Effects of Diesel Exhaust Particles and Engineered Nanoparticles on DNA Methylation of Genes Associated with Pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) in Bronchial Epithelial Cells

by

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Koç University

Graduate School of Health Sciences

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and have found that it is complete and satisfactory in all respects,
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To Curiosity and Science…

ABSTRACT

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Inhaled particles such as diesel exhaust particles (DEP) and engineered nanoparticles (NP) create an important risk for respiratory health. Studies suggest that DEP and NP induce oxidative stress, cellular toxicity, and inflammation, which can lead to airway diseases. These particles are also shown to cause DNA methylation in various cellular systems. However, their effect on genes involved in chronic obstructive pulmonary disease (COPD) are not clear. We aimed to investigate effects of DEP and NP on mRNA expression and DNA methylation of genes associated with the pathogenesis of COPD.

BEAS-2B cells were incubated with DEP, Titanium Dioxide (TiO_2) and Multi-walled Carbon Nanotubes (MWCNTs) at concentrations of 0-100 µg/mL for 6, 24, and 48 hours, and viability of the cells was assessed using MTT assay. mRNA expression of COPD-associated genes (*GCLC, CYP1B1, MUC5B, MUC5AC, FAM13A, SERPINA1*) was analysed by qRT-PCR. CpG-targeted pyrosequencing was performed to understand DNA methylation status of differentially expressed genes.

Particles did not cause a reduction in cell viability greater than 50%, except 100 µg/mL DEP at T48 hours of incubation. DEP and NP altered mRNA expression for *GCLC* and *CYP1B1* genes, which are both involved in oxidative stress mechanisms. There were no significant changes in methylation in the promoter regions of the genes. However, DNA methylation changes were observed in CpG sites located on exon 1 and intron 1. Although 50 µg/ml DEP increased DNA methylation of *GCLC* gene at T24 hours of incubation, this was decreased by MWCNT, as the exposure time increased. TiO_2 did not cause significant DNA methylation changes in *GCLC* gene. 100 µg/mL MWCNT increased DNA methylation of *CYP1B1* gene at T6 and T48 hours, whereas neither DEP nor TiO_2 caused a significant change in DNA methylation of *CYP1B1* gene.

Our findings suggest that DEP and MWCNT may play a role in the pathogenesis of COPD by altering mRNA expression and DNA methylation of *CYP1B1* and *GCLC* genes.

Keywords: Bronchial Epithelial Cells, Diesel Exhaust Particles, Engineered Nanoparticles, DNA Methylation, COPD.

ÖZETÇE

Dizel Egzoz Partiküller ve İnsan Yapımı Nanopartiküllerin Bronş Epitel Hücrelerinde Kronik Obstrüktif Akciğer Hastalığı (KOAH) ile İlişkili Genlerin DNA Metilasyonu Üzerine Etkileri

Sinem Erkan

Hücresel ve Moleküler Tıp, Yüksek Lisans

Ocak 19, 2023

Dizel egzoz partiküller (DEP) ve insan yapımı nanopartiküller (NP) gibi solunum yolu ile alınan partiküller akciğer sağlığı için önemli bir risk oluşturur. Çalışmalar DEP ve NP'lerin oksidatif stres, hücresel toksisite ve inflamasyonu indükleyerek havayolu hastalıklarının oluşumuna sebep olduklarını önermektedir. Aynı zamanda bu partiküllerin birçok hücresel sistemde DNA metilasyonuna sebep oldukları da gösterilmiştir. Ancak bu partiküllerin kronik obstruktif akciğer hastalığı (KOAH) ile ilişkilendirilen genler üzerine olan etkisi net değildir. Bu çalışmada, DEP ve NP'lerin KOAH ile ilişkili genlerin mRNA ekspresyonu ve DNA metilasyonu üzerine olan etkilerini araştırmayı amaçladık.

BEAS-2B hücreleri 0-100 µg/mL konsantrasyonlarında DEP, Titanyum Dioksit (TiO_2) ve Çok Duvarlı Karbon Nanotüpler (ÇDKNT) ile 6, 24 ve 48 saat boyunca inkübe edilmiş ve MTT testi ile hücrelerin canlılığı değerlendirilmiştir. KOAH ile ilişkili genlerin (*GCLC, CYP1B1, MUC5B, MUC5AC, FAM13A, SERPINA1*) mRNA ekspresyonları kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) ile analiz edilmiştir. Ekspresyonu değişen genlerin DNA metilasyon durumlarını anlayabilmek için CpG hedefli pirosekanslama metodu uygulanmıştır.

Partiküllerin 48 saatlik DEP 100 µg/mL inkübasyonu haricinde, %50'den fazla hücre ölümüne yol açmadığı görülmüştür. DEP ve NP'ler, ikisi de oksidatif stres mekanizmalarında yer alan genler olan *GCLC* ve *CYP1B1* mRNA ekspresyonunu değiştirmiştir. Bu genlerin promotör bölgelerinde anlamlı bir metilasyon değişimi görülmemiştir. Ancak exon 1 ve intron 1'de yer alan CpG bölgelerinde anlamlı metilasyon değişimleri gözlenmiştir. 50 µg/mL DEP'in 24 saat boyunca inkübasyonu *GCLC* geninin DNA metilasyonunu arttırmasına rağmen, ÇDKNT maruziyeti, inkübasyon süresi arttıkça DNA metilasyonunun azalmasına sebep olmuştur. TiO_2 *GCLC* geni üzerinde anlamlı bir DNA metilasyon değişimine sebep olmamıştır. 100 µg/mL MWCNT 6 ve 48 saatlik inkübasyon sonucu *CYP1B1* geninde DNA metilasyonunu arttırırken, DEP ve TiO_2 *CYP1B1* geninde anlamlı bir DNA metilasyon değişimine sebep olmamıştır.

Bulgularımız, DEP ve ÇDKNT'lerin *CYP1B1* ve *GCLC* genlerinin mRNA ekspresyonları ve DNA metilasyonlarının değişimlerine yol açarak KOAH patogenezinde rol oynayabileceklerini önermektedir.

Anahtar kelimeler: Bronş Epitel Hücreleri, Dizel Egzoz Partiküller, İnsan Yapımı Nanopartiküller, DNA Metilasyonu, KOAH.

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ABBREVIATIONS

ACS	Acute coronary syndrome
AECs	Alveolar epithelial cells
AHR	Aryl hydrocarbon receptor
ATII	Alveolar Type 2
ANOVA	Analysis of variance
AuNP	Gold nanoparticles
5-AzadC	*5*-Aza-2′-deoxycytidine
A549	Lung carcinoma epithelial cell line
BEAS-2B	Human non-tumorigenic lung epithelial cell line
BEC	Bronchial epithelial cells
BCL-2	B-cell lymphoma 2
BM	Bisulphite modification
CNT	Carbon Nanotubes
CREB3L1	CAMP Responsive Element Binding Protein 3 Like 1
CpG	Cytosine-phosphate-guanine
CSE	Cigarette smoke extract
CYP1B1	Cytochrome P450 Family 1 Subfamily B Member 1
COPD	Chronic obstructive pulmonary disease
CXCL/IL-8	Chemokine (C-X-C motif) ligand or *Interleukin 8*
DEP	Diesel exhaust particles
DEG	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DNMTs	DNA methyltransferases
dPBS	Dulbecco's phosphate-buffered saline
EPA	Environmental Protection Agency
FAM13A	Family With Sequence Similarity 13 Member A
FBS	Fetal bovine serum
FEVI	Forced expiratory volume
FVC	Forced vital capacity

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCLC	Glutamate-Cysteine Ligase Catalytic Subunit
GSH	Glutathione
GWAS	Genome-wide association studies
HEK293	Human embryonic kidney cells
HMOX1	Heme Oxygenase 1
ISR	Integrated stress response
miRNA	MicroRNA
MUC5AC	Mucin 5AC, Oligomeric Mucus/Gel-Forming
MUC5B	Mucin 5B, Oligomeric Mucus/Gel-Forming
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
MWCNT	Multi-walled carbon nanotubes
5mC	5-Methylcytosine
NE	Neutrophil elastase
Nc-RNA	Non-coding RNA
NFW	Nuclease-free water
NP	Nanoparticles
NRF2	Nuclear factor erythroid 2–related factor 2
PAHs	Polycyclic aromatic hydrocarbons
PM	Particulate matter
SERPINA1	Serpin Family A Member 1
SF	Serum-free
SPDEF	SAM Pointed Domain Containing ETS Transcription Factor
STAT6	Signal Transducer and Activator of Transcription 6
12-SV40	12-Simian virus 40
SWCNT	Single-walled carbon nanotubes
TAE	Tris-acetate-EDTA
Th	T-helper
TiO_2	Titanium dioxide

Chapter 1:

INTRODUCTION

Air pollution is a crucial health problem defined as the contamination of the indoor or outdoor environment by physical, chemical, and biological agents (World Health Organization, 2022a). Particle matters (PM) are among the most toxic elements of air pollution. They play an important role in the pathogenesis and the progression of chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). It is known that more than half of PM is traffic generated. Diesel exhaust particles (DEP) belong to this category (Wrobel, Rokita, & Maenhaut, 2000). On the other hand, engineered nanoparticles (NPs) can contribute to PM pollution, since nanotechnology, which is originated from NPs, has improved and expanded in all areas of daily life. NPs exhibit sizes ranging from 1–100 nm. There are different kind of NPs such as fullerenes, carbon nanotubes (CNT), dendrimers, quantum dots, titanium dioxide (TiO_2), gold, and silver NPs. TiO_2 are among the most widely used nanomaterials, whereas CNTs are relatively new products. Single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT) are two types of CNTs (Kobayashi, Izumi, & Morimoto, 2017). Both NPs and DEPs have very tiny diameters, so when inhaled, they reach into the distal airways and alveoli of lungs, and cause serious health problems. It has been shown that DEP and NPs induce genotoxicity, oxidative DNA damage, and inflammation in mice (Trouiller, Reliene, Westbrook, Solaimani, & Schiestl, 2009). Furthermore, NPs have been shown to have DNA methylation changes in the cells. Studies show that environmental risk factors, such as PM2.5 and biofuels, are involved in the pathogenesis of COPD through aberrant DNA methylation (Leclercq et al., 2017; Sood et al., 2010).

COPD is a major health problem worldwide, particularly in developing countries. It is the third leading cause of death worldwide and caused 3.23 million deaths in 2019 (World Health Organization, 2022b). COPD is a chronic inflammatory disease and usually manifests as combination of emphysema and chronic obstructive bronchitis. *FAM13A*, *SERPINA1*, *GCLC*, *CYP1B1*, *MUC5AC*, and *MUC5B* are the genes related to different characteristics of COPD pathogenesis, such as mucus hypersecretion, oxidative stress, and inflammation. COPD develops due to both genetic and environmental risk factors. In COPD, environmental factors lead to changes in gene expression through

epigenetic mechanisms. Of these, DNA methylation plays a significant role in the pathogenesis of various diseases including COPD (Perzel Mandell et al., 2021; Schmidl, Delacher, Huehn, & Feuerer, 2018; Vrba & Futscher, 2018). It is known that DEP and NPs cause epigenetic changes at the cellular level. However, it is not clear whether DEP or NPs cause methylation on the genes related to COPD. We hypothesize that DEP and NPs contributes to the pathogenesis of COPD by leading to DNA methylation of the genes involved in the disease pathogenesis.

Our aim was to examine whether DEPs and NPs could alter the expression of genes associated with COPD pathogenesis through DNA methylation. In line with this purpose, our goals were:

1. To investigate effects of DEP and NPs (TiO_2 and MWCNTs) on mRNA expression of the genes (*GCLC, CYP1B1, MUC5B, MUC5AC, FAM13A, SERPINA1*), in BEAS-2B bronchial epithelial cells.
2. To study DNA methylation in genes that showed significant concentration/time-dependent changes in their mRNA expression following exposure to DEP, TiO_2 and MWCNTs.

Based on the existing literature, chapter 2 provides general information on topics related to this study. Chapter 3 contains the methods that were used in the study. The results of the study are presented in chapter 4. In chapter 5, discussion of the results is provided with the limitations and the future directions of the study.

Chapter 2:
GENERAL INFORMATION

2.1 Air pollution

Air pollution is a significant health problem, defined as contamination of the indoor and outdoor environment by physical or biological agents (World Health Organization, 2022a). As the US Environmental Protection Agency (EPA) identifies, there are six different types of air pollutants: carbon monoxide, lead, nitrogen oxides, ground-level ozone, particle pollution, and sulfur oxides (Centers for Disease Control and Prevention, 2021).

2.1.1 Particulate Air Pollution

Particle air pollution includes particulate matters (PM), which are the vast components of air pollution. They are a mixture of solid particles and liquid droplets and are divided into three according to their size: Coarse particles (PM10) have a diameter of 10 μm or less, whereas fine particles have a diameter of 2.5 μm or less. On the other hand, ultrafine particles (PM0.1) have much smaller diameters (≤0.1 μm in diameter) (Li et al., 2016). The size of these particles is significant in their potential to cause health problems. Since can be inhaled easily, and can reach the lung periphery, and cause serious health problems such as decreased lung function, coughing, difficulty in breathing (US Environmental Protection Agency, 2022b). Larger particles tend to be trapped in the nose, mouth, or throat, whereas smaller particles, such as PM2.5 or PM0.1, can go deeper into the lungs (US Environmental Protection Agency, 2022b). These particles can be emitted from power plants, industries, and automobiles (US Environmental Protection Agency, 2022a).

Diesel Exhaust Particles

Traffic emissions are a significant part of urban air pollution. It is known that more than half of the total PM2.5 emissions are traffic-generated, and the majority of these particles are made by diesel exhaust particles (DEP) (Wrobel et al., 2000). DEP are a complex mixture of various organic and inorganic substances. They are discharged from the exhaust of diesel-type internal combustion engines. The structure of these particles is composed of elemental carbon atoms surrounded by polycyclic aromatic hydrocarbons

(PAH), low amounts of sulfate, nitrate, metals, and other trace elements (Wichmann, 2007). These particles contain both fine particles and ultrafine particles. Ultrafine particle size is in the range of nanoparticles (≤0.1 μm in diameter), which is why DEPare sometimes named as NPs (Li et al., 2016).

2.1.2 Engineered Nanoparticles

NPs create a risk on human health since they are widely used in nanotechnology, which is utilized in all areas of daily life. These particles are divided into two; combustion NPs and engineered NPs. Combustion are emitted from diesel exhaust, welding fumes, and coal fly ash (Sadik, 2013). They have no predetermined size, and they may exhibit undefined chemistry. The second category of anthropogenic NPs, engineered NPs exhibit specific sizes ranging from 1–100 nm (Ramakrishnan et al., 2021). They are designed with specific features such as surface properties and chemistry. Fullerenes, carbon nanotubes (CNT), dendrimers, quantum dots, TiO_2, gold, and silver NPs are examples of engineered NPs (Sadik, 2013).

Titanium Dioxide Nanoparticles (TiO_2)

TiO_2 is a metal oxide that naturally occurs in the environment. It has three polymorphic forms: anatase, rutile, and rhombic brookite. Many synthetic methods eventually lead to the formation of TiO_2 with specific physicochemical properties (Ziental et al., 2020). TiO_2 are one of the most widely used nanomaterials (Hong, Zhou, Zhou, & Wang, 2017). Because of their low cost and availability, TiO2 have been used in many industries, such as pharmaceuticals (Lingaraju et al., 2021), processed foods (Wang et al., 2020), fabrics, paints, and sunscreens (Kiss et al., 2008).

Multi-walled Carbon Nanotubes

CNTs are allotropes of carbon with properties between graphite and fullerenes. There are two types of carbon nanotubes: single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). The difference between them depends mainly on the orientation of sp^2 molecular orbital of carbon. In SWCNT, sp^2 carbon units are oriented as one, whereas MWCNT are composed of multiple concentric tubes of rolled-up graphene (Oliveira & Morais, 2018). CNTs are physically robust and resistant structures. In both aqueous and non-aqueous solutions, MWCNTs present active surface

area, chemical inertness, high strength, and low charge-transfer resistance. Because of the properties that MWCNT have, they are employed in transistors, flat-panel displays, nanoelectronics, batteries, solar cells, and other electronic components. They may also be utilized to store energy ("Applications of Multi-Walled Carbon Nanotubes," 2021).

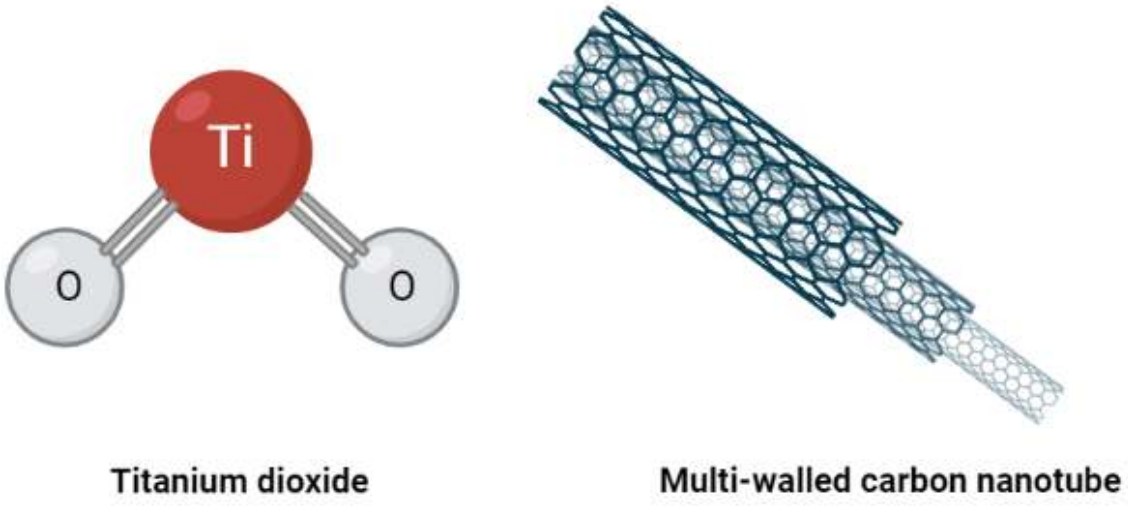

Figure 2.1: Structure of TiO_2 and MWCNT (Figure is created in Biorender.com).

2.2 Chronic Obstructive Pulmonary Diseases

2.2.1 Definition, Epidemiology and Risk factors

COPD is a chronic inflammatory disease that usually manifests as a combination of emphysema (due to the breakdown of the alveoli) and chronic obstructive bronchitis (the result of chronic inflammation of the bronchial tubes). COPD is one of the top three causes of death worldwide and caused 3.23 million deaths in 2019 (World Health Organization, 2022b). Therefore, it is a significant public health problem worldwide, especially in developing countries. Based on epidemiological studies, in 2010, there were 384 million COPD cases with an 11.7% global prevalence (Adeloye et al., 2015). It is estimated that, by 2060, there may be over 5.4 million deaths annually from COPD and similar airway diseases (Global Initiative for Chronic Obstructive Lung Disease, 2022). Although smoking is the most common cause of COPD, many studies show that non-smokers can develop airway obstruction, as well. In COPD of non-smokers, airway obstruction is mainly due to inhaled environmental pollutants (I. A. Yang, Jenkins, & Salvi, 2022). Environmental and occupational irritants such as air pollutants, biomass smoke, wood dust, and diesel exhaust contribute to the formation and progression of the disease. Aside from these risk factors, genetic abnormalities, abnormal lung development, and accelerated aging all contribute to COPD development (Global Initiative for Chronic Obstructive Lung Disease). The best known genetic risk factor COPD is α1anti-trypsin defiency. Exposure to environmental irritants may contribute to the progression of COPD through epigenetic mechanisms.

2.2.2 Pathogenesis of COPD

The airways and lung parenchyma are the key areas, where pathological alterations in COPD can be identified. COPD is a heterogeneous disease and several mechanisms play a role in its pathogenesis. The disease is characterized by proteolytic–anti-proteolytic imbalance, epigenetic alterations (Avci, Sarvari, Savai, Seeger, & Pullamsetti, 2022), lung inflammation, oxidative stress (Thimmulappa, Chattopadhyay, & Rajasekaran, 2020), mucociliary dysfunction, airway fibrosis and alveolar destruction (Barnes, 2019). Although various characteristics are involved in its pathophysiology, the pathogenesis of COPD is mainly regulated through several signaling pathways, including oxidative stress and inflammation (Kaur, Chandel, Malik, & Naura, 2022). Patients with

COPD have higher levels of oxidative stress, particularly during acute exacerbations. Exposure to air pollutants or cigarette smoke leads to the generation of reactive oxygen species (ROS) by inflammatory and epithelial cells in the lung. As a result, oxidative stress increases in airways (Barnes, 2020). It is also known that there is a positive correlation between inflammation and disease severity in COPD patients (Parris, O'Farrell, Fong, & Yang, 2019). Proteases in the lung break down connective tissues, whereas antiproteases counterbalance this action. Increased levels of numerous proteases derived from these cells and epithelial cells have been seen in COPD patients as a result of the increase in inflammatory cell numbers. The protease-mediated degradation of elastin, a primary connective tissue component in the lung parenchyma, is a principal characteristic of emphysema (Stockley, 1999). On the other hand, airway mucus hypersecretion is another important feature of COPD (Deqing Yang et al., 2021). Increased goblet cells and enlarged submucosal glands cause mucus hypersecretion, triggered by exposure to cigarette smoke and other noxious agents (Shen et al., 2018).

Epigenetic changes are also associated with susceptibility to COPD. In a genome-wide association study (GWAS), DNA methylation profiling was performed in lung tissue from COPD patients, differences in methylation loci related to lung function, asthma diagnosis, nicotine addiction, T-cell development, and other factors were identified (Agustí & Hogg, 2019).

2.2.3 COPD Related Genes and Their Roles in the Disease Pathogenesis

A number of genes including *SERPINA1, FAM13A*, *MUC5AC, MUC5B*, *GCLC,* and *CYP1B1* are reported to be involved in the pathogenesis of COPD. Of these, *SERPINA1* and *FAM13A* are susceptibility genes for COPD as reported by genetic association studies. *MUC5AC* and *MUC5B* code for proteins involved in mucus secretion, one of COPD's main pathophysiological characteristics, and two other genes (*GCLC* and *CYP1B1*) are involved in oxidative stress mechanisms, which are the central players of COPD pathogenesis. The role of these genes and their methylation status in COPD are shown in **Table 2.1.**

Table 2.1: Key roles and methylation status of COPD-related genes in pathogenesis of COPD.

Gene name	Role in COPD pathogenesis	Methylation status in COPD patients
SERPINA1	Alpha-1 antitrypsin deficiency	Hypermethylation in blood samples, hypomethylation in peripheral lung tissues of COPD patients
FAM13A	COPD susceptibility gene	-
MUC5B	Mucus hypersecretion	-
MUC5AC	Mucus hypersecretion	-
GCLC	Oxidative stress, synthesis of GSH	Hypermethylation due to cigarette smoke
CYP1B1	Oxidative stress, regulates xenobiotic metabolism and estrogen metabolism	Hypomethylation in patients with emphysema

The *SERPINA1* (serpin family A member 1) gene encodes for alpha-1 antitrypsin enzyme, which is a neutrophil elastase (NE) inhibitor. NE is a major inflammatory protease released by neutrophils and is present in the airways of patients with respiratory diseases such as COPD (Voynow & Shinbashi, 2021). Therefore, the genetic deficiency of alpha-1 antitrypsin causes diseases with respiratory symptoms similar to those in COPD patients (Miravitlles et al., 2017). Studies showed that the absence or alteration of this protein was related to the risk of developing COPD (Rotondo et al., 2021). Investigation about the DNA methylation in *SERPINA1* in COPD patients were also investigated in the literature. In a particular study, they aimed to investigate the methylation profile of *SERPINA1* in blood cells from acute coronary syndrome (ACS) patients with COPD or without COPD. They found that *SERPINA1* hypermethylation was higher in COPD patients than the patients without COPD (Rotondo et al., 2021). The study suggests an association between *SERPINA1* hypermethylation and COPD development. Sundar *et al.* have also shown that, in peripheral lung tissues of smokers and COPD patients, *SERPINA1* gene was significantly hypomethylated (Sundar &

Rahman, 2016). Although the relationship between the *SERPINA1* gene and COPD pathogenesis has been studied in genetic and epigenetic studies with COPD patients, the effect of air pollutants on the transcription of this gene has never been studied.

As identified in GWAS, *FAM13A* (Family with Sequence Similarity 13 Member A) is a COPD susceptibility gene (Hancock et al., 2010; Repapi et al., 2010). A study that checked the expression of the FAM13A gene showed increased expression of this gene in lung tissue from COPD patients (W. J. Kim et al., 2014). Additionally, another study showed that FAM13A protein levels are increased, and β-catenin protein levels are decreased in human COPD lung tissues compared with ex-smokers without COPD. These studies suggested that FAM13A may be responsible for promoting the development of human and murine emphysema. Inhibition of the β-catenin-mediated lung repair pathway can be involved in this process (Jiang et al., 2016). Moreover, there is a single cell RNA-sequencing data which proved that expression of FAM13A was significantly increased in human COPD-derived ATII cells compared to healthy ATII cells (Lin et al., 2021). In another study, FAM13A protein levels in the small airway epithelium were reported to be negatively correlated with FEV1/FVC ratio suggesting that FAM13A protein levels in the small airway epithelium are associated with COPD severity (Zhu et al., 2021). It has also been suggested that mRNA expression of FAM13A is affected by particulate exposure. In a study where 16HBE cells were treated with cigarette smoke extract (CSE), authors foun dthat *FAM13A* mRNA expression and its protein levels were significantly downregulated in COPD-derived primary alveolar epithelial cells (AECs), but not in control-derived primary AECs (Q Chen, De Vries, Boezen, & Heijink, 2020). When the methylation status of this gene was investigated, authors found that the exposure to PM10 led to a significant differential methylation in a CpG (cg00905156) in FAM13A (Gruzieva et al., 2019). However, it is not known whether DEP and NP can affect this gene's transcription or DNA methylation.

