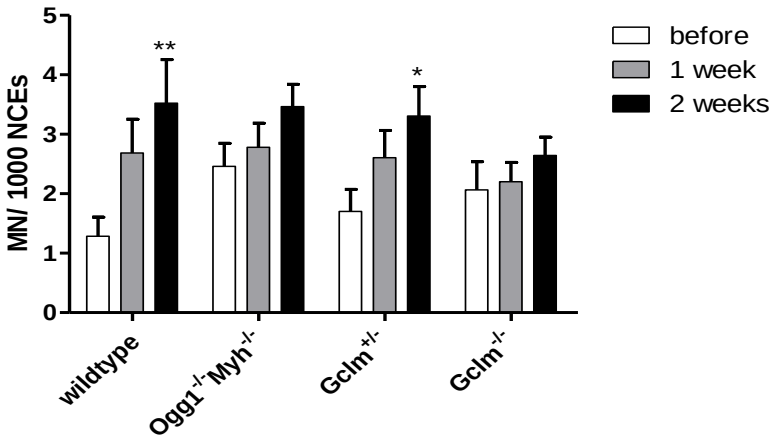


Fig. 2-4 In-vivo DNA damage assays in peripheral leukocytes A.) γH2AX foci in peripheral blood increase after 1 and 2 weeks of exposure to SSTS in all mice ($p<0.05$). n= 8 for wildtype mice and 6 for all other groups. **B.)** Micronuclei in normochromatic erythrocytes significantly increase in WT and Gclm heterozygous animals after exposure to smoke. n= 5 for wildtype mice and 6 for all other groups. *indicates $p<0.05$, **indicates $p<0.01$, and ***indicates $p<0.001$. Error bars indicate the mean ± SEM. Significance is compared to before timepoints unless otherwise indicated.

Fig.2-5. Glutathione in the peripheral blood is differentially altered in response to SSTS depending on genotype. GSH is a major antioxidant in the cell. As expected, *Gclm*$^{-/-}$ mice have decreased levels of GSH at all time points (Fig. 2-5A). Following exposure to SSTS, peripheral blood GSH levels were increased in all knockout mice over spontaneous levels (*Ogg1*$^{-/-}$*Myh*$^{-/-}$, *Gclm*$^{-/-}$, and *Gclm*$^{+/-}$) but not in wildtype mice (Fig.2-5A). GSH levels were significantly increased as early as 1 week in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ and *Gclm*$^{-/-}$ mice and after 2 weeks in *Gclm*$^{+/-}$ mice. Therefore, wildtype mice may be more efficient at regulating a stress response when exposed to sub-chronic doses of SSTS.

Fig.2-5

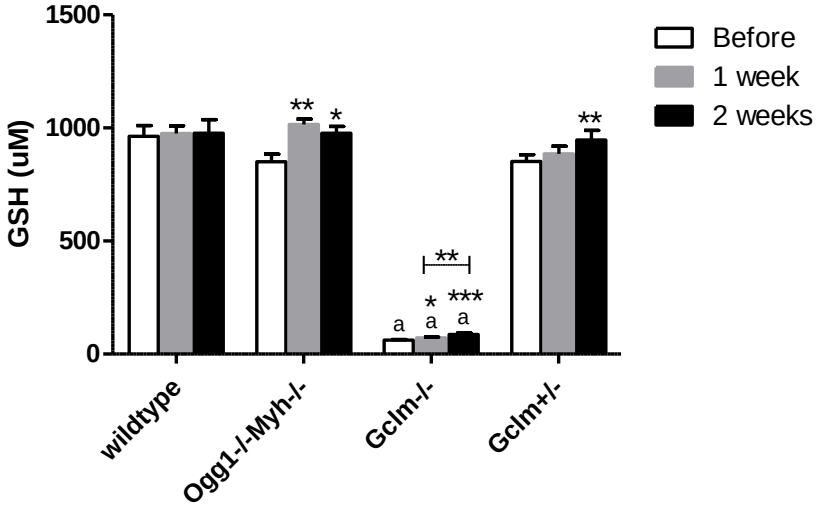
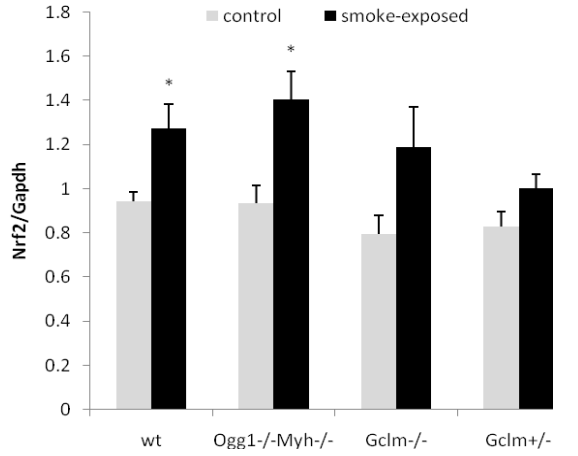


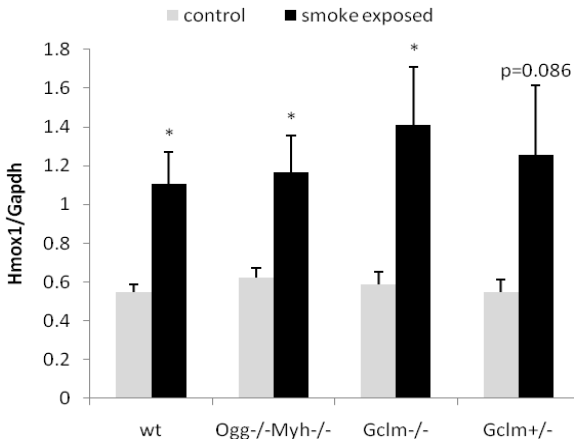
Fig. 2-5. Peripheral blood levels of GSH are differentially altered after SSTS exposure. Peripheral blood GSH levels are differentially altered depending on genotype. GSH levels in *Gclm*$^{-/-}$ mice are significantly lower than all other mice at all timepoints. n= 8 for wildtype mice and 6 for all other groups. *indicates $p<0.05$, **indicates $p<0.01$, and ***indicates $p<0.005$. 'a' indicates $p<0.005$ comapred to all other genotypes. Error bars represent the mean ± SEM.

Fig.2-6. Gene expression of proteins involved in oxidative stress response and GSH regulation are altered in lungs after exposure to SSTS. After 2 weeks of exposure to SSTS, *Nrf2* expression is significantly increased in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ and wildtype mice (Fig.2-6A). Nrf2 is a transcription factor upregulated in response to oxidative stress and smoke [118, 119]. *Hmox* is regulated by Nrf2 and is increased in all mice (Fig.2-6B). This data is consistent with previous results showing increased *Nrf2* and *Hmox* in response to smoke in wildtype mice [118].

Fig.2-6

A.

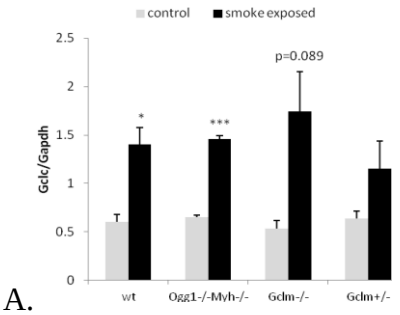




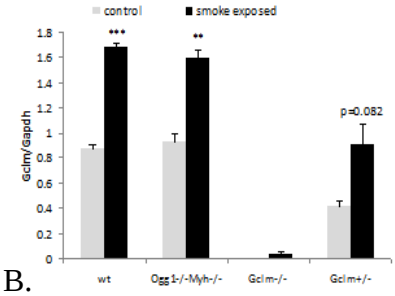
B.

Fig.2-6 Gene expression of *Nrf2* and *Hmox* proteins involved in oxidative stress response in the lung are upregulated in response to SSTS. A.) *Nrf2* expression changes, n=3 for each group. **B.)** *Hmox* expression changes, n=5 for each group. *indicates $p<0.05$. Error bars represent the mean ± SEM.

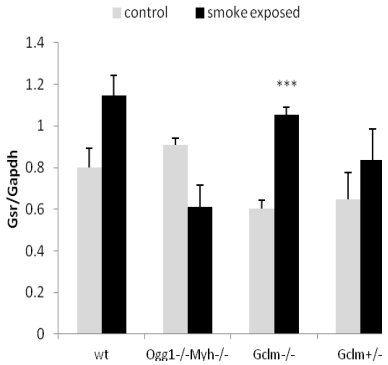
Fig.2-7.Since we found altered levels of GSH in the blood, we also looked at expression of genes involved in GSH homeostasis. Although McConnachie *et al* found a spontaneous increase of *Gclc* in the liver of *Gclm*$^{-/-}$ mice [115], we did not see a similar increase in the lungs. Following exposure to smoke, however, *Gclc* expression was upregulated in *Ogg1*$^{-/-}$ *Myh*$^{-/-}$, *Gclm*$^{-/-}$, and wildtype mice (Fig. 2-7A). In addition, *Gclm* expression was increased in response to SSTS in all mice except the Gclm-deficient mice (Fig.2-7B). Other genes involved in the GSH regulation include Glutathione reductase (*Gsr*), responsible for reducing GSSG to 2 GSH units and glutathione-S-transferases (*Gstα4*) which use GSH for detoxification. We found that *Gsr* is significantly upregulated in *Gclm*$^{-/-}$ mice (Fig.2-7C). We also found that *Gstα4* is significantly upregulated in *Gclm*$^{-/-}$ mice (Fig.2-7D). Therefore, mice primarily responded to SSTS exposure by up-regulating genes involved in oxidative stress response and there are slight differences depending on genotype.



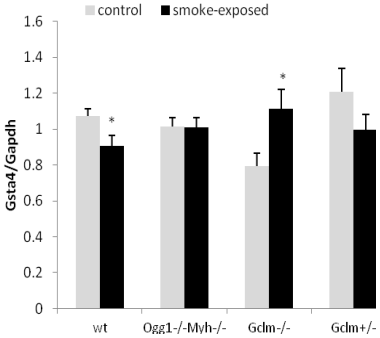
A.



B.



C.



D.

Fig.2-7. E**xpression of genes which affect GSH in the lung in response to SSTS. A.)** *Gclc* expression changes, n=3 for each group. **B.)** *Gclm* gene expression changes, n=3 for each group. **C.)** *Gsr* expression changes, n=3 for each group. **D.)** *Gstα4* expression changes, n=5 for each group. *indicates $p<0.05$, ** indicates $p<0.01$, ***indicates $p<0.001$. Error bars represent the mean ± SEM.

Fig.2-8.*Ogg1*$^{-/-}$*Myh*$^{-/-}$ double knockout mice exhibit decreased survival after exposure to B[*a*]P by oral gavage. B[*a*]P and other polyaromatic hydrocarbons (PAHs) are thought to be the cause of the carcinogenic effects of exposure to coal tars, soots, and related materials [106]. B[*a*]P has also been shown to induce tumor formation at the site of application in rodents [106]. Metabolism of BaP to the major carcinogen BaP-diolepoxide , forms reactive oxygen species including superoxide, H2O2, •OH radicals and semiquinone. [120].These ROS induce DNA damage and can cause modifications to guanine residues to form the common 7,8-dihydro-8-oxoguanine (8oxo-dG) mutagenic legion .This specific legion is normally recognized by the Ogg1 enzyme. [121].Furthermore, (8oxo-dG) legions readily mispair with adenines to form transversion mutations, these mispaired adenines are removed by the MYH protein.[122] In this current work *Ogg1*$^{-/-}$Myh$^{-/-}$ and wildtype mice were exposed to100 mg/kg B[*a*]P twice per week for 1 month by oral gavage. *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice exposed to B[*a*]P have a significantly decreased median lifespan compared to B[*a*]P-exposed wildtype mice (Fig.2-8A, $p<0.005$). In addition, *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice gavaged with B[*a*]P had a shorter median lifespan than *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice gavaged with corn oil only (Fig.2-8B, $p<0.005$). These data show that the *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice have increased susceptibility to a major component found in cigarette smoke.

Fig.2-8

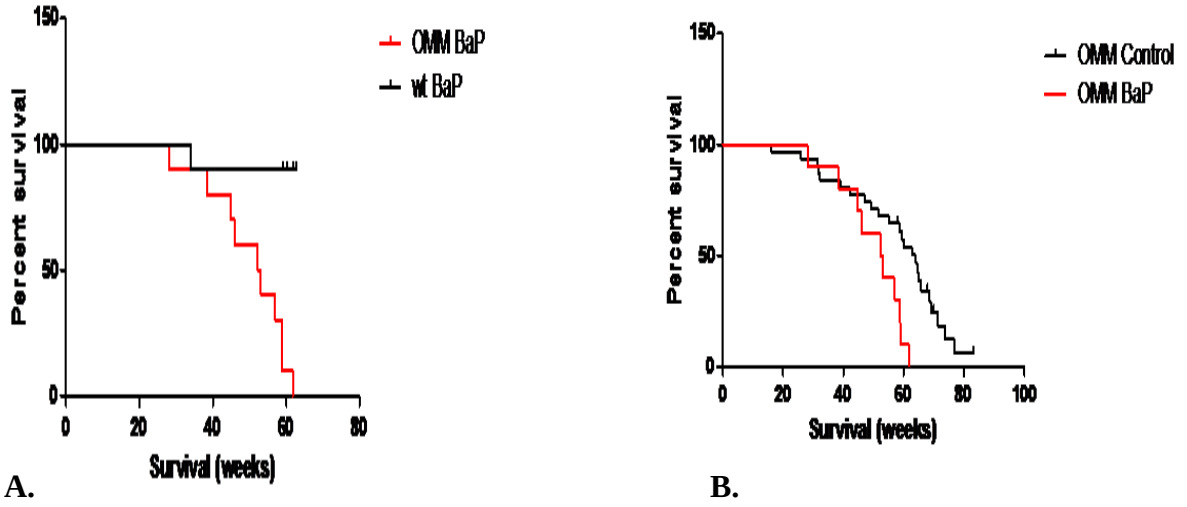


Fig.2-8. Percent survival in Wildtype and *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice after Benzo (a) pyrene gavaging A.) Percent survival in weeks in wildtype (black line) and *Ogg1*$^{-/-}$*Myh*$^{-/-}$(red line) mice after benzo (a) pyrene gavaging. **B.)** Percent survival in weeks in control *Ogg1*$^{-/-}$*Myh*$^{-/-}$(black line) and benzo (a) pyrene gavaged *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice (red line). n=10 for wildtype mice and n=9 for *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice. $p < 0.005$ for both curves.

Discussion

We examined susceptibility to cigarette smoke extract and SSTS in mice deficient in the repair of oxidative DNA damage and in mice with decreased levels of GSH. In *ex vivo* CSE experiments we found that cells from *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice had significantly higher amounts of chromosomal aberreations and single stranded breaks after 24-hour incubations assessed via micronucleus and comet assays, respectively. After a 3-hour CSE incubation we also observed a significant increase in DNA double-strand breaks assessed by γH2AX foci formation in peripheral blood lymphocytes. *In vivo* experiments showed that DNA double-strand breaks were increased in response to SSTS in all groups of mice. GSH homeostasis, however, was

differentially regulated in *Ogg1*$^{-/-}$*Myh*$^{-/-}$, *Gclm*$^{-/-}$, and *Gclm*$^{+/-}$ mice compared to wildtype mice. Although in our *in vivo* experiments we did not observe a significant increase in DNA damage as measured by the comet assay after a two week SSTS exposure in all mice, a significant increase in *ex vivo* mouse lymphocytes suggests that the cells of the DNA repair deficient mice are more sensitive than that of wildtype mice exposed to CSE. There may be a dose or time-dependency which is important in differentiating these mice *in vivo*, as alluded to by the decreased γH2AX foci formation after 2 weeks of SSTS exposure in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice. In addition, *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice were significantly more sensitive to B[*a*]P, a constituent of cigarette smoke.

In our experiments SSTS induced DNA double-strand breaks in peripheral blood cells in all mice after 1 week of exposure to SSTS, similar to what has been seen in cells exposed to smoke [123]. Levels of DNA double-strand breaks decreased significantly in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice after 2 weeks of exposure and remained approximately the same or slightly decreased in all other mice indicating i) an increase in DNA repair capacity and/or ii) that SSTS-induced γH2AX foci had reached a saturation point so that there was an equilibrium between DNA damage and repair by 1 week. Although we found significant increases in DNA damage as measured by the micronucleus assay only in wildtype and *Gclm*$^{+/-}$ mice, in all groups of mice micronuclei levels seemed to increase over time. In support of this, slight but significant increases in peripheral blood micronuclei has been shown previously [124]. Since γH2AX foci form in response to DNA double-strand break recognition, and may signal repair, the lack of a significant increase in micronuclei could be indicative of proficient repair of double-strand breaks, or an indication that the measurement of micronuclei is not as sensitive after 1 and 2 weeks of exposure to SSTS. Micronuclei have been shown to increase 30 hours after a 1 day exposure to SSTS [125]. Micronuclei gradually accumulate in circulating blood until they reach a maximum level at 48-

72 hours, then decrease [126]. Therefore, if repair capacity increases, circulating levels of micronuclei may decrease after 1-2 weeks of SSTS exposure. Single-strand breaks and hOGG1-induced single-strand breaks in peripheral blood lymphocytes also did not significantly increase in response to SSTS. It is possible that upregulation of protective enzymes such as DNA repair proteins prevented increases in DNA damage after 1 week of exposure since it was shown that *HO-1* (*Hmox1*) and *Ogg1* were upregulated as early as 4 days after exposure to diesel exhaust particles [127]. We also show an increase in *Hmox1* after SSTS exposure. Although *Ogg1* could not be upregulated in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice, there are several proteins which could help repair DNA damage [75].

