Applying a new technique, the interferon gamma liposomal delivery system to improve drug delivery in the treatment of Lung Cancer

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Applying a new technique, the interferon gamma liposomal delivery system to improve drug delivery in the treatment of Lung Cancer

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Abstract

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Keywords: interferon gamma; naked and liposome forms; DNA damage; genotoxic; genoprotective, human lymphocytes; lung cancer; healthy individuals.

Lung cancer is one of the main causes of death worldwide, with most patients suffering from an advanced unresectable or metastatic non-small cell lung cancer. The mortality trends are mostly related to patterns of tobacco use, specifically from cigarettes. Tobacco is the basic etiological agent found as a consequence of the inhalation of tobacco smoke. Published data show the use of interferons (IFNs) in the treatment of lung tumours due to their potential in displaying antiproliferative, anti-angiogenic, immunoregulatory, and proapoptotic effects. Type1 IFNs have been employed as treatments for many types of cancer, both for haematological cancers and solid tumours. The IFN-γ (naked) functions as an anticancer agent against various forms of cancer. Hence, this study aimed to investigate the genoprotective and genotoxic effects of IFN-γ liposome (nano) on 42 blood samples from lung cancer patients, compared to the same sample size from healthy individuals. The effectiveness of IFN- γ liposome against oxidative stress was also evaluated in this study. A concentration of 100U/ml of IFN-γ liposome was used to treat the lymphocytes in: Comet and micronucleus assays, Comet repair, Western blotting and real-time polymerase chain reaction (qPCR) were based on a preliminary test for the optimal dose. The lymphocytes from lung cancer patients presented with higher DNA damage levels than those of healthy individuals. IFN-γ liposome was not found to induce

any DNA damage in the lymphocytes. Also, it caused a significant reduction in DNA damage in the lymphocytes from lung cancer patients in; Comet, Comet repair and micronucleus assays. Furthermore, the 100U/ml of IFN-γ liposome significantly reduced the oxidative stress caused by H_2O_2 and appeared to be effective in both groups using the Comet and micronucleus assays. Results from; Comet, Comet repair and micronucleus assays were consistent.

The data obtained indicated that IFN-γ in both forms (naked INF-γ and INF-γ nano-liposome) may potentially be effective for the treatment of lung cancer and showed the ability of IFN-γ liposome to reduce the DNA damage more than the naked form.

The IFN-γ in both forms has also shown anti-cancer potential in the lymphocytes from lung cancer patients by regulating the expression of p53, p21, Bcl-2 at mRNA and protein levels by up-regulating the p53 and p21 to mediate cell cycle arrest and DNA repair in lung cancer patients.

The findings of this study are consistent with the view that the naked IFN-γ and liposome could have a significant role in cancer treatment, including lung cancer.

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List of Abbreviations

1

1,2-dioleoyl-3-trimethylammoniopropane (DOTAP)
1,2-dioleoyl-sn-glycero-3-phsphoethanolamine (DOPE)
1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DPPG)
1640 Medium (RPMI-1640)

8

8-Hydroxydeoxyguanosine (8-OH-Gua)
8-Oxoguanine glycosylase (OGG1)

A

Acquired immune deficiency syndrome (AIDS)
Adenine (A)
Alkali labile sites (ALS)
All-trans retinoic acid (ATRA)
alpha-1 antitrypsin (AAT)
Antigen presenting cells (APCs)

B

Base excision repair (BER)
Binucleated (BiNC)
Bis(chloromethyl) ether (BCME)
Bovine serum albumin (BSA)
Bradford Royal Infirmary (BRI)

C

Cdkn1a (p21)
Cell counting kit 8 (CCK8)
Cholesteryl hemisuccinate (CHEMS)
Chronic obstructive pulmonary disease (COPD)
Cyclin dependent kinase inhibitor (CDKI)
Cyclin-dependentkinase 2 (CDK2)
Cytochalasin B (Cyto-B)
Cytochrome P-450 (*CYP*)
Cytokinesis block micronucleus assay (CBMN)
Cytosine (C)