Airway mucus hypersecretion is one of the most critical features of COPD and also a crucial factor in morbidity and mortality of the disease (Phillips, James, & Lethem, 1997). Mucus contains mucins, which have important roles in the protection of airway epithelium. The change in the structure of the mucin and its rheologic features have been associated with mucus hypersecretion. Over the past decade, the structure of oligomeric O-linked glycoproteins expressed as mucin has been better understood with the studies carried out. The gel-forming mucins predominantly found in airway mucus are Mucin 5AC (MUC5AC) and mucin 5B (MUC5B), both which are essential regulators for the

production and secretion of mucins in COPD patients. MUC5AC is considered the most important one, because its overproduction in COPD results in mucus obstruction. A study showed that MUC5AC protein expression is significantly higher in the bronchiolar epithelium of patients with COPD (Caramori et al., 2004).

It is also known that the expression of *MUC5AC* is changed under exposure to irritants such as air pollution (PM2.5), tobacco smoking, and biomass fuel exposure (Young et al., 2007). In a study where they exposed human airway epithelial cells to DEP, *MUC5AC* and *MUC5B* expression were significantly increased (Na, Kim, Choi, Bae, & Song, 2019). In another study, gene expression of *MUC5B* was higher in TiO_2 exposed adult mice, than in controls (Ma et al., 2019). To determine whether DNA methylation played a role in regulating *MUC5AC* expression, Yamada et al. treated cell lines that have low *MUC5AC* expression with 5-aza-2′-deoxycytidine (5-AzadC), a DNA-demethylating agent. They showed that 5-AzadC, *MUC5AC* expression, which was significantly increased, led to hypomethylation of the CpG islands near the promoter region of the *MUC5AC* gene (Yamada et al., 2010).

Glutamate Cysteine Ligase Catalytic Subunit (GCLC) Gene

Similar to airway mucus hypersecretion, oxidative stress is another important feature of chronic obstructive pulmonary disease. *GCLC* regulates the synthesis of glutathione (GSH), a critical antioxidant in the airways (MacNee, 2000). GSH is synthesized in a two-step process. The first stpe is catalyzed by glutamate cysteine ligase (GCL), where The bond between glutamate and cysteine is mediated, forming γ-glutamylcysteine (γ-GC). the secon step is catalyzed by glutathione synthetase (GSS). In this step lysine is added to formed γ-GC. GCL is composed of two subunits: GCLC and GCLM. (Franklin et al., 2009). GCLC gene is located on chromosome 6 at position p12. Although GCLC is expressed in almost any tissue, including lungs, its mRNA level in liver tissues is the highest. Cigarette smoke causes oxidative stress and depletes glutathione (GSH) levels. Nuclear erythroid-related factor 2 (NRF2) is involved in the glutamate-cysteine ligase catalytic subunit (GCLC) transcriptional regulation. A study found that the gene expression of NRF2 and *GCLC* significantly decreased after a 2-month cigarette smoke exposure comparing to the controls. In another study (Zheng et al., 2019), they showed DEP exposure induced GCLC expression and showed time course effect, both at protein and gene level. Different types of oxidants and free radicals are

implicated in the pathogenesis of COPD. Therefore, the induction of antioxidant genes is an important approach in the protection against environmental factor-induced oxidative stress (Dianat et al., 2018). Other than transcriptional changes, it was shown that in COPD patients, GCLC gene was hypermethylated as a result of chronic cigarette smoke exposure. Cheng et al.,2016 analyzed DNA methylation on GCLC promoter in clinical lung biopsy specimens from current-smoker, ex-smoker, and never-smoker patients with or without COPD. They found that the DNA methylation level of the GCLC promoter of cigarette smoking groups was significantly higher than non-smoking groups. The authors also checked the mRNA levels of GCLC in the lungs and concluded that mRNA levels were correlated with the level of GCLC promoter hypermethylation. Expression of GCLC mRNA and GSH synthesis in these clinical samples and human bronchial epithelial (BEAS-2B) cells stimulated by cigarette-smoke extract (CSE) was evaluated. CSE-treated BEAS-2B cells had hypermethylation of the GCLC gene, mRNA downregulation, and a decreased intracellular GSH level. When they checked the effect of methylation inhibitor, 5-aza-2'-deoxycytidine, they saw that GCLC expression was restored in CSE-treated BEAS-2B cells (Cheng et al., 2016).

Cytochrome p450 Family 1 Subfamily B Member 1 (CYP1B1) Gene

CYP1B1 is another gene involved in oxidative stress mechanisms. It is located on chromosome 2 at position p21. This gene encodes a member of the cytochrome P450 superfamily of enzymes. The encoded enzymes localizes to the endoplasmic reticulum and metabolizes procarcinogens such as PAHs (Ni Li, Zhou, Du, Wei, & Chen, 2011). In a study they found that the expression levels of *CYP1B1* were found to be upregulated in COPD samples compared to controls. *CYP1B1* gene also encodes proteins that are localized in the lungs; thus, it is thought that it may activate COPD-associated compounds (Danlei Yang, Yan, Hu, & Wang, 2020). *CYP1B1* gene also regulates xenobiotic metabolism and estrogen metabolism (Sowers, Wilson, Kardia, Chu, & McConnell, 2006). In a study it was found that the methylation level of *CYP1B1* was inversely correlated with lung function and the radiographic severity of emphysema in females. Therefore, hypomethylation of this gene is thought to be related to the reduction of lung function and severity of radiographic emphysema in females (Wan et al., 2015).

2.3 Effects of Air Pollution on Airway Diseases

Exposure to air pollutants can affect various systems in human body. However, the most significant effect is seen on the respiratory system, since the respiratory tract is the first site of exposure to inhaled pollutants. As epidemiological and mechanistic studies have shown, there is a close relationship between air pollution and chronic airway diseases such as COPD (Duan, Hao, & Yang, 2020).

2.3.1 Particulate Air Pollution

Particulate matters (PM) can reach different regions in the respiratory tract according to their sizes. PM10 can enter the upper airways and cause detrimental effects. On the other hand, finer particles, such as PM2.5 and PM0.1 can enter the terminal bronchioles and alveoli. These tiny particles can have an impact on numerous organ systems when they cross the blood-air barrier and enter the blood (Xing, Xu, Shi, & Lian, 2016). So far, many studies have investigated the adverse effects of PM2.5 on the respiratory system and the underlying mechanisms.

Epidemiological studies suggest that short-term exposure to PM leads to COPD exacerbations (Y.-T. Huang et al., 2021). In a study from China, short-term exposure to PM2.5 were reported to lead to increased airway inflammation and respiratory symptoms in COPDs (Z.-H. Chen et al., 2016). The authors suggested that an increase in PM-induced airway inflammation in COPD patients, might be the reason behind the exacerbation of the disease. Some studies also suggest that PM2.5 particles comprise bacteria and bacteria-derived components and exposure to these particles is thought to alter the microbiome in the distal airway of COPD patients, which could cause exacerbation of the disease (Ni, Chuang, & Zuo, 2015). Furthermore, long-term exposure to low concentration of PM2.5 (10 $\mu g/m^3$) was associated with reduced FEV1 and FVC, and accelerated rate of lung function decline. PM2.5 (10 $\mu g/m^3$) was also associated with increased COPD prevalence in healthy (non-smoker) adult men and women in the Northeastern United States (Rice et al., 2015). These epidemiological studies have shown a close relationship between ambient PM exposures and COPD.

DEP are significant components of PM2.5. They can induce airway hyperresponsiveness (AHR) and inflammation and are associated with cardiorespiratory mortality and the aggravation of the disease (B.-G. Kim et al., 2016). It was also shown that DEPs can induce acute AHR and acute asthma exacerbations independent of their

effects on allergic sensitization (Hao, Comier, Wang, Lee, & Nel, 2003). Long-term exposure to air pollution causes chronic respiratory disease, and chronic inhalation of DEPs leads to the development of cough and sputum (Pope, Dockery, & Schwartz, 1995). As the dose of DEP exposure increases, an increase in airway inflammation, a higher cytokine response, and more severe lung fibrosis is seen, which suggests that environmental reduction of DEP exposure is important for patients with airway diseases (B.-G. Kim et al., 2016). Experimental studies have shown that diesel particles impair host defense by suppressing, e.g., macrophage and epithelial cell function (Rivas-Santiago et al., 2015; Sarkar et al., 2012). Another study showed that, in primary bronchial epithelial cells from COPD and non-COPD patients, diesel exhaust (DE) exposure led to increased expression of the oxidative stress response gene *HMOX1* (long terms) and *CXCL/IL-8* (long terms) and activation of the integrated stress response (ISR) (Zarcone et al., 2016).

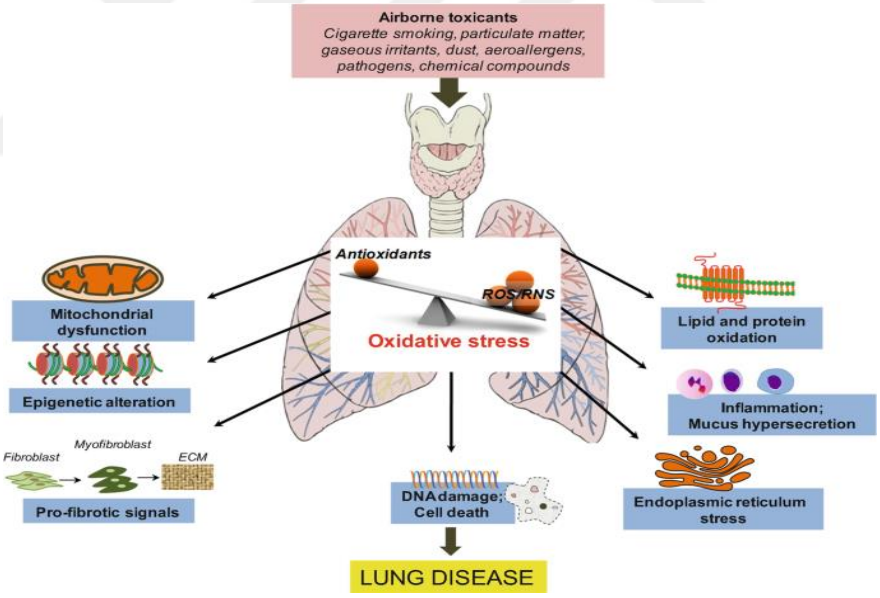

Figure 2.2: Effects of airborne toxicants on lung diseases.

(Thimmulappa et al., 2020)

2.3.2 Engineered Nanoparticles

NP have tiny diameters, so they can quickly enter almost any tissue and cell in the body and cause undesirable effects (Bonner et al., 2010; Oberdörster, Oberdörster, & Oberdörster, 2005). After inhaling NP, they can be deposited throughout the entire respiratory system, from the nose to the inside the lung. Because of the larger surface area of the lungs, NP have a primary entry route. Larger particles (more than 10 μm) tend to be deposited in the upper part of the respiratory system and can be removed quickly by the body through coughing and sneezing. Unfortunately, smaller nanoscale particles may reach the gas exchange surface areas and remain there for a more extended period (Asmatulu, Asmatulu, & Yourdkhani, 2009).

The mechanism behind the NP penetration is not fully understood. However, it is believed that NP can be absorbed by lung cells that could lead to long-term consequences such as airway inflammation, bronchitis, asthma, emphysema, lung cancer, neurodegenerative diseases, and cardiovascular effects (Kumar, 2006). A study showed for the first time that TiO_2 induced clastogenicity, genotoxicity, oxidative DNA damage, and inflammation *in vivo* in mice (Trouiller et al., 2009). In animal studies, CNTs induced sustained inflammation, genetic damage, fibrosis, and lung cancer following long-term inhalation (Card, Zeldin, Bonner, & Nestmann, 2008). MWCNTs and SWCNTs have strong chemical stability, and when inhaled, they cannot be easily dissolved in the body, which could damage cells, DNA, and surrounding tissues (Service, 2005).

2.4 Epigenetic Mechanisms, Air Pollution and COPD

COPD is a chronic disease that progresses with damage in mucociliary dysfunction, lung inflammation, airway fibrosis, and alveolar destruction (Global Initiative for Chronic Obstructive Lung Disease). Harmful particles and gases taken by inhalation are responsible for the emergence and development of the disease. Besides all these, structural genetic factors and epigenetic mechanisms related to environmental exposures are also influential in the progression of this disease.

2.4.1 DNA Methylation

Epigenetics investigate the changes in gene expression that occur through alterations in the chromosome rather than in the DNA sequence (Berger, Kouzarides, Shiekhattar, & Shilatifard, 2009). Although epigenetic mechanisms do not directly affect DNA sequence, they regulate a related gene expression through chemical modifications of DNA bases and changes to the chromosomal superstructure, where DNA is packaged. Three different epigenetic mechanisms are identified: (i) DNA methylation, (ii) histone modifications, and (iii) non-coding RNA (ncRNA)-associated gene silencing. DNA methylation occurs primarily at cytosine in cytosine-guanine dinucleotides (CpG). A methyl group is added to carbon number 5 in cytosine to form 5-methylcytosine (5mC). This process is catalyzed by DNA methyltransferase (DNMT) enzymes (*DNMT1, DNMT3A, DNMT3B)*. This methyl group can be removed, and this process is called demethylation. Typically, methylation turns genes “off,” and demethylation turns genes “on” (Centers for Disease Control and Prevention, 2022). CpG islands within promoter regions are common targets for epigenetic DNA methylation. In the promoter region, methylated cytosines recruit gene suppressor proteins and reduce interaction between the DNA and transcription factors (Moore, Le, & Fan, 2013). Cytosine methylation is due to the formation of heterochromatin and after this methylation process, the nucleosome tightens and prevents transcriptional machinery from interacting with the DNA (McMahon, Karunasena, & Ahuja, 2017). All these DNA methylation processes lead to gene silencing. Hypermethylation of CpG islands in gene promoters usually leads to the downregulation of gene expression, whereas hypomethylation leads to upregulation of the gene (Gros et al., 2012).

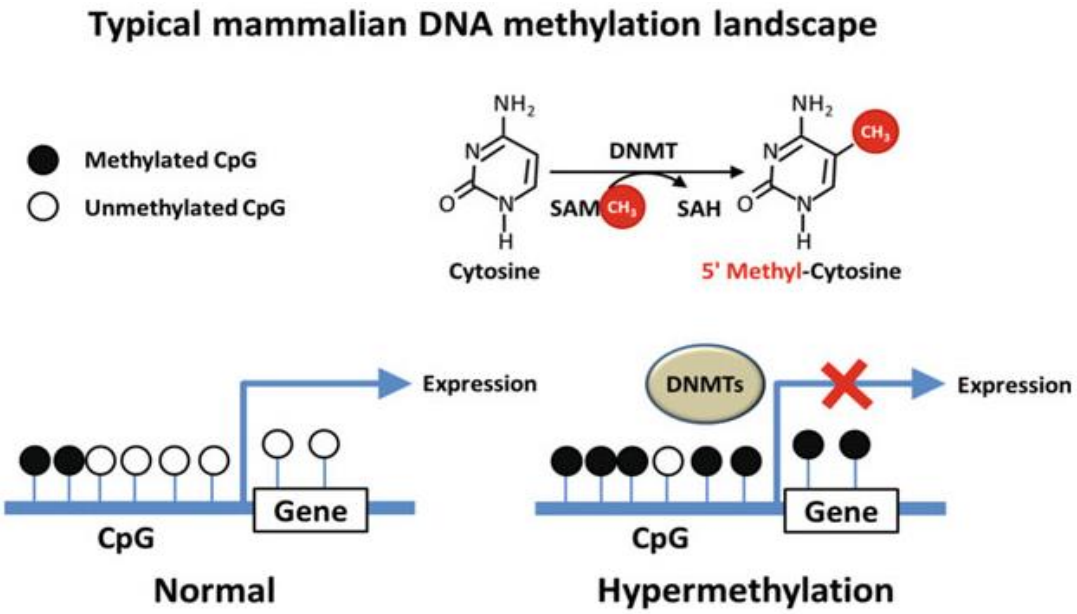

Figure 2.3: Mechanism of DNA methylation.

(X. Chen et al., 2020)

2.4.2 Role of DNA Methylation in COPD

Since COPD is affected by genetic and environmental factors, the role of epigenetics is important in COPD pathogenesis. DNA methylation is the one of the epigenetic mechanisms that has been extensively studied in COPD.

Morrow et al. analyzed the whole genome DNA of homogeneous lung tissue samples from patients with COPD (Morrow et al., 2018). In another study, Vucic et al. analyzed genome-wide methylation and gene expression on airway epithelial cells obtained from COPD patients and concluded that aberrant DNA methylation was associated with expression variation of genes and pathways involved in COPD pathogenesis. The nuclear factor, erythroid two like 2 (Nrf2) pathway that mediates oxidative stress response, was one of the most affected pathways (Vucic et al., 2014).

A large-scale gene-specific investigation of DNA methylation demonstrated that COPD was associated with DNA methylation of 349 CpG sites in two family-based cohorts and *SERPINA1* hypomethylation was significantly associated with COPD and phenotypes of lung dysfunction (Qiu et al., 2012). In another study performed by Wan et al., authors collected buccal brushes DNA samples from COPD patients with current and former smoking history. They found that the methylation level of *CYP1B1* was inversely

correlated with lung function and the radiographic severity of emphysema in females. CYP1B1 plays a role in xenobiotic metabolism, probably contributing to COPD development (Wan et al., 2015). In another study, where the authors checked the DNA methylation level of the glutamate-cysteine, the promoter of GCLC gene in cigarette smoking groups was significantly higher than those of non-smoking groups. The mRNA levels of GCLC in the lungs were correlated with the level of GCLC promoter hypermethylation. This was also confirmed by an *in-vitro* study (Cheng et al., 2016).

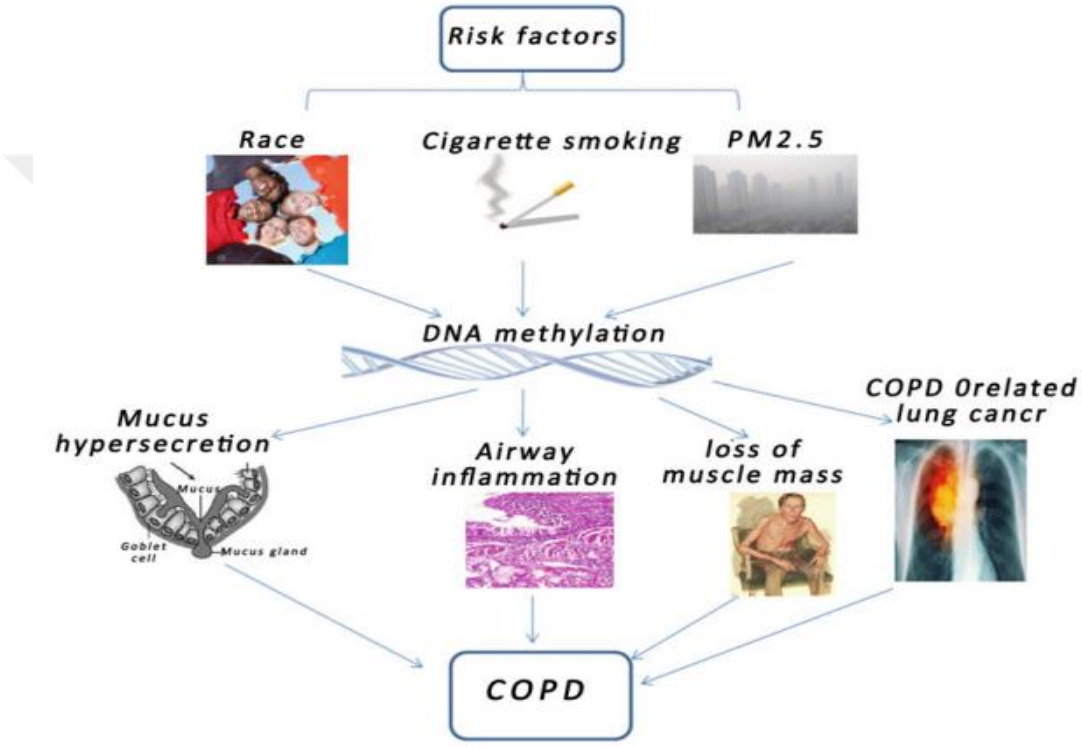

Figure 2.4: Risk factors involved in the relationship between DNA methylation and COPD.

(X. Chen et al., 2020)

2.4.3 Air Pollution and DNA Methylation

As shown by the studies in the literature, it is known that DNA methylation is involved in the pathogenesis of COPD. Environmental risk factors are also involved in the disease progression, through aberrant DNA methylation. It was found that air pollution, especially PM2.5, could induce hypermethylation of p16 gene promoter and decrease DNMTs activity in primary bronchial epithelial cells derived from COPD patients (Leclercq et al., 2017). In another study, it was found that the expression of DNMTs can be affected by cigarette smoking, too. While DNMTs mediate DNA methylation, cigarette smoking could affect the expression of target genes via differential methylation induced by abnormal expression of DNMTs (Sundar & Rahman, 2016).

Several studies showed that DEP exposure cause DNA methylation changes different cellular systems. In a study where they collected bronchial epithelial cells (BECs), and exposed them with DEP, they observed that DEP significantly altered bronchial epithelial cell global DNA methylation (Clifford et al., 2017). In another study performed on mice models, chronically inhaled exposure to DEP altered DNA methylation levels of T-helper (Th) genes *in* vivo (Liu et al., 2008).

In a study performed with SWCNTs and MWCNTS on human 16HBE14 bronchial epithelial cells, gene-specific cytosine DNA methylation was found (Öner et al., 2020). In another study of human bronchial epithelial (BEAS-2B) cells, prolonged treatment with silica NP at a low non-cytotoxic concentration of 5 μg/mL, resulted in marked DNA hypermethylation. This was evident by a predominant number (1973) of hypermethylated CpG loci over 223 hypomethylated CpG loci. Hypermethylation of the CREB3L1 and BCL-2 gene promoters was correlated with significant down-regulation of gene expression (Zou et al., 2016). It was also shown that not only epithelial cells have DNA methylation changes when exposed with particles, MRC5 lung fibroblast cells also show reduced global DNA methylation following 0.5 or 4 μg/mL TiO_2-NPs exposure for 24 and 48 h (Patil, Gade, & Deobagkar, 2016 ?). Sooklert et al. found that global DNA methylation is reduced in 72h after human kidney embryonic HEK293 cells were exposed to 100 μg/mL of gold NPs (AuNPs) (Sooklert et al., 2019). Moreover, Blanco et al. reported increased global cytosine DNA methylation in response to the exposure of human lung adenocarcinoma A549 cells to 200 μg/mL Ag-NPs for 72 h (Blanco et al., 2017).

2.4.4 COPD and Airway Epithelium

Airway epithelium make the first line of defence by forming a physical barrier against inhaled irritants. Airway epithelial cells play a crucial role in orchestrating the cellular mechanisms involved in the regulation of the innate and adaptive immune response. Epithelial cells participate in the inflammatory response and re-modelling phenomena of lung tissue during respiratory diseases (Albano, Montalbano, Gagliardo, Anzalone, & Profita, 2022).

2.4.5 Bronchial Epithelial Cells (BEAS-2B)

The bronchial epithelial cell (BEAS-2B) line was established from normal human bronchial epithelium obtained from a non-cancerous individual (Reddel et al., 1988). Transfection with the adenovirus 12-SV40 hybrid virus was performed in the establishment process. Cell line was immortalized via consecutive cell passaging. Since then, it has been used in many studies as an in-vitro cell model for studying the molecular mechanisms behind respiratory diseases (Ha, Ham, Lee, & Choi, 2007; Vallabani et al., 2011; Vergaro et al., 2016).

Chapter 3:

METHODS

3.1 Culture of Bronchial Epithelial Cells (BEAS-2B) and Incubation with Particles

BEAS-2B cells were used for the experiments in this project. Experiments were conducted between passage numbers P25 and P30 to prevent the effect of cell age on the results.