Since GSH is the most abundant small molecule antioxidant in the cell and its homeostasis is associated with several health effects [113, 128], we measured circulating GSH levels. In response to SSTS exposure, we found that peripheral blood GSH levels significantly increased in *Ogg1*$^{-/-}$*Myh*$^{-/-}$, *Gclm*$^{-/-}$, and *Gclm*$^{+/-}$ mice but not wild type mice. Since *Ogg1*$^{-/-}$*Myh*$^{-/-}$ and wildtype mice in principle have equivalent abilities to regulate GSH, this was somewhat surprising. An increase in GSH in response to stress has been seen previously [74, 129], and specifically in a human lung cell line in response to cigarette smoke extract [130]. Since GSH regulation is important in many parameters of human health, changes in GSH levels may indicate disruption of normal processes [113, 128]. Different levels of peripheral blood GSH may be explained by differential regulation of enzymes involved in GSH metabolism as found in the lungs, in addition to Nrf2, which is a transcription factor shown to upregulate *Gclc* and *Gclm* [129, 131, 132]. Although *Gclc* is up-regulated in the lungs of wildtype, *Ogg1*$^{-/-}$*Myh*$^{-/-}$ and *Gclm*$^{-/-}$ mice, Gclm is considered to be more involved in stress response [129]. Therefore, it is not surprising that *Gclm* is induced in all mice besides the knockout mouse. *Gsr*, important in

reducing GSSG, is upregulated in *Gclm*$^{-/-}$ mice. Since these mice lack GSH sythesizing capacity, this may be a compensatory mechanism in *Gclm*$^{-/-}$ mice in response to SSTS. *Gclm*$^{-/-}$ mice were also the only group of mice to upregulate *Gstα4*. *Gstα4* is a glutathione transferase which has been shown to detoxify 4-HNE [133], a product of lipid peroxidation. Therefore, an increase in *Gstα4* in *Gclm*$^{-/-}$ mice may indicate the presence of oxidative stress.

The fact that mice respond to cigarette smoke with increased production of GSH and GSH metabolizing enzymes and possibly DNA repair functions might explain why it is difficult to induce cigarette smoke induced lung cancer in mice [134, 135]. Of the small rodents, hamsters, rats, and mice have all been used to study effects of smoke. In many cases a small difference in smoke-exposed versus non-exposed animals was found, however the degree of carcinogenesis was relatively low [135]. Newer models of smoke-induced lung cancer have been developed. One uses a high exposure concentration (100-250 TPM/m^3) for up to 30 months which led to a significant induction of lung tumors and cancer in mice and rats [136-138]. However, smoke exposed mice lived longer than control mice and had a significantly delayed onset of other types of cancer [136] which is in agreement with our *in vivo* studies, showing an induction of defense enzymes and antioxidants which can reduce systemic genotoxicity.

In relation to smoking induced lung cancer the strongest and most widely studied carcinogens are the PAHs [106]. B[*a*]P, one of the most extensively studied PAH present in cigarette smoke, has been shown to induce tumors at the site of application in rodents [106]. Metabolism of PAHs causes genotoxic intermediates that can lead to ROS mediated G to T transversion mutations caused by elevated 8-OHdG adenine mispairing.[111, 120] These mutations are recognized and removed by the Ogg1, and MYH enzymes, respectively. Furthermore, this specific DNA alteration is elevated in peripheral leukocytes and lung tissue in smokers and lung cancer

patients, making it advantageous to use base excision reapair deficient animals to study ROS mediated DNA damage caused by B[*a*]P exposure. In our experiments we gavaged wildtype and *Ogg1*$^{-/-}$*Myh*$^{-/-}$ double knockout mice with 100 mg/kg B[*a*]P. Although extensive pathology was not conducted on these animals we observed a marked decrease in overall survival proportions in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ double knockout mice gavaged with B[*a*]P compared to B[*a*]P-gavaged wildtype and sham-exposed *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice. In support of these observations, [139] describes an increased risk of lung cancer in individuals with polymorphisms in DNA damage repair exposed to PAHs, including B[*a*]P, [139]. B[*a*]P DNA adducts are primarily repaired by nucleotide excision repair, but our results indicate that a certain proportion is also repaired by the base excision repair pathway or that B[*a*]P metabolites lead to damage which requires the base excision repair pathway.

Mechanistically the modes of cigarette smoke extract induced genotoxicity are not fully elucidated. Many studies suggest oxidative stress plays a major role in the genotoxic response [140-143]. However, these and many other aspects of cigarette smoke-induced carcinogenesis and tumorogenesis require further study. Our *ex vivo* results suggest an increased susceptibility to cigarette smoke induced genotoxicity in cells from base excision repair deficient mice compared to untreated wildtype mice. These data further identify the importance of proper DNA repair and removal of genotoxic insults from noxious environmental agents like cigarette smoke extract.

In conclusion, this study has observed a significant difference in *ex vivo* CSE-induced damage in base excision repair deficient mice compared to wildtype mice, differentially regulated GSH homeostasis in *Ogg1*$^{-/-}$*Myh*$^{-/-}$, *Gclm*$^{-/-}$, and *Gclm*$^{+/-}$ mice compared to wildtype mice, and decreased survival proportions after exposure to B[*a*]P in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice compared

to wildtype mice. These results are in agreement with epidemiological data showing an increased susceptibility to lung carcinogenesis in people with polymorphisms in *OGG1* and *MYH* [7] [121]. More studies using genetically modified animal models may expedite our understanding of cigarette smoke-induced cancer susceptibility.

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Chapter 3: Assessing Phenotypic Biomarkers of DNA Damage to Determine Susceptibility to Develop Lung Cancer

Abstract

Lung cancer is the leading cause of death from cancer in both men and women in the United States and causes more deaths than the next three most common cancers combined (colon, breast, and prostate). Nearly 90 percent of individuals who develop lung cancer are smokers, yet only 10-15 percent of lifetime smokers will develop the disease.This statistic suggests that some individuals are more susceptible to developing lung cancer than others. In this work utilizing markers of DNA double strand breaks, oxidative DNA damage, and inflammation induced protein damage we sought to establish phenotypic tools to assess if individuals are more susceptible to cigarette smoke extract induced genotoxicity. In spite of our very small sample size we found statistically significant results correlating age and race to susceptibility to develop DNA damage, as well as, positive yet non-significant trends in gender, family history of cancer, pack years of cigarettes smoked, lung cancer difference to non-lung cancer, and individuals with history of other cancers.

Introduction

Lung cancer is the leading cause of death from cancer in both men and women in the United States. [21, 22] Lung cancer causes more deaths than the next three most common cancers combined (colon, breast and prostate). [23, 24] Nearly 90 percent of individuals who develop lung cancer are smokers, yet only 10-15 percent of lifetime smokers will develop the disease. [22, 25] What is further perplexing is the notion that it is not well understood why some individuals are more susceptible to smoking induced lung cancer. This suggests that other factors concomitant with history of smoking may be involved in susceptibility to develop carcinogenesis in these select individuals. It is well documented that factors such as inflammation, other

confounding diseases, age, genetic polymorphisms, race, sex, family history of cancer, and other environmental exposures may play a major contributing role factor to cancer incidence in these individuals.[144-147] Furthermore, there are few phenotypic tools available to determine susceptibility to lung cancer opening the door to pioneering new technologies in disease susceptibility and early prognosis. [148] In this work we utilized markers of DNA damage and repair in peripheral leukocytes in the presence of cigarette smoke extract as an indication of increased susceptibility in individuals with known lung cancer. To further strengthen our analysis we combined our data with the known characteristics of confounding diseases, age, race, sex, family history of cancer, and other environmental exposures in each individual. In spite of our very small sample size we found statistically significant results correlating age and race to increased genotoxicity as well as positive yet non-significant trends in gender, family history of cancer, pack years of cigarettes smoked, lung cancer difference to non-lung cancer, and individuals with history of other cancers. This information will serve to further elucidate if these factors will contribute to the genotoxic susceptibility of disease.

Materials and Methods

Inclusion/Exclusion Criteria. In the current study the amount of cigarette smoke extract induced genotoxicity was assessed in a heterogeneous cancer population of 30 patients comprising 24 former smokers 2 current smokers and 4 non-smokers. Inclusion criteria were as follows both men and women of all races and ethnic groups were eligible; individuals who were Age ≥18 years; had the ability to provide consent; had concurrent illness including COPD; had no known HIV or tuberculosis; non-smokers who have smoked <100 cigarettes in their lifetime; and smokers and former smokers at risk for lung cancer who are scheduled for a bronchoscopy were included in the study. Pregnant females; individuals with contraindications to fiberoptic

bronchoscopy including hemodynamic instability; severe obstructive airway disease (as determined by spirometry); unstable angina, congestive heart failure; respiratory failure/hypoxemia; inability to protect airway; prior radiotherapy or chemotherapy to lungs or mediastinum; altered level of consciousness; or who inability to understand the consent form either due to mental status or language barriers were excluded from the study. The sample identity was blinded to the laboratory investigators and we accounted for age, gender and smoking status in the study design.

Blood Collection. For exposure to CSE a vein on the inside of the patient's elbow or the back of the patient's wrist was used for blood sampling. A tourniquet (tight band) was placed around the upper arm of the individual and the skin over the vein is usually cleaned with an antiseptic wipe. A needle is then inserted into the vein through the cleaned skin. The needle is connected either to a syringe, or directly to vacuumed sealed purple capped K2/K3 EDTA-coated tubes. (Sarstedt Aktiengesellschaft & Co., Numbrecht). After the required amount of blood, approximately 2-3 milliliters, is taken from the vein the needle is removed. The small wound is pressed on with cotton wool for a few minutes to stop the bleeding and prevent bruising.

Whole blood CSE exposure. Frozen stocks of cigarette smoke extract were supplied by the lab of Andrew Dannenberg at Cornell University as described previously [116]. Concentrated 40.3 puffs/mL cigarette smoke extract was diluted to a working solution of 5 puffs/mL with PBS. 5 puffs/mL cigarette smoke extract (CSE) was administered directly into whole peripheral blood to a final concentration of 1 puff/mL CSE and allowed to incubate in a shaking 37°C incubator for 3,6, or 24 hours.

Immunofluorescence. To determine the amount DNA damage after CSE incubation whole peripheral blood was administered to erythrocyte lysis buffer, cells were laid over poly -D-lysine-coated coverslips and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) at room temperature as described previously [51]. Subsequently, cells were permeabilized with 0.5% Triton X-100 (Sigma), followed by 5 rinses in PBS. Blocking was done in aluminum-covered plates overnight at 4°C in 10% FBS. Coverslips were then incubated for 1 hour at room temperature with mouse anti-phospho-Histone H2A.X (JW301Upstate) Temecula, CA at a dilution of 1:400, Mouse anti–8-oxoguanine clone 483.15 (Upstate, MAB3560) at 1:250 or Rabbit anti-nitrotyrosine (Upstate, 06-284) at 1:200 then rinsed with 0.1% Triton X-100. Following a second 10% FBS blocking, cells were stained with FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:150 and (1:200) for 1 hour at room temperature for samples with γH2AX primary and with 8-oxoguanine primary antibody respectively. Alexa 594-conjugated anti-rabbit IgG (Jackson ImmunoResearch) (1:200) was used for samples that were incubated with nitrotyrosine primary antibody. Coverslips were mounted onto slides using VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA). Foci were analyzed on a Zeiss automated microscope. At least 120 cells were counted per sample and cells with more than four distinct foci in the nucleus were considered positive for γH2AX.[52] Cells that exhibited elevated fluroscent intensity compared to background were considered positive for 8-oxoguanine, and Nitrotyrosine respectively. Positive cells were determined on a Zeiss automated microscope. Apoptotic cells, which have an approximate 10-fold increased in nuclear foci in damaged cells, were not included in analyses [52, 53]. Statistical analysis was done using a linear mixed model with repeated measures nested within an individual using STATA statistical analysis software.

Statistical analyses. Statistical analyses were done using bi-variate and multi-variate linear mixed models with repeated measures nested within an individual. According to (Afifi, A. A, Virginia Clark, and Susanne May. Computer-aided Multivariate Analysis. 4th ed. Boca Raton, Fla.: Chapman & Hall/CRC, 2004). Bi-variant interactions are considered interactions between two distinct variables to determine if any combination of factor levels can have a different linear effect on the dependent variable. (Afifi, A. A, Virginia Clark, and Susanne May. Computer-aided Multivariate Analysis. 4th ed. Boca Raton, Fla.: Chapman & Hall/CRC, 2004) Suggest these interaction models were assessed to determine if the linear relationship between a covariate and the dependent variable changes for different levels of a factor. Additionally, multi-variate linear mixed models were conducted to estimate a model with more than one outcome variable. Linear Mixed Models were utilized due to the ability to assess correlated and non-constant variability. Futhermore, these models provide the flexibility to assess not only the mean of a response variable, but its covariance structure as well. To further strengthen our data within these models categorical predictors of age and pack years smoke were assessed. The dependent variables were my genotoxic readouts of γH2AX, 8-oxoguanine, and nitrotyrosine. Measurements of each DNA damage parameter were conducted in each individual and assessed using STATA statistical analysis software.

Table. 3-1. Patient Demographical

Patient ID #	Age	Gender	Smoking Status	Pack years Smoked	Cancer Family History	Exposure History	Previous Cancer History	Race	Lung Cancer Diagnosis

541	79	Male	Former	25	Yes	N/A	Prostate	White	Yes
542	78	Female	Former	40	N/A	N/A	N/A	Black	Yes
543	64	Male	Current	28	Yes	N/A	N/A	Black	Yes
544	66	Male	Former	31	Yes	Asbestos/ Agent Orange	N/A	Black	Yes
545	86	Male	Former	46	Yes	N/A	No	White	Yes
546	87	Male	Former	30	Yes	N/A	Skin Cancer	White	Yes
549	63	Male	Never	0	Yes	N/A	Prostate	White	No
550	70	Male	Former	76	None	Acid fumes	N/A	Asian	Yes
553	66	Female	Former	5	Yes	N/A	N/A	White	Other
554	70	Male	Former	40	None	N/A	N/A	White	Yes
555	79	Male	Former	1	Yes	N/A	Prostate	White	Yes
556	54	Female	Never	0	Yes	N/A	Invasive Thymoma	White	Other
557	55	Female	Never	0	N/A	N/A	Ovarian	White	Other
558	67	Female	Never	0	Yes	N/A	N/A	White	Other
559	58	Male	Former	4	Yes	N/A	Bladder	White	Yes
561	68	Female	Former	9	N/A	N/A	N/A	White	Yes
564	61	Female	Former	45	Yes	N/A	Basal cell Skin	White	Yes
565	55	Male	Current	41	Yes	Asbestos	N/A	White	Yes
568	85	Male	Former	39	Yes	N/A	N/A	White	Yes
570	79	Male	Former	57	Yes	Asbestos	Transitional bladder	Hispanic	Yes
571	69	Male	Former	52	None	Asbestos	Bladder	White	No
572	43	Male	Former	22	Yes	N/A	N/A	White	No

573	40	Female	Never	0	None	N/A	N/A	Asian	Yes

Table 3-1. This table shows the parmeters that were assessed as well as the demographical information for each patient.

Results

Bi-variant modeling was used to assess if age, race, sex, past cancer history, pack years smoked ,familial history of cancer, and history of harmful exposure are positive predictors of cigarette smoke extract induced γH2AX, 8-oxoguanine, and Nitrotyrosine formation in peripheral leukocytes over time. These assays were conducted in individuals with and without lung cancer that were exposed to cigarette smoke extract. In all assays percent positive cells were assessed in peripheral white blood cells via fluorescent microscopy.

Markers of DNA damage

H2AX is a member of the histone H2A protein family and becomes rapidly phosphorylated in presence of a DNA damaging event.[62] This rapid phosphorylation causes recruitment of DNA repair proteins to the site of the break and is detectable by specific antibodies to γH2AX. The formation of γH2AX a marker of double stranded breaks in peripheral leukocytes were counted in each individual.

8-oxoguanine is a mutagenic lesion caused by the interaction of a reactive oxygen species to DNA that causes G:C to T:A transversion mutations during replication[58] Induction of 8-oxoguanine in peripheral leukocytes is an indication of increased ROS mediated DNA damage.

Nitrotyrosine is a biochemical marker for inflammation that is formed from nitric oxide-induced peroxynitrite interacting with other reactive nitrogen species to tyrosine residues of proteins [58, 149]

Age

Fig3-1. **Age of the individual has been shown to be a contributing factor in cancer incidence.**[150] We examined the role age plays in the assessment of the increased genotoxic susceptibility. At baseline formation of γH2AX are highest in individuals who are in their 80's (light blue line) followed closely by individuals in their 70's (gold line), 60's (dark green line), 50's (red line) and the lowest for individuals who are in their 40's (dark blue line). Upon administration of cigarette smoke extract to the peripheral blood all groupings flipped causing a disordinal interaction. This interaction is described by observing individuals with highest baseline γH2AX foci formation, individuals depicted by (light blue line), have the lowest amount of γH2AX foci formed at all other time points, and the lowest baseline γH2AX formation individuals depicted by (dark blue line) have the highest amount of γH2AX foci formed at all other time points. The contribution of age was a significant predictor of γH2AX induced genotoxicity at $p<0.0247$. This suggests that age is a strong predictor of γH2AX induced genotoxicity. N=2 (dark blue line),N=4 (red line), N=8 (dark green line),N=6 (gold line), and N=3 (light blue line), respectively.individuals who are in their 40's (dark blue line).