D

Daunorubicin (DNR)
Deoxyribonucleic acid (DNA)
Dibutyl phthalate in xylene (DPX)
Dichloromethane (DCM)
Dimethyl sulfoxide (DMSO)
Dioleoylphosphatidylethanolamine (DOPE)
Distearoylphosphatidylethanolamine
DNA damage response (DDR)
DNA repair capacity (DRC)
Donkey Anti-Rabbit IgG H&L (HRP)
Double-strand breaks (DSBs)
Drug delivery systems (DDSs)
Dynamic light scattering (DLS)

E

Enhanced chemiluminescence (ECL)
Enhanced permeability and retention (EPR)
Environmental Tobacco Smoke (ETS)
Epidermal growth factor receptor (EGFR)
Escherichia coli (e-coli)
Ethidium bromide (EtBr)
Extensive stage (ES)

F

Fetal bovine serum (FBS)
Food and drug administration (FDA)
Formamidopyrimidine DNA glycosylase (FPG)

G

General Lifestyle Survey (GLS)
Giant unilamellar vesicles (GUV)
Glutathione S-transferase (GST)
Guanine (G)

H

Homologous recombination (HR)
Human 8-oxoguanin glycosylase (hOGG1)
Human epidermal growth factor receptor (HER)
Hydrogen peroxide (H_2O_2)
Hydroperoxyl radical (HO_2)
Hydroxyl radical ($OH^\bullet$)

I

Idiopathic pulmonary fibrosis (IPF)
IFN regulatory factor 1 (IRF-1)
IFN-γ-activated sequences (GAS)
Insertion/deletion loops (IDLs)
Interferons (INFs)
International Agency for Research on Cancer (IARC)
Union for International Cancer Control (UICC)
Interstitial lung disease (ILD)

L

Large unilamellar vesicles (LUV)
Limited stage (LS)
Low melting point agarose (LMP)

M

Major histocompatibility complex (MHC)
Micro gel electrophoresis (MGE)
Micronuclei (MNi)
Micronucleus FISH (Fluorescence in situ hybridisation)
Mismatch repair (MMR)
Mitogen-activated protein kinase (MAPK) pathway
Mitomycin C (MMC)
Mitoxantrone (MXT)
Mononucleated cells (MonoNC)
Multilamellar vesicles (MLV)
Multinucleated (MultiNC)
Multinucleated cells (MultiNC)
Multivesicular vesicles (MVV)

N

N-[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA)
N-acetyltransferase (NAT)
Natural killer (NK) cells
Negative control (NC)
NOT SIGNIFICANT (ns)
Non-Homologous end joining (NHEJ)
Non-small cell lung cancer (NSCLC)
Normal melting point agarose (NMP)
Nuclear buds (NBUDs)
Nuclear division index (NDI)
Nucleoplasmic bridges (NPBs)

Nucleotide excision repair (NER)

O

Oligolamellar vesicle (OLV)
Olive tail moment (OTM)
One-Way analysis of variance (ANOVA)

P

Particulate matter (PM)
Peripheral blood lymphocyte (PBL)
Pharmaceutical ingredients (APIs)
Phosphatidylinositol 3' kinase (PI3K)–AKT pathway
phosphoinositide 3-kinase (PI3K)/AKT
Phythaemagglutinin (PHA)
Polydispersity index (PDI)
Polymer polyethylene glycol (PEG)
Polymerase Chain Reaction (PCR)
Polyvinylidene fluoride (PVDF)
Positive control (PC)
Potassium chloride (KCl)
Proliferating cell nuclear antigen (PCNA)
Protein kinase C (PKC)
Proteins and peptides (PPs)

Q

Quantification cycle (Cq)
Quantitative reverse transcription PCR (qRT-PCR)

R

Reactive oxygen and nitrogen species (RONS)
Reactive oxygen species (ROS)
Real-time PCR (RT-PCR)
Retinoblastoma gene (RB1)
Reversed phase high-pressure liquid chromatography (RP-HPLC)
Roswell Park Memorial Institute (RPMI)

S

Signal transducer and activator of transcription 1
Single cell gel electrophoresis (SCGE)
Single strand breaks (SSBs)
Sister chromatin exchange (SCE)
Small cell lung cancer (SCLC)
Small unilamellar vesicles (SUV)