3.1.1 BEAS-2B Cell Line Cultures

BEAS-2B cells (ATCC, CRL-9609 Wesel, Germany) in RPMI-1640 (Sigma Life Science) medium containing 10% fetal bovine serum (FBS) (Biowest, S181H-500) and 1% penicillin/streptomycin (Biowest, L0022-100) were incubated at 37°C with 5% CO_2 in 75 cm^2 culture flasks (Corning, Thermo). Culture medium was changed every two days. Cells that reached 80% confluence were used in the experiments. An example image for the BEAS-2B cells in culture is shown in **Figure 3.1.**

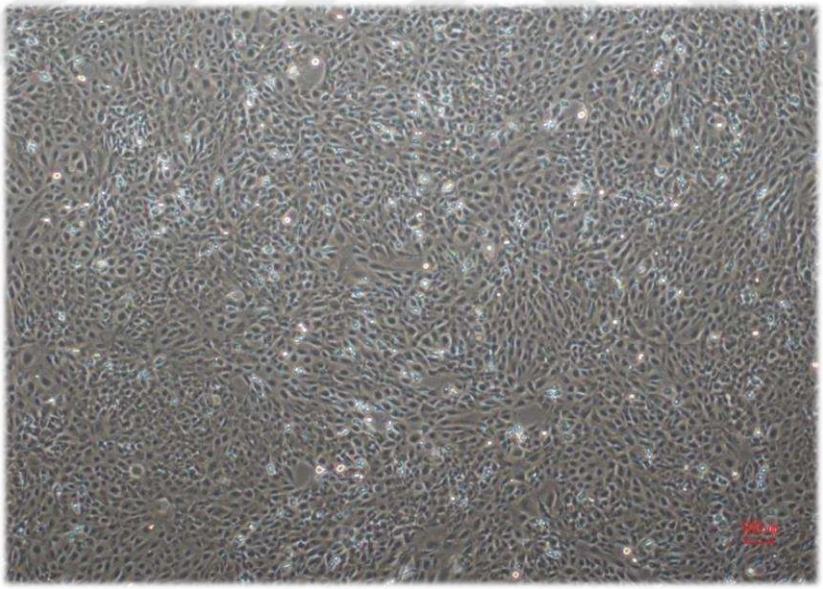

Figure 3.1: Light microscopy image of %80 confluence BEAS-2B cells.

When cells reached %80 confluence, they were washed with 1X Dulbecco's Phosphate Buffered Saline (1X dPBS) (Biowest, L0615-500), to prevent the inactivation of trypsin that will be used later on. After washing, cells were dis-attached from the bottom by adding 3 ml of 0.25% Trypsin-EDTA (1X) (Gibco) and incubated at 37°C with 5% CO_2

for 4 minutes. The cells were checked under the microscope to see if they were dis-attached. RPMI medium (6ml) containing 10% fetal bovine serum was added to inactivate trypsin, and the cells were transferred into 15 ml of falcon. Following centrifugation at 400G for 4 minutes, the supernatant was discarded and the pellet was resuspended in appropriate amounts of RPMI complete medium. The cell suspension was prepared in trypan blue (160 µl of RPMI complete medium, 20 µl of cell suspension and 20 µl of trypan blue) and homogenized by pipetting. Ten µl from the suspension was transferred to hemocytometer (Marienfeld, Germany) and live cells were counted under the inverted microscope (Olympus CK40, Japan).

3.1.2 Preparation of Particle Suspensions

DEP were a gift from Dr. H. Takano (National Institute for Environmental Studies, Tsukuba, Japan) and engineered NPs (TiO_2 and MWCNT) were provided by Günter Oberdörster (University of Rochester, USA). All three particles were prepared freshly in serum-free (SF) medium at a concentration of 1mg/mL as a stock solution before experiments. The stock solutions were vortexed for 30s and then sonicated using an ultrasonic sonicator (Becton Dickinson) for 30 minutes to prevent aggregation of the particles. The image for dissolved particles in SF medium is shown in **Figure 3.2.** Later on, solutions of particles were diluted in SF medium to get the study concentrations of 10, 50, and 100 µg/mL.

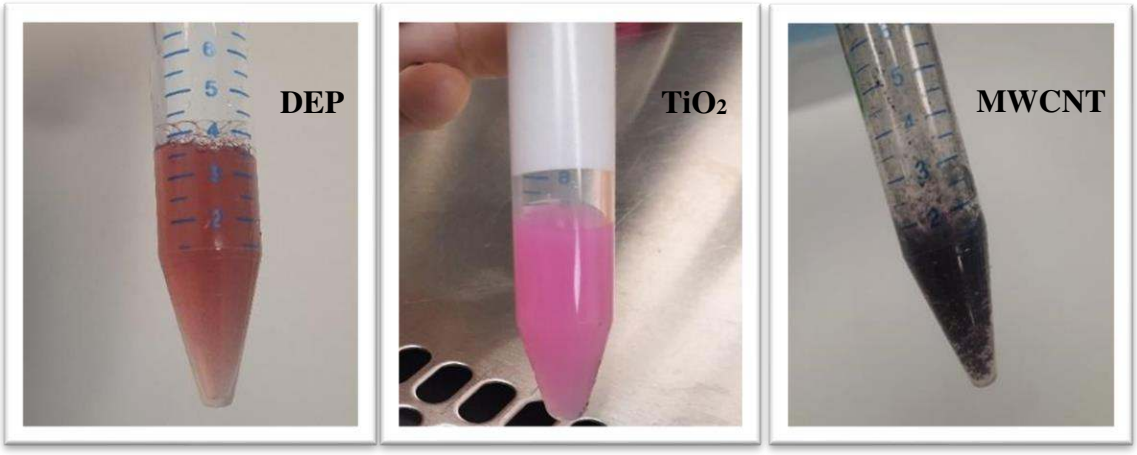

Figure 3.2: Images of DEP, TiO_2 and MWCNT solutions.

3.1.3 Incubation of BEAS-2B Cultures with Particles for DNA and RNA isolation

BEAS-2B cells were seeded in 6-well plates (n=3 biological replicates for each concentration) at a density of $2x10^5$ cell/well and kept for 24-26 hours to reach %70-80 confluency. Cells were washed with 1X dPBS (Biowest, L0615-500), and then the medium was changed to SF, containing %99 RPMI-1640 (Sigma Life Science) medium and 1% penicillin/ streptomycin (Biowest, L0022-100). Cells were incubated for another 24 hours at 37°C under 5% CO_2 condition. SF medium was removed and cells were washed with 1X dPBS (Biowest, L0615-500) to remove dead cells from the environment. Cells were then treated with particles at 10, 50 and 100 µg/mL concentrations. Each experimental set-up had a control group (0 µg/mL, only SF medium). Cells were incubated with the particles for 6, 24 and 48 hours at 37°C under 5% CO2 environment. RNAs were collected for qPCR experiments, whereas DNAs were collected for pyrosequencing.

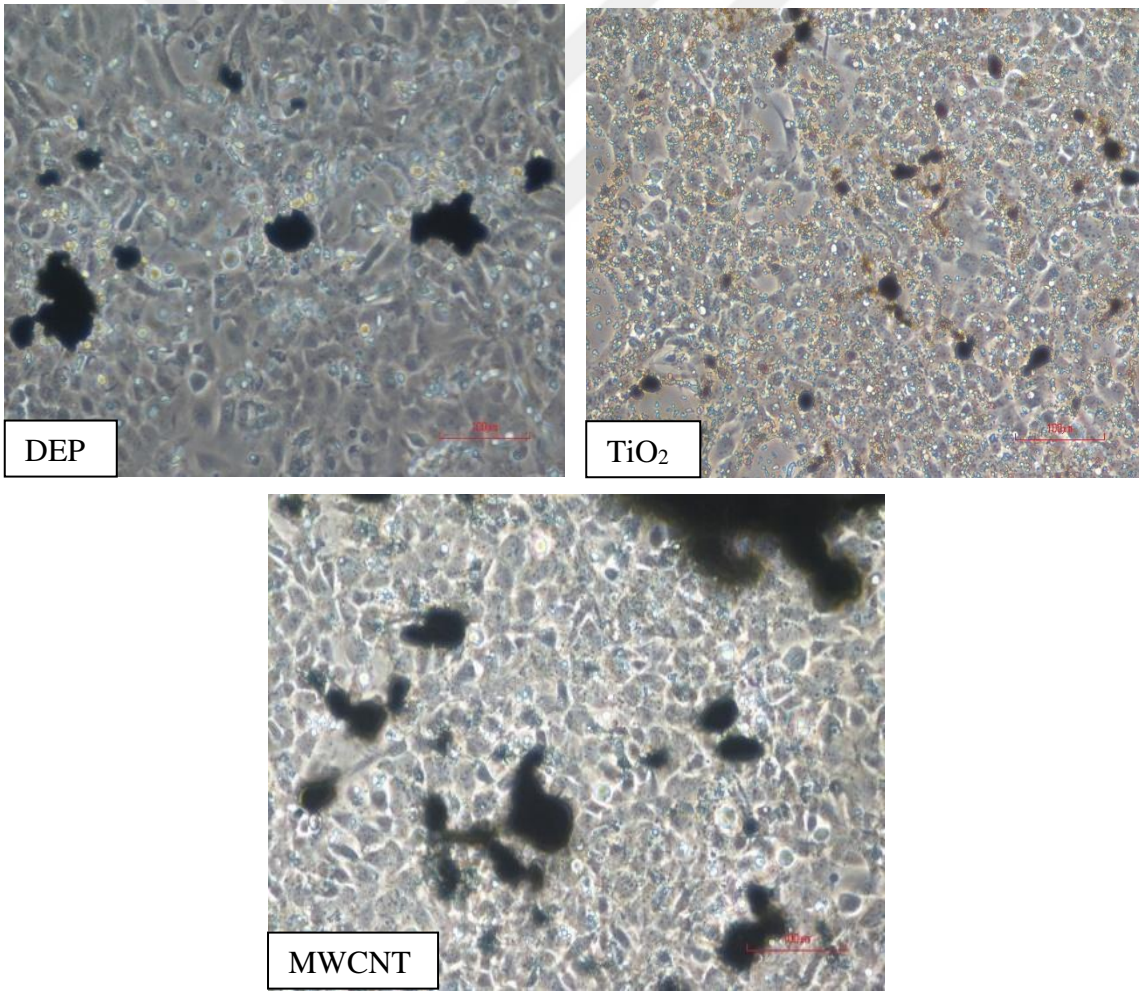

Figure 3.3: Light microscopy images of particle treated BEAS-2B cells.

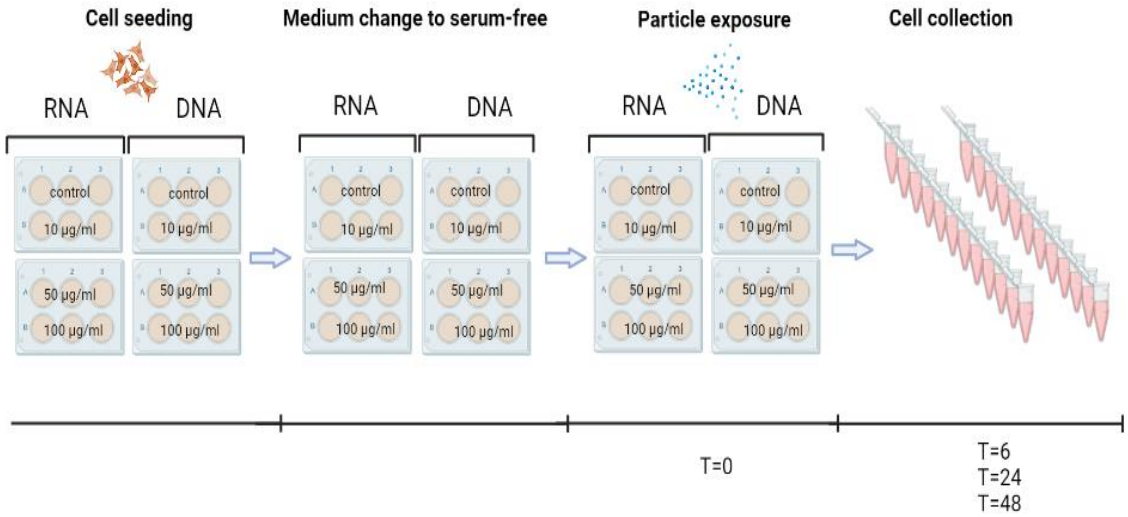

Figure 3.4: Schematic representation of the cell culture protocol.

(Figure is created in BioRender.com).

3.2 Cell Viability Assay

Viability of cells was assessed using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). BEAS-2B cells were seeded into 24-well plates (n=5 biological replicates for each concentration) at a density of $5*10^4$ cell/well and waited until cells reached to 70-80% confluency. Medium was then changed to SF RPMI-1640 (Sigma Life Science) containing 1% penicillin/ streptomycin (Biowest, L0022-100). The cells were incubated for another 24 hours at 37°C under 5% CO_2 condition. SF medium was removed and cells were treated with particles at 10, 50 and 100 µg/mL concentrations. Each experimental set-up had a control group (0 ug/mL, only SF medium). Cells were incubated with the particles for 6-24 and 48 hours at 37°C and 5% CO2 environment. Foloowing the incubation, 500 µl of MTT solution (1 mg/mL) dissolved in PBS was added into the wells, and cells were incubated for 1 hour 37°C with 5% CO_2. After 1 hour, the MTT solution was removed and replaced with dimethyl sulphoxide (DMSO). The change in color was read at 540 nm using a colorimetric plate reader.

3.3 Quantitative Polymerase Chain Reaction (qRT-PCR)

3.3.1 Primer Design for qRT-PCR

Primers for qRT-PCR were designed by using NCBI Primer Blast. The list of the primers is shown in **Table 3.1.**

Table 3.1: Primer sequences used in qRT-PCR.

Genes	Primer sequence (5'→ 3')	Amplicon size(bp)
GAPDH	Forward: ATGGAAATCCCATCA	57
	Reverse: CGCCCCACTTGATTT	
FAM13A	Forward: TGCTCATGTACCCCAAGTCAG	223
	Reverse: CTTGTCTCTCCCATGTCGAAC	
GCLC	Forward: GAGGTCAAACCCAACCCAGT	92
	Reverse: AAGGTACTGAAGCGAGGGTG	
MUC5B	Forward: AGCTGTAACTGCACACCCAG	165
	Reverse: GATGATGGTGCCGTTGCTTC	
CY1P1B1	Forward: TGAGTGCCGTGTGTTTCGG	197
	Reverse: GTTGCTGAAGTTGCGGTTGAG	
MUC5AC	Forward: AGTGTGCCTGCGTCTACAAC	178
	Reverse: ACCGTGTATTGCTTCCCGTC	
SERPINA1	Forward: GAAAGGTGGGACATTGCTGC	78
	Reverse: CCCCAAAATGCCTGATGCTG	

3.3.2 Validation of Primers by Gradient PCR

Gradient PCR was performed in order to determine the optimum annealing temperature for primers. For 5 different temperatures (54°C, 56°C, 58°C 60°C, 62°C), samples were prepared. Negative controls were also studied for each primer to check if there was any contamination. Total volume of 20 µl reaction mix was prepared. Each tube contained 10 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 0.8 µl from 10 µM primer mix and 8.2 µl nuclease-free water (NFW). Complementary DNA (cDNA) (1 µl) was added at the end. cDNA concentration in each tube was 25 ng. Negative controls did not contain any cDNA. NFW (9.2 µl) was added to those tubes. All samples were run in the thermal cycler (Veriti 96 Well Thermal Cycler, Applied

Biosystems), in different temperatures. Reaction conditions for thermal cycler are shown in **Table 3.2.**

Table 3.2: Reaction conditions for thermal cycler.

Stages	Temperature	Time duration	Cycle(s)
Stage 1	95 °C	2 minutes	1
Stage 2	95 °C	30 seconds	40
	Different Temperatures	30 seconds	
	72 °C	1 minute	
Stage 3	72 °C	10 minutes	1
	4 °C	∞	

After this period, the samples were run on %1.5 agarose gel. Agarose (Sigma Life Science, A9539-500G) (1.8 g) was dissolved in 120 ml of 1X TAE (prepared from TAE 50x, Eco-Tech ClearBand, TAE1000) and 5 µl of ethidium bromide was added. Then, the samples were added to the gel (10 µl from each tube). GeneRuler 50 bp of DNA ladder (Thermo Scientific) was added to the first well by mixing with 6X DNA Loading Dye (Thermo Scientific). The gel electrophoresis was started at 70V and continued for 30 minutes. At the end of the run, the gel was visualized by the ChemiDoc Imaging System (Bio-Rad). The temperature that gave the best band intensity was chosen for each primer.

3.3.3 RNA isolation

For the gene expression analysis, after incubation times were over, supernatants were discarded from 6 well plates and 300 µl of RNA lysis buffer (Zymo Research) was added directly to the cells. Cells were collected with a cell scraper, transferred into a 1.5 ml eppendorfs and then stored at -80°C until RNA isolation. Total RNA was extracted from cells using an RNA isolation kit (Quick-RNA MiniPrep Plus, R1058), according to the manufacturer's instructions. RNA concentration and purity was determined by Nanodrop spectrophotometer (Thermo Fisher Scientific, 2000c). Samples with an A260/280 and A230/260 ratios of 2.0-2.2 were transcribed into cDNA. Isolated RNAs were stored at -80°C until they were used.

3.3.4 Complementary DNA (cDNA) Synthesis

cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad, #1708891). PCR reactions were performed using the thermal cycler (T100 thermal cycler, Bio-Rad) with following conditions: 25°C for 5 min; reverse transcription step at 46°C for 20 min and Reverse Transcription inactivation at 95°C for 1 min. Each sample was diluted with nuclease free ultra-pure water (Eco-Tech, ClearBand) to get 25 ng of cDNA in 2 µl (for each qRT-PCR plate well). Diluted cDNAs were stored at -20 for further use.

3.3.5 Quantitative Real-Time Polymerase Chain Reaction

Amplification of target genes was performed using specific primers by the SYBR Green method in Applied Biosystems QuantStudio 7 Flex qPCR instrument. An amount of 2 QuantiNova SYBR Green PCR mix was used in the experiments. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the expression levels of different genes and the relative cDNA ratio was calculated using the value of threshold cycles (Ct). Final concentration of 0.3 µM forward and reverse primers were used in 10 µl total reaction. The amplification efficiency between the target and the reference GAPDH genes was compared by using the 2(-Delta Delta C(T), 2–ΔΔCt) method. Each qRT-PCR plate was run using 3 biological replicates for each concentration (0,10,50 and 100 µg/mL) and 2 technical replicates (duplicates) for each sample. Reaction conditions are shown in **Table 3.3.**

Table 3.3: Reaction conditions for qRT-PCR.

Stages	Temperature	Time duration	Cycle (s)
Hold Stage	95 °C	2 minutes	1
PCR Stage	95 °C	5 seconds	40
	Different temperatures	19 seconds	
Melt Curve Stage	95 °C	5 seconds	1
	65 °C	1 minute	
	97 °C	15 seconds	
Hold Stage	40 °C	10 minutes	1

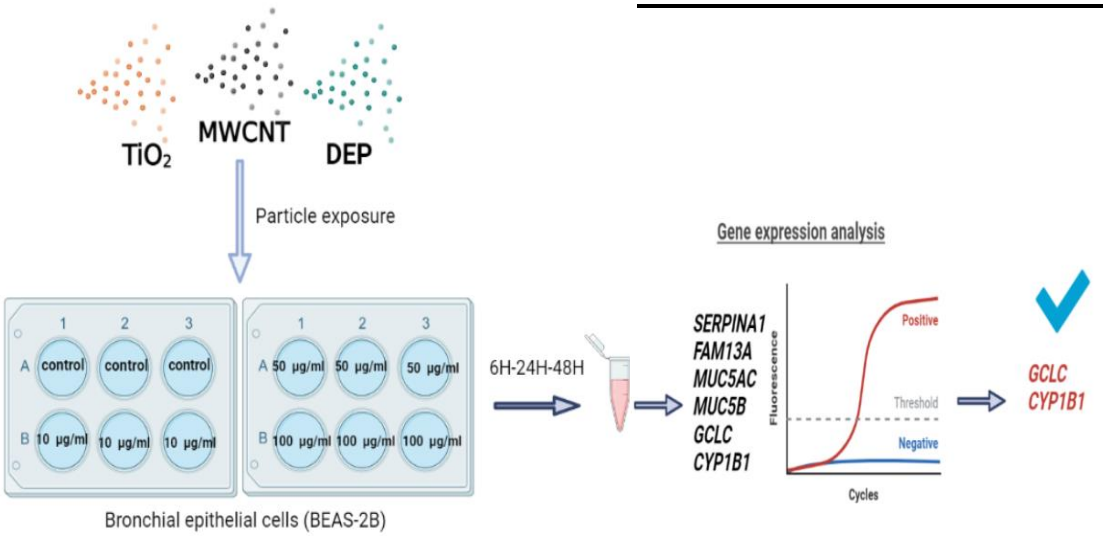

Figure 3.5: Genes differentially expressed after particle exposure. (Figure is created in BioRender.com).

3.4 Pyrosequencing

3.4.1 Assay Design for Methylation Analysis

The genes, whose expression changed significantly after exposure with all 3 particles (DEP, TiO_2 and MWCNT) and for three different time points (6H-24H-48H), were chosen for further analysis. Pyrosequencing assays for *GCLC* and *CYP1B1* genes were designed (Designed reports for each locus are shown in **Appendix A**).

3.4.2 Validation of Assays

PCR was performed in order to see if the primers work efficiently. Samples were prepared for 2 different temperatures (55°C and 60°C), and then were were run with both Q solution and without Q solution in parallel in the thermal cycler (Veriti 96 Well Thermal Cycler, Applied Biosystems). The condition that gives the best band intensity on the agarose gel was chosen, and pyrosequencing validation was performed in those conditions. Agarose gel image of the primers is shown in **Figure 3.6.** A condition of 60°C without q solution was chosen for primers.

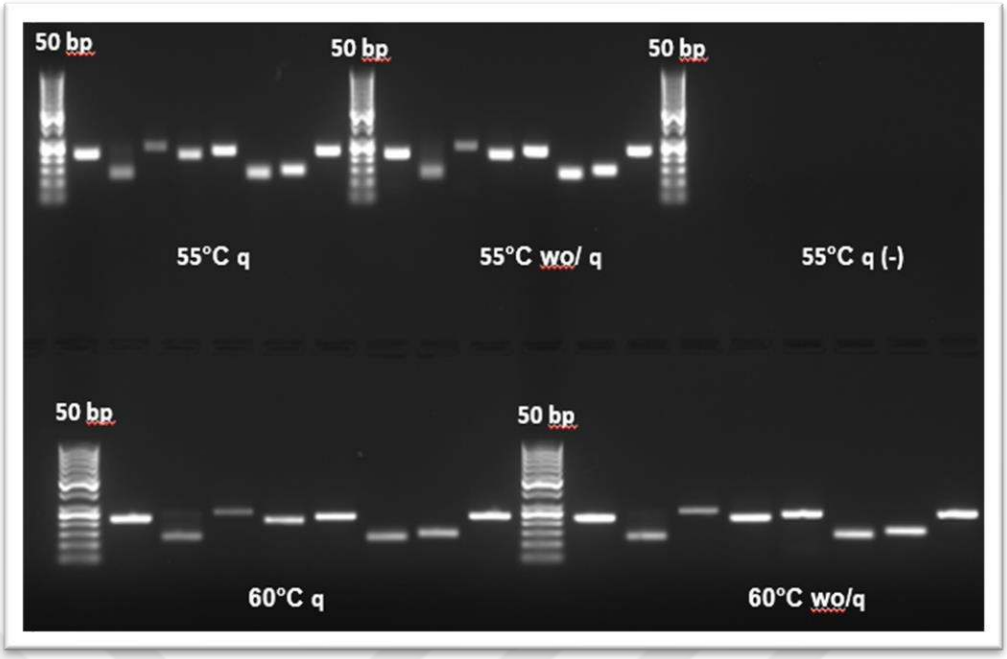

Figure 3.6: Agarose gel image of pyrosequencing primers.

(The bands given in each condition belongs to *GCLC.1-2, GCLC.3, GCLC.4-5, CYP1B1.1, CYP1B1.3, CYP1B1.5, CYP1B1.6-7, CYP1B1.8-10,* respectively). Primers were named according to the CpG positions they include.

Primers were used for further experiments, only if they had R^2 greater than 0.95. (Pyrosequencing validation results of the primers were shown in **Appendix B).** CpG regions 2 and 3 for *GCLC* gene and regions 8, 9 and 10 for *CYP1B1* gene were eliminated, since they failed in pyrosequencing analysis.

3.4.3 Genomic DNA Isolation

For methylation analysis of the selected genes, after incubation times were over, supernatants were discarded from 6-well plates, and 220 µl of Biofluid buffer and proteinase K mix (200 µl biofluid buffer, 20 µl proteinase K) was added directly to the cells. Cells were collected with a cell scraper, transferred into 1.5 ml eppendorf tubes and then stored in -80°C until DNA isolation. Biological replicates of the treatments were pooled in each of the 3 independent experiments. Genomic DNA was extracted from cells using a DNA isolation kit (Quick-DNA MiniPrep Plus, D4069) according to the manufacturer's instructions. Concentrations and purities of DNAs were measured with the NanoDrop spectrophotometer (Thermo Fisher Scientific, 2000c). Samples with an A260/280 and A230/260 ratios of 180-2.2 were stored at -80°C until bisulfite modification.

3.4.4 Bisulfite Modification

1000 ng of gDNA was subjected to bisulfite conversion reaction using EZ-DNA Methylation Lightning kit (Zymo Research, USA, D5030). The amount of gDNA was completed 20 µl with nuclease-free water. 130 µl of CT conversion reagent was added to 1000 ng DNA samples in a PCR tube. Samples were then placed in a thermal cycler with the following conditions: 98°C for 10 minutes, 64°C for 2.5 hours and 4°C ∝. Bisulfite modification was performed according to manufacturer's instructions. At the end of the process, DNAs were eluted in 10 µl of M-elution buffer and stored at -80°C for further experiments.

3.4.5 Pyro-PCR

Pyro step was applied to BM (Bisulfite modificated) DNAs, using *GCLC* and *CYP1B1* forward and reverse primers (Pyromark PCR kit, 978703). PCR reactions were prepared according to the **Table 3.4**, by using Qiagen PyroMark-PCR kit (Pyromark PCR kit, 978703).