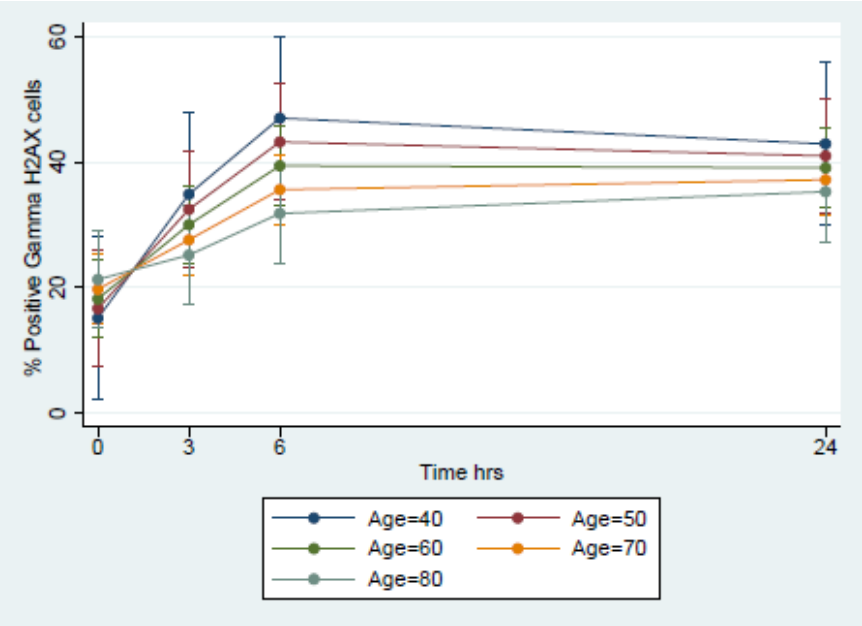

Fig3-1. The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and the age of the individual. This depicts the role age has on susceptibility to develop γH2AX foci in peripheral leukocytes. N=2 (dark blue line),N=4 (red line), N=8 (dark green line),N=6 (gold line), and N=3 (light blue line), respectively.individuals who are in their 40's (dark blue line). $p<0.0247$

A measure of ROS induced genotoxicity via 8-oxoguanine staining showed that at baseline there was a very little difference in positive 8-oxoguanine staining in all age groups as indicated by the almost single data point. After 3hrs of cigarette smoke extract incubation individuals who were in the 40's age range (dark blue line) had the highest amount of 8-oxoguanine staining. This trend persisted throughout the 24hr time course as did the lowered amount of 8-oxoguanine staining observed in the 50's (red line), 60's (dark green line), 70's (gold line), 80's (light blue line) year old groupings, respectively. The suggests that contribution of age being a indicator of 8-oxoguanine induced DNA damage was trending towards significant at $p=0.0759$. N=2 (dark blue line),N=4 (red line), N=8 (dark green line),N=6 (gold line), and N=3 (light blue line).

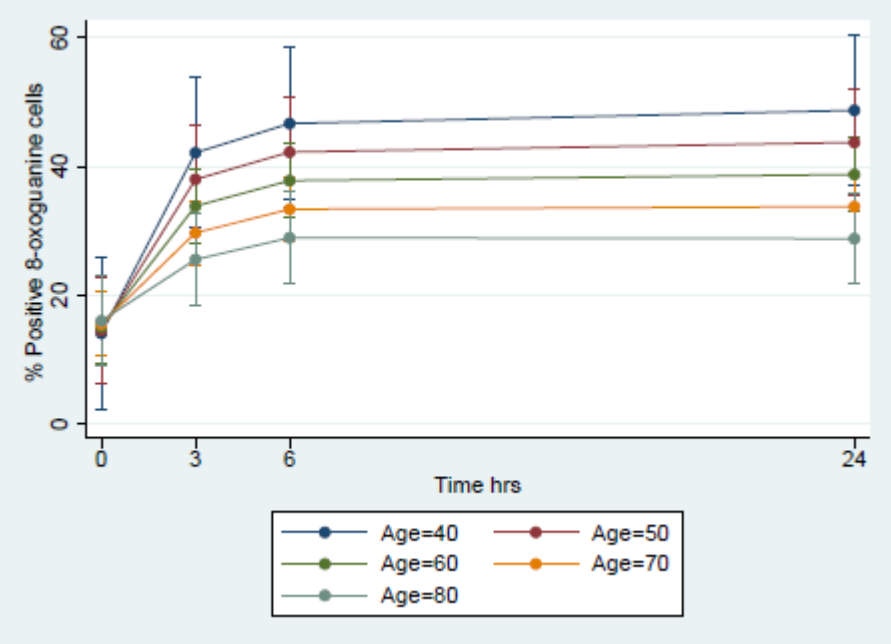

Fig3-2. The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and age of the individual. This depicts the role age has on susceptibility to develop 8-oxoguanine positive cells in peripheral leukocytes. N=2 (dark blue line),N=4 (red line), N=8 (dark green line),N=6 (gold line), and N=3 (light blue line), respectively.individuals who are in their 40's (dark blue line). P=0.0759

As a measure of inflammation induced genotoxicity we measured nitrotyrosine staining and observed similar trends of what was present in γH2AX, and 8-oxoguanine. Individuals who were in the 40's age range (dark blue line) had the highest amount of nitrotyrosine staining. Followed by 50's (red line), 60's (dark green line), 70's (gold line), 80's (light blue line) year old groupings, respectively. Although this observation was not significant at p=0.2179 we observed fairly consistent trends with the above mentioned biomarkers.

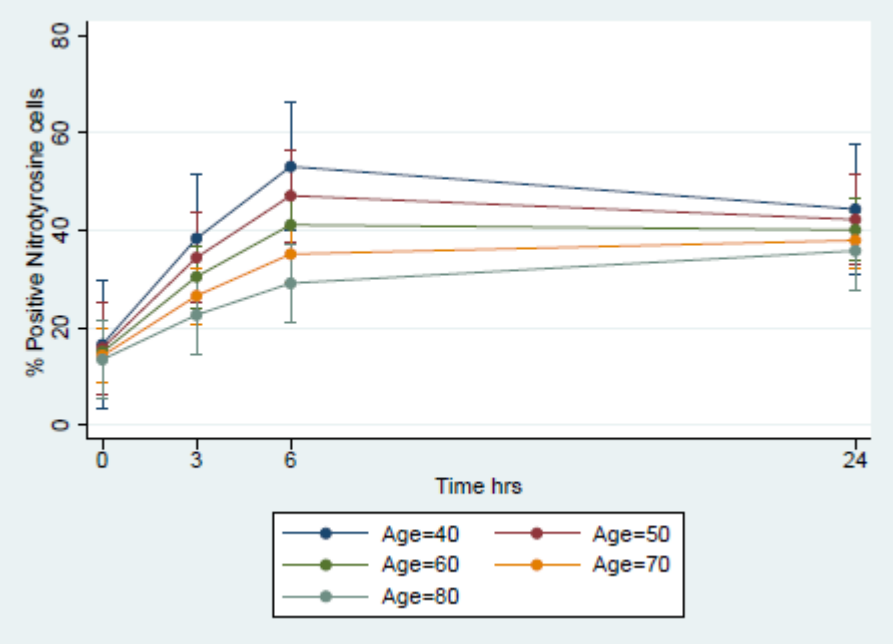

Fig3-3. The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and age of the individual. This depicts the role age has on susceptibility to develop Nitrotyrosine positive cells in peripheral leukocytes. N=2 (dark blue line),N=4 (red line), N=8 (dark green line),N=6 (gold line), and N=3 (light blue line), respectively.individuals who are in their 40's (dark blue line). p=0.2179

Smoking History Measured by Pack Years

Fig.3-4.Smoking is a known is cause of lung cancer. Smoking is currently responsible for a third of all cancer deaths in many Western countries.[107] It has been estimated that every other smoker will be killed by tobacco. [107] We examined the effect of prolonged smoking on DNA damage susceptibility. Assessing the amount of DNA double strand breaks in peripheral leukocytes via γH2AX foci formation we indentified at baseline individuals who smoked for 30-39yr smoking history (gold line) had the highest amount of positive γH2AX cells, individuals 1-10yrs smoking history (burgundy line) had the next highest baseline amount of double stranded breaks, followed 40-49yr smoking history (light blue line), individuals who smoked 20-29yrs (dark green line), and never smokers (dark blue line), and followed finally by individuals who smoked 50+yrs smoking history of (red line). At 3hrs and 6hrs all groups show an induction of cigarette smoke extract mediated DNA damage.

However, this induction did not persist in individuals with a 40-49yr smoking history (light blue line), and never smokers (dark blue line) who had modest decrease at this 24hr timepoint. Individuals with 50+yrs smoking history (red line) and individuals who smoked 20-29yrs (dark green line) had a steady state induction of γH2AX at 24hrs compared to the 6hr timepoint. 1-10yrs smoking history (burgundy line) and 30-39yr smoking history (gold line) experienced induction of γH2AX at 24hrs compared to the 6hr timepoint. Overall this bivariant model was not significant at p=0.3034 n=5 never smokers (dark blue line), n=3 1-10yrs smoking history (burgundy line), n=3 20-29yrs smoking history (dark green line), n=3 30-39yr smoking history (gold line), n=6 40-49yr smoking history (light blue line), n=3 50+yrs smoking history (red line).

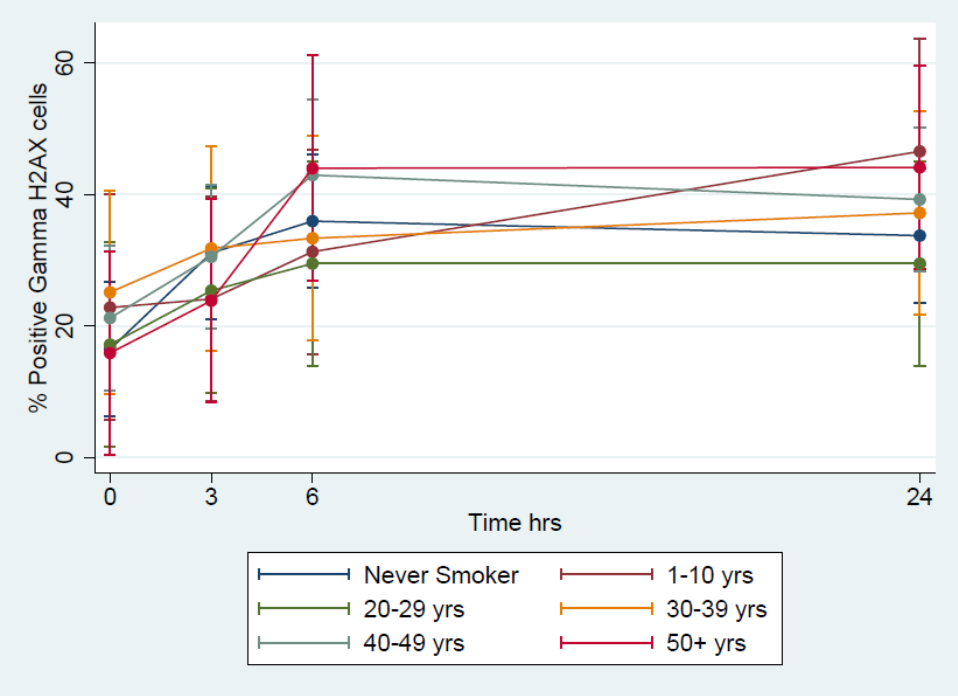

Fig.3-4. The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and smoking history of the individual. This depicts the role smoking history has on susceptibility to develop γH2AX foci in peripheral leukocytes. n=5 never smokers (dark blue line), n=3 1-10yrs smoking history (burgundy line), n=3 20-29yrs smoking history (dark green line), n=3 30-39yr smoking history (gold line), n=6 40-49yr smoking history (light blue line), n=3 50+yrs smoking history (red line). p=0.3034

Fig.3-5 Using the marker of oxidative DNA damage we observed at baseline there were little differences between individuals who had longer or shorter smoking histories. Individuals who smoked for 40-49yrs (light blue line) exhibited the highest amount of positively stained 8-oxoguanine peripheral leukocytes compared to other groups. After 3hrs of cigarette smoke extract incubation there was an induction in all groups. The highest induction at 3hrs of ROS induced DNA damage occurred in individuals who were grouped as never smokers (dark blue line) this group remained the highest throughout the experiment. The next highest induction occurred in individuals who had a 40-49yr smoking history (light blue line), followed by individuals who had at 30-39yr smoking history (gold line), individuals who smoked 1-10yrs (burgundy line), individuals who smoked 20-29yrs (dark green line), and lastly individuals who smoked 50+yrs of (red line). At 6hrs there was a slight decrease in the amount of 8-oxoguanine positively stained cells in never smokers (dark blue line) yet this group remained the highest amongst others. Individuals who had a smoking history of 1-10yrs (burgundy line) also exhibited a decrease from its previous 3hr timepoint. All other groups exhibited increases in the amount of genotoxicity exhibited at 6hrs of cigarette smoke extract incubation. The highest induction at 6hrs of positively stained cells was observed in individuals who had a smoking history of 50+yrs of (red line) compared to previous timepoints.

At 24hrs an induction of positively stained cells was observed in individuals who were grouped as never smokers (dark blue line), followed by individuals who had at 30-39yr smoking history (gold line), and individuals who smoked 1-10yrs smoking history (burgundy line). Decreases at 24hrs from the previous 6hr timepoint occurred in individuals who smoked 20-29yrs (dark green line), followed finally by individuals who smoked 50+yrs smoking history of (red line). Overall this bivariant model was not significant at p=0.5604 n=5 never smokers (dark blue line), n=3 1-10yrs smoking history (burgundy line), n=3 20-29yrs smoking history (dark green line), n=3 30-39yr smoking history (gold line), n=6 40-49yr smoking history (light blue line), n=3 50+yrs smoking history (red line).

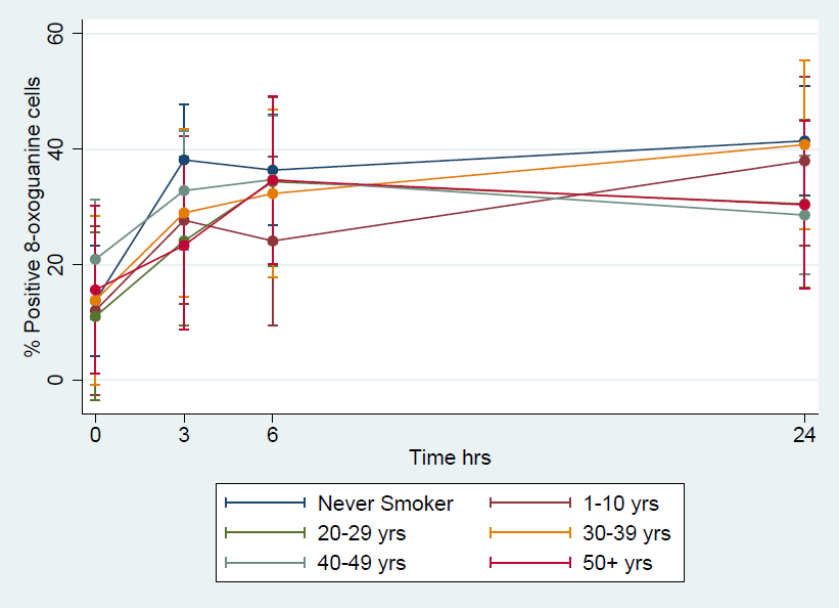

Fig.3-5 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and smoking history of the individual. This depicts the role smoking history has on susceptibility to develop 8-oxoguanine positive cells in peripheral leukocytes. n=5 never smokers (dark blue line), n=3 1-10yrs smoking history (burgundy line), n=3 20-29yrs smoking history (dark green line), n=3 30-39yr smoking history (gold line), n=6 40-49yr smoking history (light blue line), n=3 50+yrs smoking history (red line). p=0.5604

Fig.3-6 Using the marker of nitrotyrosine to assess the amount of DNA damage. We observe at baseline all groups have relatively similar amounts of positive nitrotyrosine staining. At 3hrs we observed an induction in all groups, yet the highest induction occurred in never smokers (dark blue line) and 40-49yr (light blue line). Individuals with a smoking history of 30-39yr (gold line), had the next highest induction of nitrotyrosine positively stained cells followed by 50+yrs (red line), 1-10yrs (burgundy line), and finally 20-29yrs (dark green line). At 6hrs of cigarette smoke extract incubation individuals with 1-10yrs smoking history (burgundy line) exhibited the highest amount of positively stained cells, followed by never smokers (dark blue line), individuals with 50+yrs smoking history (red line), 20-29yrs smoking history (dark green line), and lastly individuals with a 30-39yr (gold line) exhibited a decrease in positive staining compared to the 3hr timepoint. At 24hrs individuals with a smoking history of 40-49yr (light blue line) had the highest positively stained cells, followed by never smokers (dark blue line), individuals with 50+yrs (red line) ,individuals with a 30-39yr (gold line), and finally 20-29yrs (dark green line) had the least amount of positively stained cells at this time point. Overall this bivariant model was not significant at p=0.8061 n=5 never smokers (dark blue line), n=3 1-10yrs smoking history (burgundy line), n=3 20-29yrs smoking history (dark green line), n=3 30-39yr smoking history (gold line), n=6 40-49yr smoking history (light blue line), n=3 50+yrs smoking history (red line).