Sodium dodecyl sulphate-polyacrylamide gel (SDS- PAGE)
Standard error of the mean (S.E.M)
Strand breaks (SBs)
Superoxide ($O_2^{\bullet-}$)

T

T helper cell type 1 (Th1)
Technicalgrade chloromethyl methyl ether (CMME)
Thymine (T)
Translesion DNA synthesis (TLS)
Transmission electron microscopy (TEM)
Trifluoroacetic acid (TFA)
Tris buffered saline containing Tween 20 (TBS-T)
Tuberculosis (TB)
Tumour-node-metastasis (TNM)
Tyrosine kinase inhibitors (TKIs)

W

Western blot assay (WB)

CHAPTER ONE: Introduction

1 Introduction

1.1 Cancer

Cancer is considered an important cause of death worldwide and the second leading cause of death globally, which cause 9.6 million deaths in 2018 and more than 166.000 deaths in the UK a year (Cancer Research UK 2019b).

Cancer is a complex human genetic disease that causes a rapid transformation of normal cells into cancerous tumour cells, which invade healthy tissues damaging their normal function (Bray et al. 2018). Complex factors contribute to the development of cancer; studies have linked the disease to the growing population, ageing, and socioeconomic development (Bray et al. 2018).

The development of cancer is a multi-stage process, which occurs as a result of mutations. Such mutations emerge from errors in deoxyribonucleic acid (DNA) repair and replication. Defective repair of DNA damage caused by non-repair or misrepair of DNA modifications may contribute to an accumulation of successive mutations within a cell, which increases the risk of cancer (Liu et al. 2019). Notably, an alteration in any of three particular types of genes such as tumour suppressor genes, oncogenes, and stability genes , will cause tumourigenesis. The activation of oncogenes can occur from gene amplification, chromosomal translocations, or subtle intragenic mutations that influence the main residues to

regulate the gene output activity, whilst the opposite occurs in tumour-suppressor genes by genetic alterations. Mutations decrease the activity of the gene product. Mutations in tumour-suppressor genes and oncogenes work similarly at a physiological level, as they both enhance the neoplastic mechanisms by elevating the number of tumour cells. This is achieved through various processes; the induction of cell birth, the suppression of cell death; by arresting of the cell-cycle (Cameron and Neil 2004; Vogelstein and Kinzler 2004).

Stability genes enhance carcinogenesis differently when mutated. This includes mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes that are repairing for subtle mistakes made through normal DNA replication or exposure to mutagens (Vogelstein and Kinzler 2002).

One of the widely acknowledged properties of tumour cells is their ability to produce new vasculature to enhance the 'angiogenic switch' by increasing the factors that increase blood vessel formation and decreasing the molecules that inhibit blood vessel production (Weinberg and Hanahan 2000; Lugano et al. 2020). Further more the hallmarks of cancer includes the sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, activating invasion and metastasis. In an update published in 2011 ("Hallmarks of cancer: the next generation"), Weinberg and Hanahan proposed two new hallmarks: abnormal metabolic pathways and evasion of the immune system, and two enabling characteristics: genome instability, and inflammation (Hanahan and Weinberg 2011) .

1.1.1 Lung Cancer

The term lung cancer encompasses epithelial cancers in the mucosa of the bronchi, sometimes in the parenchyma of the lung, which exists in the trachea (windpipe), bronchi (airways) or lung air sacs (alveoli) (Travis 2002; Lumb 2016). Primary lung cancer originates from the lung cells, whereas a secondary origin occurs as a result of metastasise into the lungs from other organs such as colorectal cancer, prostate cancer, etc. (Stella et al. 2019). Lung cancer accounts for roughly thirteen percent of the annual cancer cases worldwide. It is additionally the second most common type of cancer in both males and females, accounting for 13% of all new cancer cases in the UK in 2017 (Parsons et al. 2010; Cancer Research UK 2019a). However, its incidence is greater in males than females (Ferlay et al. 2015).