Table 3.4: PyroMark PCR reaction.

Reagents	Volume	Final concentration
PyroMark-PCR Master Mix	12.5 µl	1x
Coral Load	2.5 µl	1x
Primer mix	2 µl	0.2 µM
Nuclease-free water	6 µl	-
BM DNA	2 µl	200 ng

Prepared PCR reactions were placed in thermal cycler with following conditions shown in **Table 3.5.**

Table 3.5: Pyro-PCR reaction conditions.

Step	Temperature	Duration	Cycle(s)
Initial PCR activation step	95°C	15 minutes	1
Denaturation	95°C	15 seconds	50
Annealing	60°C	30 seconds	
Extension	72°C	30 seconds	
Final Extension	72°C	5 minutes	
Cooling	4°C	∝	

After the procedure was over, all the samples were loaded into 2% agarose gel, and run at 100 volts for 30 minutes. Only after checking their band intensities, samples were processed with the pyrosequencing experiment.

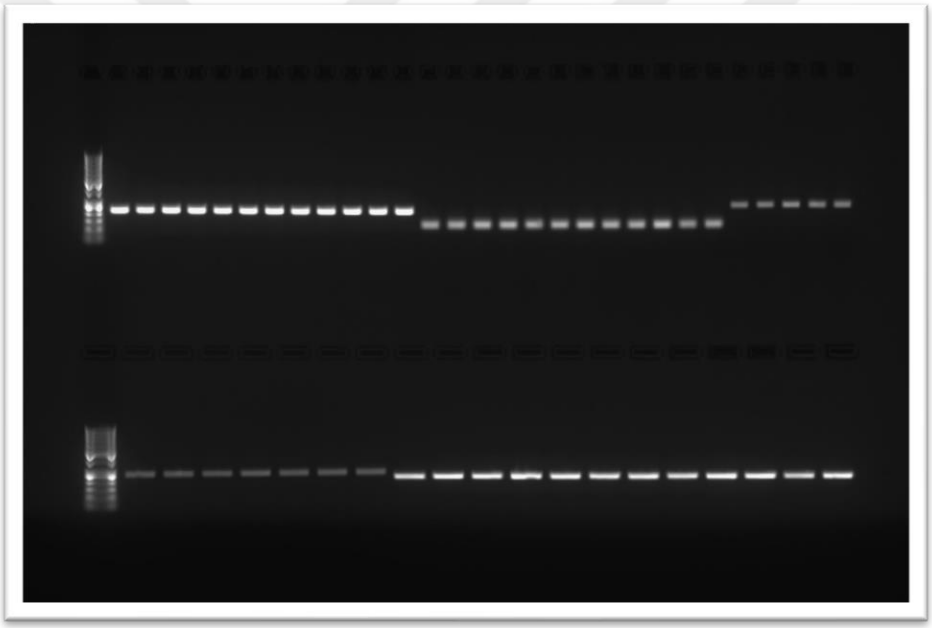

Figure 3.7: Representative image for agarose gel images of PCR samples.

3.4.6 Pyrosequencing

Ten µl of PCR product was added into binding mixture containing 2 µl of streptavidin coated magnetic Sepharose beads (GE17-5113-01/GE HealthCare), 38 µl binding buffer (Qiagen, PyroMark Binding buffer, 979006) and 30 µl high purity water. The PCR-bead mixture was shaken at 1400 rpm for 15 minutes. Then biotinylated PCR product was captured using filter probes and washed with 70% ethanol for 5 seconds, denaturation buffer (Qiagen, PyroMark Denaturation solution, 979007) for 5 seconds and

finally 1x wash buffer (Qiagen, Pyromark Wash Buffer, 979008) for 10 seconds. DNA product-bead mixture was annealed (Qiagen, Pyromark Annealing Buffer, 979009) with sequencing primers at 80^{o}C for 2 minutes and let it cool at room temperature for 7 minutes (38,4 µl annealing buffer and 1,6 µl sequencing primer in each well). Unmethylated and methylated EpiTect DNA control (Qiagen, Epitect control DNA set, 59695) and negative template were used for each run. The PyroMark Q96-ID cartridge was loaded with substrate, enzyme, and nucleotides (Qiagen, Pyromark Q96 Gold Reagents, 972804) according to the volumes required for each plate set-up. DNA methylation percentages were calculated by the PyroMark software.

3.5 Statistical Analysis

Before analysis, data were tested for normality using D'Agostino and Pearson omnibus normality testa, then one-way analysis of variance (ANOVA) or Kruskal Wallis tests were performed accordingly. Comparisons between groups were performed using Kruskal–Wallis/Dunn's multiple comparison tests in non-parametric data, whereas ANOVA/Dunnett's multiple comparison tests or Tukey's multiple comparison tests were used for parametric data. P values less than 0.05 were considered significant. GraphPad 9.3.1 was used for the statistical analysis.

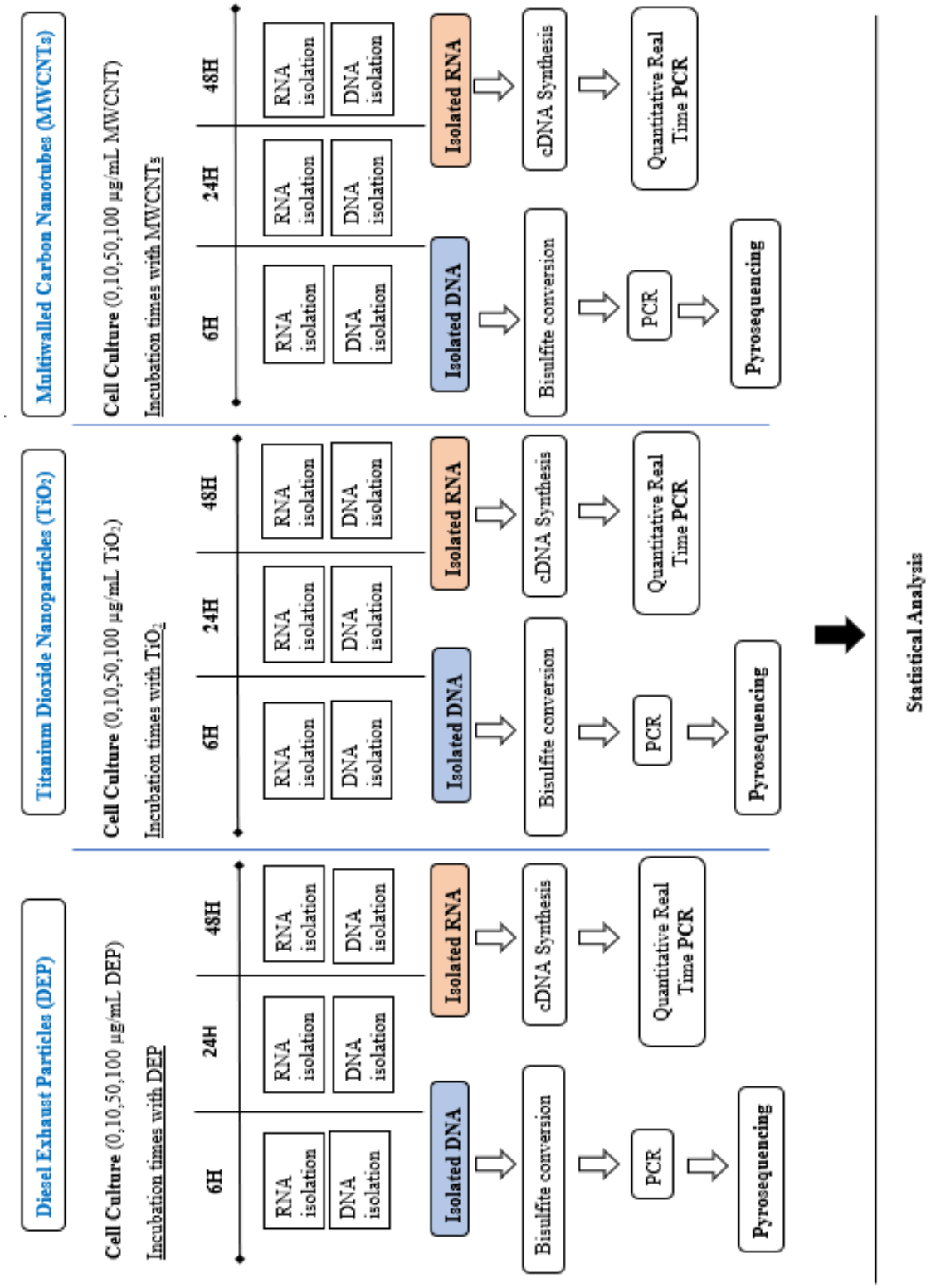

Figure 3.8: Overview of the experimental set-up.

Chapter 4:

RESULTS

4.1 Cell Viability Analysis

In the first part of the project, viability of cells was assessed with MTT, to ensure that the concentration of the particles to be used in further experiments did not cause a high death rate among the BEAS-2B cells. Data were normalized to time-matched controls and represent mean ± SEM or median ± interquartile range of three independent experiments (n=5 biological replicates in each independent experiment). Each exposure groups in specific time intervals were compared with the control group (0 µg/mL).

4.1.1 Effect of DEP, TiO_2 and MWCNT on the Viability of BEAS-2B Cultures

10 µg/mL DEP caused a significant decrease (p=0.0074), whereas 50 µg/mL DEP caused a significant increase (p= 0.0392) in the cell viability, at 6th hours (**Figure 4.1a**). When cells were incubated with DEP for 24 hours, 10 µg/mL (p=0.0006) and 100 µg/mL (p<0.0001) caused a significant decrease in the cell viability **(Figure 4.1b).** It was seen that 50 µg/mL (p=0.0110) and 100 µg/mL (p<0.0001) DEP caused a significant decrease at 48th hours. 100 µg/mL DEP concentration was seen to be toxic for BEAS-2B cells, for 48th hours of incubation **(Figure 4.1c).**

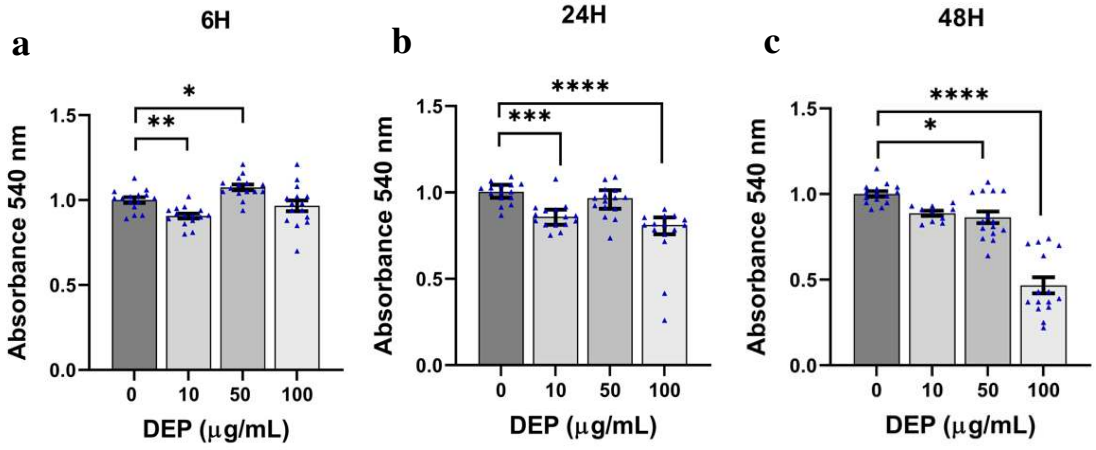

Figure 4.1: The effect of DEP on the viability of BEAS-2B cultures. (a) 6 hours, (b) 24 hours, and (c) 48 hours of incubation with increasing concentrations of DEP. Data were given as mean ± SEM (a, c) and median ± interquartile range (b). *p <0.05, **p < 0.01, ***p < 0.001 and ****p< 0.0001.

Effect of TiO_2 in the viability of BEAS-2B cultures were similar to the effect of DEP. 10 µg/mL TiO_2 caused a significant decrease in the viability at 6th hours ($p<0.0001$) **(Figure 4.2a).** Interestingly, 24 hours of incubation with TiO_2 particles did not cause any significant effect on the viability of the cells **(Figure 4.2b).** At 48th hours, TiO_2 caused a significant decrease as the concentration of the particles increased. 10 µg/mL ($p=0.0009$), 50 µg/mL ($p<0.0001$) and 100 µg/mL ($p<0.0001$) TiO_2 caused a significant decrease in the cell viability **(Figure 4.2c).**

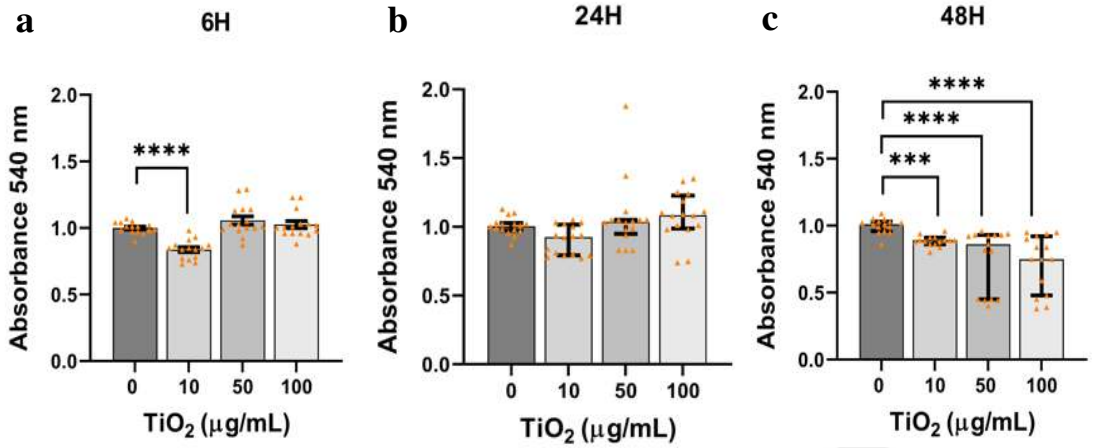

Figure 4.2: The effect of TiO_2 on the viability of BEAS-2B cultures. (a) 6 hours, (b) 24 hours, and (c) 48 hours of incubation with increasing concentrations of TiO_2. Data were given as mean ± SEM (a) and median ± interquartile range (b, c). *p <0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

A similar pattern was seen in the MWCNT treated BEAS-2B cells. At 6th hours, only 10 µg/mL MWCNT caused a significant decrease ($p= 0.0002$) **(Figure 4.3a).** All three concentrations caused a significant decrease in the cell viability at 24th hours, but it was not dose dependent. 10 µg/mL ($p=0.0002$), 50 µg/mL ($p=0.0199$), and 100 µg/mL ($p=0.0098$) significantly decreased the cell viability **(Figure 4.3b).** At 48th hour a significant decrease in cell viability was seen in 10 µg/mL MWCNT ($p= 0.0013$) and 100 µg/mL MWCNT ($p<0.0001$) **(Figure 4.3c).**

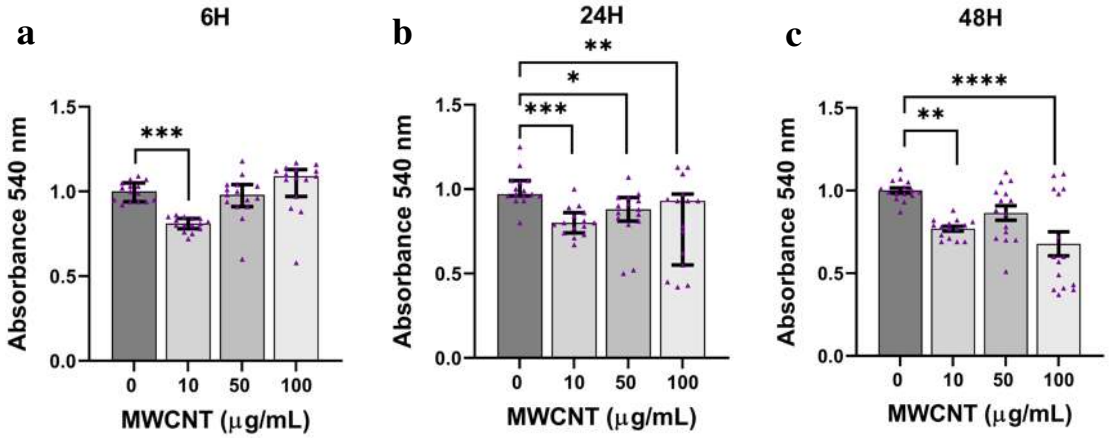

Figure 4.3: The effect of MWCNT on the viability of BEAS-2B cultures. (a) 6 hours, (b) 24 hours, and (c) 48 hours of incubation with increasing concentrations of MWCNT. Data were given as mean ± SEM (c) and median ± interquartile range (a, b). *p <0.05, **p< 0.01, ***p < 0.001 and ****p < 0.0001.

The viability results allowed the decision for the concentrations of 10 µg/mL, 50 µg/mL and 100 µg/mL, which did not show a greater than 50% reduction in cell viability, except for 48 hours of incubation with 100 µg/mL DEP.

4.2 qRT-PCR mRNA Gene Expression Results

mRNA expression of COPD related genes *(GCLC, CYP1B1, MUC5B, MUC5AC*, *FAM13A* and *SERPINA1*) were analyzed with qRT-PCR, as fold change relative to GAPDH mRNA expression. Data were shown as the combination of three independent experiments (n=3 biological replicates in each independent experiment). The exposure groups in each time point (6, 24 and 48 hours) were compared to control group (0 µg/mL). The differences between exposure concentrations in different time were also analyzed within each other.

4.2.1 The mRNA Expression of COPD Related Genes in DEP Treated Samples

DEP increased GCLC mRNA expression at 50 µg/mL (p=0.0019, p=0.0046) and 100 µg/mL (p=0.0008, p=0.0001), at 6 and 24 hours, respectively. On the other hand, only 50 µg/mL (p=0.0444) DEP increased *GCLC* expression at 48th hours. When it was checked whether incubation times caused any significant gene expression changes, at 48th hours, 50 µg/mL DEP (p=0.0177) and 100 µg/mL DEP (0.0004) caused a significant decrease in gene expression when compared with 24th hours. Additionaly, 48 hours of DEP incubation decreased *GCLC* expression at 50 µg/mL (p=0.0019), compared to 6 hours **(Figure 4.4a)**.

DEP incubation increased CYP1B1 mRNA expression at 10 µg/mL (p=0.0186) and at 50 µg/mL (p=0.0176), at 6th hours. At 24 hours, DEP incubation increased *CYP1B1* expression at the concentration of 100 µg/mL (p=0,0244), whereas at 48 hours, 50 µg/mL DEP (p=0.0001) caused a significant increase in the gene expression. 24 hours of 10 µg/mL DEP (p=0.0077) incubation significantly decreased *CYP1B1* expression compared to 6 hours **(Figure 4.4b)**.

50 µg/mL DEP (p=0.0071) significantly decreased MUC5B mRNA expression at 24 hours. When compared with 24 hours, 48 hours of incubation with 50 µg/mL DEP (p=0.0175) resulted in increased expression of *MUC5B* gene **(Figure 4.4c)**.

DEP did not cause any significant changes in the mRNA expressions of MUC5AC, FAM13A and SERPINA1 genes, when time points are analyzed individually **(Figure 4.4d-f)**. On the other hand, when time points are compared within each other, 24 hours of incubation with 50 µg/mL (p=0.0386) DEP caused a significant decrease in *SERPINA1* gene expression, compared to 6 hours. Controversially, incubation with 50 µg/mL DEP (p=0.0024) for 48 hours caused a significant increase in gene expression, compared to 24 hours of incubation **(Figure 4.4f)**.

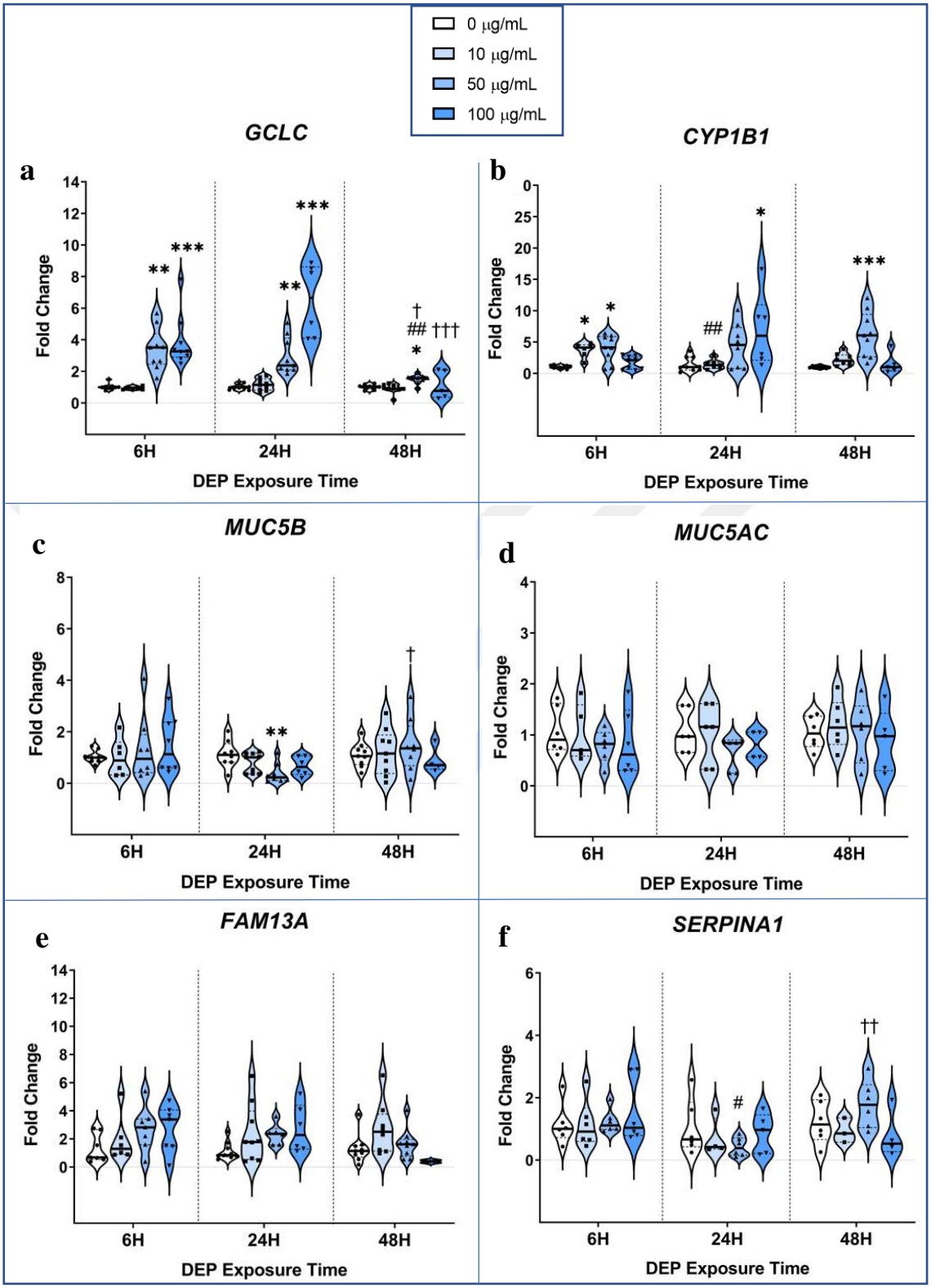

Figure 4.4: Effect of DEP on mRNA expression of COPD related genes.

mRNA expressions of (a) GCLC, (b) CYP1B1, (c) MUC5B, (d) MUC5AC, e) FAM13A, and f) SERPINA1, after incubation with increasing concentrations of DEP, for 6, 24 and 48 hours. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to 0 µg/mL in each time point. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to 6H, $^{\dagger}p < 0.05$, $^{\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ compared to 24H.

4.2.2 The mRNA Expression of COPD Related Genes in TiO_2 Treated Samples

TiO_2 significantly increased mRNA expression of *GCLC* at concentrations of 10 µg/mL (p=0.0045), 50 µg/mL (p<0.0001) and 100 µg/mL (p=0,0008) at 6 hours, whereas only 50 µg/mL (p=0.0298) TiO_2 increased the gene expression at 24 hours. On the other hand, the expression of *GCLC* was decreased at concentrations of 10 µg/mL (p=0.0001), 50 µg/mL (p=0.0038) and 100 µg/mL (p= <0,0001), at 48 hours. Exposure to TiO_2 for 48 hours in each concentration were compared with 6 and 24 hours and a significant decrease in the gene expression were observed **(Figure 4.5a).**

100 µg/mL TiO_2 (p= 0.0301) significantly increased the expression of *CYP1B1* at 6 hours whereas at 24 hours, 50 µg/mL TiO_2 (p= 0.0471) caused a significant increase. TiO_2 decreased the expression of *CYP1B1* at all concentrations (p<0.0001) at 48 hours. At 24 hours, 100 µg/mL TiO_2 decreased the gene expression compared to 6 hours (p=0.0102). 48 hours of TiO_2 at concentrations of 10 µg/mL (p=0.0001), 50 µg/mL (p=0.0014) and 100 µg/mL (p=0.0009) µg/mL caused a significant decrease in gene expression when compared with 6 hours. Additionally, 48 hours of TiO_2 at concentrations of 10 µg/mL (p= 0.0269) and 50 µg/mL (p=0.0028) caused a significant decrease in the gene expression when compared to 24 hours **(Figure 4.5b)**.