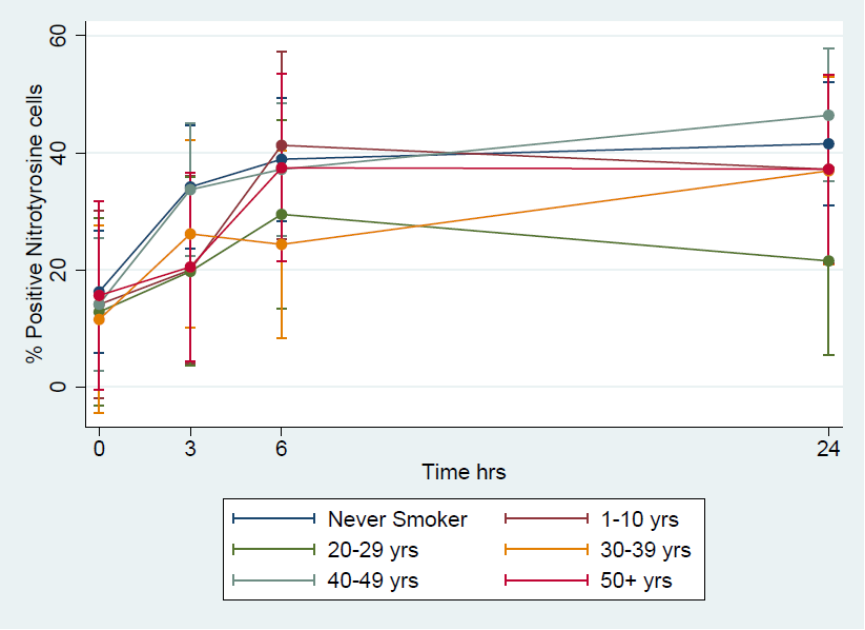

Fig.3-6 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and smoking history of the individual. This depicts the role smoking history has on susceptibility to develop Nitrotyrosine positive cells in peripheral leukocytes. n=5 never smokers (dark blue line), n=3 1-10yrs smoking history (burgundy line), n=3 20-29yrs smoking history (dark green line), n=3 30-39yr smoking history (gold line), n=6 40-49yr smoking history (light blue line), n=3 50+yrs smoking history (red line). p=0.8061

Race

Fig.3-7 Racial demographic has been shown to be key predictor to aggressiveness and prevalence of lung cancer incidence in individuals.[151, 152] We examined the role race plays in the assessment of the increased genotoxic susceptibility. Using γH2AX as indication marker of double strand breaks we assessed the role race has γH2AX foci formation. We observed at baseline individuals of Hispanic (non-black) ethnicity (green line) has the highest amount of γH2AX foci formation followed by individuals of White (gold line), Black (red line), and Asian (blue line) ethnicities, respectively.

At 3hrs of cigarette smoke extract incubation there is a near two fold increase of γH2AX foci formed in Hispanic (non-black) ethnic individuals followed by slight inductions in all ethnicities except Blacks. At 6hrs there was a near two fold induction of γH2AX foci formed in Asians while all other ethnic groups observed slight inductions. There was a persistent increase in γH2AX foci formed as time progressed in all racial groups except Asians where a near two-fold decrease of γH2AX foci formed was observed. These observations were not significant p=0.2465. White (gold line) n=17, Black (red line) n=3 Asian (blue line) n=2, and Hispanic (green line) n=1.

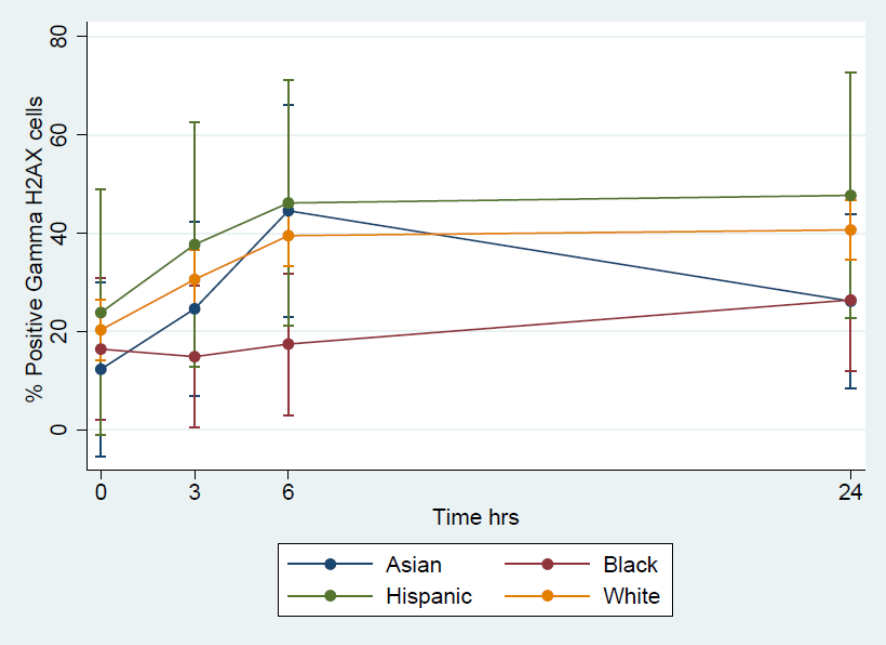

Fig.3-7 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and smoking history of the individual. This depicts the role race has on susceptibility to develop γH2AX foci positive cells in peripheral leukocytes. White (gold line) n=17, Black (red line) n=3 Asian (blue line) n=2, and Hispanic (green line) n=1. p=0.2465

Fig.3-8. In 8-oxoguanine staining we observed minor differences in staining between racial groups. At 3hrs we observe slight inductions of positively stained 8-oxoguanine cells in all ethnic groups. At 6hrs we see inductions of positively stained cells in Hispanic (green line) and Whites (gold line) ethnicities, and slight decreases in Asian (blue line) and Black (red lines) ethnicities respectively. At 24hrs there is an induction present from 6hrs in Whites (gold line) and Blacks (red line) ethnicities, and a decrease in Hispanic (green line) and Asian (blue line) individuals. These observations were not significant p=0.4108. White (gold line) n=17, Black (red line) n=3 Asian (blue line) n=2, and Hispanic (green line) n=1.

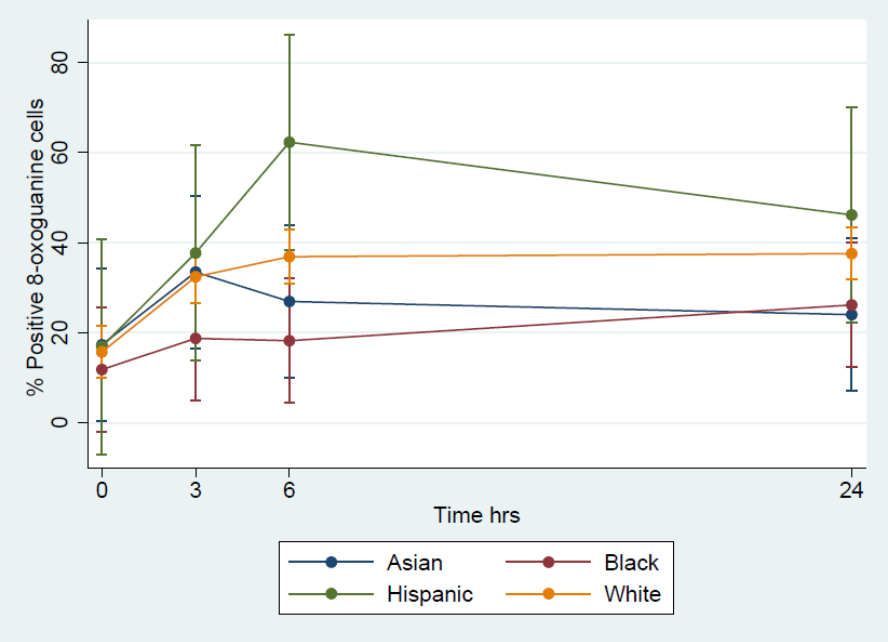

Fig.3-8 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and smoking history of the individual. This depicts the role race has on susceptibility to develop 8-oxoguanine positive cells in peripheral leukocytes. White (gold line) n=17, Black (red line) n=3 Asian (blue line) n=2, and Hispanic (green line) n=1. p=0.4108

Fig.3-9 Using the Nitrotyrosine as an indicator of inflammation. We observed that at baseline Asian (blue line) and White (gold line) have slightly higher amounts positively stained nitrotyrosine cells compared to other ethnic groups. At 3hrs of cigarette smoke extract incubation we observe an increase in positively stained nitrotyrosine cells in all ethnic groups. This increase persists after 6hr cigarette smoke extract induction but is observed highest in Hispanic individuals (green line). At 24hrs of cigarette smoke extract incubation there is an continuous induction of positive nitrotyrosine stained cells compared to 6hrs in White (gold line) and Black (red line) individuals. Conversely in Hispanic (green line) and Asian (blue line) individuals there is a decrease at 24hr time point compared to the 6hrs time point. These observations were significant at $p<0.0243$. White (gold line) n=17, Black (red line) n=3 Asian (blue line) n=2, and Hispanic (green line) n=1.

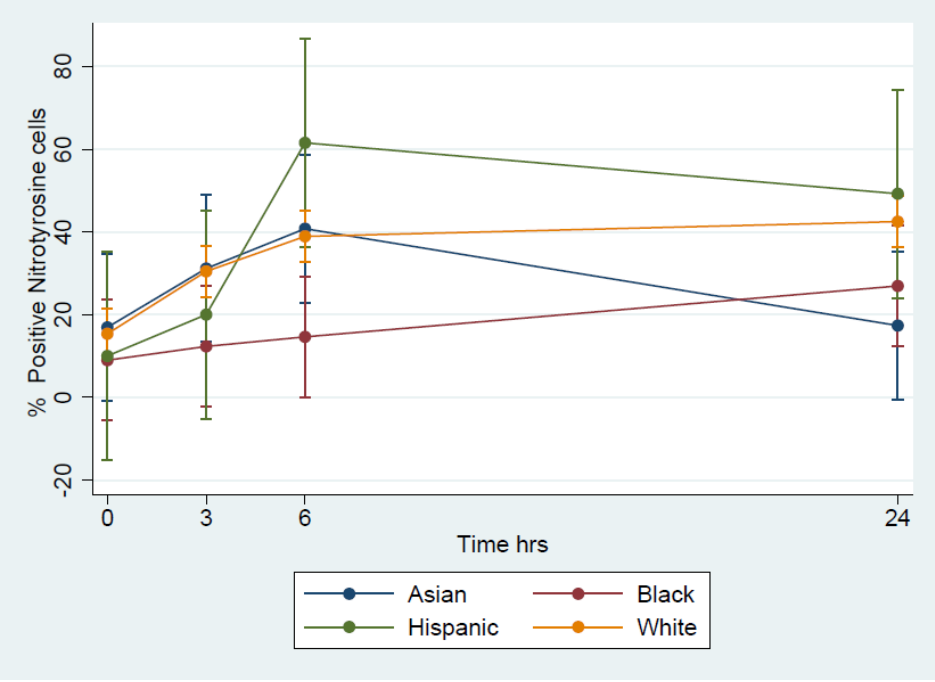

Fig.3-9 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and smoking history of the individual. This depicts the role race has on susceptibility to develop Nitrotryosine positive cells in peripheral leukocytes. White (gold line) n=17, Black (red line) n=3 Asian (blue line) n=2, and Hispanic (green line) n=1. $p<0.0243$

Gender

Fig.3-10. It has been well documented that there are sex disparities in cancer that make gender a significant variable in increase cancer incidence.[153, 154] We assessed the effect gender had on susceptibility to markers of genotoxicity. We observed using the biomarker of γH2AX that at baseline males (red line) had a slight increase in γH2AX foci formation compared to females (blue line) this trend persists throughout the initial 6hr cigarette smoke extract incubation. At 24hr incubation of cigarette smoke extract incubation there is a change in the initial trend and there is a slight induction of γH2AX foci formation in females and a slight reduction in foci formation in males. p=0.6369. Males (red line) n=15, females (blue line) n=8.

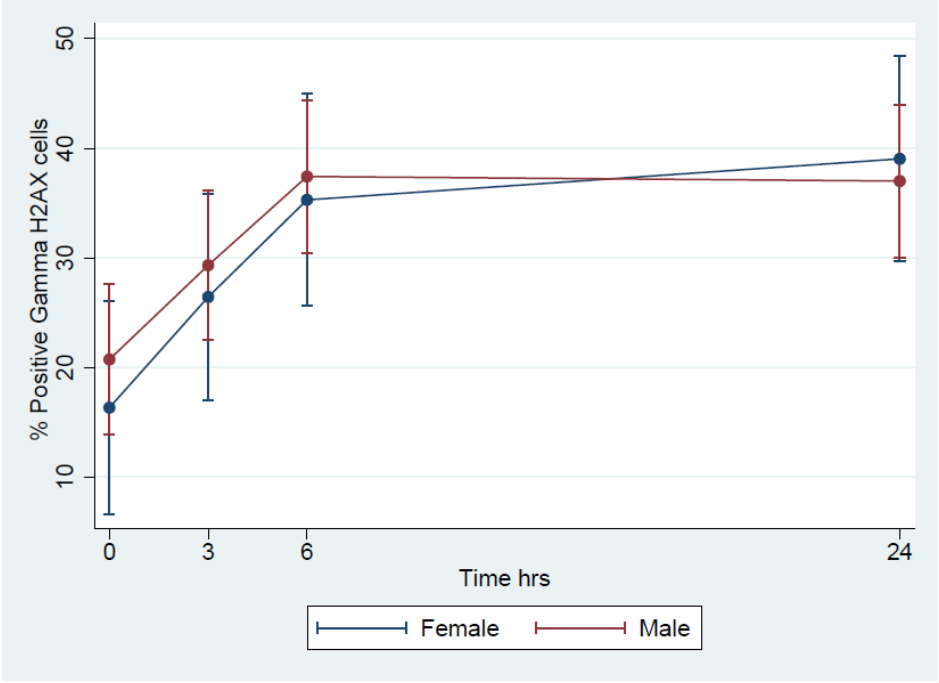

Fig.3-10 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and smoking history of the individual. This depicts the role gender has on susceptibility to develop γH2AX foci positive cells in peripheral leukocytes. Males (red line) n=15, females (blue line) n=8. p=0.6369

Fig.3-11. In 8-oxoguanine staining we observed minor differences in staining between gender groups .With slightly more positively stained 8-oxoguanine cells in males (red line) at baseline compared to females (blue line). This trend switches upon administration of cigarette smoke extract and a higher than 2-fold induction of 8-oxoguanine positively stained cells is observed in females (blue line) at 3hr time point compared to baseline. This induction persists at 6hrs and reaches the highest point at 24hrs of cigarette smoke extract incubation in females (blue line). There was an induction of positively stained 8-oxoguanine cells in males (red line) at 3 and 6hrs of cigarette smoke extract incubation compared to baseline however this increase was lower at all time points after baseline compared to females(blue line). This induction was highest at 6hr cigarette smoke extract incubation compared to baseline and slightly decreases after 24hr incubation in males (red line). Overall this was not significant p=0.2501 males (red line) n=15, females (blue line) n=8.

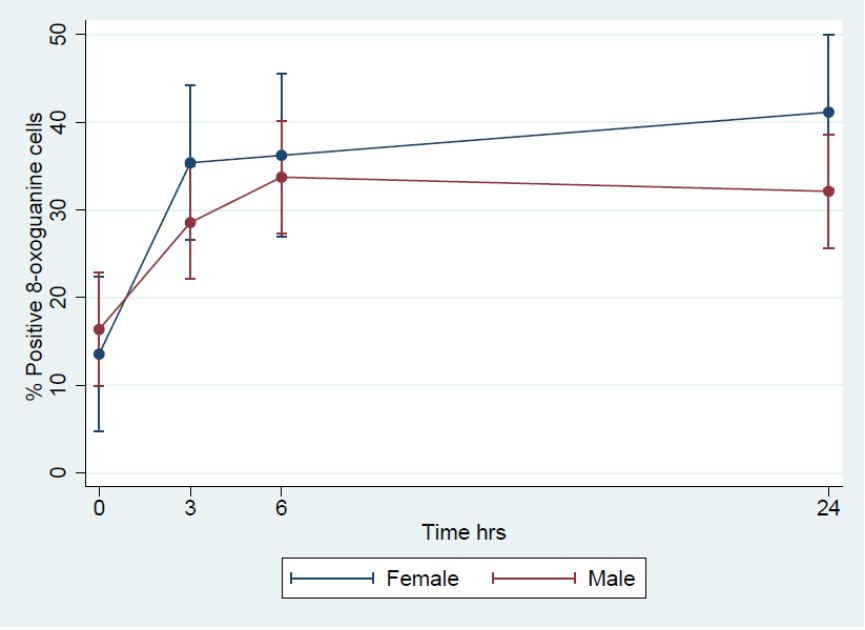

Fig.3-11 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and gender of the individual. This depicts the role gender has on susceptibility to develop 8-oxoguanine positive cells in peripheral leukocytes. Males (red line) n=15, females (blue line) n=8. p=0.2501

Fig.3-12 We observe a similar trend of positively stained nitrotyrosine cells as was seen in 8-oxoguanine staining. A minor difference in staining between gender groups with slightly more positively stained nitrotyrosine cells in males (red line) at baseline compared to females (blue line) was observed. There were more positively stained nitrotyrosine cells at 3hrs in females (blue line) compared males (red line). This increase was sustained at 6hrs and 24hrs. The highest amount of positively stained nitrotyrosine cells was present at 6hrs in females (blue line) and slightly decreased at 24hrs. There was a slight induction at 6hrs in males (red line) this increase was highest at 24hrs in these individuals. Overall this was not significant difference between these groups at p=0.2033 males (red line) n=15, females (blue line) n=8.