Interestingly, while the rate of lung cancer in males in the UK has fallen by 47% between the years of 1975 and 2006, from 113 to 61 per 100,000, the rate in females has risen by 65% during the same period, from 23 to 38 per 100,000 (Riaz et al. 2012). In the UK, lung cancer is the leading reason of cancer death, with approximately 35 000 deaths a year. The diagnostic peaks for lung cancer are between the ages of 73 and 84 (Chivima 2015), and the overall 5-year survival rate is lower than 10%, largely due to the majority of patients presenting with a late-phase of the disease, a point when treatment has little impact on survival (Ali et al. 2015). Incidence rates are elevated in Europe and Northern America and are lowest in some areas of Africa (Ferlay et al. 2010).

1.1.1.1 Risk factors for lung cancer

The following sections focus on the factors that cause lung cancer.

1.1.1.1.1 Smoking

Cigarette smoking is the most significant factor for lung cancer (Koutsokera et al. 2013). Moreover, the repercussions of smoking have been associated with other cancers, including cancers of the oral cavity and pharynx, oesophageal cancer, stomach cancer and pancreatic cancer (Dela Cruz et al. 2011) . Tobacco smoke is a complex, reactive mixture containing an estimated 5,000 chemical compounds. Seventy-three of this compounds classified as carcinogenic and more than 20 compounds are lung carcinogens . The polycyclic aromatic hydrocarbons (PAHs) are the most toxic and carcinogenic compounds in tobacco smoke that cause DNA-damaging and a significant source of toxic chemicals mediated disease in humans (Talhout et al. 2011). Tobacco smoke has been associated with causing cancer by binding to DNA. It forms mutagenic DNA adducts causing genetic mutations, epigenetic changes and oxidative stress (Stewart and Wild 2014). The inactive carcinogenic chemicals in tobacco smoke are activated by P-450 cytochrome enzymes which causes them to bind directly to the DNA forming DNA adducts. Reactive metabolites are able to bind to macromolecules such as protein and DNA causing a disruption of their normal function. Some of these binding results in adverse health effect (Hecht 2012). The direct impact of tobacco smoke on DNA is evident through the presence of DNA adducts which causes DNA miscoding. During the DNA replication, if the DNA adducts remain unrepaired , this subsequently causes the DNA polymerase enzymes to process the adducts incorrectly and result in permanent mutation . Some mutations are directly linked to carcinogens in cigarettes and are observed in somatic mutations; for instance, O^6-methylguanine adducts causes G→A transitions. These mutations are associated with *KRAS* oncogene in lung cancer,

as well as the *TP53* gene; this is also observed in cigarette smoke-induced cancers. As a result of metabolic activated PAHs both *KRAS* and *TP53* mutations are observed in lung cancer, which demonstrates that DNA damage caused by tobacoo smoking (Centers for Disease et al. 2010; Tsay et al. 2018). The PAH-DNA adduct formation levels are higher in smokers than non-smokers (Kanwal et al. 2017). Cigarette smoking became prevalent in the United Kingdom within the early 20th century, with the uptake occurring about 20 years earlier in males than females. The trend in smoking peaked in the 1940s in males and in the late 1960s in females, steadily declining since then (Edwards 2004). In 2013, the rates of smoking estimated by the General Lifestyle Survey (GLS) among men and women in the UK were 20% and 19%, respectively (Ali et al. 2015). In the UK, lung cancer incidence in men was 70%, and 55% of lung cancer in women was due to tobacco smoking (O'Keeffe et al. 2018).

Studies have shown that cigarette smoking is the main risk factor in lung cancer patients, current smokers have a 15-fold increase in the risk of death from lung cancer than lifelong non-smokers (Doll et al. 2005). On the other hand, this risk decreases significantly in individuals who cease smoking before middle age (Peto 2000). There is a linear dose-response relationship between cigarette consumption and lung cancer mortality. People who smoke fewer cigarettes daily for a longer duration have an increased risk of lung cancer than those who smoke more cigarettes for a shorter term (Watanabe 2016). In addition to voluntary smoking, lung cancer can also be caused by second-hand smoke (passive smoking) (Kim et al. 2014). The risk of lung cancer is also elevated following exposure to Environmental Tobacco Smoke (ETS) (Sakai et al.); an estimated

14% to 15% of lung cancers, among persons who have never smoked, are caused by exposure to ETS (Parkin 2011).