The mRNA expression of MUC5B was significantly increased at 6 hours at the concentrations of 50 µg/mL (p<0.0001) and 100 µg/mL (p=0.0020). The gene expression was decreased at 48 hours at the concentration of 10 µg/mL (p=0.0160). When concentrations of TiO_2 at 24 hours and 48 hours were compared to 6 hours, a significant decrease in the gene expression were also observed **(Figure 4.5c)**.

Similar to its effect on *MUC5B* gene expression, 50 µg/mL TiO_2 (p=0.0137) increased MUC5AC mRNA expression at 6 hours. At 48 hours, 10 µg/mL (p=0.0223) and 100 µg/mL (p=0.0389) caused a significant decrease in the gene expression. In addition, 10 µg/mL (p=0.0144) and 100 µg/mL (p=0.0049) at 48 hours caused a significant decrease compared to 24 hours. Moreover, 10 µg/mL TiO_2 at 48^{th} hours decreased the gene expression compared to 6 hours (p=0.0019) **(Figure 4.5d)**.

The mRNA expression of FAM13A was only decreased at 100 µg/mL concentration (p=0.0020) at 48 hours. There was also a significant decrease in the gene expression at this concentration, when compared to 24 hours (p=0.0169) **(Figure 4.5e)**.

50 µg/mL (p=0.0162) TiO_2 caused a significant increase in SERPINA1 mRNA expression at 6^{th} hours. On the other hand, at 48^{th} hours, gene expression significantly

decreased after 50 µg/mL (p=0.0343) and 100 µg/mL (p=0.0005) TiO_2 exposure. Additionally, 50 µg/mL (p=0.0052) and 100 µg/mL (p=0.0148) TiO_2 at 48 hours decreased the gene expression compared to 6 hours. Moreover, 100 µg/mL TiO_2 at 48 hours decreased the gene expression when compared to 24 hours (p=0.0033) **(Figure 4.5f)**.

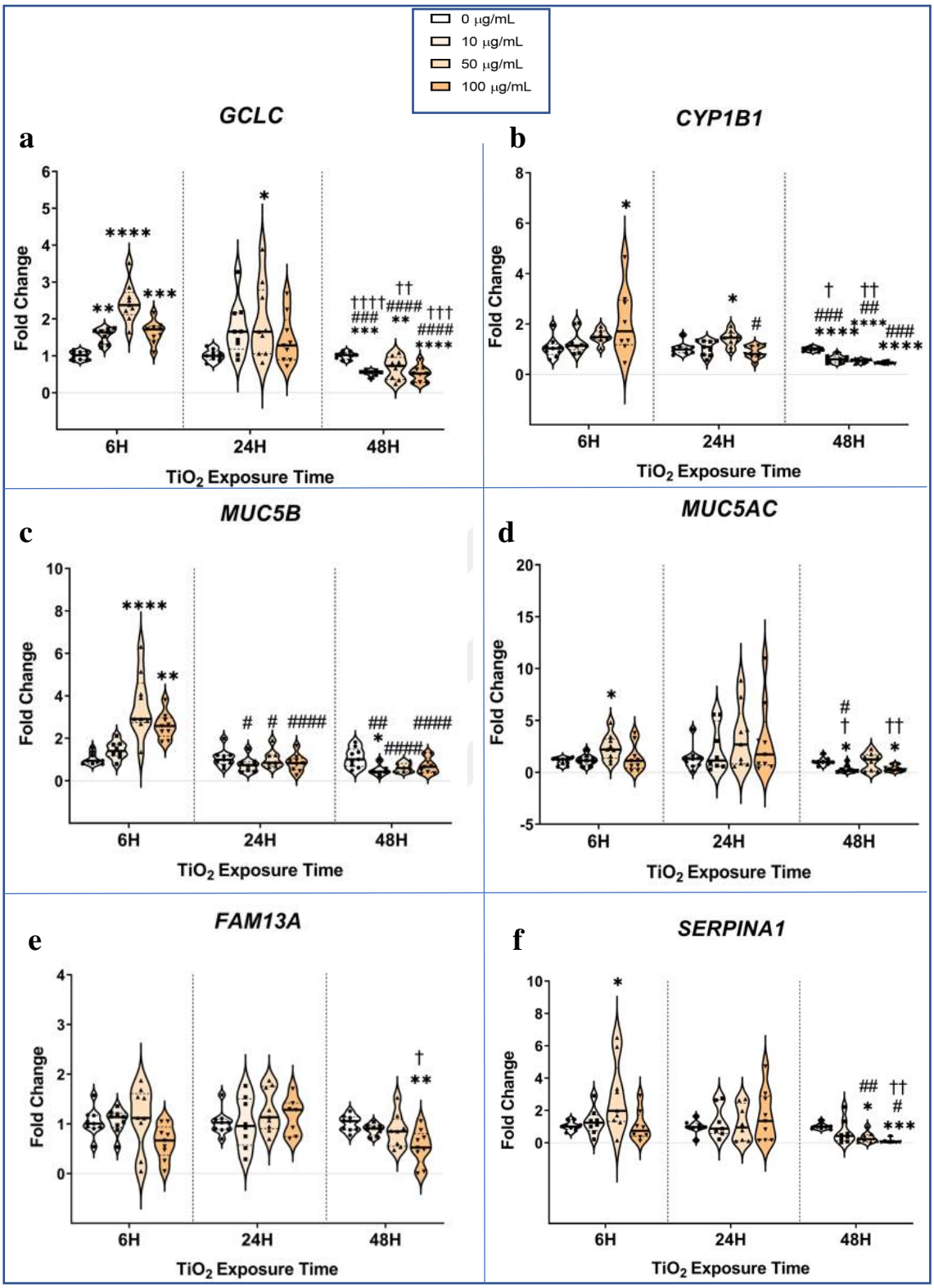

Figure 4.5: Effect of TiO_2 on mRNA expression of COPD related genes.

mRNA expressions of (a) GCLC, (b) CYP1B1, (c) MUC5B, (d) MUC5AC, (e) FAM13A, and (f) SERPINA1, after incubation with increasing concentrations of TiO_2 for 6, 24 and 48 hours. *p<0.05, **p < 0.01, ***p < 0.001 compared to 0 µg/mL in each time point. $^{\#}$p <0.05, $^{\#\#}$p< 0.01, $^{\#\#\#}$p < 0.001 compared to 6H, †p <0.05, †p < 0.01, †††p < 0.001 compared to 24H.

4.2.3 The mRNA Expression of COPD Related Genes in MWCNT Treated Samples

50 µg/mL MWCNT (p=0.0036) significantly increased GCLC mRNA expression at 6 hours. Additionally, 50 µg/mL (p<0.0001) and 100 µg/mL (p=0.0054) MWCNT also caused a significant increase in the gene expression at 24 hours. 50 µg/mL MWCNT at 24 hours increased the gene expression compared to 6 hours, but the gene expression at the same concentration was decreased at 48 hours **(Figure 4.6a)**.

Exposure to 100 µg/mL MWCNT decreased the mRNA expression of CYP1B1 at 6 (p=0.0063), 24 (p=0.0416) and 48 hours (p<0.0001). At 48 hours, lower concentrations such as 10 µg/mL (p=0.0379) and 50 µg/mL (p=0.0027) significantly increased the gene expression. In addition, 10 µg/mL TiO_2 at 48 hours increased *CYP1B1* expression compared to 6 (p=0.0335) and 24 hours (p=0.0345) **(Figure 4.6b)**.

MWCNT increased the mRNA expression of MUC5B at the concentration of 50 µg/mL (p=0.0042) at 6 hours, whereas 100 µg/mL TiO_2 decreased the gene expression at 24 hours. At 24 hours, 50 µg/mL (p=0.0253) and 100 µg/mL (p=0.0341) MWCNT significantly decreased gene expression compared to 6 hours **(Figure 4.6c)**.

MWCNT did not cause any significant effect on the mRNA expression of MUC5AC **(Figure 4.6d)**.

MWCNT significantly increased the mRNA expression of FAM13A at the concentrations of 10 µg/mL (p=0.0132) and 50 µg/mL (p=0.0044) at 48 hours **(Figure 4.6e)**.

Exposure to MWCNT had no significant effect on the mRNA expression of SERPINA1 **(Figure 4.6e)**.

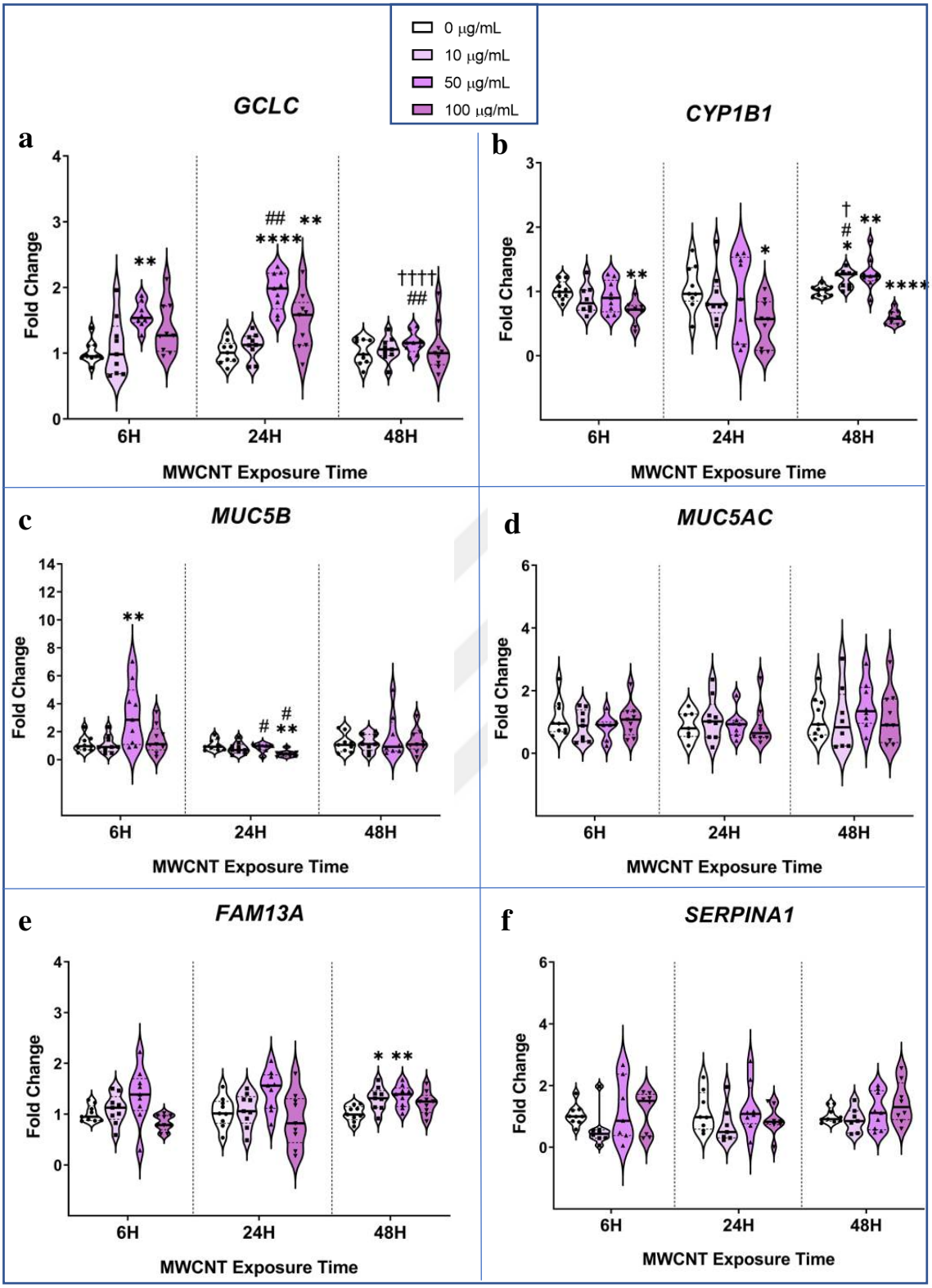

Figure 4.6: Effect of MWCNT on mRNA expression of COPD related genes. mRNA expressions of (a) GCLC, (b) CYP1B1, (c) MUC5B, (d) MUC5AC, (e) FAM13A, and (f) SERPINA1, after incubation with increasing concentrations of MWCNT, for 6, 24 and 48 hours. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to 0 µg/mL in each time point. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to 6H, $^{\dagger}p < 0.05$, $^{\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ compared to 24H.

4.3 Pyrosequencing Analysis

GCLC and *CYP1B1* were two genes whose expression significantly changed the most, after incubation with all three particles (DEP, TiO_2 and MWCNT). To understand if these transcriptional changes were related with epigenetic mechanisms, we analyzed the DNA methylation of these genes after they were exposed with particles. Pyrosequencing assays for *GCLC* and *CYP1B1* genes were designed and assays that have the highest score were chosen. The exposure groups in each time point (6, 24 and 48 hours) were compared to control group (0 µg/mL). The differences between exposure concentrations in different time were also analyzed within each other.

4.3.1 Methylation Profile of GCLC gene

Three CpG sites in *GCLC* gene were checked for DNA methylation analysis (CpG1, CpG3 and CpG5).

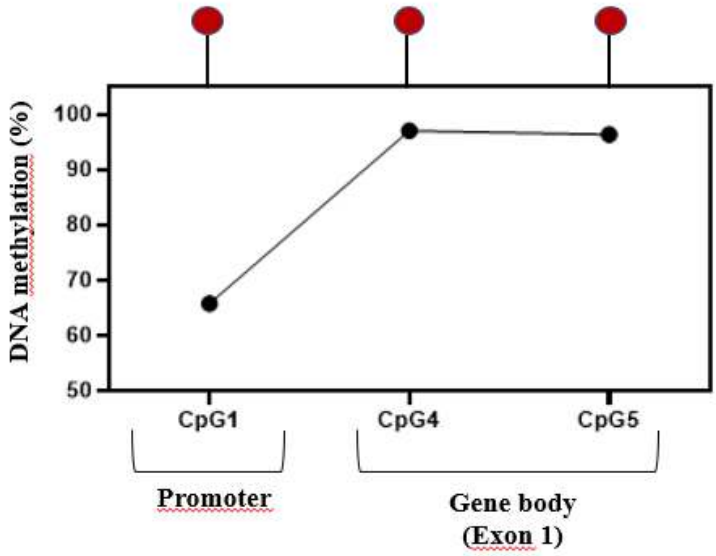

Figure 4.7: CpG methylation dynamics in *GCLC* in non-exposed (control) BEAS-2B cells.

DNA methylation of different CpG sites in *GCLC* gene in non-exposed (control) groups was shown in **Figure 4.7.** 6H was selected as baseline and each dot shows the average CpG methylation of control groups in this exposure group. The genomic locations of CpG sites were also shown in the figure.

DEP had no significant effect on DNA methylation of CpG position 1 **(Figure 4.7a)**. At 24 hours, increased DNA methylation of CpG position 4 was observed at the concentration of 50 µg/mL (p= 0.0382) **(Figure 4.7b)**. When it was compared with 6 hours, a significant increase in DNA methylation was also seen (p=0,0338) **(Figure 4.7b)**. Incubation with 10 µg/mL DEP (p=0.0328) and 100 µg/mL DEP (p=0.0328) at 48 hours increased DNA methylation of CpG position 5, when compared with 6 hours **(Figure 4.7c)**.

TiO_2 did not cause any DNA methylation changes in any of the CpG positions in *GCLC* gene **(Figure 4.8)**.

When exposed with MWCNT, there were not any significant changes in the time points individually, when they compared with the control group. On the other hand, 50 µg/mL MWCNT (p=0.0211) at 24 hours decreased DNA methylation of CpG position 4, when compared to 6 hours **(Figure 4.9b)**. Additionaly, incubation with 10 µg/mL MWCNT (p=0.0338) at 24 hours decreased DNA methylation in CpG position 5, compared to 6 hours **(Figure 4.9c)**.

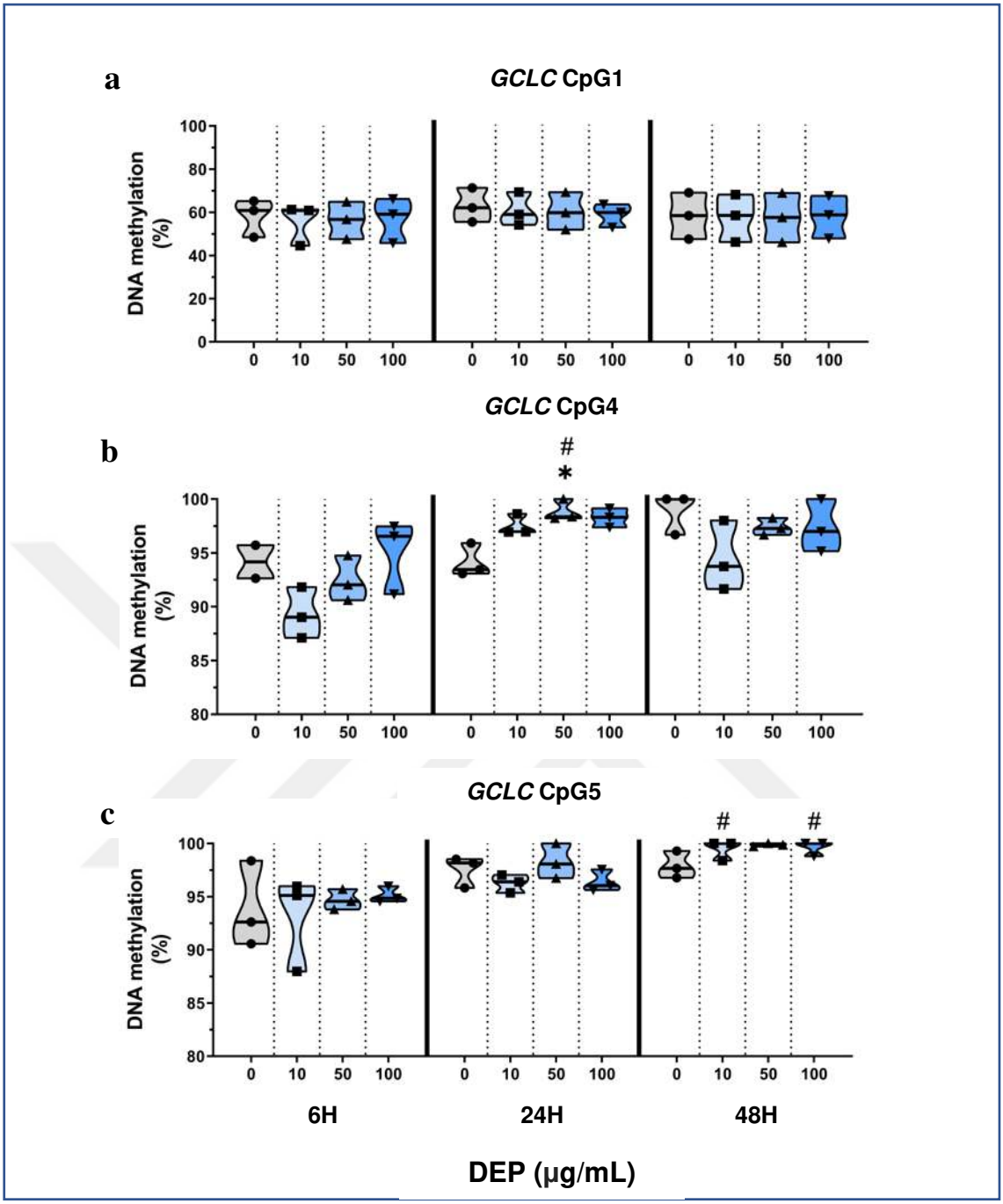

Figure 4.8: Effect of DEP on DNA methylation of *GCLC* gene.

GCLC methylation at (a) CpG position 1,(b) CpG position 4, (c) CpG position 5. *p<0.05 compared to 0 µg/mL in each time point. #p<0.05 compared to 6H.

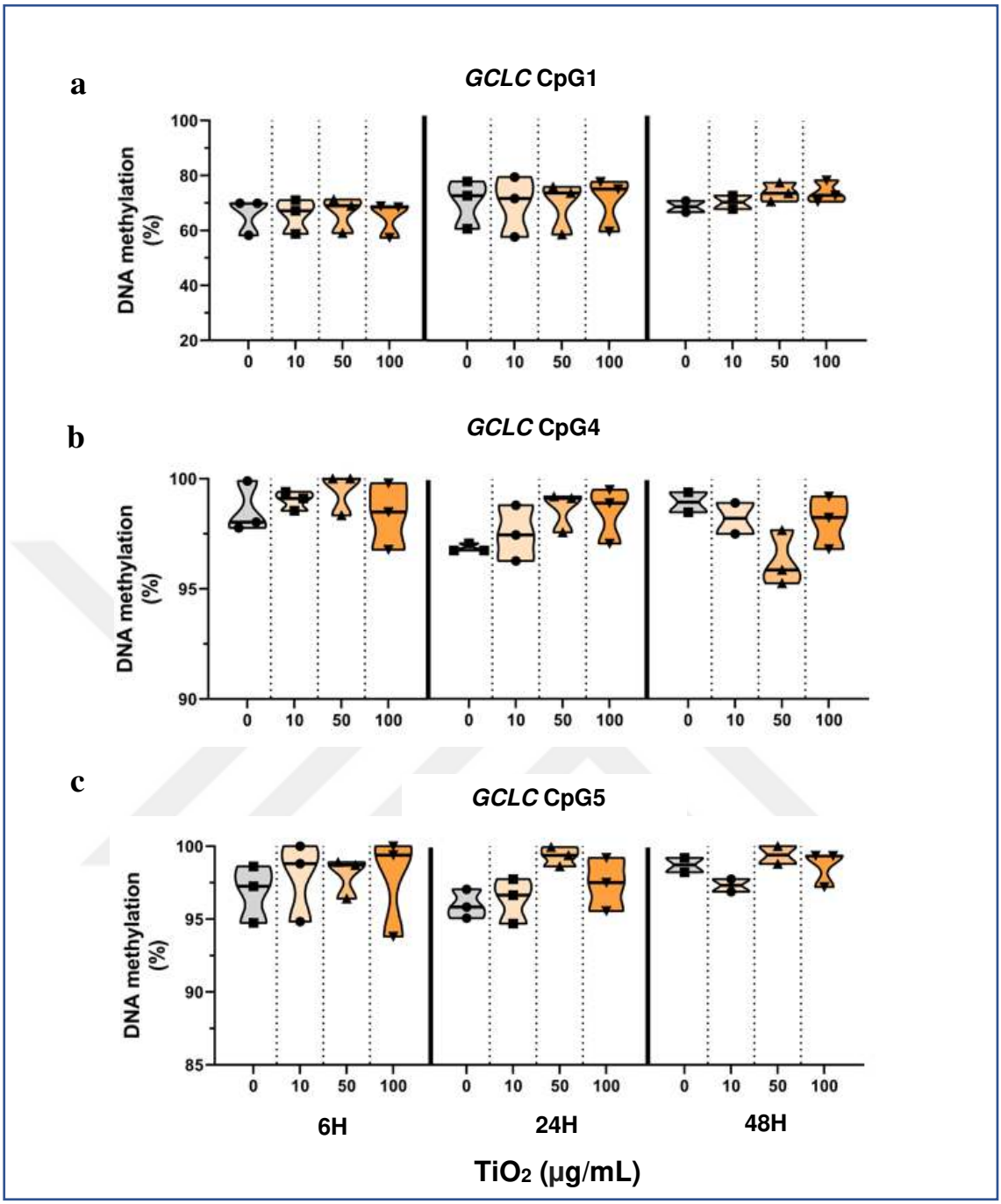

Figure 4.9: Effect of TiO_2 on DNA methylation of GCLC gene. *GCLC* methylation at (a) CpG position 1, (b) CpG position 4, (c) CpG position 5.