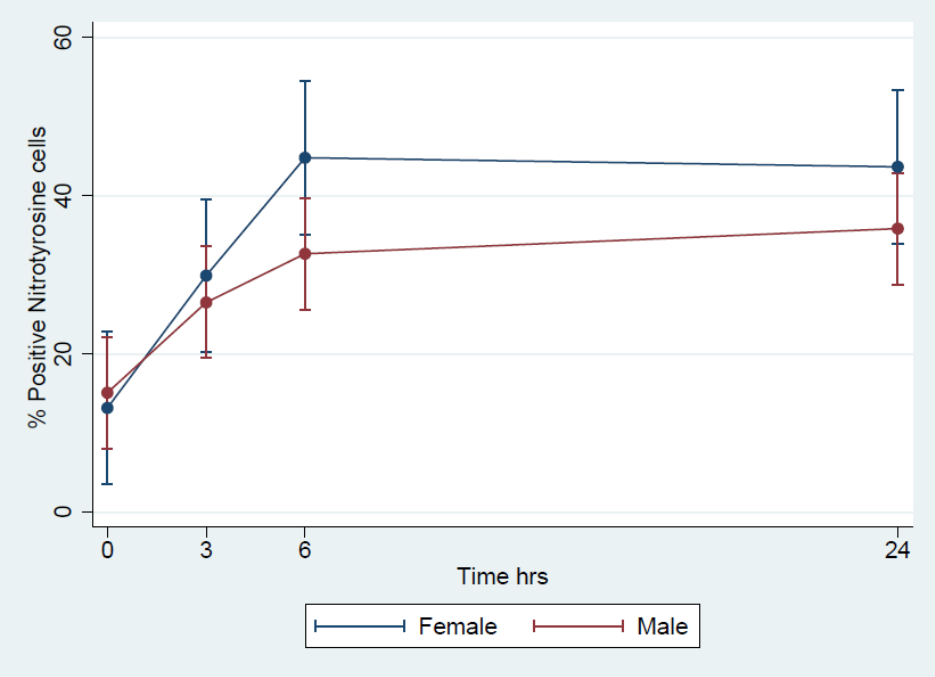

Fig.3-12 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and gender of the individual. This depicts the role gender has on susceptibility to develop Nitrotyroe positive cells in peripheral leukocytes. Males (red line) n=15, females (blue line) n=8. p=0.2033

Past Cancer History

Fig.3-13.We determined if individuals with a previous history of cancer influenced the incidence of cigarette smoke extract induced genotoxicity.Assessing the marker of γH2AX we observe at baseline individuals with no previous cancer history (blue line) have slightly higher amounts of γH2AX foci formed in the peripheral leukocytes than that of individuals who have a previous history of cancers (red line). Conversely, at 3hrs, 6hrs, and 24hrs of cigarette smoke extract incubation individuals with a history of cancer (red line) exhibited higher amounts of γH2AX foci compared to individuals with no previous cancer history (blue line). Overall this was not significant difference between these groups at p=0.2832 individuals with no previous cancer history (blue line) n=13, individuals who have a previous history of cancers (red line) n=10.

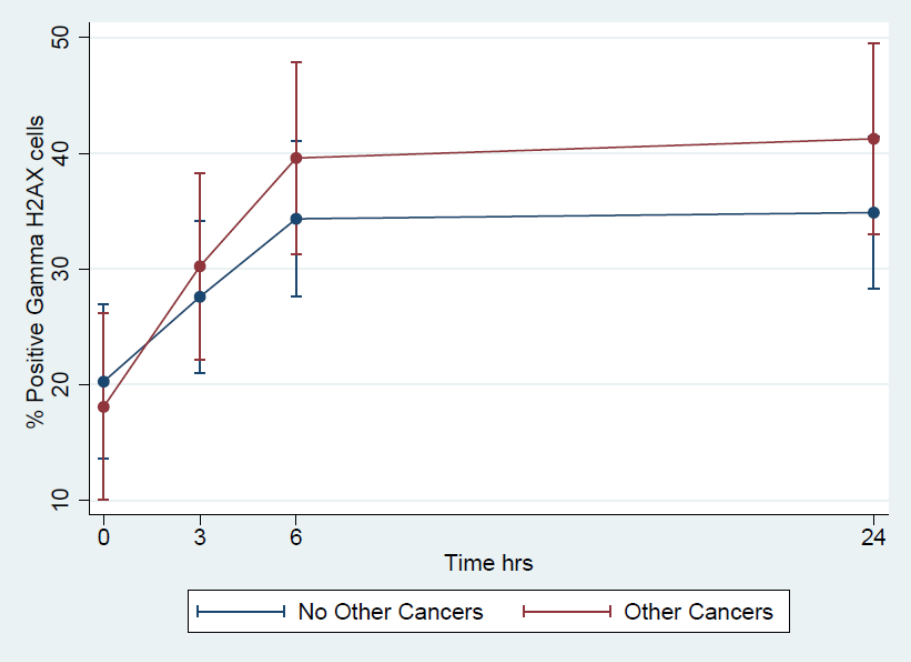

Fig.3-13. The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and cancer history of the individual. This depicts the role family has on susceptibility to develop γH2AX foci cells in peripheral leukocytes. Individuals with no previous cancer history (blue line) n=13, individuals who have a previous history of cancers (red line) n=10 p= 0.2832

Fig.3-14.Using 8-oxoguanine as a measurement of oxidative DNA damage we observe at baseline individuals with no previous cancer history (blue line) have slightly higher amounts of γH2AX foci formed in the peripheral leukocytes than that of individuals who have a previous history of cancers (red line). After 3hrs of cigarette smoke extract incubation both groups have an induction of cigarette smoke extract DNA damage yet more damage is observed in individuals who have a previous history of cancers (red line) compared individuals with no previous cancer history (blue line). This trend continues after 6hr cigarette smoke extract incubation. At 24hr cigarette smoke extract incubation a decrease in the amount of DNA double strand breaks occurs in individuals who have a previous history of cancers (red line) and an increase is observed in individuals with no previous cancer history (blue line). Overall this was not significant difference between these groups at p=0.1529 individuals with no previous cancer history (blue line) n=13, individuals who have a previous history of cancers (red line) n=10.

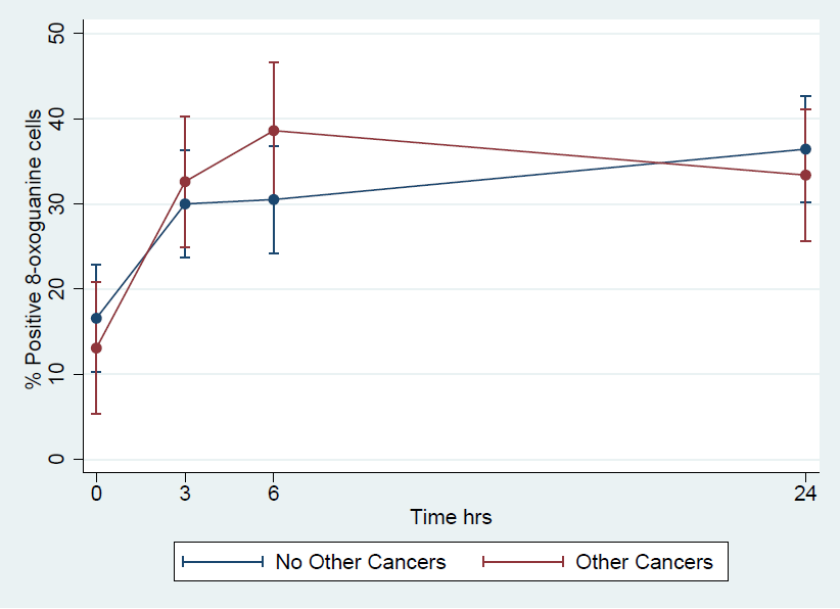

Fig. 3-14. The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and cancer history of the individual. This depicts the role having a history of other cancers has on susceptibility to develop. Individuals with no previous cancer history (blue line) n=13, individuals who have a previous history of cancers (red line) n=10.p=0.1529

Fig.3-15. Using the nitrotyrosine marker of inflammation. We observe at baseline that individuals who have a previous history of cancers (red line) have more positively compared to individuals with no previous cancer history (blue line). At 3hrs of cigarette smoke extract there was an induction of positively stained nitrotyrosine cells in both groups. At this time point individuals with no previous cancer history (blue line) have higher amounts of positively stained cells compared to individuals who have a previous history of cancers (red line). This trend continues after 6hr cigarette smoke extract incubation. At 24hrs cigarette smoke extract incubation there is a modest induction of both groups, yet a decrease was observed in individuals with no previous cancer history (blue line) compared to 6hr incubation. Conversely, there was an increase observed in individuals who have a previous history of cancers (red line) compared to 6hr incubation timepoint. There was no significant difference between these groups at p=0.4311 individuals with no previous cancer history (blue line) n=13, individuals who have a previous history of cancers (red line) n=10.

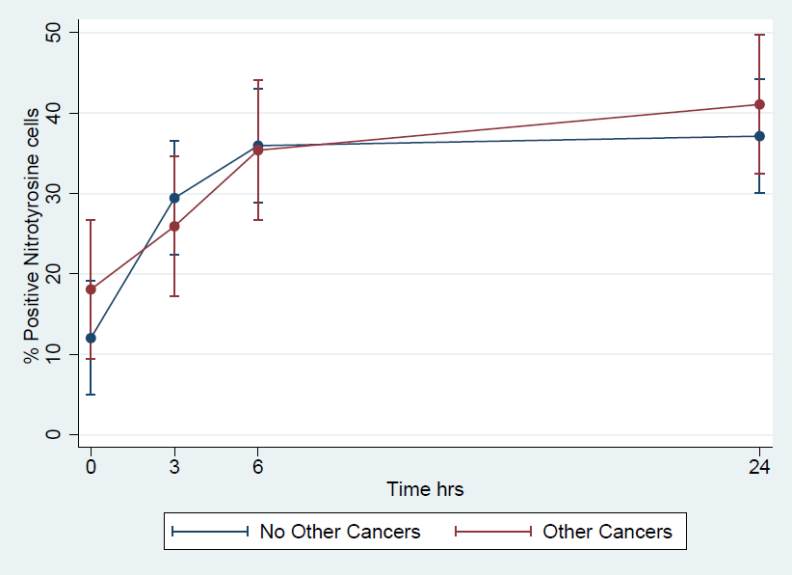

Fig.3-15 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and cancer history of the individual. This depicts the role having a history of other cancers has on susceptibility to develop Nitrotyrosine positive cells in peripheral leukocytes. Individuals with no previous cancer history (blue line) n=13, individuals who have a previous history of cancers (red line) n=10. p=0.4311

Fig.3-16. To demonstrate the contribution of having a previous history of specific cancers to the susceptibility of DNA damage we assessed DNA double strand breaks via γH2AX. This assessment was conducted in patients with a previous history of other cancers compared to individuals with no history of other cancers. We observe that individuals with no other cancer history (blue line), bladder cancer (green line), and prostate cancer (red line) had similar γH2AX induction. Individuals who had a history of basal cell skin cancer (burgundy line) and invasive thymoma (gold line) had the next highest baseline amount of positive γH2AX cells. Individuals with ovarian cancer (light blue line) had the lowest baseline amount of positively stained cells. There was an induction of γH2AX foci formation at 3hrs in all groups. At 6hrs cigarette smoke extract incubation there was an induction in all groups excluding individuals with prostate cancer (red line) who exhibit a slight decrease. The highest amounts of γH2AX foci formation occurring in individuals with bladder cancer (green line) followed by basal cell skin cancer (burgundy line), invasive thymoma (gold line), no other cancer history (blue line), and ovarian cancer (light blue line). There was only a modest induction of γH2AX at 24hrs in individuals with ovarian cancer (light blue line) all other individuals had a relatively consistent amount of γH2AX positive cells as the previous 6hr time point. Overall this was not significant p=0.1196. n=13 no other cancer history (blue line), n=2 bladder cancer (green line), n=1 ovarian cancer (light blue line),n=1 basal cell skin cancer (burgundy line), n=1 invasive thymoma (gold line), and n=3 prostate cancer (red line).

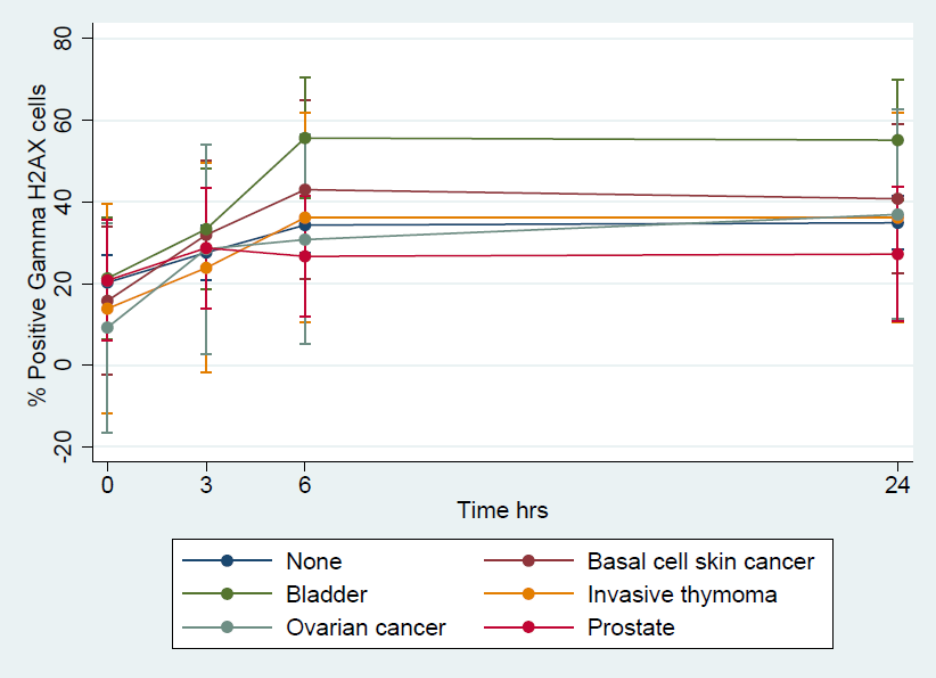

Fig.3-16.The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and contribution of having a previous history of specific cancers to the susceptibility to develop γH2AX foci . n=13 no other cancer history (blue line), n=2 bladder cancer (green line), n=1 ovarian cancer (light blue line),n=1 basal cell skin cancer (burgundy line), n=1 invasive thymoma (gold line), and n=3 prostate cancer (red line). p=0.1196

Fig.3-17.Using 8-oxoguanine staining we observed relatively low amounts of positively stained cells at baseline levels. At 3hrs there was an induction of γH2AX foci formation at 3hrs in all groups. The highest increase was a higher than two-fold induction of positively stained cells in individuals with ovarian cancer (light blue line) followed closely by individuals with invasive thymoma (gold line), and bladder cancer (green line). Individuals with no other cancer history (blue line), basal cell skin cancer (burgundy line), and prostate cancer (red line) also exhibited increased 8-oxoguanine staining at 3hrs but this induction was lowered than the previous mentioned cancer subtypes.

Individuals with ovarian cancer (light blue line) followed closely by individuals with invasive thymoma (gold line), and bladder cancer (green line) cancer subtypes exhibited a modest increase in positively stained cells at 6hrs after cigarette smoke extract incubation. Individuals with no other cancer history (blue line), and prostate cancer (red line) exhibited relatively unchanged amounts of positively stained cells at 6hrs. A slight decrease of positively stained cells at 6hrs was observed in individuals with basal cell skin cancer (burgundy line). There was a slight increase at 24hr cigarette smoke extract incubation in positively stained cells in individuals with invasive thymoma (gold line), no other cancer history (blue line), and in individuals with basal cell skin cancer (burgundy line). Individuals with bladder cancer (green line) and prostate cancer (red line) exhibited slight decreases in positively stained 8-oxoguanine cells at 24hrs. Overall this was not significant p=0.3633.n=13 no other cancer history (blue line), n=2 bladder cancer (green line), n=1 ovarian cancer (light blue line), n=1 basal cell skin cancer (burgundy line), n=1 invasive thymoma (gold line), and n=3 prostate cancer (red line).

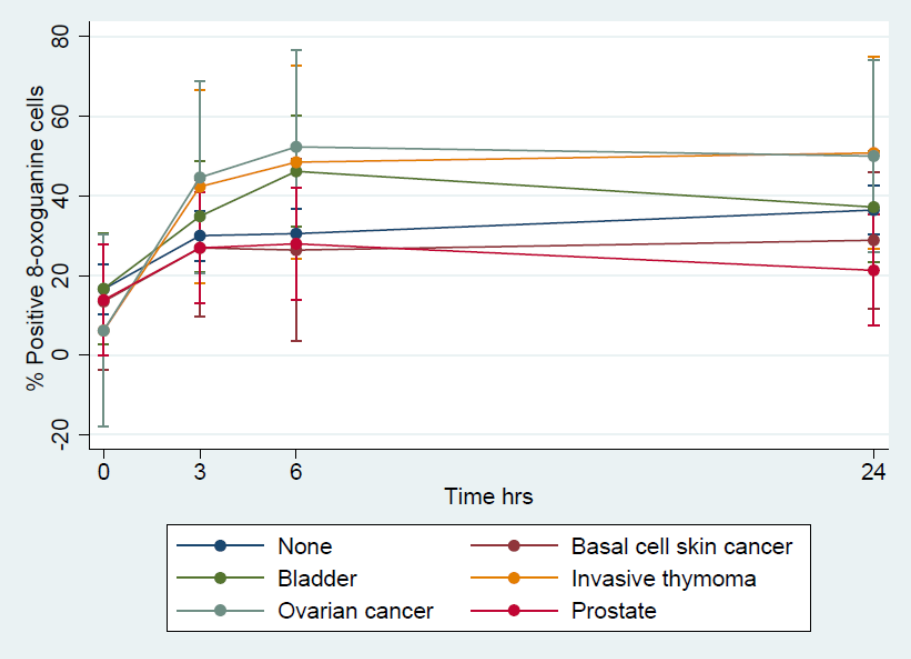

Fig.3-17. The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and contribution of having a previous history of specific cancers to the susceptibility to develop positive 8-oxoguanine cells . n=13 no other cancer history (blue line), n=2 bladder cancer (green line), n=1 ovarian cancer (light blue line),n=1 basal cell skin cancer (burgundy line), n=1 invasive thymoma (gold line), and n=3 prostate cancer (red line). p=0.3633

Fig.3-18 Assessing the nitrotryosine marker of inflammation. We see at baseline that prostate cancer (red line) and bladder cancer have the highest amount of positively stained cells followed basal cell skin cancer (burgundy), invasive thymoma (gold line), no other cancer history (blue line). Individuals with ovarian cancer (light blue line) had the lowest amount of positively stained cells. At 3hrs there were inductions in all cancer subtypes except prostate cancer. At 6hrs there were inductions in all cancer subtypes. The most positively stained cells were seen in individuals who had invasive thymoma (gold line), followed closely by bladder cancer (green line), and ovarian cancer (light blue line). Individuals with no other cancer history (blue line), and in individuals with basal cell skin cancer (burgundy line), and prostate cancer (red line) exhibited inductions of positively stained nitrotyrosine cells that were lower than the higher two cancer subtypes. At 24hrs invasive thymoma (gold line), bladder cancer (green line), and with basal cell skin cancer (burgundy line) exhibited slight inductions of positively stained cells compared to 6hrs. Individuals with ovarian cancer (light blue line), no other cancer history (blue line), and prostate cancer (red line) did not exhibit inductions at 24hr cigarette smoke extract compared to 6hrs. Overall this was not significant p=0.3734.n=13 no other cancer history (blue line), n=2 bladder cancer (green line), n=1 ovarian cancer (light blue line), n=1 basal cell skin cancer (burgundy line), n=1 invasive thymoma (gold line), and n=3 prostate cancer (red line).