The International Agency for Research on Cancer (IARC) concluded that passive smoking is carcinogenic to humans, with an increased risk of 30% for men and 20% for women within never smokers who are exposed to ETS from their partner (Kim et al. 2014; Ye et al. 2014). Children who were early exposure and known as a passive smoker could also be at high risk of developing lung cancer later in life. A previous study presented that 9 million American children aged less than 5 years suffer from passive smoking (Kanwal et al. 2017).

Passive smoking puts people at higher risk of having tobacco smoking related diseases such as lung cancer and may also increase the risk of other types of cancers such as larynx (voice box) and pharynx (upper throat) cancers (ASH 2017).

1.1.1.1.2 Occupational exposures

Exposure to occupational carcinogen plays an important role in increasing lung cancer, and the respiratory system is the most susceptible site of occupationally-acquired malignancy (Gustavsson et al. 2000; Uguen et al. 2017). The most commonly implicated occupational agent in lung cancer aetiology is asbestos, which increases lung cancer risk among smokers and non-smokers (Dela Cruz et al. 2011; Järvholm and Aström 2014).

Ten percent of lung cancer deaths in men and 5% deaths in women worldwide in 2000 were due to occupational factors and asbestos (Consonni et al. 2010). Other occupational and environmental agents that may increase the risk of lung cancer include Bis (chloromethyl) ether (BCME) and technical-grade

chloromethyl methyl ether (CMME) (Spyratos et al. 2013). Owing to their lung carcinogenicity, the use of these chemicals has been diminished substantially in the United States and worldwide. Sulfur mustard, or mustard gas, can cause occupational exposure in the military sector, which may occur during its storage and deliberate destruction. Further factors resulting in occupational exposure may include Coal-tar pitch, a soot carbonaceous by-product of the incomplete conversion of fossil fuel or other carbon-containing materials (e.g. paper, plastics), and diesel engine exhausts (Field and Withers 2012).

1.1.1.1.3 Radon gas

Radon is a colourless, odourless radioactive gas produced from the decay of naturally occurring uranium. After the uranium decay in soil and rock, the radon gas produced and seep up through the ground and spread into the air and dissolve in ground water and accumulate indoors in buildings as well as in underground mines (Demoury et al. 2013). The radon gas is considered the second main cause of lung cancer after smoking (Krstić 2017). The alpha radiations produced by solid radioactive products of radon known as radon progeny when inhaled into the lungs cause cellular DNA damage and, eventually, lung cancer (Gaskin et al. 2018). The risk of lung cancer associated with radon exposure is significantly higher for smokers than for never-smokers. It is estimated that more than 85% of radon-related lung cancer deaths are in smokers (Lantz et al. 2013).

1.1.1.1.4 Air pollution

Outdoor air pollution is a mixture of air pollution sources containing a variety of hazardous air pollutant, which produced from transportation, factories, agriculture and industrial burning of waste, and has been related to the elevation of lung cancer incidence and mortality (Raaschou-Nielsen et al. 2011; Cao and Chen 2019). Fine particulate matter (PM) increases the risk of global lung cancer mortality to 8% (Pope III et al. 2002). PM2.5, defined as particles less than 2.5μm in diameter suspended in the atmosphere, has caught much attention due to its ability to depositi deep into the respiratory tract and cause adverse health effects even at a low concentration level in the air (Fajersztajn et al. 2013). It includes a mixture of liquid and solid particles, such as, black carbon, metals, sulfate, nitrate, PAHs and automobile exhaust particles (Li et al. 2018).

IARC concluded through different studies a positive correlation between PM2.5 with lung cancer and classified as a Group I carcinogen (Loomis et al. 2013; Hamra et al. 2014).

A study suggested that some PM organic components have the potential to trigger oxidative damage to DNA, lipids, and proteins (Hanzalova et al. 2010). Furthermore, in another study, it was demonstrated that PM2.5 could lead to the release of ROS in the inflammatory response; this then causes for ROS-mediated DNA damage response to be activated. Organic extracts (mostly benzo(a)pyrene and c-PAH contents) from PM2.5 can lead to oxidative DNA damage in humans (Chu et al. 2015). The exposure of HBE cells to 200 and 500 μg/ml PM2.5, 970 and 492 gene alterations were detected in a study (Ding et al. 2014).