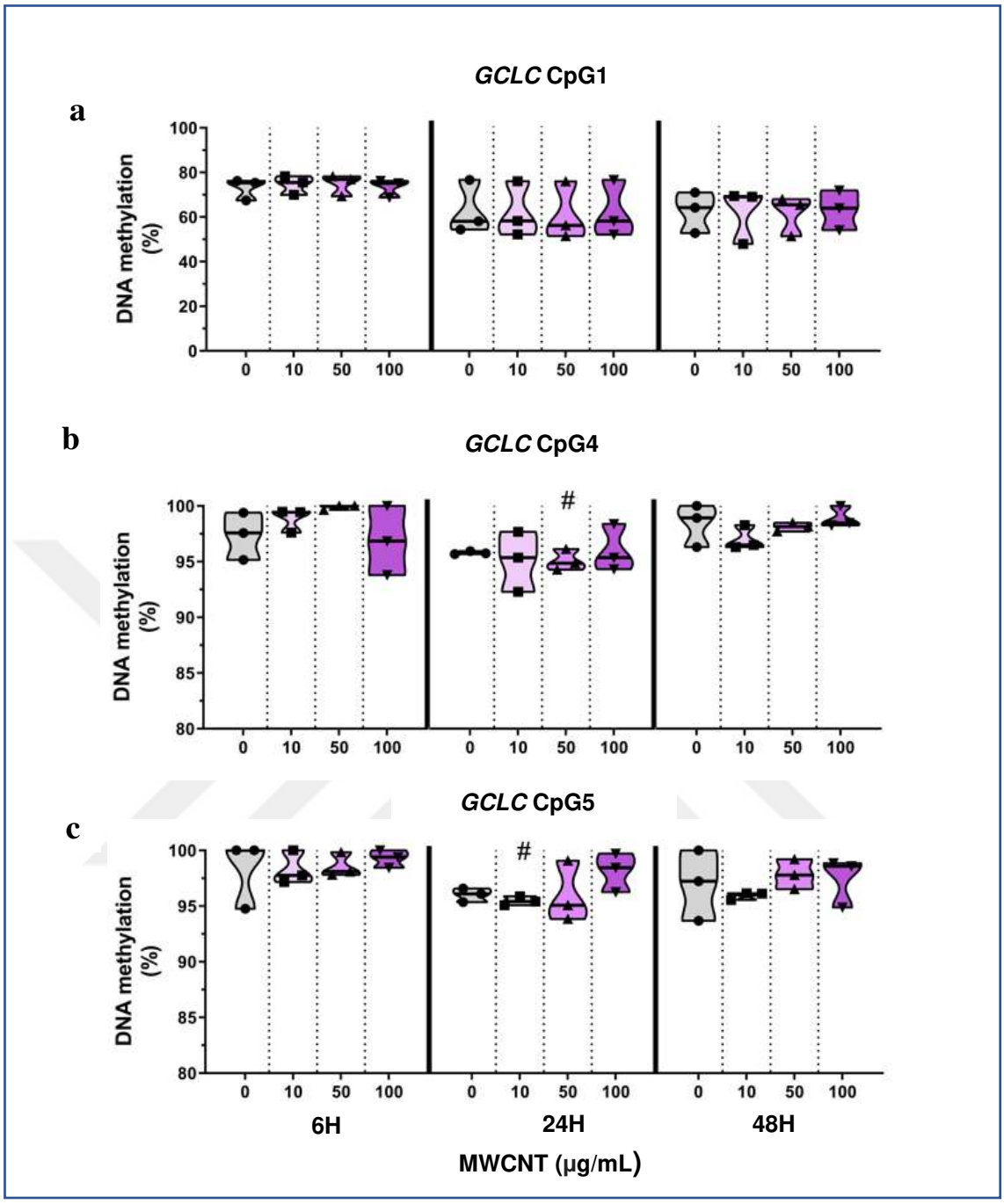

Figure 4.10: Effect of MWCNT on DNA methylation of GCLC gene. *GCLC* methylation at (a) CpG position 1, (b) CpG position 4, (c) CpG position 5. $^{\#}p < 0.05$ compared to 6H.

4.3.2 Methylation Profile of CYP1B1 gene

Five CpG sites in *CYP1B1* gene were checked for DNA methylation analysis (CpG1, CpG3, CpG5, CpG6 and CpG7).

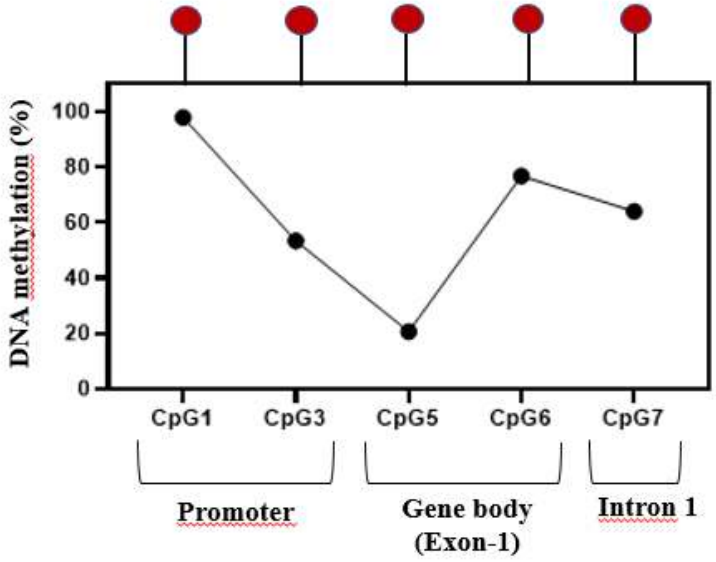

Figure 4.11: CpG methylation dynamics in *CYP1B1* in non-exposed (control) BEAS-2B cells.

DNA methylation of different CpG sites in *CYP1B1* gene in non-exposed (control) groups was shown in **Figure 4.11.** 6H was selected as baseline and each dot shows the average CpG methylation of control groups in this exposure group. The genomic locations of CpG sites were also shown in the figure.

DEP did not cause any significant changes in DNA methylation of *CYP1B1* gene, in any of the CpG positions shown in **Figure 4.10**.

TiO_2 also did not cause any significant changes in DNA methylation of *CYP1B1* gene (**Figure 4.11**).

MWCNT increased DNA methylation of two CpG positions in *CYP1B1* gene, at different time points. At 48 hours, 100 μg/mL MWCNT (p=0.0197) caused increased DNA methylation of CpG position 5 (**Figure 4.12c**). At 6 hours, 100 μg/mL MWCNT (p=0.0276) increased DNA methylation of CpG position 7 (**Figure 4.12e**).

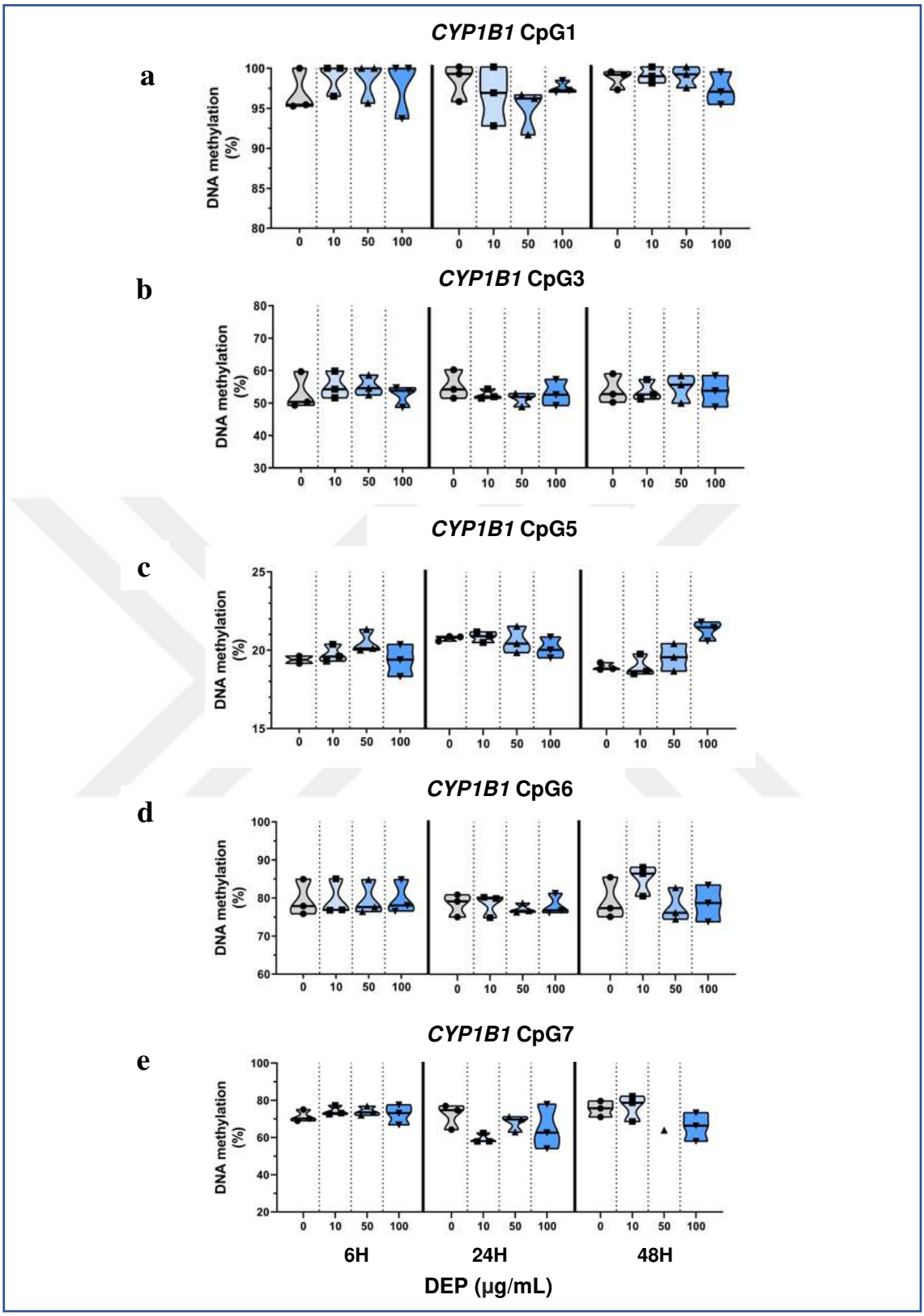

Figure 4.12: Effect of DEP on DNA methylation of *CYP1B1* gene. *CYP1B1* methylation at (a) CpG position 1, (b) CpG position 3, (c) CpG position 5, (d) CpG position 6, (e) CpG position 7.

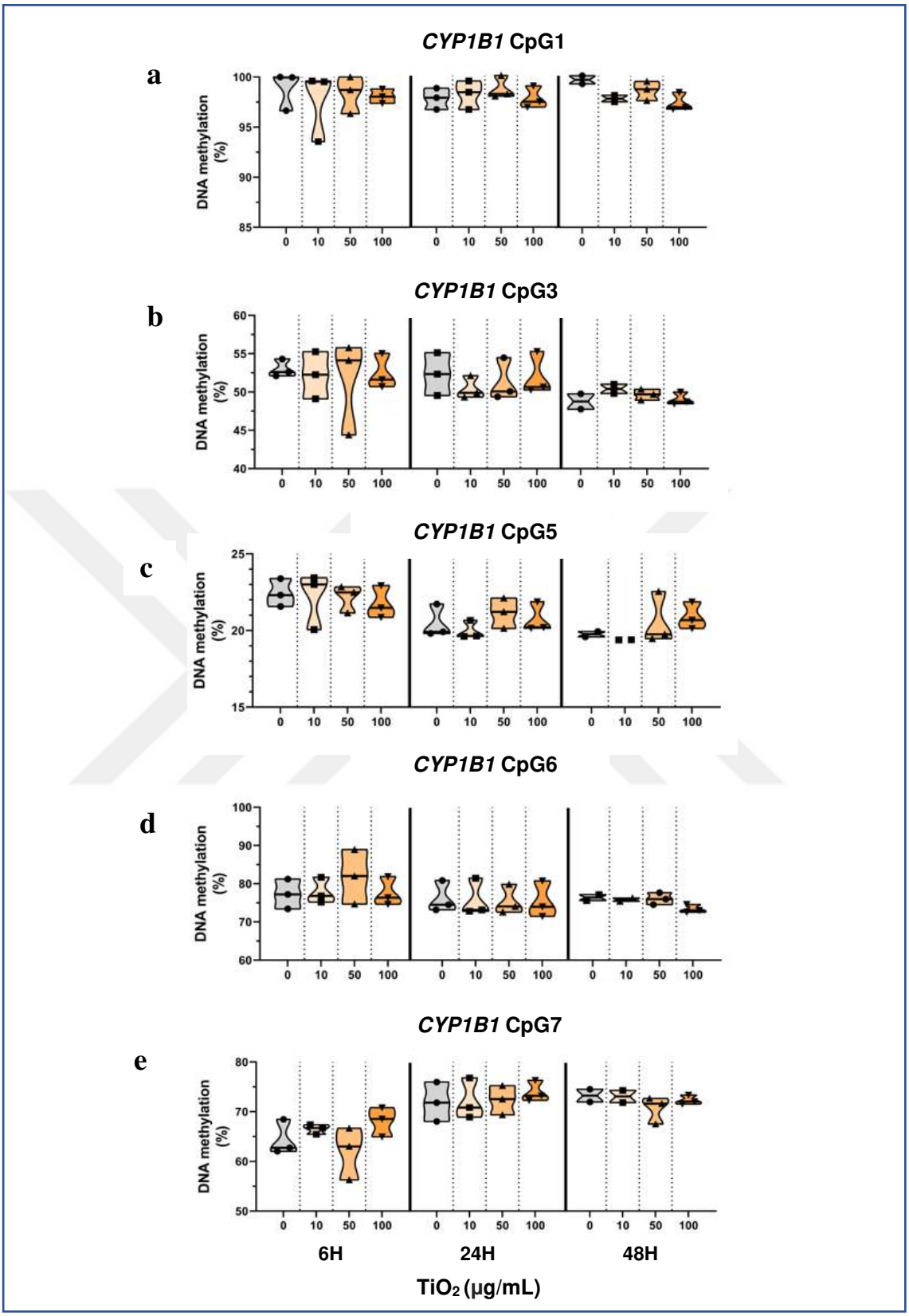

Figure 4.13: Effect of TiO_2 on DNA methylation of *CYP1B1* gene. *CYP1B1* methylation at (a) CpG position 1, (b) CpG position 3, (c) CpG position 5, (d) CpG position 6, (e) CpG position 7.

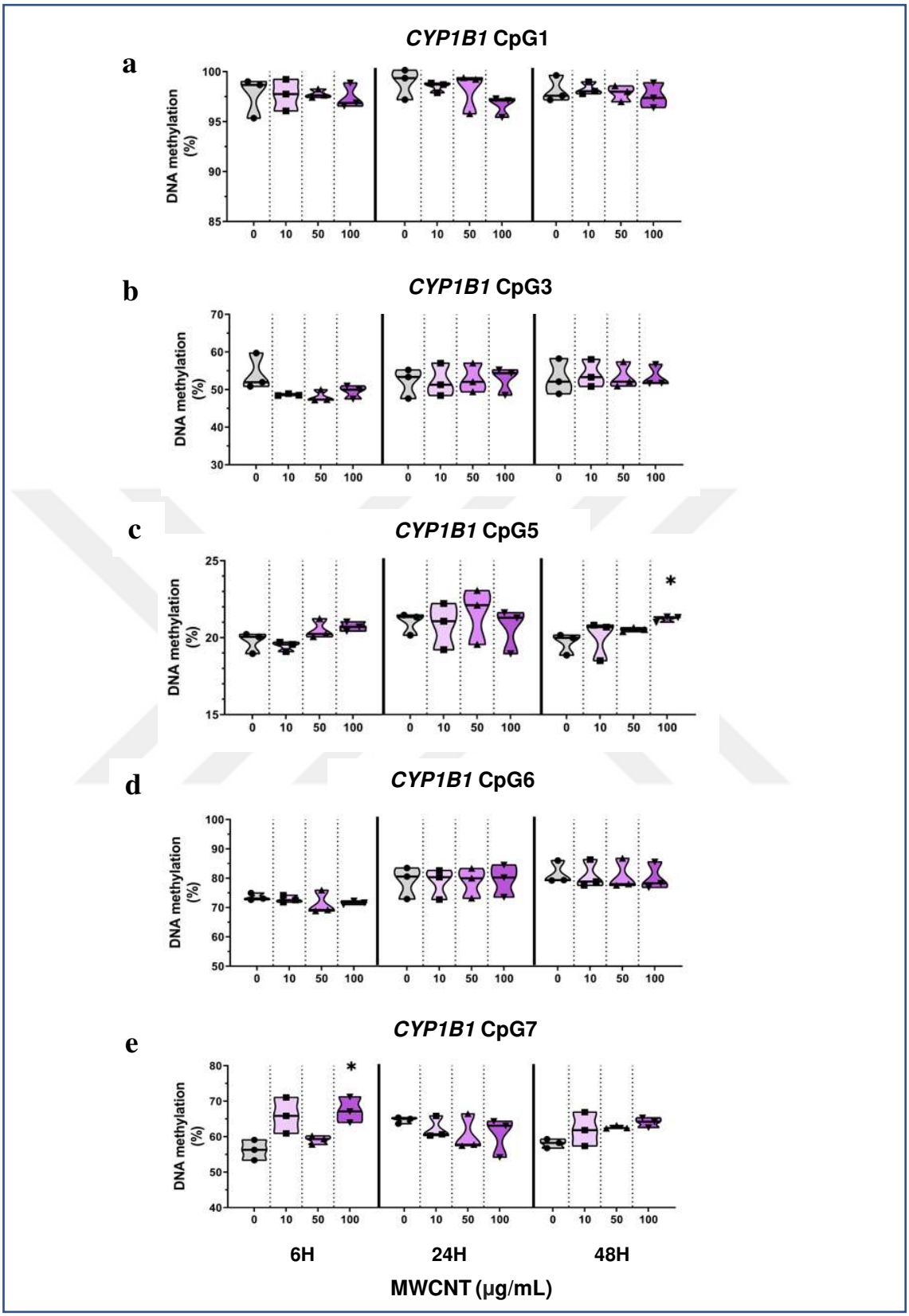

Figure 4.14: Effect of MWCNT on DNA methylation of *CYP1B1* gene. *CYP1B1* methylation at (a) CpG position 1, (b) CpG position 3, (c) CpG position 5, (d) CpG position 6, (e) CpG position 7. *P <0.05 compared to 0 µg/mL in each time point.

Chapter 5:
DISCUSSION

Many factors are involved in the pathogenesis of COPD, including epigenetic mechanisms (Morrow et al., 2018), abnormal lung development, aging and genetic factors (Global Initiative for Chronic Obstructive Lung Disease). It is known that environmental pollutants such as particulate matters induce epigenetic changes such as DNA methylation, histon modifications and microRNAs (miRNAs) (Syed, Hew, Kohli, Knowlton, & Nadeau, 2013). Among these mechanisms, DNA methylation is mostly studied in COPD pathogenesis. Our previous studies have shown that DEP can suppress the ciliary beating frequency of bronchial epithelial cells, increases the inflammator response and affects the viability, proliferation and apoptosis of these cells (Bayram, Devalia, Khair, et al., 1998; Bayram, Devalia, Sapsford, et al., 1998; Bayram et al., 2006). However, the effect of DEP, TiO_2 and MWCNT on the DNA methylation of COPD related genes is an area that has not been studied exclusively, which is why we decided to focus on this gap. DNA methylation is an epigenetic mechanism that regulates gene transcription (Zhang, Lang, & Zhu, 2018). Therefore, here in this project we checked the effect of DEP and NP (TiO_2 and MWCNT) on the mRNA expression of literature-based selected COPD associated genes and checked the DNA methylation status of two of the most upregulated genes (*GCLC* and *CYP1B1*), upon exposure. To our knowledge, effect of DEP and NP on DNA methylation status of these two COPD associated genes were not studied in the literature.

Airway epithelial cells form the first line of cellular defense toward inhaled irritants. That is why we performed our experiments on bronchial epithelial cell line (BEAS-2B). Before moving further into experiments, we analyzed the toxicity of these three particles so that the concentrations for the particle exposure and incubation times were not lethal to cells. We treated BEAS-2B cell with each particles at the concentrations of 0, 10, 50 and 100 µg/mL. for 6, 24 and 48 hours. 0 µg/mL was our control group (only serum-free medium). The variety of the exposure concentrations and incubation times was the other reason that made our study unique.

Our previous studies had shown that DEP and NP can effect cell viability (Bayram, Fakili, Gogebakan, Bayraktar, & Ekinci, 2010). Here we evaluated the effect of DEP, TiO_2 and MWCNT on the viability of BEAS-2B cultures. We observed a similar

pattern for all three particles in each time points. DEP caused a significant decrease in cell viability at 6 hours only at 10 μg/mL, whereas it caused a significant increase at 50 μg/mL, which we couldn't explain the reason. At 24 and 48 hours, exposure to DEP significantly decreased the cell viability. Even though there was a decrease in the cell viability at the given time points, only exposure to 100 μg/mL DEP for 48 hours was found to be toxic for the cells. Similarly, exposure to TiO_2 and MWCNT suppressed the cell viability in each time interval, except at 24 hours, TiO_2 had no significant effect. Therefore, exposure concentrations for TiO_2 and MWCNT were found to be non-toxic at the chosen concentrations and incubation times. In a study where they checked the effect of DEP on human bronchial epithelial cells, they concluded that 10 μg/mL and 50 μg/mL concentrations did not show a greater than 30% reduction in cell viability, which was consistent with our data. On the other hand 100 μg/mL was found to be toxic for cells (Frias et al., 2020). Although we saw a similar effect, the incubation time for DEP in the mentioned study was shorter than ours (2 hours). The reason we obtained a similar result between different time incubations would be due to the components of culture medium. In the mentioned study they used LHC-9 growth medium supplemented with 10% fetal bovine serum whereas we cultured our cells in serum-free RPMI medium.

The genes we focused in our study (*GCLC, CYP1B1, MUC5B, MUC5AC, FAM13A* and *SERPINA1*) were selected through a detailed literature screening. We checked whether the mRNA expression of these genes would be affected by particle exposure or not, in order to move on with DNA methylation analysis.

Oxidative stress is a major driving force in COPD pathogenesis. It has been seen that there is a marked increase in oxidative stress in the lungs of patients with COPD. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that regulates multiple antioxidant genes and, thereby, protects the lungs against oxidant damage (Barnes, 2020). When we examined the mRNA expression of Glutamate cysteine ligase catalytic (GCLC) –a rate-limiting enzyme in the synthesis of glutathione, an important endogenous antioxidant- we found that incubation with each particle increased the gene expression at 6 and 24 hours at the concentration of 50 μg/mL, indicating that particle exposure causes increased oxidative stress. Because *GCLC* is frequently increased in response to oxidative stress, the obtained result was expected. On the other hand, at 48 hours, each concentration of TiO_2 incubation led to a decrease in the gene expression when compared with the control. The mechanism behind this may be that the longer incubation times with TiO_2 may result in more increased oxidation and the

antioxidant response of the bronchial epithelial cells may be limited so that the gene expression decreases (Natarajan et al., 2016). This speculation can also be supported by the decreased expression of *GCLC* at 48 hours in each exposure group (10, 50 and 100 μg/mL), when compared with 6 and 24 hours.

Since all three particles influenced GCLC expression and different transcriptional changes were observed at various times, we decided to investigate if epigenetic mechanisms were responsible for any of these transcriptional changes. To our knowledge this was the first study to ever check the influence of both DEP and NP on the DNA methylation status of oxidative stress related gene -*GCLC*- as time and dose-dependently.

DNA methylation is an epigenetic mechanism involved in the regulation of genes by adding or removing a methyl group at the fifth carbon of the pyrimidine ring of cytosine to form 5mC. Most 5mCs are found at CpG sites and these formed 5mC are found in many regions in the genome including repetitive sequences, gene bodies, and intergenic regions. If CpG sites located in a gene body are hypermethylated, gene expression is increased. On the other hand, if CpG sites located in promoters or enhancers are hypermethylated, these regions become heterochromatic and they are not bound by transcription activators. This leads to transcriptional silencing (Jang, Shin, Lee, & Do, 2017).

In a study investigating the role of *GCLC* methylation in COPD, it was found that smoking increased the levels of DNA methylation in the GCLC promoter in both human lung tissue and in vitro BEAS-2B cells. Besides, it was found in this study that the hypermethylation seen in the GCLC promoter was responsible for the downregulation of GCLC mRNA in lung tissues and glutathione (GSH) in plasma. Additionally, it has been reported that cigarette smoke extract decreased GCLC mRNA levels and intracellular GSH levels in BEAS-2B cells (Cheng et al., 2016). In another study, it was reported that *GCLC* promoter methylation levels decreased after lead exposure and this resulted in increased *GCLC* gene expression and GSH and glutathione S-transferase (GST) levels as an epigenetic response to maintain cellular redox balance in 109 workers working in the automotive industry (Devóz et al., 2021). In this direction, as mentioned above, it is seen that the hypermethylation occurring in the promoter of this gene does not increase the gene's expression but, on the contrary, causes it to decrease.

In order to check whether there were any epigenetic mechanisms behind the expression changes, we evaluated the methylation alterations on three CpG sites on promoter and exon 1 (gene body) regions of *GCLC* gene (CpG1, CpG4 and CpG5) after

DEP, TiO_2, and MWCNT incubation for 6, 24, and 48 hours. DEP did not cause significant methylation changes in the promoter region of GCLC gene (CpG1). However, we observed that 50 µg/mL DEP increased DNA methylation in CpG position 4, at 24 hours of incubation, compared to control group. According to our qPCR results, at this time point and concentration, the mRNA expression of GCLC was increased significantly, when compared to control. As mentioned above, this may be due to the location of CpG position 4, which is located in the gene body (exon 1). Although studies investigating the relationship between DNA methylation in exon 1 and gene expression is controversial, as it was suggested in another study, DNA methylation of the gene body increases gene expression and that the rapid establishment of this is dependent on the presence of the DNMT3B (X. Yang et al., 2014), which may be checked later in further studies to provide more evidence in our case.

50 µg/mL DEP also led to an increase in DNA methylation at 24 hours, compared to 6 hours, but we did not see any significant changes in the mRNA expression of *GCLC* between the 6^{th} and 24^{th} hours. Here, we saw a dose dependent methylation increase (50 µg/mL vs 0. µg/mL) positively correlated with transcription of the gene but the time dependent methylation change did not alter any transcriptional change. Here, we can speculate that, longer incubation times with particles may result in decreased or increased methylation levels that do not affect the transcription of the gene. To understand if this is the case, further investigations are needed. At CpG position 5, 10 and 100 µg/mL concentrations of DEP also caused increased DNA methylation of the gene at 48 hours compared to 6 hours. When we evaluated whether it was consistent with mRNA expression, we did not see any significant changes at 48 hours, compared to 6 hours. CpG position 5 in *GCLC* gene is located at the end of exon 1, therefore the effect of the methylation change may not be reflected in the gene expression change because it may be far from the binding site of transcription factors.