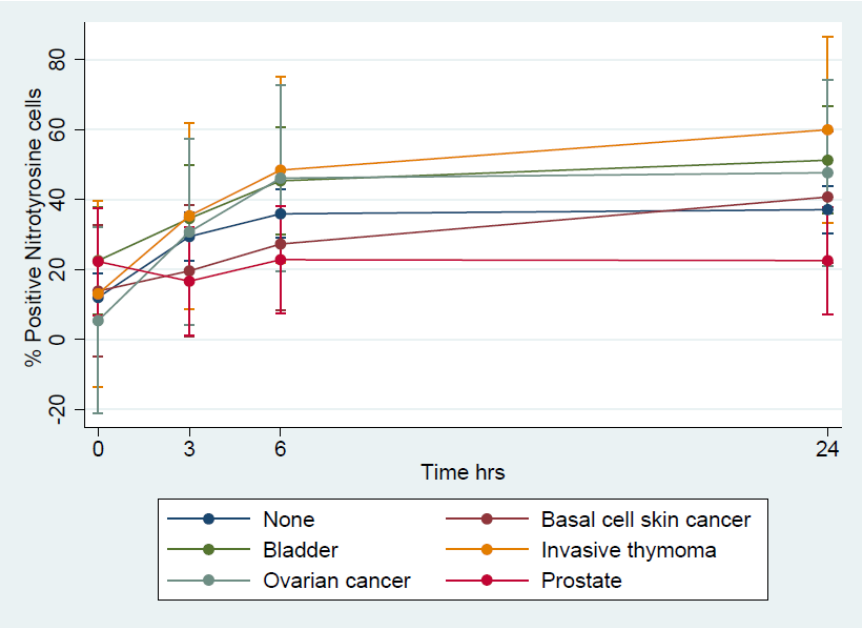

Fig.3-18. The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and contribution of having a previous history of specific cancers to the susceptibility to develop positive nitrotyrosine cells . n=13 no other cancer history (blue line), n=2 bladder cancer (green line), n=1 ovarian cancer (light blue line),n=1 basal cell skin cancer (burgundy line), n=1 invasive thymoma (gold line), and n=3 prostate cancer (red line). p=0.3734

Family History

Fig.3-19.Family history of lung cancer is an established risk factor for lung cancer.[155, 156] We determined if individuals with a known family history have an increase susceptibility to cigarette smoke induced genotoxicity.When assessing the effects family history has on the genotoxic marker γH2AX we observe that individuals who have a family history (red line) of cancer have higher baseline γH2AX foci formation compared to individuals who do not have a family history of cancer (blue line). This higher γH2AX foci formation persists after 3hr, 6hr,and 24hr cigarette smoke extract incubation in (red line) compared to individuals who do not have a history of cancer (blue line). Although this trend was consistent overall this was not significant p=0.8388. n=7 individuals with no family history of cancer (blue line) and n=16 individuals who have a family history (red line)

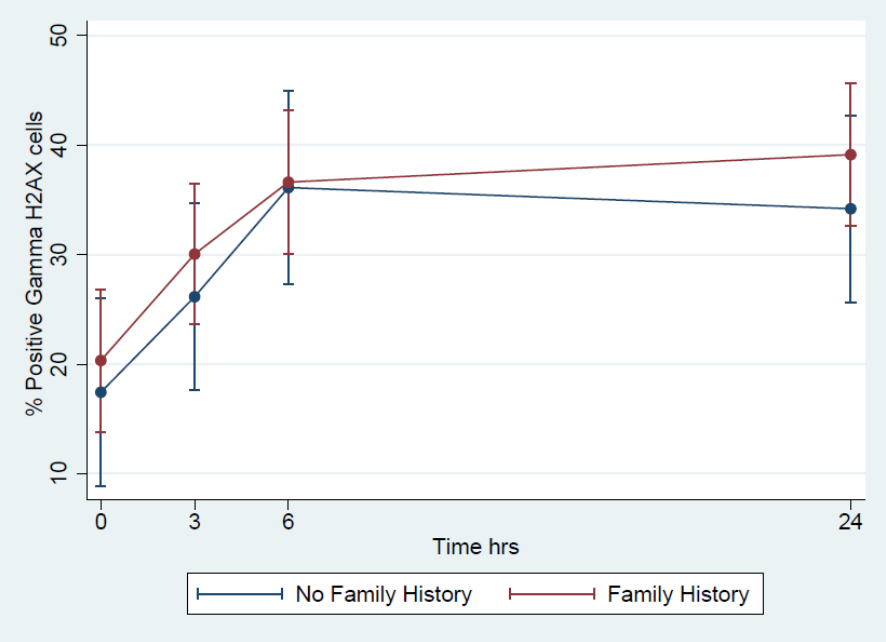

Fig.3-19 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and family history of the individual. This depicts the role family has on susceptibility to develop γH2AX positive cells in peripheral leukocytes. No family history n=7 individuals with no family history of cancer (blue line) and n=16 individuals who have a family history (red line), p=0.8388

Fig.3-20.Using the indication of ROS mediated DNA damage we observe that at baseline the amount of positively stained 8-oxoguanine stained cells are similar for both groups of individuals, yet individuals with a family history (red line) exhibit slightly higher 8-oxoguanine staining compared to individuals with no family history of cancer (blue line). At 3hrs we observed an induction in positively stained cells for both groups, but individuals with no family history of cancer (blue line) has slightly more positive cells than individuals with a family history (red line). This trend is inversed at 6hr and 24hrs and individuals with a family history of other cancers (red line) exhibit elevated 8-oxoguanine stained cells compared to individuals with no family history of cancer (blue line). Overall this was not significant p=0.2363. n=7 individuals with no family history of cancer (blue line) and n=16 individuals who have a family history (red line)

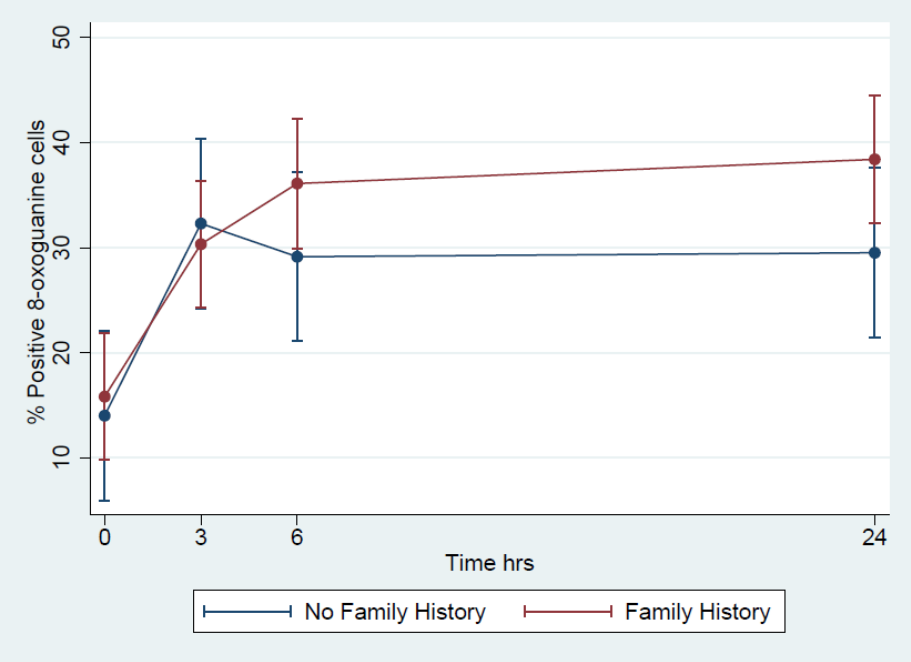

Fig.3-20 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and family history of the individual. This depicts the role family has on susceptibility to develop 8-oxoguanine positive cells in peripheral leukocytes. No family history n=7 individuals with no family history of cancer (blue line) and n=16 individuals who have a family history (red line). p=0.2363

Fig.3-21 With the nitrotyrosine marker. We observe that at baseline individuals with no family history of cancer (blue line) have slightly higher amounts of positively stained nitrotyrosine cells compared to individuals with a family history (red line). This trend continues until 6hr incubation with cigarette smoke extract where we see that individuals with a family history (red line) exhibits a slight increase in nitrotyrosine positive cells compared to individuals with no family history of cancer (blue line). The highest induction of positive cells in individuals with a family history (red line) occurs after 24hr incubation with cigarette smoke extract. Conversely a slight decrease can be observed in individuals with no family history of cancer (blue line) at this time point. Overall this was not significant p=0.5312 n=7 individuals with no family history of cancer (blue line) and n=16 individuals who have a family history (red line).

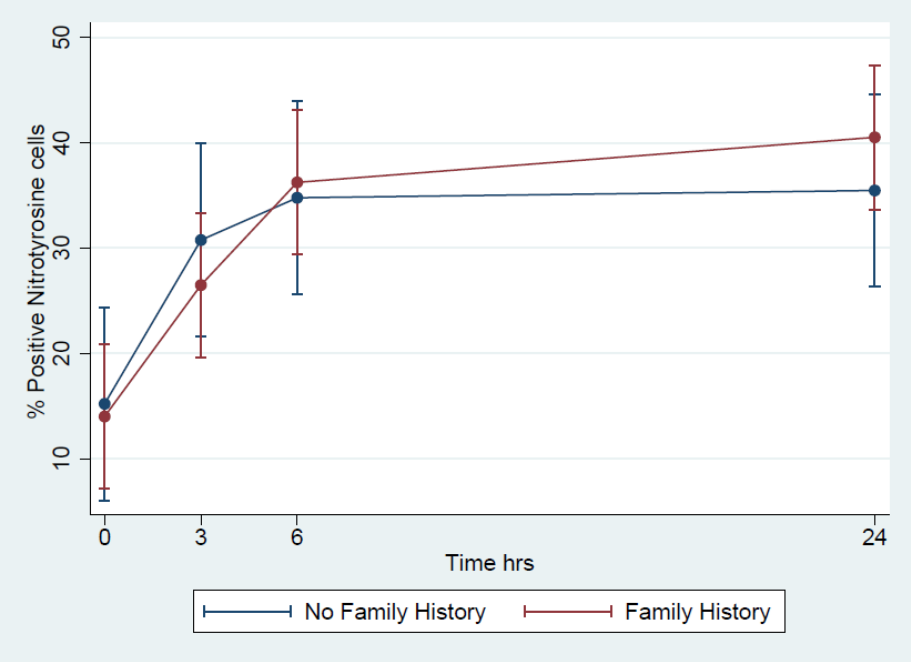

Fig.3-21 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and family history of the individual. This depicts the role family has on susceptibility to develop 8-oxoguanine positive cells in peripheral leukocytes. No family history n=7 individuals with no family history of cancer (blue line) and n=16 individuals who have a family history (red line). p=0.5312

Exposure History

Fig.3-22.Exposure to hazardous airborne chemicals has been shown to cause lung cancer.[157] Furthermore concomitant exposure of noxious chemicals and smoke has also been link to higher lung cancer incidence.[158]. We therefore sought to establish if exposure history of noxious chemicals combined with a history of smoking increased individuals susceptibility to cigarette smoke extract genotoxicity. With the γH2AX marker we observe that at baseline individuals with no history of previous chemical exposure (blue line) have higher positive γH2AX foci formation than individuals who do have a history of previous chemical exposure (red line). A similar trend of induction of γH2AX foci formation is observed in both groups at 3hr and 6hrs.

Yet, individuals with no history of previous chemical exposure (blue line) persistently have more γH2AX foci being formed in peripheral leukocytes compared to individuals who do have a history of previous chemical exposure (red line). At 24hrs of cigarette smoke extract incubation individuals who do have a history of previous chemical exposure (red line) exhibit a slight increase of γH2AX foci formation compared to baseline individuals with no history of previous chemical exposure (blue line). This observation was not significant p=0.6823. n=18 for individuals with no history of previous chemical exposure (blue line), n=5 for individuals who do have a history of previous chemical exposure.

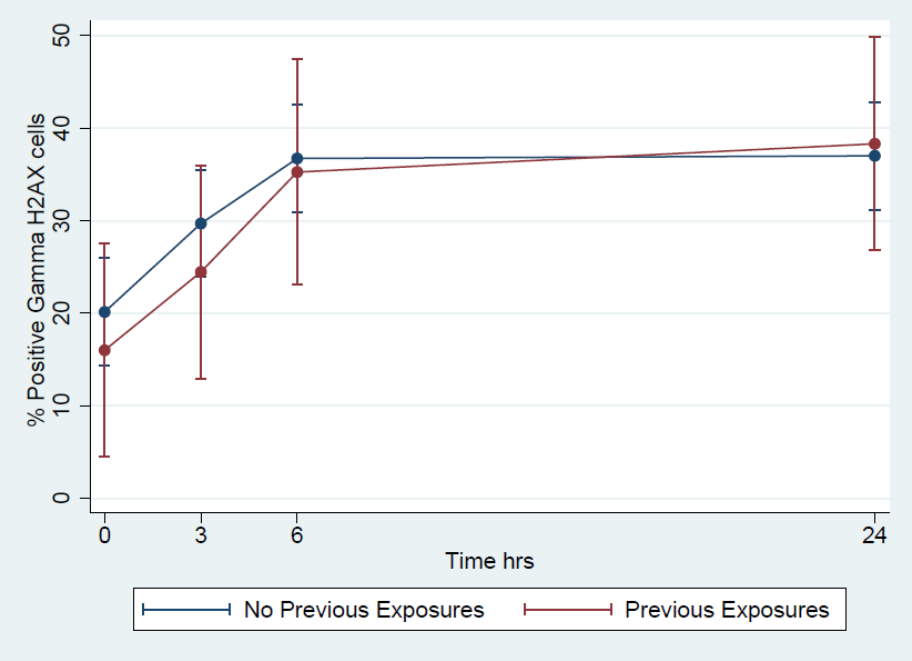

Fig.3-22 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and previous exposure history of the individual. This depicts the role previous exposure has on susceptibility to develop γH2AX foci. n=18 for individuals with no history of previous chemical exposure (blue line), n=5 for individuals who do have a history of previous chemical exposure.

Fig.3-23.With the marker of ROS induced DNA damage we observed that at baseline individuals who do have a history of previous chemical exposure (red line) exhibit slightly higher amounts of 8-oxoguanine stained cells compared to individuals with no history of previous chemical exposure (blue line). At 3hrs of cigarette smoke extract induction an inverse in this trend occurs and individuals with no history of previous chemical exposure exhibit a higher than two fold induction in positively stained cells compared to baseline amounts while only a modest observation can be seen in individuals who do have a history of previous chemical exposure (red line). At 6hrs of cigarette smoke extract incubation the trend reverts and individuals who do have a history of previous chemical exposure (red line) have slightly more positively stained 8-oxoguanine cells compared to individuals with no history of previous chemical exposure (blue line). At 24hrs of cigarette smoke extract both groups of individuals have similar amounts of positively stained cells. This marker yielded observations that were not significant p=0.5104. n=18 for individuals with no history of previous chemical exposure (blue line), n=5 for individuals who do have a history of previous chemical exposure.