The *in vivo* study demonstrated that mice treated with PM2.5 reported a mutation in 57 genes (14 upregulated, and 43 downregulated). This suggests that these

mutations occur as a result to a possible exogenous stimulus, metabolic processing and an inflammatory immune pathway, possibly involving the MAPK signaling pathway. These studies provide a preliminary basis to direct further investigation of oncogene activation in lung cancer following PM2.5 exposure (Zhou et al. 2015). *p53* is an important regulatory gene and plays many roles in cell proliferation, DNA damage repair and apoptosis (Cai et al. 2013). A study was conducted to show the impact of PM2.5 on inducing p53 mutation using human alveolar epithelial BEAS-2B cells were constantly exposed to low dose of PM2.5 for 10 days. It was elucidated that the PM2.5 is able to trigger p53 promoter methylation by increasing DNA (cytosine-5-)-methyltransferase 3β (DNMT3B) methylation levels, resulting in p53 inactivation contributes to the pathogenesis of lung cancer (Ellis et al. 1997; Cai et al. 2013).

Indoor air pollution is the main factor to develop lung cancer in never smokers, especially in women and children, due to various indoor activities such as passive smoking, solid fuel burning, and poor ventilation systems in rural households, and also may happen from building materials and wall coverings in urban cities (Behera and Balamugesh 2005; Mu et al. 2013).
Poor indoor air ventilation in a developing country includes exposure to fumes from high-temperature cooking, which is considered as an important cause of lung cancer (Malhotra et al. 2016).

1.1.1.1.5 Family history of lung cancer

Numerous studies found that the first relatives of cancer patients are associated with 1.3-6.0 higher risk of lung cancer both in smokers and non-smokers. It

suggests that the possibility of familial lung cancer could be because of shared environmental factors (e.g. indoor and outdoor air pollution) , genetic factors and similar lifestyles (e.g. smoking habit and diet) (Lissowska et al. 2010). The risk of lung cancer increased five-fold in persons aged less than 60 years if first-degree relatives were diagnosed with early-onset lung cancer and are considered to be linked to genetic factors (multiple genetic loci and interactions) (Cassidy et al. 2006; Schwartz et al. 2018).

Researchers from Japan analysed nine members of a large family suffering from autosomal dominant lung adenocarcinoma. They have concluded that the novel HER2 mutation is potentially oncogenic, with the possibility of causing hereditary and sporadic lung adenocarcinoma (Kanwal et al. 2017).

1.1.1.1.6 Pre-existing chronic respiratory diseases

Many acquired respiratory diseases have been demonstrated to increase susceptibility to lung cancer. Multicentre observation research concluded that about 28.2% of newly diagnosed lung cancer patients had latent tuberculosis (TB) infection (Fan et al. 2014). Another cohort study showed that TB was approximately higher 11-fold in lung cancers patients compared to non-tuberculosis (Yu et al. 2011).

The lung fibrosis and inflammation from TB could induce DNA damage, which may elevate the risk of lung cancer (Liang et al. 2009; Brenner et al. 2011), and

the excess lung cancer risk appeared to be concentrated mainly within the first 3 years after the diagnosis of tuberculosis (Leung 2016).

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease that targets the lower airways and lung parenchyma. Noxious gases and particle inhalation such as cigarette smoke are oxidative stress from the environment. They have been found to have the main role in the ageing process of lung cells leading to COPD (Boukhenouna et al. 2018).

The main pathological changes associated with COPD are increased mucus-producing cells, airways inflammation, airflow limitation and lung parenchyma destruction. COPD symptoms include; shortness of breath, rapid lung function deterioration, coughing, and sputum production (Higham et al. 2019). The alpha-1 antitrypsin (AAT) deficiency is the main genetic cause of COPD, which leads to the destruction of the elastic lung tissues by elastase and subsequently earlier onset of COPD (Brode et al. 2012).