After TiO_2 exposure, substantial differences in mRNA expression between exposure groups were observed; however, the methylation at the CpG sites of *GCLC* gene that we examined showed no alterations. Although it may appear that DNA methylation had no involvement in the transcriptional alterations seen in *GCLC* after exposure to TiO_2, more CpG sites are required to be examined to gain a better understanding of the epigenetic impact of TiO_2 nanoparticles.

Similar to DEP, MWCNT did not cause significant methylation changes in the promoter region of *GCLC* gene. At CpG position 4, 50 µg/mL MWCNT decreased DNA

methylation in GCLC's gene body at 24 hours, compared to 6 hours. According to our qPCR results, mRNA expression of the gene was increased at 24 hours compared to 6 hours. CpG position 4 is located in the gene body and as we mentioned above, DNA methylation in the gene body may be the reason behind the increase in gene expression as we have seen in 50 µg/mL DEP-incubated cells at 24 hours. Interestingly, here we see a different pattern. There may be other reasons why the effect of CpG position 4 methylation on GCLC mRNA expression exhibits a different expression profile between DEP and MWCNT. Among these, the chemical composition of MWCNT and DEP and the activation of different cellular response mechanisms can be considered. MWCNT is derived from inert carbon. However, DEP can contain elementary and organic carbon as well as polycyclic hydrocarbons. Therefore, the two different particles may have had different effects on *GCLC* expression and CpG methylation. In different studies, it can be seen that smoking and lead heavy metal have an opposite effect on *GCLC* gene expression and methylation change (Cheng et al., 2016; Devóz et al., 2021).

At CpG position 5, 10 µg/mL MWCNT decreased DNA methylation of the gene at 24 hours compared to 6 hours, but the mRNA expression did not change. Again, as we mentioned above, this may be related with the location of CpG position 5 which is at the end of exon 1.

The other gene that we studied, which is important in COPD pathogenesis was *CYP1B1*. In a study where they performed functional enrichment analysis for the differentially expressed genes in COPD patients, they found that *CYP1B1* was included in the top 10 DEG-compound pairs. The expression level of this gene was significantly upregulated in COPD patients (Danlei Yang et al., 2020). It is a key enzyme involved in the metabolism of exogenous and endogenous substrates, many of which include carcinogenic compounds. Many studies have demonstrated a role for *CYP1B1* in the bioactivation of pro-carcinogens, such as polycyclic aromatic hydrocarbons (PAHs). These PAHs act via the AhR (aryl hydrocarbon receptor) complex (Falero-Perez, Song, Sorenson, & Sheibani, 2018). We expected to see changes in the mRNA expression of CYP1B1, after particle exposure. As expected, we saw that 50 µg/mL DEP incubation increased CYP1B1 mRNA expression at 6 and 48 hours, whereas at 24 hours, there was a significant increase only at 100 µg/mL. On the other hand, 24 hours of 10 µg/mL DEP incubation significantly decreased *CYP1B1* expression compared to 6 hours.

Similary, incubation with TiO_2 increased *CYP1B1* expression at 6 (100 µg/mL) and 24 hours (50 µg/mL), whereas at 48 hours, TiO_2 decreased the expression of *CYP1B1* at

all concentrations. The exposure groups at 48 hours were also significantly decreased when compared with 6th and 24th hours. TiO_2 incubation had also caused the same effect in GCLC mRNA expression, in this exposure group.

Contreversely there was a different expression pattern in MWCNT treated cells. At 6 hours and 24 hours, there was a decreasing trend which was significant at 100 µg/mL concentration. At 48 hours the gene expression was increased at lower concentrations such as 10 and 50 µg/mL whereas at 100 µg/mL a significant decrease was also seen when compared to control. 10 µg/mL TiO_2 at 48 hours also increased *CYP1B1* expression when compared to 6th and 24th hours. Several reports show that PM and AHR-mediated induction of CYP enzymes results in excessive generation of reactive oxygen species (ROS), causing oxidative stress. Furthermore, it was observed that exposure to PM and PAHs induce inflammatory responses and may lead to chronic inflammatory diseases such as COPD (Vogel, Van Winkle, Esser, & Haarmann-Stemmann, 2020). Here we showed that both DEP and NP altered the expression of *CYP1B1* at different time points and concentrations, which may result in oxidative stress responses, leading to progress in COPD pathogenesis.

In order to check whether there were any epigenetic mechanisms behind the expression changes in *CYP1B1* gene, we analyzed 5 CpG sites on promoter, exon 1 (gene body), and intron 1 (intronic) sites of *CYP1B1* gene (CpG1, CpG3, CpG5, CpG6 and CpG7). After substantial differences in mRNA expression between exposure groups were observed after DEP and TiO_2 exposure, we saw that neither DEP nor TiO_2 cause DNA methylation changes at any of the CpG sites selected. We can interpret this result in two ways: the transcriptional changes we observed after incubation with the particles, may not be related with DNA methylation changes. Even though this may be true, we cannot be sure of this since we only checked 5 CpG sites in *CYP1B1* gene. More CpG sites are required to be examined to gain a better understanding of the epigenetic impact of these nanoparticles.

On the other hand, MWCNT did not cause significant methylation changes in the promoter region of *CYP1B1* gene, however, at 48 hours, 100 µg/mL MWCNT increased DNA methylation of the *CYP1B1* gene at CpG position 5. At this condition we had seen that mRNA expression of *CYP1B1* was decreased. At this condition we had seen that mRNA expression of CYP1B1 was decreased. 100 µg/mL MWCNT at 6 hours also increased DNA methylation at CpG position 7. This increase in DNA methylation was again consistent with decrease in mRNA expression This increase in DNA methylation

was again consistent with decrease in mRNA expression. CpG position 5 is located in the gene body. As it was suggested, transcriptional gene silencing is associated with methylation of CpG sites and CpG islands located near the transcription start sites (TSS) of genes (Jones, 2012). This may explain the reason behind this negative correlation between gene expression and DNA methylation. The methylation in these CpG sites may prevent binding of the transcription factor leading to decreased expression of the nearby genes, if they are located in the proximity of key transcription factor binding sites. On the other hand, CpG position 7 is located in intron 1. In our study, 100 μg/ml MWCNT decreased *CYP1B1* gene expression compared to the control, while it increased DNA methylation in the CpG 7 region, compared to the control. In a study where they evaluated the relationship between methylation of CpG sites located in different regions and gene expression, they found out that the gene expression was inversely correlated with DNA methylation levels in the first intron (Anastasiadi, Esteve-Codina, & Piferrer, 2018).

Other than the most upregulated genes *GCLC* and *CYP1B1*, we also checked for the expression of Mucin genes *MUC5B* and *MUC5AC*, which are the major mucins produced in the airways, therefore involved in COPD pathogenesis (Ridley & Thornton, 2018).

As mentioned before in the previous chapters, chronic obstructive pulmonary disease is a heterogenous disease characterized by chronic bronchitis and emphysema. Airway mucus hypersecretion is one of the important pathological features of chronic obstructive pulmonary disease. Inhaled environmental pollutants have a major role here. In response to these inhaled pollutants abnormalities is mucin production been seen in some studies (Hogg, Paré, & Hackett, 2017). In a recently published study, it is suggested that MUC5AC hyperconcentration in the airways might represent an important pathobiological element of COPD progression (Radicioni et al., 2021). In another study, they found out that when exposed to excessive, long-term stimulation of pollutants, submucosal glands secrete MUC5B to promote the expression of STAT6 (Signal transducer and activator of transcription 6) and SPDEF (SAM Pointed Domain Containing ETS Transcription Factor), which leads to goblet cell differentiation and mucus production (X. Huang et al., 2022). These studies show the importance of mucin genes on the disease pathogenesis. Here we checked if DEP, TiO_2 and MWCNT would affect the mRNA expression of MUC5B and MUC5AC. We saw that three of the particles induced the mRNA expression of *MUC5B* gene. On the other hand, DEP and MWCNT had no effect on *MUC5AC* gene expression. However, similar to its effect on MUC5B

mRNA expression, 50 µg/mL TiO_2 increased MUC5AC mRNA expression at 6 hours. At 48 hours, 10 and 100 µg/mL TiO_2 caused a significant decrease in the gene expression. When compared to 24 hours, 10 and 100 µg/mL TiO_2 at 48 hours also caused a significant decrease. 10 µg/mL TiO_2 at 48 hours also decreased the gene expression compared to 6 hours. What we can interpret from here is that, at early exposure time (6 hours), inhaled TiO_2 particles might lead to transcriptional activation leading to increased expression of *MUC5AC* and *MUC5B* gene, whereas as the incubation time with particles increases, this effect reverses and results in decreased mRNA expression.

We have also found that the mRNA expression of FAM13A was affected by particle exposure too. *FAM13A* is a susceptibility gene which is oftenly expressed in airway epithelium. It was shown to be involved in emphysema susceptibility, regulation of mitochondrial function (Hawkins & Mora, 2017) and β-catenin stability (Jiang et al., 2016). However, it is still not is not understood how altered expression or function contributes to the development of COPD. In a study they found out that, *FAM13A* levels were significantly decreased upon 24-h exposure to 20% CSE in COPD-derived primary human AECs, but not in control-derived AECs (Qing Chen et al., 2021). When we checked the effect of TiO_2 on FAM13A mRNA expression, we saw that 100 µg/mL TiO_2 incubation led to significant decrease in the mRNA expression, at 48 hours. TiO_2 is applied to the cigarette filter material as a whitening agent. Therefore, the similar effect we observed here might be relatable with this. Oppositely, MWCNT significantly increased FAM13A mRNA expression. DEP showed no effect on the mRNA expression of FAM13A.

SERPINA1 is another gene known to be associated with COPD pathogenesis (Repapi et al., 2010). We found out that DEP and TiO_2 induce the gene expression at different time points, whereas MWCNT had no effect on the mRNA expression of SERPINA1.

Although particles caused transcriptional changes in MUC5B, MUC5AC, FAM13A and SERPINA1, they were not consistent as we saw in *GCLC* and *CYP1B1* gene expressions. Therefore, they were not chosen for DNA methylation analysis.

We are aware of the fact that there were some limitations of our study. The biggest limitation that we faced was that we weren't be able to repeat these experiments in primary epithelial cells. Since this was not a long term project, we performed these experiments on bronchial epithelial cell line (BEAS-2B). Even though it is a generally

preferred cell line to study the effects of inhaled pollutants on molecular mechanisms, it would have better to use primary epithelial cell cultures derived from COPD patients since it would give us more idea about the in vivo effects of these particles. Limited time also prevented me from performing supplementary experiments such as checking the mRNA expressions of DNA methyltransferases together with their activity. Other than these limitations, for the budget reasons we only evaluated limited number of CpG sites. More CpG sites need to be evaluated to have a clearer idea on the role of these particles in DNA methylation related to COPD pathogenesis.

Even though the limitations we faced during this study is of great importance, we believe that our results will have an important contribution to the literature, since we checked the effect of DEP and NP (TiO_2 and MWCNT) on the mRNA expression of COPD-related genes, dose and time dependently. Other than that, to our knowledge, this was the first study to ever check the DNA methylation status of *GCLC* and *CYP1B1* genes. Using three different pollutants (DEP, TiO_2, MWCNT) in three different concentrations (10,50,100 µg/mL) and for three incubation times (6, 24 and 48 hours), our results gives us a well-rounded idea about the effects of these inhaled particles.

To sum it up, particles did not cause a reduction in cell viability greater than 50%, except 100 µg/mL DEP at T48 hours of incubation. DEP and NP altered mRNA expression for *GCLC* and *CYP1B1* genes, which are both involved in oxidative stress mechanisms. There were no significant changes in methylation in the promoter regions of the genes. However, DNA methylation changes after DEP and MWCNT exposure were observed in CpG sites located on exon 1 and intron 1.

Our findings suggest that DEP and MWCNT may play a role in the pathogenesis of COPD by altering mRNA expression and DNA methylation of *CYP1B1* and *GCLC* genes.

For future directions, the limitations we mentioned above should and will be taken into consideration. In some exposure groups, mRNA expressions of MUC5B, MUC5AC, FAM13A and SERPINA1 was also induced. The DNA methylation status of these genes are also planned to check in our further studies. Here in this study, we only checked the mRNA expression of the genes but for further studies, their protein levels in the cells should also be checked to see if there was a correlation between them. DNA methylation is catalyzed by DNA methyltransferases. To establish whether the observed changes in DNA methylation are accompanied by persistent changes in expression of the known DNMT genes (*DNMT1, DNMT3A, DNMT3B),* analysing mRNA levels of these genes from the particle exposed and non-exposed (control) cells is another important approach needed to be kept in mind, as well as checking their activity. On the other hand, since DNA methylation is just one regulatory epigenetic mechanism, further studies are clearly needed in order to investigate the effect of these particles on other epigenetic mechanisms such as histon modifications.

BIBLIOGRAPHY

Adeloye, D., Chua, S., Lee, C., Basquill, C., Papana, A., Theodoratou, E., . . . Campbell, H. (2015). Global and regional estimates of COPD prevalence: Systematic review and meta–analysis. *Journal of global health, 5*(2).

Agustí, A., & Hogg, J. C. (2019). Update on the pathogenesis of chronic obstructive pulmonary disease. *New England Journal of Medicine, 381*(13), 1248-1256.

Albano, G. D., Montalbano, A. M., Gagliardo, R., Anzalone, G., & Profita, M. (2022). Impact of Air Pollution in Airway Diseases: Role of the Epithelial Cells (Cell Models and Biomarkers). *International journal of molecular sciences, 23*(5), 2799.

Anastasiadi, D., Esteve-Codina, A., & Piferrer, F. (2018). Consistent inverse correlation between DNA methylation of the first intron and gene expression across tissues and species. *Epigenetics & chromatin, 11*(1), 1-17.

Applications of Multi-Walled Carbon Nanotubes. (2021). Retrieved from https://nanografi.com/blog/applications-of-multiwalled-carbon-nanotubes/

Asmatulu, R., Asmatulu, E., & Yourdkhani, A. (2009). *Toxicity of nanomaterials and recent developments in the protection methods.* Paper presented at the SAMPE Fall Technical Conference, Wichita.

Avci, E., Sarvari, P., Savai, R., Seeger, W., & Pullamsetti, S. S. (2022). Epigenetic Mechanisms in Parenchymal Lung Diseases: Bystanders or Therapeutic Targets? *International journal of molecular sciences, 23*(1), 546.

Barnes, P. J. (2019). Small airway fibrosis in COPD. *The International Journal of Biochemistry & Cell Biology, 116*, 105598.

Barnes, P. J. (2020). Oxidative stress-based therapeutics in COPD. *Redox biology, 33*, 101544.

Bayram, H., Devalia, J. L., Khair, O. A., Abdelaziz, M. M., Sapsford, R. J., Sagai, M., & Davies, R. J. (1998). Comparison of ciliary activity and inflammatory mediator release from bronchial epithelial cells of nonatopic nonasthmatic subjects and atopic asthmatic patients and the effect of diesel exhaust particles in vitro. *Journal of Allergy and Clinical Immunology, 102*(5), 771-782.

Bayram, H., Devalia, J. L., Sapsford, R. J., Ohtoshi, T., Miyabara, Y., Sagai, M., & Davies, R. J. (1998). The effect of diesel exhaust particles on cell function and

release of inflammatory mediators from human bronchial epithelial cells in vitro. *American journal of respiratory cell and molecular biology, 18*(3), 441-448.

Bayram, H., Fakili, F., Gogebakan, B., Bayraktar, R., & Ekinci, E. (2010). Effects Of Diesel Exhaust Particles (DEP) On The Viability Of Human Airway Epithelial Cells Treated In Serum. In *A16. HEALTH EFFECTS ASSOCIATED WITH DIESEL EXPOSURE* (pp. A1034-A1034): American Thoracic Society.

Bayram, H., Ito, K., Issa, R., Ito, M., Sukkar, M., & Chung, K. F. (2006). Regulation of human lung epithelial cell numbers by diesel exhaust particles. *European Respiratory Journal, 27*(4), 705-713.

Berger, S. L., Kouzarides, T., Shiekhattar, R., & Shilatifard, A. (2009). An operational definition of epigenetics. *Genes & development, 23*(7), 781-783.

Blanco, J., Lafuente, D., Gómez, M., García, T., Domingo, J. L., & Sánchez, D. J. (2017). Polyvinyl pyrrolidone-coated silver nanoparticles in a human lung cancer cells: time-and dose-dependent influence over p53 and caspase-3 protein expression and epigenetic effects. *Archives of toxicology, 91*(2), 651-666.

Bonner, J. A., Harari, P. M., Giralt, J., Cohen, R. B., Jones, C. U., Sur, R. K., . . . Zhu, J. (2010). Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *The lancet oncology, 11*(1), 21-28.

Caramori, G., Di Gregorio, C., Carlstedt, I., Casolari, P., Guzzinati, I., Adcock, I., . . . Chung, K. (2004). Mucin expression in peripheral airways of patients with chronic obstructive pulmonary disease. *Histopathology, 45*(5), 477-484.

Card, J. W., Zeldin, D. C., Bonner, J. C., & Nestmann, E. R. (2008). Pulmonary applications and toxicity of engineered nanoparticles. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 295*(3), L400-L411.

Centers for Disease Control and Prevention. (2021). Air Quality. Retrieved from https://www.cdc.gov/air/pollutants.htm

Centers for Disease Control and Prevention. (2022). Genomics & Precision Health. Retrieved from https://www.cdc.gov/genomics/disease/epigenetics.htm

Chen, Q., De Vries, M., Boezen, M., & Heijink, I. (2020). The role of the COPD susceptibility gene FAM13A in barrier function and pro-inflammatory responses of human airway epithelial cells. In: Eur Respiratory Soc.

Chen, Q., de Vries, M., Nwozor, K. O., Noordhoek, J. A., Brandsma, C.-A., Boezen, H. M., & Heijink, I. H. (2021). A protective role of fam13a in human airway

epithelial cells upon exposure to cigarette smoke extract. *Frontiers in physiology*, 819.

Chen, X., Yan, F., Lin, X., Shi, L., Wang, X., & Zeng, Y. (2020). DNA methylation in chronic obstructive pulmonary disease. *Single-cell Sequencing and Methylation*, 83-98.

Chen, Z.-H., Wu, Y.-F., Wang, P.-L., Wu, Y.-P., Li, Z.-Y., Zhao, Y., . . . Mao, Y.-Y. (2016). Autophagy is essential for ultrafine particle-induced inflammation and mucus hyperproduction in airway epithelium. *Autophagy, 12*(2), 297-311.

Cheng, L., Liu, J., Li, B., Liu, S., Li, X., & Tu, H. (2016). Cigarette smoke-induced hypermethylation of the GCLC gene is associated with COPD. *Chest, 149*(2), 474-482.

Clifford, R. L., Jones, M. J., MacIsaac, J. L., McEwen, L. M., Goodman, S. J., Mostafavi, S., . . . Carlsten, C. (2017). Inhalation of diesel exhaust and allergen alters human bronchial epithelium DNA methylation. *Journal of Allergy and Clinical Immunology, 139*(1), 112-121.

Devóz, P. P., Dos Reis, M. B., Gomes, W. R., Maraslis, F. T., Ribeiro, D. L., Antunes, L. M. G., . . . Barbosa Jr, F. (2021). Adaptive epigenetic response of glutathione (GSH)-related genes against lead (Pb)-induced toxicity, in individuals chronically exposed to the metal. *Chemosphere, 269*, 128758.

Dianat, M., Radan, M., Badavi, M., Mard, S. A., Bayati, V., & Ahmadizadeh, M. (2018). Crocin attenuates cigarette smoke-induced lung injury and cardiac dysfunction by anti-oxidative effects: the role of Nrf2 antioxidant system in preventing oxidative stress. *Respiratory research, 19*(1), 1-20.

Duan, R.-R., Hao, K., & Yang, T. (2020). Air pollution and chronic obstructive pulmonary disease. *Chronic diseases and translational medicine, 6*(04), 260-269.

Falero-Perez, J., Song, Y.-S., Sorenson, C. M., & Sheibani, N. (2018). CYP1B1: A key regulator of redox homeostasis. *Trends in cell & molecular biology, 13*, 27.

Franklin, C. C., Backos, D. S., Mohar, I., White, C. C., Forman, H. J., & Kavanagh, T. J. (2009). Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. *Molecular aspects of medicine, 30*(1-2), 86-98.

Frias, D. P., Gomes, R. L. N., Yoshizaki, K., Carvalho-Oliveira, R., Matsuda, M., Junqueira, M. d. S., . . . Conceição, P. R. d. (2020). Nrf2 positively regulates

autophagy antioxidant response in human bronchial epithelial cells exposed to diesel exhaust particles. *Scientific reports, 10*(1), 1-13.

Global Initiative for Chronic Obstructive Lung Disease. *Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease 2022 report*. Retrieved from

Gros, C., Fahy, J., Halby, L., Dufau, I., Erdmann, A., Gregoire, J.-M., . . . Arimondo, P. B. (2012). DNA methylation inhibitors in cancer: recent and future approaches. *Biochimie, 94*(11), 2280-2296.

Gruzieva, O., Xu, C.-J., Yousefi, P., Relton, C., Merid, S. K., Breton, C. V., . . . Ladd-Acosta, C. (2019). Prenatal particulate air pollution and DNA methylation in newborns: an epigenome-wide meta-analysis. *Environmental health perspectives, 127*(5), 057012.

Ha, M.-H., Ham, S.-Y., Lee, D.-H., & Choi, J. (2007). In vitro toxicity assay using human bronchial epithelial cell, Beas-2B, for the screening of toxicological risk of dioxin-like compounds sampled from small sized Korean waste incineration plants. *Chemosphere, 70*(1), 20-28.

Hancock, D. B., Eijgelsheim, M., Wilk, J. B., Gharib, S. A., Loehr, L. R., Marciante, K. D., . . . Barr, R. G. (2010). Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. *Nature genetics, 42*(1), 45-52.

Hao, M., Comier, S., Wang, M., Lee, J. J., & Nel, A. (2003). Diesel exhaust particles exert acute effects on airway inflammation and function in murine allergen provocation models. *Journal of Allergy and Clinical Immunology, 112*(5), 905-914.

Hawkins, G. A., & Mora, A. L. (2017). FAM13A, a fatty acid oxidation switch in mitochondria. friend or foe in chronic obstructive pulmonary disease pathogenesis? In (Vol. 56, pp. 689-691): American Thoracic Society.

Hogg, J. C., Paré, P. D., & Hackett, T.-L. (2017). The contribution of small airway obstruction to the pathogenesis of chronic obstructive pulmonary disease. *Physiological reviews*.

Hong, F., Zhou, Y., Zhou, Y., & Wang, L. (2017). Immunotoxic effects of thymus in mice following exposure to nanoparticulate TiO2. *Environmental toxicology, 32*(10), 2234-2243.

Huang, X., Guan, W., Xiang, B., Wang, W., Xie, Y., & Zheng, J. (2022). MUC5B regulates goblet cell differentiation and reduces inflammation in a murine COPD model. *Respiratory research, 23*(1), 1-12.

Huang, Y.-T., Chen, C.-C., Ho, Y.-N., Tsai, M.-T., Tsai, C.-M., Chuang, P.-C., & Cheng, F.-J. (2021). Short-term effects of particulate matter and its constituents on emergency room visits for chronic obstructive pulmonary disease: A time-stratified case-crossover study in an urban area. *International Journal of Environmental Research and Public Health, 18*(9), 4400.

Jang, H. S., Shin, W. J., Lee, J. E., & Do, J. T. (2017). CpG and non-CpG methylation in epigenetic gene regulation and brain function. *Genes, 8*(6), 148.

Jiang, Z., Lao, T., Qiu, W., Polverino, F., Gupta, K., Guo, F., . . . Castaldi, P. J. (2016). A chronic obstructive pulmonary disease susceptibility gene, FAM13A, regulates protein stability of β-catenin. *American journal of respiratory and critical care medicine, 194*(2), 185-197.

Jones, P. A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics, 13*(7), 484-492.

Kaur, M., Chandel, J., Malik, J., & Naura, A. S. (2022). Particulate matter in COPD pathogenesis: An overview. *Inflammation Research*, 1-19.

Kim, B.-G., Lee, P.-H., Lee, S.-H., Kim, Y.-E., Shin, M.-Y., Kang, Y., . . . Park, C.-S. (2016). Long-term effects of diesel exhaust particles on airway inflammation and remodeling in a mouse model. *Allergy, asthma & immunology research, 8*(3), 246-256.

Kim, W. J., Lim, M. N., Hong, Y., Silverman, E. K., Lee, J.-H., Jung, B. H., . . . Park, Y. B. (2014). Association of lung function genes with chronic obstructive pulmonary disease. *Lung, 192*(4), 473-480.

Kiss, B., Bíró, T., Czifra, G., Tóth, B. I., Kertész, Z., Szikszai, Z., . . . Hunyadi, J. (2008). Investigation of micronized titanium dioxide penetration in human skin xenografts and its effect on cellular functions of human skin-derived cells. *Experimental dermatology, 17*(8), 659-667.

Kobayashi, N., Izumi, H., & Morimoto, Y. (2017). Review of toxicity studies of carbon nanotubes. *Journal of occupational health*, 17-0089-RA.

Kumar, C. S. (2006). *Nanomaterials: toxicity, health and environmental issues* (Vol. 5): St. Martin's Press.