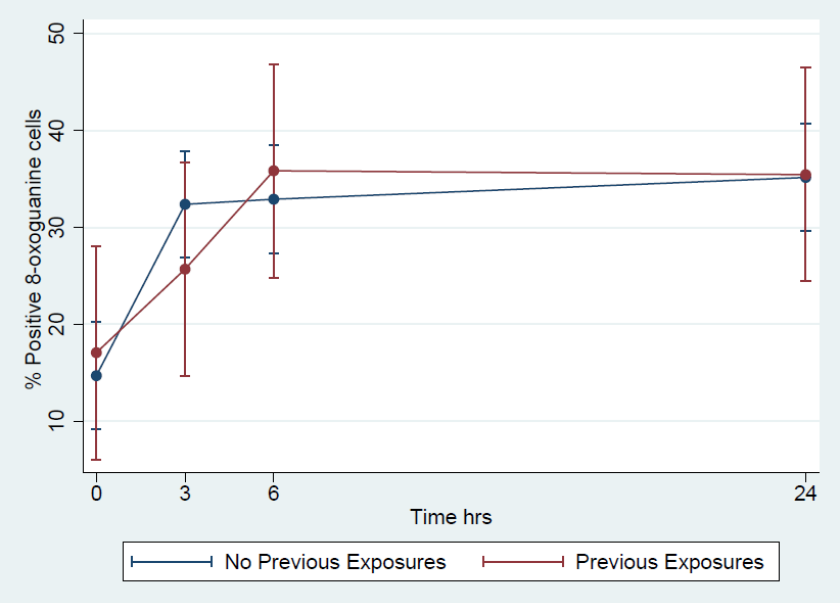

Fig.3-23 The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and previous exposure history of the individual. This depicts the role previous exposure has on susceptibility to develop positive 8-oxoguanine. n=18 for individuals with no history of previous chemical exposure (blue line), n=5 for individuals who do have a history of previous chemical exposure, p=0.5104

Fig.3-24.Measurements of positively stained cells using the inflammation marker nitrotyrosine show that at baseline individuals with no history of previous chemical exposure (blue line) have a slight increase in nitrotyrosine stained cells compared to individuals who do have a history of previous chemical exposure (red line). At 3hr cigarette smoke extract incubation both groups exhibit increases in positively stained nitrotyrosine cells. Yet, the induction in individuals with no history of previous chemical exposure (blue line) is two-fold higher than baseline levels conversely only a modest induction of positively stained cells are observed in individuals who do have a history of previous chemical exposure (red line) at the same time point. At 6hrs, and 24hrs there are slight inductions in both groups. At 6hr time point individuals with no history of previous chemical exposure (blue line) have a small increase in positive staining compared to individuals who do have a history of previous chemical exposure (red line).At 24hrs it is observed that individuals who do have a history of previous chemical exposure (red line) having slightly more positively stained nitrotyrosine cells compared to individuals with no history of previous chemical exposure (blue line). The results of this observations had data that were not significant p=0.6032. n=18 for individuals with no history of previous chemical exposure (blue line), n=5 for individuals who do have a history of previous chemical exposure.

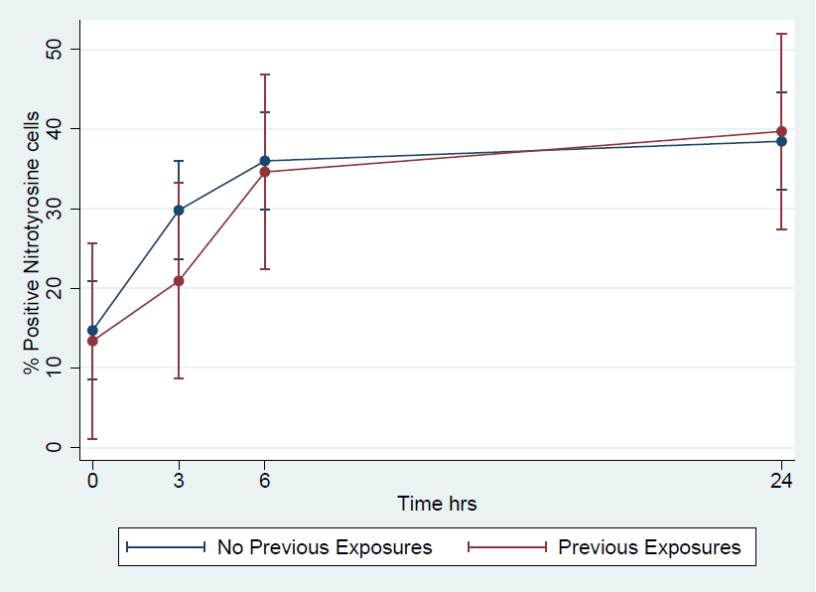

Fig. 3-24.The assessment of the bi-variant interaction between time of cigarette smoke extract incubation and previous exposure history of the individual. This depicts the role previous exposure has on susceptibility to develop positive nitrotyrosine. n=18 for individuals with no history of previous chemical exposure (blue line), n=5 for individuals who do have a history of previous chemical exposure, p=0.6032

Multi-variate Models of Interaction

Fig.3-25. Multi -variate modeling assessing models of interaction between time of extract incubation and age while controlling for sex, race, and pack years smoked.After assessing our bivariant interactions we now wanted to establish our larger interaction models while controlling for variables that could have a collinear influence on our interaction. We assess γH2AX foci formation in the newest interaction model. At baseline we observe that there is little variation between the age groups. After administering cigarette smoke extract we detect an induction of positively stained cells in all groups at three hours.

The group that exhibited the highest accumulation of double strand breaks were the individuals who are in their 40's (dark blue line), followed by individuals who are in their 50's (red line), individuals who are in their 60's (dark green line), individuals in their 70's (gold line), and finally individuals who are in their 80's (light blue line) exhibited the lowest amount of γH2AX foci formation in peripheral leukocytes. This trend persisted throughout the duration of the study at subsequent time points. After controlling for the variables of sex, race , smoking history measured by pack years in the patients a statistically significant positive interaction at $p<0.0240$ occurs between incubation time and age. This suggests that an individual's age will positively affect the amount of accrued genotoxicity. This model also yielded a near statistically significant results in our controlled variable, race at $p=0.0795$. n=2 individuals who were in their 40's (dark blue line), individuals who were in their 50's n=4 (red line), individuals who were in their 60's n=8 (dark green line), individuals who were in their 70's n=6 (gold line), and individuals who were in their 80's n=3 (light blue line).

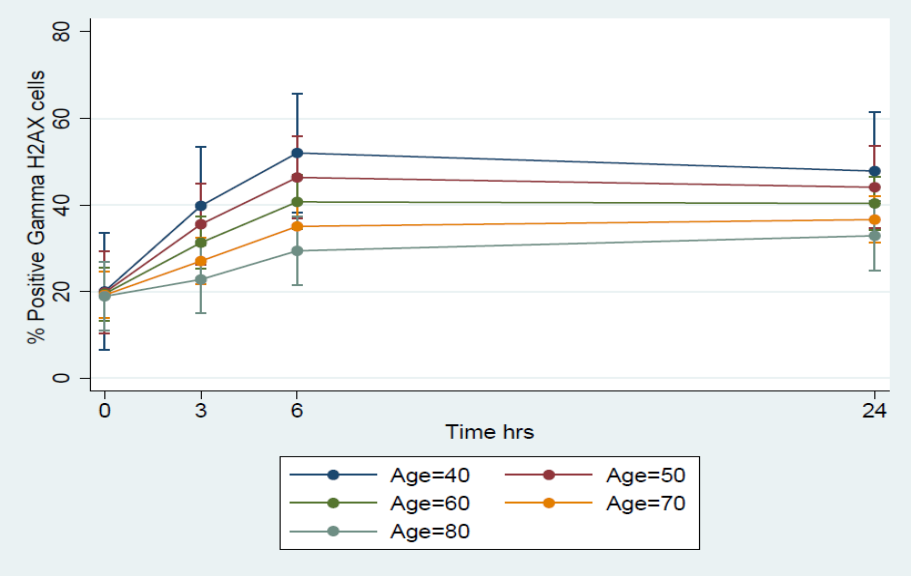

Fig.3-25. Multi -variant modeling assessing models of interaction between time of cigarette smoke extract incubation and age while controlling for sex, race, and pack years smoked. n=2 individuals who were in their 40's (dark blue line), individuals who were in their 50's n=4 (red line), individuals who were in their 60's n=8 (dark green line), individuals who were in their 70's n=6 (gold line), and individuals who were in their 80's n=3 (light blue line). Significant positive interaction at $p<0.0240$ occurs between incubation time and age. A near statistically significant results in our controlled variable, race at $p=0.0795$

Fig.3-26. Assessing reactive oxygen species induced DNA damage in our multivariant model we observed a very similar trend of DNA damage as our marker of double strand breaks. Very little distinguishing properties between the groups at baseline occurred. At 3hrs, a clear induction in all groups occurred. The highest induction at this time point occurred in individuals who are in their 40's (dark blue line), followed by individuals who are in their 50's (red line), individuals who are in their 60's (dark green line), individuals in their 70's (gold line), and finally individuals who are in their 80's (light blue line) exhibited the lowest percent of positive cells. This observation is trending towards significant at, $p= 0.0749$. This multivariant model also yielded a statistically significant race variable at $p<0.0012$. n=2 individuals who were in their 40's (dark blue line), individuals who were in their 50's n=4 (red line), individuals who were in their 60's n=8 (dark green line), individuals who were in their 70's n=6 (gold line), and individuals who were in their 80's n=3 (light blue line).

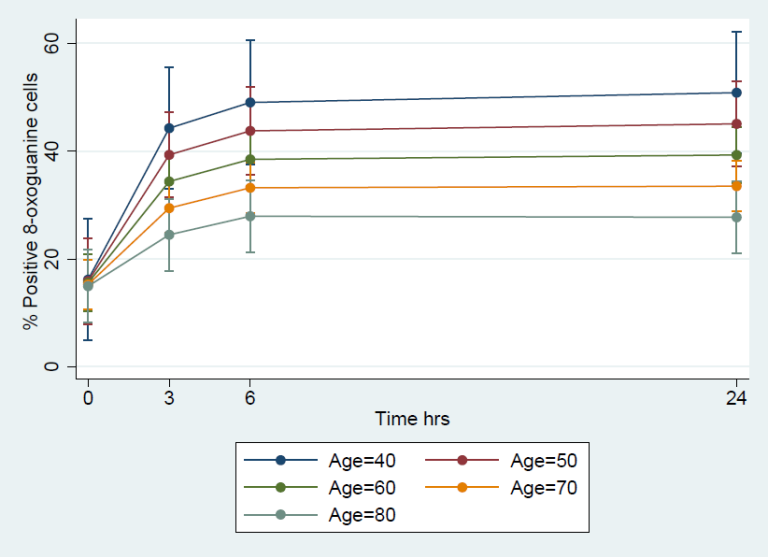

Fig. 3-26.Multi -variate modeling assessing models of interaction between time of cigarette smoke extract incubation and age while controlling for sex, race, and pack years smoked. n=2 individuals who were in their 40's (dark blue line), individuals who were in their 50's n=4 (red line), individuals who were in their 60's n=8 (dark green line), individuals who were in their 70's n=6 (gold line), and individuals who were in their 80's n=3 (light blue line). Nearly significant positive interaction at p= 0.0749 occurs between incubation time and age. A statistically significant result was found in our controlled variable, race at p<0.0012.

Fig.3-27.This marker of inflammation yielded results that were similar to the previously mentioned markers of DNA damage. At every timepoint throughout the incubation the highest induction occurred in individuals who are in their 40's (dark blue line), followed by individuals who are in their 50's (red line), individuals who are in their 60's (dark green line), individuals in their 70's (gold line), and finally individuals who are in their 80's (light blue line) exhibited the lowest percent of positive cells. This observation was not significant at, p= 0.2179. Conversely, this multivariant model also yielded a statistically significant race variable at p<0.0012. n=2 individuals who were in their 40's (dark blue line), individuals who were in their 50's n=4 (red line), individuals who were in their 60's n=8 (dark green line), individuals who were in their 70's n=6 (gold line), and individuals who were in their 80's n=3 (light blue line).

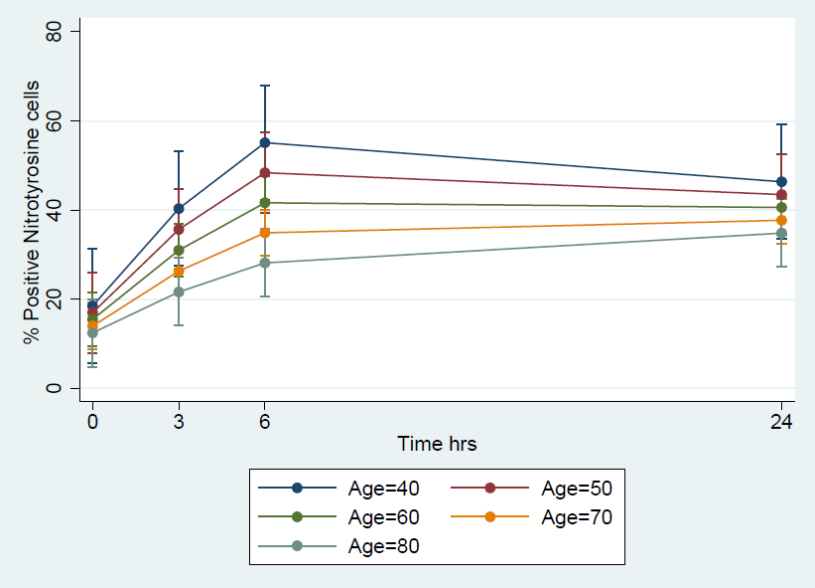

Fig.3-27 Multi -variant modeling assessing models of interaction between time of cigarette smoke extract incubation and age while controlling for sex, race, and pack years smoked. n=2 individuals who were in their 40's (dark blue line), individuals who were in their 50's n=4 (red line), individuals who were in their 60's n=8 (dark green line), individuals who were in their 70's n=6 (gold line), and individuals who were in their 80's n=3 (light blue line). p= 0.2179 is between the time and age interaction. A statistically significant result was found in our controlled variable, race at p<0.0012.

History of lung cancer

Fig.3-28.Multi -variate modeling assessing models of interaction between time of extract incubation and history of lung cancer while controlling for sex, race, and pack years smoked. Assessing cigarette smoke extract induced γH2AX between individuals who have lung cancer and individuals who do not have confirmed lung cancer. We observe at baseline individuals with lung cancer (blue line) have higher amounts of positively stained γH2AX compared to individuals who do not have confirmed lung cancer (red line). This trend persisted and for 3 and 6hrs of cigarette smoke extract incubation. On the other hand at 24hrs individuals with no confirmed lung cancer (red line) exhibited an induction of positively stained γH2AX foci formed in peripheral leukocytes than individuals with lung cancer (blue line) individuals with lung cancer (blue line). This interaction was trending towards significant at p=0.0971. n=17 for invidivuals with lung cancer (blue line), n=7 with no confirmed lung cancer (red line).

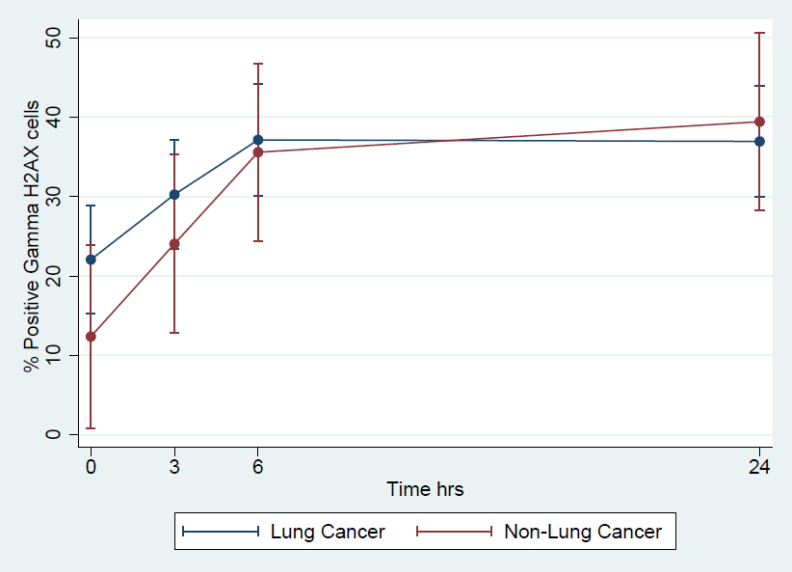

Fig .3-28.Multi -variant modeling assessing models of interaction between time of extract incubation and history of lung cancer while controlling for sex, race, and pack years smoked. Individuals with lung cancer (blue line) n=17, individuals with lung cancer n=7 (red line). This interaction was trending towards significant at p=0.0971.

Fig.3-29 Using 8-oxoguanine as a marker of DNA damage we detect an induction in the baseline amounts of individuals with lung cancer (blue line) have higher amounts of positively stained γH2AX compared to individuals who do not have confirmed lung cancer (red line). This trend occurs at 3, and 6hr cigarette smoke extract induced DNA damage. As observed with γH2AX an inverse of increased genotoxicity occurs and individuals who do not have confirmed lung cancer (red line) exhibit the highest induction of positively stained cells at 24hrs cigarette compared individuals who do not have confirmed lung cancer (red line). This interaction was significant at p=0.0136. n=17 for invidivuals with lung cancer (blue line), n=7 with no confirmed lung cancer (red line).

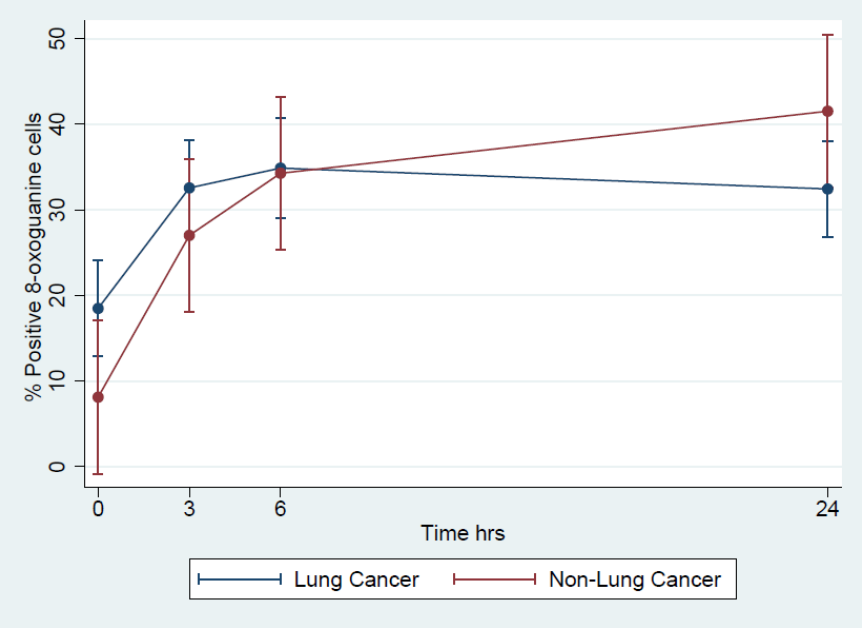

Fig .3-29 Multi -variant modeling assessing models of interaction between time of extract incubation and history of lung cancer while controlling for sex, race, and pack years smoked. Individuals with lung cancer (blue line) n=17, individuals with lung cancer n=7 (red line). This interaction was significant at p=0.0136.

Fig.3-30.Using the marker of nitrotyrosine we observe individuals with a history of lung cancer (blue line) exhibit an induction of positively stained cells at baseline compared to individuals who do not have confirmed lung cancer (red line). At 3hrs individuals with a history of lung cancer (blue line) remain higher than individuals who do not have confirmed lung cancer (red line). At 6hrs this trend changes and individuals who do not have confirmed lung cancer (red line) have higher amounts of positively stained nitrotyrosine cells compared to individuals with a history of lung cancer (blue line). At 24hrs of cigarette smoke extract incubation both groups remain relatively unchanged from the previous 6hr timepoint. This interaction was not significant at p=0.3828.

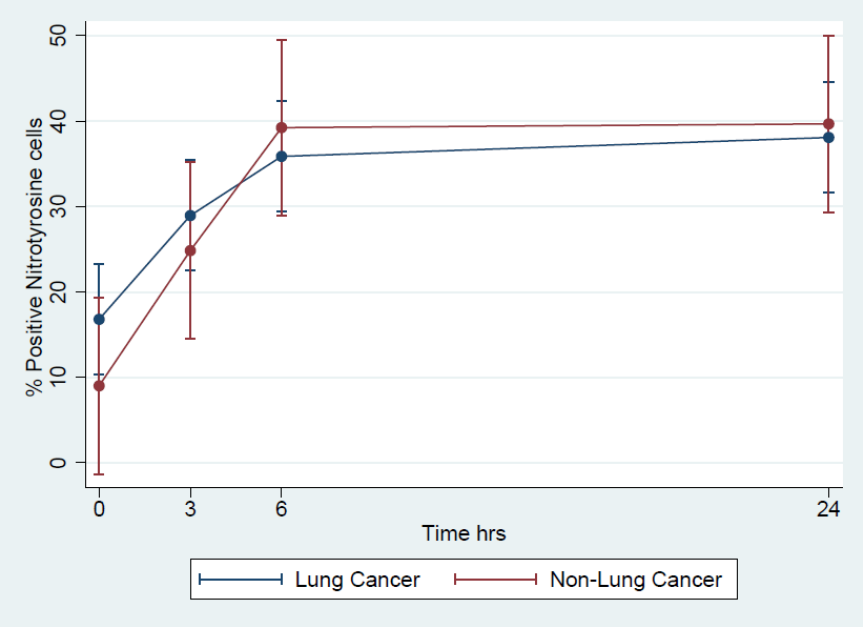

Fig .3-30Multi -variant modeling assessing models of interaction between time of extract incubation and history of lung cancer while controlling for sex, race, and pack years smoked. Individuals with lung cancer (blue line) n=17, individuals with lung cancer n=7 (red line). This interaction was significant at p=0.3828.

Discussion

Lung cancer is the leading cause of death from cancer in both men and women in the United States. [21, 22] . Although cigarette smoking is the predominating cause of lung cancer incidence only a subset of smoking individuals develop the disease. This suggests that genetic modulation of prominent factors may be leading to susceptibility in these individuals. [159] In this work we sought to establish biomarkers of susceptibility by assessing cigarette smoke extract induced DNA damage in patients with or without lung cancer. Peripheral blood leukocytes of these individuals were utilized and markers of DNA double strand breaks, reactive oxygen species induced DNA damage, and damage to nitrotyrosine residues caused by inflammation were assessed. As further contributions of susceptibility we assessed the contributions of age, race, gender, past cancer history, smoking history measured as pack years smoked, family cancer history, and previous chemical hazardous exposure history of these individuals that may lead to DNA damage. Cigarette smoke contains over 6000 chemicals many of which are known carcinogenic agents.[148] The carcinogenic compounds present in cigarette smoke are heterogeneous in nature and cause various intrinsic changes to the composition of many tissues in which they interact. Upon metabolism of many of the carcinogenic constituents in cigarette smoke water-soluble extracts of cigarette smoke are formed in some body compartments, such as blood, saliva, or fluid lining alveolar spaces, these extracts can contain active carcinogenic metabolites and can act on both cellular and extracellular compartments.[160]. Thus addition of cigarette smoke extract into peripheral blood recapitulates a natural smoking environment and serves as a great tool to assess genotoxicity. In this work we utilized bi-variate and multi-variate models to assess if the age of an individual concomitantly with cigarette smoke extract incubation increases genotoxic susceptibility. Age of the individual

has been shown to be a contributing factor in cancer incidence.[150] In addition according to 2009 statistics from the center for disease control the risk of developing lung cancer increases in age and is higher in men than it is in women. Furthermore, the center for disease control depicts that at the age of 60 there is an expected 2.27% and 1.72% increase of men and women to develop lung cancer sometime over a 10year span, respectively. In our study we found that a positive interaction occurred between age and time of cigarette smoke extract incubation in both our bi-variant and multi-variant statistical models. Unexpectedly we observed a significant increase in the amount of positive γH2AX foci in younger individuals compared to older individuals. We also indentified a near significant increase in 8-oxoguanine staining and a non-significant trend in nitrotryosine staining depicting similar increases in DNA damage in younger individuals. One plausible explanation to this observation may lie in the fact that these individuals have built up less of a resistance to the components of cigarette smoke thus causing an increased response upon recognition of the many DNA damaging compounds present in the cigarette smoke extract. Another possible explanation to the increase susceptibility to DNA damage in this group may be due to the fact only two individuals fell within this grouping or that one of the individuals who did fall within this group was non-smoking. This non-smoking status may render individuals more susceptible to components of cigarette smoke than smoking, or former smoking individuals. To investigate this we assessed the effect that smoking history has on DNA damage. Smoking tobacco is the major etiological risk factor for lung cancer development in current or former smokers.[161] Although smoking is the most prevalent cause of lung cancer 15% of lung cancer patients have never smoked and lung cancer in these non-smoking individuals comprise the seventh leading cause of mortality amongst solid tumors.[162] We sought to determine the interaction that smoking history has on our biomarkers of DNA

damage. Using all three markers we see that non-smokers had high to moderate amounts of DNA damage that persisted throughout cigarette smoke extract incubation. We also see a trend that individuals with a longer smoking history had a tendency to clear the cigarette smoke extract induced DNA damage at faster rates than that of never smoking individuals. A possible rationale to this trend is that current and former smokers may have increased enzymes that have a familiarity with cigarette smoke related damage, thus the recognition and removal of the perturbation is faster. Conversely never smoking individuals do not have an up-regulation of these enzymes making the clearing of the damage occur at a slower rate than smoking or former smoking individuals. Furthermore, it is well established that genetic modulation of important detoxifying enzymes renders an individual to become more susceptible to lung cancer.[121, 144, 163, 164]. Although not assessed in our work it is a possibility that individuals who do not properly remove cigarette smoke induced perturbations may be due to faulty repair systems rendering them more susceptible. It is established that individuals of certain racial demographics have been shown to exhibit increased association of lung cancer. [151, 152]. Although we only observed a significant increase in one of the biomarkers in our bi-variant interaction model when race is controlled for in our larger multi-variant model we see the significant interaction race has on 8-oxoguanine, and nitrotyrosine induced DNA damage and a near significant induction of γH2AX. These data show that race has a significant interaction on susceptibility to cigarette smoke induced DNA damage. When assessing the specific races it is shown that individuals of Hispanic ethnicity have the highest induction of DNA damage in all three biomarker followed by individuals who have White ethnicity, Asian ethnicity, and lastly Black ethnicity. It is established that cigarette smoke and its many components can induce reactive oxygen species that cause mutagenic legions that are normally repaired by specific DNA repair proteins.[121, 139,

165].Recently it has been shown that SNPs in base excision repair genes in Hispanic and Black individuals increase the risk of developing lung cancer.[7]. Although the prevalence of lung cancer is second highest in Whites one study reveals that these individuals averaged the most lung cancer related surgical operations leading to a lower mortality rate than other ethnic groups with lower socialeconomic status. [147] A possible explanation of the relatively low increase in DNA damage in this group may be attributed to the higher amount of surgical procedures conducted. An increase of operation may be leading to better disease prognosis due to the fact that resection of tumors may cause a change in microenvironment thus slowing down rate of new tumor formation and causing a decrease in DNA damage. It is established that individuals of Asian decent have relatively low smoking prevalence and lung cancer incidence. [166] Our data corroborates with this and in our study we observed modest increases in cigarette smoke extract induced DNA damage in these individuals. Another plausible explanation to this modest increase may lie in the normal dietary intake of these individuals. [167] Describes that the protective impact of lifelong or early exposure to soy-derived isoflavones were associated with a 27% of risk reduction in lung cancer individuals. This increase of protection by a high-soy diet could possibly substantiate the modest cigarette smoke extract induced genotoxicity seen in this racial group. Surprisingly in our study Black individuals consistently had lower amounts of cigarette smoke extract induced DNA damage. Yet, it is well established that Blacks have higher lung cancer incidence than any other racial group.[152]. Black men have the highest incidence of lung cancer as well as the highest mortality.[168] In a recent study, despite Black smokers having higher plasma cotinine per individual cigarette smoked exposure to nicotine and carcinogens per individual cigarette as assessed by urine biomarkers was similar or lower in Blacks compared to Whites.[151] This may suggest that Black individuals although may smoke more may have

lower exposure to cigarette carcingoens due more frequent cigarettes but less intense smoking habits or higher clearance rates of the carcinogens. Our data corroborates with this study due to the consistently low amounts of cigarette smoke extract induced genotoxicity in this racial group. Furthermore, Polymorphisms in DNA damage and repair genes may offer a plausible explanation for the observed modulation in response to genotoxicity in Blacks as well as other racial groups. In conjunction with race it has been well documented that there are sex disparities in cancer that make gender a significant variable in increased cancer incidence.[153, 154]. We examined the role gender plays in the assessment of the increased genotoxic susceptibility. On average lung cancer incidence is increased in males compared to women. Furthermore women exhibit higher relative rates of certain smoking related lung cancer types than men.[20] In our study we observed that in two of our biomarkers of DNA damage women have increased susceptibility to cigarette smoke induced DNA damage. In corroboration of our data [169] describe that women exhibit increased susceptibility to tobacco smoke carcinogens, which may be attributed to smoking-induced bulky/hydrophobic DNA adducts due to an increased expression of CYP1A1 in their lungs, compared to men. This may offer an explanation of the trend we observe of increased damage in women compared to men. Prolonged exposure to cigarette smoke is associated with the formation of cancer in various positions within the body of men and women who are smokers and non-smokers.[170, 171] Knowing the well documented effects of cigarette smoke and its extracts we assessed two bi-variant models to determine if there was a purported susceptibility to cigarette smoke extract induced DNA damage amongst lung cancer or non lung cancer individuals in one bi-variant model or individuals with a previous cancer history or individuals who do not have a previous cancer history in another. We observed that people with lung cancer displayed higher amounts of early susceptibility to DNA damage in

all our biomarkers although this early damage waned as time progressed. These data depict a temporal susceptibility that may be present in lung cancer individuals and may suggest a target of therapeutic intervention. We also saw individuals with other cancers or a previous cancer history exhibited higher susceptibility to DNA damage in two of our three biomarkers. This data lead us to further interrogate of the individuals with a reported history of other cancer what cancer types would lead to increased susceptibility to cigarette smoke extract induced DNA damage. In all three biomarkers we see that individuals with a history of bladder cancer remained near the top of the list of highest induced amounts of cigarette smoke extract induced DNA damage. We also observed a trend in 2 of the 3 biomarkers that individuals with a history of invasive thymoma or ovarian cancer also demonstrated an increased susceptibility to cigarette smoke extract induced DNA damage. As further validation of the observed trend in individuals with history of cancer recent studies show an association between smoking and cancer of the paranasal sinuses, nasopharynx, adenocarcinoma of the esophagus, myeloid leukemia, oral cavity, pharynx, larynx, pancreas, urinary bladder, ovaries, and renal pelvis.[107, 171] Furthermore [172] describes an increased risk associated with developing lung cancer in smoking individuals who have bladder cancer. Collectively our data accord with previous data and accurately links common smoking related cancers and susceptibility to cigarette smoke extract induced. The observation of cigarette smoke extract induced DNA damage in individuals with a history of other cancers lead us to investigate if there is a similar trend of susceptibility in individuals with a family lung cancer or other cancers. Furthermore, it is documented that a family history of lung cancer or other cancers is an established risk factor for lung cancer.[155, 156] We determined if individuals with a known family history of cancer have an increase susceptibility to cigarette smoke induced genotoxicity. Our results depict a trend that individuals

with a family history of cancer consistently had higher cigarette smoke extract induced DNA damage in all our biomarkers. In further support of these results it is shown that individuals with a family history of lung cancer or previous pulmonary diseases have a two- to threefold increased risk for developing this disease. [173] This is further supported by another study that indentified a positive family history of any cancer was associated with over 2-fold risk of developing lung cancer than negative family history. [174]. Our data are additive to these previous studies due to our assessment of genotoxicity suggests individuals with a family history of cancer or previous lung disease may have more susceptibility to cigarette smoke induced DNA damage. In concurrence with a family history of cancer and other parameters, we assessed if individuals with a history of previous chemical exposure exhibited a susceptibility to cigarette smoke extract induced DNA damage. Exposure to hazardous airborne chemicals has been shown to cause lung cancer.[157] Furthermore concomitant exposure of noxious chemicals and smoke has also been link to higher lung cancer incidence.[158].In our experiments we do not see a significant difference nor a trend in our bi-variant models assessing if previous chemical exposure positively interacts with cigarette smoke extract induced DNA damage.

In conclusion the results of our study shows significant interactions of age and race on cigarette smoke extract induced DNA damage and positive trends in sex, previous personal cancer history, family history of cancer, and smoking history that is associated with lung cancer. Furthermore, in establishing our biomarkers we were able to detect increased susceptibility to cigarette smoke induced DNA damage in individuals with varying disease history and smoking status. This provides evidence for using these genotoxic assays as biomarkers in determining susceptibility to lung cancer in individuals, and opens the door to these biomarkers being used as

possibly pre-screening tools to lung cancer predisposition and susceptibility in smoking and non-smoking individuals.

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Conclusions

The goal of this dissertation was to elucidate the role genotoxic susceptibility plays in pulmonary diseases of the mouse and human, and to assess if cigarette smoke extract induced genotoxicity present in peripheral leukocytes can be utilized as a biomarker of lung cancer. In chapter 1 we assessed the contribution of the key immunological modulator interleukin-13 role in asthma induced genotoxicity. Recent studies have shown the ability of ROS present in asthma to cause genotoxicity at the site of inflammation. Yet none have attempted to elucidate IL-13's role in this genotoxic response nor have any assessed systemic genotoxicity present in models of asthma. In our work we found genotoxicity present at the site of inflammation by increase in transcripts of DNA damage as well as inductions of single and double strand breaks and chromosomal abberations in peripheral leukocytes. We furthermore observed increases of oxidative DNA damage present in peripheral leukocytes of transgenic mice. We also characterized a late IgE response and chronic nuetrophilic infiltration in serum and BAL fluid, respectively.

Collectively these data implicate the major role of IL-13 beyond the current scope of understanding and points to the possibility of it being a key modulator of both the immune response in asthma but also genotoxicity. This undoubtedly opens the door to further exploring IL-13 as a immune target for not only asthma but for other inflammatory related diseases of the lung and beyond.

In chapter 2 we sought to establish the role genetic modulation of important DNA repair and antioxidant response proteins play in determining the susceptibility to develop cigarette smoke extract and sidestream tobacco smoke induced DNA damage. It is well characterized that deficiencies in DNA repair and antioxidant capcity lead to increase

susceptibility [175] Conversely, it is not well understood mechanistically how the process occurs. In this study we utilized animals deficient in base excision repair and gluthathione antioxidant response. We observed a significant difference in *ex vivo* CSE-induced damage in base excision repair deficient mice compared to wildtype mice, differentially regulated GSH homeostasis in *Ogg1*$^{-/-}$*Myh*$^{-/-}$, *Gclm*$^{-/-}$, and *Gclm*$^{+/-}$ mice compared to wildtype mice, and decreased survival proportions after exposure to B[*a*]P in *Ogg1*$^{-/-}$*Myh*$^{-/-}$ mice compared to wildtype mice. These results are in agreement with epidemiological data showing an increased susceptibility to lung carcinogenesis in people with polymorphisms in *OGG1* and *MYH* [7] [121].

In chapter 3 we sought to establish if genotoxicity present in peripheral leukocytes can be utilized as biomarkers to lung cancer. Using bi- and -multi variate models we were able to assess various interactions to determine the contribution to cigarette smoke extract induced genotoxicity. Although we were limited by our small sample size , the results of our study shows significant interactions of age and race on cigarette smoke extract induced DNA damage and positive trends in sex, previous personal cancer history, family history of cancer, and smoking history that is associated with lung cancer. One interesting observation that was found was in two of our biomarkers individuals who did not have a smoking history were highly susceptible to cigarette smoke extract induced DNA damage. This information could possible lead to the pursuit of elucidating what could cause non-smoking individuals to be susceptible to cigarette smoke extract induced DNA damage. This area of interest could be advantageous and could delineate many of the mechanistic questions that are present in the field. Moreover in support of this *Thun et.al* [170] suggests that understanding what environmental and/or biological cues that cause these individuals to be more susceptible in principle should be easier to obtain in

individuals who do not have a history of smoking because they are void of many of confounding issues that would normally have to be taken into account. In addition clinical studies show non-smokers who develop lung cancer have a different molecular profile of cancer that respond better to target therapy than smoking individuals.[21, 170] The afore mentioned reasons amongst others make the establishment of biomarkers of lung cancer and other lung diseases a priority to improving human health and disease prognosis moving forward.