Leclercq, B., Platel, A., Antherieu, S., Alleman, L., Hardy, E., Perdrix, E., . . . Happillon, M. (2017). Genetic and epigenetic alterations in normal and sensitive COPD-diseased human bronchial epithelial cells repeatedly exposed to air pollution-derived PM2. 5. *Environmental Pollution, 230*, 163-177.

Li, N., Georas, S., Alexis, N., Fritz, P., Xia, T., Williams, M. A., . . . Nel, A. (2016). A work group report on ultrafine particles (American Academy of Allergy, Asthma & Immunology): Why ambient ultrafine and engineered nanoparticles should receive special attention for possible adverse health outcomes in human subjects. *Journal of Allergy and Clinical Immunology, 138*(2), 386-396.

Li, N., Zhou, Y., Du, L., Wei, M., & Chen, X. (2011). Overview of Cytochrome P450 1B1 gene mutations in patients with primary congenital glaucoma. *Experimental eye research, 93*(5), 572-579.

Lin, X., Li, Y., Gong, L., Yun, J. H., Xu, S., Tesfaigzi, Y., . . . Zhou, X. (2021). Tempo-spatial regulation of the Wnt pathway by FAM13A modulates the stemness of alveolar epithelial progenitors. *EBioMedicine, 69*, 103463.

Lingaraju, K., Basavaraj, R., Jayanna, K., Bhavana, S., Devaraja, S., Swamy, H. K., . . . Naika, H. R. (2021). Biocompatible fabrication of TiO2 nanoparticles: Antimicrobial, anticoagulant, antiplatelet, direct hemolytic and cytotoxicity properties. *Inorganic Chemistry Communications, 127*, 108505.

Liu, J., Ballaney, M., Al-Alem, U., Quan, C., Jin, X., Perera, F., . . . Miller, R. L. (2008). Combined inhaled diesel exhaust particles and allergen exposure alter methylation of T helper genes and IgE production in vivo. *Toxicological Sciences, 102*(1), 76-81.

Ma, Y., Guo, Y., Ye, H., Huang, K., Lv, Z., & Ke, Y. (2019). Different effects of titanium dioxide nanoparticles instillation in young and adult mice on DNA methylation related with lung inflammation and fibrosis. *Ecotoxicology and Environmental Safety, 176*, 1-10.

MacNee, W. (2000). Oxidants/antioxidants and COPD. *Chest, 117*(5), 303S-317S.

McMahon, K. W., Karunasena, E., & Ahuja, N. (2017). The roles of DNA methylation in the stages of cancer. *Cancer journal (Sudbury, Mass.), 23*(5), 257.

Miravitlles, M., Dirksen, A., Ferrarotti, I., Koblizek, V., Lange, P., Mahadeva, R., . . . Roche, N. (2017). European Respiratory Society statement: diagnosis and treatment of pulmonary disease in α1-antitrypsin deficiency. *European Respiratory Journal, 50*(5).

Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology, 38*(1), 23-38.

Morrow, J. D., Glass, K., Cho, M. H., Hersh, C. P., Pinto-Plata, V., Celli, B., . . . Washko, G. (2018). Human lung DNA methylation quantitative trait loci colocalize with chronic obstructive pulmonary disease genome-wide association loci. *American journal of respiratory and critical care medicine, 197*(10), 1275-1284.

Na, H. G., Kim, Y.-D., Choi, Y. S., Bae, C. H., & Song, S.-Y. (2019). Diesel exhaust particles elevate MUC5AC and MUC5B expression via the TLR4-mediated activation of ERK1/2, p38 MAPK, and NF-κB signaling pathways in human airway epithelial cells. *Biochemical and biophysical research communications, 512*(1), 53-59.

Natarajan, K., Gottipati, K. R., Berhane, K., Samten, B., Pendurthi, U., & Boggaram, V. (2016). Proteases and oxidant stress control organic dust induction of inflammatory gene expression in lung epithelial cells. *Respiratory research, 17*(1), 1-19.

Ni, L., Chuang, C.-C., & Zuo, L. (2015). Fine particulate matter in acute exacerbation of COPD. *Frontiers in physiology, 6*, 294.

Oberdörster, G., Oberdörster, E., & Oberdörster, J. (2005). Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environmental health perspectives, 113*(7), 823-839.

Oliveira, T. M., & Morais, S. (2018). New generation of electrochemical sensors based on multi-walled carbon nanotubes. *Applied Sciences, 8*(10), 1925.

Öner, D., Ghosh, M., Coorens, R., Bové, H., Moisse, M., Lambrechts, D., . . . Hoet, P. H. (2020). Induction and recovery of CpG site specific methylation changes in human bronchial cells after long-term exposure to carbon nanotubes and asbestos. *Environment international, 137*, 105530.

Parris, B. A., O'Farrell, H. E., Fong, K. M., & Yang, I. A. (2019). Chronic obstructive pulmonary disease (COPD) and lung cancer: common pathways for pathogenesis. *Journal of thoracic disease, 11*(Suppl 17), S2155.

Patil, N. A., Gade, W., & Deobagkar, D. D. (2016). Epigenetic modulation upon exposure of lung fibroblasts to TiO2 and ZnO nanoparticles: alterations in DNA methylation. *International journal of nanomedicine, 11*, 4509.

Perzel Mandell, K. A., Eagles, N. J., Wilton, R., Price, A. J., Semick, S. A., Collado-Torres, L., . . . Szalay, A. S. (2021). Genome-wide sequencing-based

identification of methylation quantitative trait loci and their role in schizophrenia risk. *Nature communications, 12*(1), 1-12.

Phillips, G. J., James, S. L., & Lethem, M. I. (1997). Rheological properties and hydration of airway mucus. In *Airway Mucus: Basic Mechanisms and Clinical Perspectives* (pp. 117-147): Springer.

Pope, C. A., Dockery, D. W., & Schwartz, J. (1995). Review of epidemiological evidence of health effects of particulate air pollution. *Inhalation toxicology, 7*(1), 1-18.

Qiu, W., Baccarelli, A., Carey, V. J., Boutaoui, N., Bacherman, H., Klanderman, B., . . . Lomas, D. A. (2012). Variable DNA methylation is associated with chronic obstructive pulmonary disease and lung function. *American journal of respiratory and critical care medicine, 185*(4), 373-381.

Radicioni, G., Ceppe, A., Ford, A. A., Alexis, N. E., Barr, R. G., Bleecker, E. R., . . . Hansel, N. N. (2021). Airway mucin MUC5AC and MUC5B concentrations and the initiation and progression of chronic obstructive pulmonary disease: an analysis of the SPIROMICS cohort. *The Lancet Respiratory Medicine, 9*(11), 1241-1254.

Ramakrishnan, S., Mahawer, S. K., Prasad, M., Chaudhary, M., Kumar, A., & Govindasamy, P. (2021). Nanomaterials in integrated methods for soil remediation (biological/physiological combination processes). In *Nanomaterials for Soil Remediation* (pp. 445-462): Elsevier.

Reddel, R. R., Ke, Y., Gerwin, B. I., McMenamin, M. G., Lechner, J. F., Su, R. T., . . . Harris, C. C. (1988). Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer research, 48*(7), 1904-1909.

Repapi, E., Sayers, I., Wain, L. V., Burton, P. R., Johnson, T., Obeidat, M. e., . . . Vitart, V. (2010). Genome-wide association study identifies five loci associated with lung function. *Nature genetics, 42*(1), 36-44.

Rice, M. B., Ljungman, P. L., Wilker, E. H., Dorans, K. S., Gold, D. R., Schwartz, J., . . . Mittleman, M. A. (2015). Long-term exposure to traffic emissions and fine particulate matter and lung function decline in the Framingham heart study. *American journal of respiratory and critical care medicine, 191*(6), 656-664.

Ridley, C., & Thornton, D. J. (2018). Mucins: the frontline defence of the lung. *Biochemical Society Transactions, 46*(5), 1099-1106.

Rivas-Santiago, C. E., Sarkar, S., Cantarella IV, P., Osornio-Vargas, Á., Quintana-Belmares, R., Meng, Q., . . . Watson, J. G. (2015). Air pollution particulate matter alters antimycobacterial respiratory epithelium innate immunity. *Infection and immunity, 83*(6), 2507-2517.

Rotondo, J. C., Aquila, G., Oton-Gonzalez, L., Selvatici, R., Rizzo, P., De Mattei, M., . . . Martini, F. (2021). Methylation of SERPINA1 gene promoter may predict chronic obstructive pulmonary disease in patients affected by acute coronary syndrome. *Clinical Epigenetics, 13*(1), 1-11.

Sadik, O. A. (2013). Anthropogenic nanoparticles in the environment. *Environmental Science: Processes & Impacts, 15*(1), 19-20.

Sarkar, S., Song, Y., Sarkar, S., Kipen, H. M., Laumbach, R. J., Zhang, J., . . . Schwander, S. (2012). Suppression of the NF-κB pathway by diesel exhaust particles impairs human antimycobacterial immunity. *The Journal of Immunology, 188*(6), 2778-2793.

Schmidl, C., Delacher, M., Huehn, J., & Feuerer, M. (2018). Epigenetic mechanisms regulating T-cell responses. *Journal of Allergy and Clinical Immunology, 142*(3), 728-743.

Service, R. F. (2005). Calls rise for more research on toxicology of nanomaterials. *Science, 310*(5754), 1609-1609.

Shen, Y., Huang, S., Kang, J., Lin, J., Lai, K., Sun, Y., . . . Cai, S. (2018). Management of airway mucus hypersecretion in chronic airway inflammatory disease: Chinese expert consensus (English edition). *International Journal of Chronic Obstructive Pulmonary Disease, 13*, 399.

Sood, A., Petersen, H., Blanchette, C. M., Meek, P., Picchi, M. A., Belinsky, S. A., & Tesfaigzi, Y. (2010). Wood smoke exposure and gene promoter methylation are associated with increased risk for COPD in smokers. *American journal of respiratory and critical care medicine, 182*(9), 1098-1104.

Sooklert, K., Nilyai, S., Rojanathanes, R., Jindatip, D., Sae-Liang, N., Kitkumthorn, N., . . . Sereemaspun, A. (2019). N-acetylcysteine reverses the decrease of DNA methylation status caused by engineered gold, silicon, and chitosan nanoparticles. *International journal of nanomedicine, 14*, 4573.

Sowers, M. R., Wilson, A. L., Kardia, S. R., Chu, J., & McConnell, D. S. (2006). CYP1A1 and CYP1B1 polymorphisms and their association with estradiol and estrogen

metabolites in women who are premenopausal and perimenopausal. *The American journal of medicine, 119*(9), S44-S51.

Stockley, R. (1999). Neutrophils and protease/antiprotease imbalance. *American journal of respiratory and critical care medicine, 160*(supplement_1), S49-S52.

Sundar, I. K., & Rahman, I. (2016). Gene expression profiling of epigenetic chromatin modification enzymes and histone marks by cigarette smoke: implications for COPD and lung cancer. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 311*(6), L1245-L1258.

Syed, A., Hew, K., Kohli, A., Knowlton, G., & Nadeau, K. C. (2013). Air pollution and epigenetics. *Journal of Environmental Protection, 4*(08), 114.

Thimmulappa, R. K., Chattopadhyay, I., & Rajasekaran, S. (2020). Oxidative stress mechanisms in the pathogenesis of environmental lung diseases. In *Oxidative Stress in Lung Diseases* (pp. 103-137): Springer.

Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P., & Schiestl, R. H. (2009). Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. *Cancer research, 69*(22), 8784-8789.

US Environmental Protection Agency. (2022a). EPA Region 1. Retrieved from https://www3.epa.gov/region1/eco/uep/particulatematter.html#:~:text=EPA%20groups%20particle%20pollution%20into,micrometers%20in%20diameter%20and%20smaller

US Environmental Protection Agency. (2022b). Particulate Matter (PM) Pollution. Retrieved from https://www.epa.gov/pm-pollution/particulate-matter-pm-basics#PM

Vallabani, N., Mittal, S., Shukla, R. K., Pandey, A. K., Dhakate, S. R., Pasricha, R., & Dhawan, A. (2011). Toxicity of graphene in normal human lung cells (BEAS-2B). *Journal of biomedical nanotechnology, 7*(1), 106-107.

Vergaro, V., Aldieri, E., Fenoglio, I., Marucco, A., Carlucci, C., & Ciccarella, G. (2016). Surface reactivity and in vitro toxicity on human bronchial epithelial cells (BEAS-2B) of nanomaterials intermediates of the production of titania-based composites. *Toxicology in Vitro, 34*, 171-178.

Vogel, C. F., Van Winkle, L. S., Esser, C., & Haarmann-Stemmann, T. (2020). The aryl hydrocarbon receptor as a target of environmental stressors–Implications for pollution mediated stress and inflammatory responses. *Redox biology, 34*, 101530.

Voynow, J. A., & Shinbashi, M. (2021). Neutrophil elastase and chronic lung disease. *Biomolecules, 11*(8), 1065.

Vrba, L., & Futscher, B. W. (2018). A suite of DNA methylation markers that can detect most common human cancers. *Epigenetics, 13*(1), 61-72.

Vucic, E. A., Chari, R., Thu, K. L., Wilson, I. M., Cotton, A. M., Kennett, J. Y., . . . Brown, C. J. (2014). DNA methylation is globally disrupted and associated with expression changes in chronic obstructive pulmonary disease small airways. *American journal of respiratory cell and molecular biology, 50*(5), 912-922.

Wan, E. S., Qiu, W., Carey, V. J., Morrow, J., Bacherman, H., Foreman, M. G., . . . DeMeo, D. L. (2015). Smoking-associated site-specific differential methylation in buccal mucosa in the COPDGene study. *American journal of respiratory cell and molecular biology, 53*(2), 246-254.

Wang, Y., Sauvat, A., Lacrouts, C., Lebeau, J., Grall, R., Hullo, M., . . . Chevillard, S. (2020). TiO2 nanomaterials non-controlled contamination could be hazardous for normal cells located in the field of radiotherapy. *International journal of molecular sciences, 21*(3), 940.

Wichmann, H.-E. (2007). Diesel exhaust particles. *Inhalation toxicology, 19*(sup1), 241-244.

World Health Organization. (2022a). Air pollution. Retrieved from https://www.who.int/health-topics/air-pollution#tab=tab_1

World Health Organization. (2022b). Chronic obstructive pulmonary disease (COPD). Retrieved from https://www.who.int/news-room/fact-sheets/detail/chronic-obstructive-pulmonary-disease-(copd)#:~:text=Chronic%20obstructive%20pulmonary%20disease%20(COPD)%20is%20the%20third%20leading%20cause,3.23%20million%20deaths%20in%202019.

Wrobel, A., Rokita, E., & Maenhaut, W. (2000). Transport of traffic-related aerosols in urban areas. *Science of the Total Environment, 257*(2-3), 199-211.

Xing, Y.-F., Xu, Y.-H., Shi, M.-H., & Lian, Y.-X. (2016). The impact of PM2. 5 on the human respiratory system. *Journal of thoracic disease, 8*(1), E69.

Yamada, N., Nishida, Y., Yokoyama, S., Tsutsumida, H., Houjou, I., Kitamoto, S., . . . Yonezawa, S. (2010). Expression of MUC5AC, an early marker of pancreatobiliary cancer, is regulated by DNA methylation in the distal promoter

region in cancer cells. *Journal of hepato-biliary-pancreatic sciences, 17*(6), 844-854.

Yang, D., Xu, D., Wang, T., Yuan, Z., Liu, L., Shen, Y., & Wen, F. (2021). Mitoquinone ameliorates cigarette smoke-induced airway inflammation and mucus hypersecretion in mice. *International Immunopharmacology, 90*, 107149.

Yang, D., Yan, Y., Hu, F., & Wang, T. (2020). CYP1B1, VEGFA, BCL2, and CDKN1A affect the development of chronic obstructive pulmonary disease. *International Journal of Chronic Obstructive Pulmonary Disease, 15*, 167.

Yang, I. A., Jenkins, C. R., & Salvi, S. S. (2022). Chronic obstructive pulmonary disease in never-smokers: risk factors, pathogenesis, and implications for prevention and treatment. *The Lancet Respiratory Medicine*.

Yang, X., Han, H., De Carvalho, D. D., Lay, F. D., Jones, P. A., & Liang, G. (2014). Gene body methylation can alter gene expression and is a therapeutic target in cancer. *Cancer cell, 26*(4), 577-590.

Young, H. W., Williams, O. W., Chandra, D., Bellinghausen, L. K., Pérez, G., Suárez, A., . . . Moghaddam, S. J. (2007). Central role of Muc5ac expression in mucous metaplasia and its regulation by conserved 5′ elements. *American journal of respiratory cell and molecular biology, 37*(3), 273-290.

Zarcone, M. C., Duistermaat, E., van Schadewijk, A., Jedynska, A., Hiemstra, P. S., & Kooter, I. M. (2016). Cellular response of mucociliary differentiated primary bronchial epithelial cells to diesel exhaust. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 311*(1), L111-L123.

Zhang, H., Lang, Z., & Zhu, J.-K. (2018). Dynamics and function of DNA methylation in plants. *Nature reviews Molecular cell biology, 19*(8), 489-506.

Zheng, X., Wang, G., Bin, P., Meng, T., Niu, Y., Yang, M., . . . Dai, Y. (2019). Time-course effects of antioxidants and phase II enzymes on diesel exhaust particles-induced oxidative damage in the mouse lung. *Toxicology and Applied Pharmacology, 366*, 25-34.

Zhu, J., Wang, F., Feng, X., Li, B., Ma, L., & Zhang, J. (2021). Family with sequence similarity 13 member A mediates TGF-β1-induced EMT in small airway epithelium of patients with chronic obstructive pulmonary disease. *Respiratory research, 22*(1), 1-13.

Ziental, D., Czarczynska-Goslinska, B., Mlynarczyk, D. T., Glowacka-Sobotta, A., Stanisz, B., Goslinski, T., & Sobotta, L. (2020). Titanium dioxide nanoparticles: prospects and applications in medicine. *Nanomaterials, 10*(2), 387.

Zou, Y., Li, Q., Jiang, L., Guo, C., Li, Y., Yu, Y., . . . Sun, Z. (2016). DNA hypermethylation of CREB3L1 and Bcl-2 associated with the mitochondrial-mediated apoptosis via PI3K/Akt pathway in human BEAS-2B cells exposure to silica nanoparticles. *PloS one, 11*(6), e0158475.

APPENDIX A: DESIGNED REPORTS FOR PYRO-PRIMERS

Table A.1: PYRO-primer sequences.

GCLC.1-2			Score: 82 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	F1	AGTAAGGGTAAGAAATATTAGATGTAGGG	29	61.6	34.5
PCR	R1	ACCAAATCTAACAAATAACTCATTATTCC	29	59.1	27.6
Sequencing	S1	TTAAATTTTGTGTTGAATTGTAA	23	43.3	17.4
Target Polymorphisms	Position1, Position2				
Sequence to Analyze	TATTYGGTGT TGGAGGTGGA GTTTGGTGGG AGGTGATTGG ATTATGYGGG TAGATTTTTT ATGAATGGTT TA				

GCLC.4-5			Score: 77 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	F1	TGTTAGATTTGGTAGTTAAAGAGTGT	26	59.0	30.8
PCR	R1	ATTAATCCACTCTCACATTACTATCA	26	58.1	30.8
Sequencing	S1	AGGTTATTTTAGAGTTAGAAG	21	44.5	28.6
Target Polymorphisms	Position4, Position5				
Sequence to Analyze	TYGTTATGTT TTTTGGATAG TTTGTAGAAT YGTGAGTTAA TTAAATTTTT TTTTTT				

CYP1B1.1			Score: 87 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	F1	TGGAGGTATTAAGTATAGAGTAAGTTATTT	30	57.9	26.7
PCR	R1	TCATAATTATAACTTTCCCCAACTCTTCA	29	59.8	31.0
Sequencing	S1	GAATAAATATAGTTTATGTAGAATG	25	42.5	20.0
Target Polymorphisms	Position1				
Sequence to Analyze	TYGTTAATGT TTTAATATTT TTTTTAGT				

CYP1B1.3			Score: 82 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	F1	AGGTATGTGGTAATGTTTTTAAAGATATG	29	59.3	27.6
PCR	R1	CTAACATAAACAACCTCCTTTCAT	24	59.0	33.3
Sequencing	S1	TTTTAAAGATATGTTTTGGTAGT	23	44.6	21.7
Target Polymorphisms	Position3				
Sequence to Analyze	TAAGAATGTA YGGGTTTTTA TAAAGAGATT AATAATA				

CYP1B1.5			Score: 81 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	F1	AAGAAAAGGGTAAGGGAGAATAT	23	58.1	34.8
PCR	R1	TCTAACATAAACAACCTCCTTTCAT	25	59.2	32.0
Sequencing	S1	TTTAGAAAATAGAAAGGATGG	21	44.3	28.6
Target Polymorphisms	Position5				
Sequence to Analyze	TAYGATTTTT TGATGAAAGG AGGTTGTTT				

CYP1B1.6-7			Score: 84 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	F1	AGGAGGTTGTTTATGTTAGAAGA	23	58.1	34.8
PCR	R1	CCCTACCAAACCAACACCCTTAAATTATT	29	58.5	37.9
Sequencing	S1	AAGAAGTTTAGATTTTTTATAGTG	24	44.0	20.8
Target Polymorphisms	Position6, Position7				
Sequence to Analyze	AYGAGGGGAG AATAAAAAAG TTGTAATATT AGGYGGGTAG TTTTAGTTTT TTTTTTTAG				

APPENDIX B: PYRO VALIDATION RESULTS OF CPG REGIONS

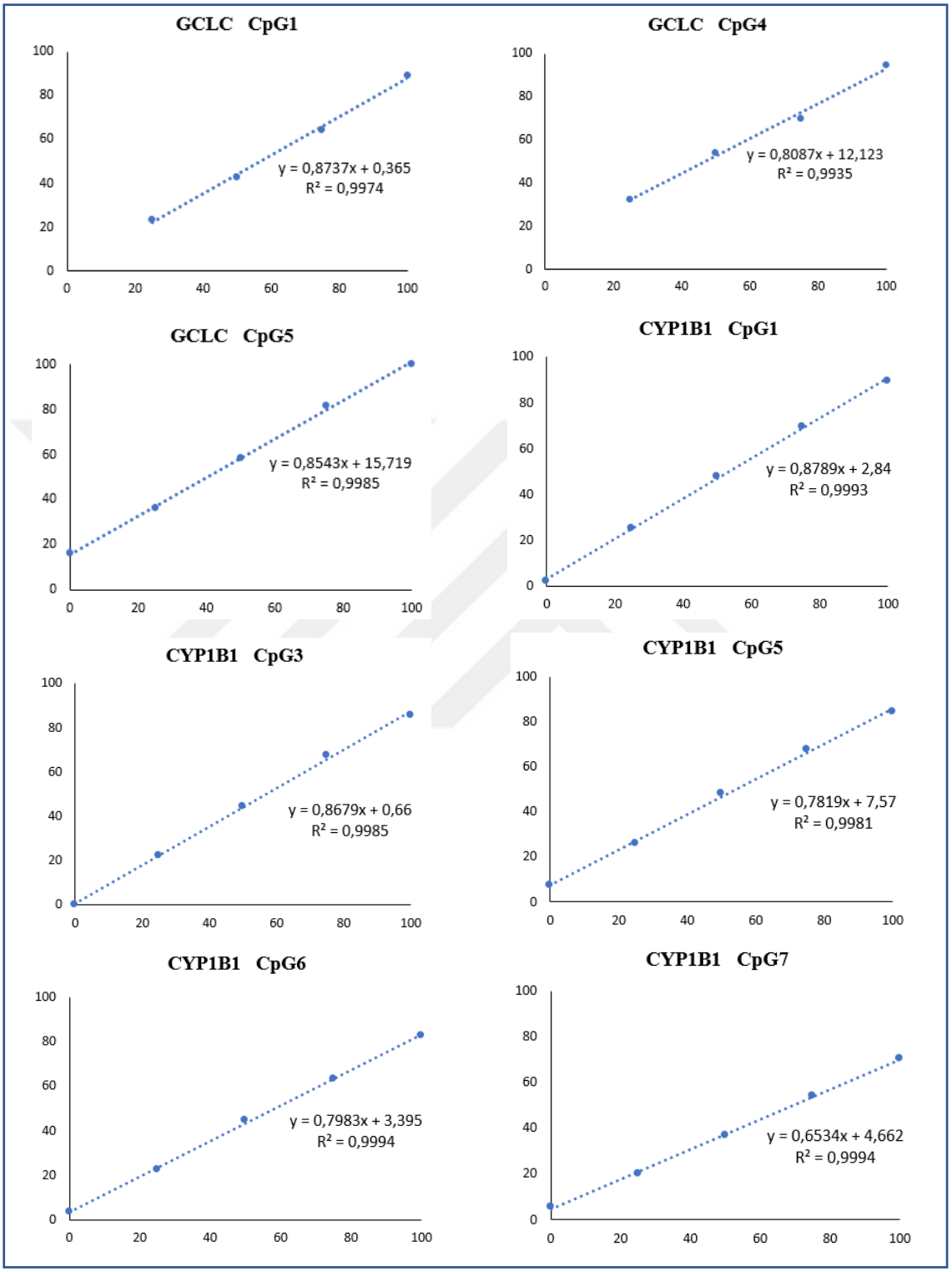

Figure B.1: Pyro validation results of CpG positions in GCLC and CYP1B1 genes.