Increased lung carcinoma risk was also implicated in patients with obstructive lung diseases like COPD and fibrotic lung diseases (Liang et al. 2009; de-Torres et al. 2015). Different studies have demonstrated that having moderate-to-severe COPD increases the risk of lung carcinoma up to 4.5-fold (Punturieri et al. 2009). Despite lung carcinoma and COPD being mainly caused by tobacco smoking, additionally, evidence also indicates an increase in lung cancer incidence in patients already diagnosed with COPD even after fixing the confounding effect of cigarette smoking (Parris et al. 2019). Many studies showed that the presence of emphysema and airflow obstruction confer a significant risk of developing lung cancer, especially squamous cell carcinoma and small cell lung cancer (SCLC)

(Dai et al. 2017). A study demonstrated that co-occurrence of chronic bronchitis and emphysema had a stronger positive association with lung cancer than chronic bronchitis alone (Denholm et al. 2014).

Furthermore, the increased risk of lung cancer was implicated in patients with interstitial lung disease (ILD), represented by idiopathic pulmonary fibrosis (IPF) and connective tissue disease-related ILD. Many studies have presented a high incidence of lung cancer in patients with ILD, ranging from 5.5% to 20.4% (Enomoto et al. 2016).

Previous studies have indicated a high incidence of lung cancer in IPF, and some have identified its risk factors. Ozawa and colleagues involved 103 IPF patients without lung cancer at the time of their initial diagnosis concluded that lung cancer's cumulative incidence was increased as the duration of follow up increased (3.3%, 15.4% and 54.7% at 1, 5 and 10 years, respectively). The median period was 120.0 months from IPF initial diagnosis to the presence of lung cancer (Ozawa et al. 2009).

The pooled analysis from the International Lung Cancer Consortium reported that patients who had a history of pneumonia increased the risk of lung cancer a-1.57 fold (Brenner et al. 2012).

A meta-analysis of asthma and lung cancer showed the relationship between asthma and lung cancer, which presented that asthma increased the risk of lung cancer (Qu et al. 2017).

1.1.1.1.7 Age and lung cancer

Due to increasing life expectancy and the increased risk of cancer with ageing, lung cancer is a common disease in older individuals. More than half of lung

cancer cases are diagnosed over the age of 65 (Maione et al. 2010) and the median age at diagnosis of lung cancer patients is between 64 – 70 years of age (Owonikoko et al. 2007). The age at diagnosis of lung cancer relies on the country health policy (Torre et al. 2016). If elderly patients do not receive diagnostic procedures in cases of suspected lung cancer, the median age is likely to be lower than in a country where elderly patients are thoroughly investigated (Blanchon et al. 2006; Tas et al. 2013).

1.1.2 Classification of lung cancer

Lung cancer can be classified mainly into two types. Small cell lung cancer (SCLC), also called oat-cell carcinoma and non-small cell lung cancer (NSCLC). SCLC accounts for nearly 15% of all lung cancer cases, whereas NSCLC accounts for about 85% (Herbst et al. 2008). Based on microscopic appearance, the SCLC is smaller than NSCLC and is characterized by rapidly dividing tumour cells. Often, SCLC results in the expansion of widespread metastases (Nall 2017). The development of SCLC encompasses two stages: the limited stage (LS) and the extensive stage (ES). If tumour growth is limited to a single lung and the lymph nodes close to that lung, the cancer is defined as LS. If cancer has spread beyond these regions, it is ES (Argiris and Murren 2001; Lemjabbar-Alaoui et al. 2015).

NSCLC is divided into the three following subtypes: squamous cell cancer, which is also called epidermoid carcinoma, adenocarcinomas, and large cell lung cancer. These cancers compose 20%, 38.5 % and 2.9% of all lung cancers, respectively (Dela Cruz et al. 2011).

These subtypes altogether comprise the distinct classification of NSCLC for the following reasons: (1) they ordinarily co-exist within a tumour, (2) it is difficult to distinguish large-cell carcinomas from adenocarcinomas and squamous cell lesions that are poorly differentiated, and (3) all these types have the same treatment regimens and better prognosis when lung cancer is diagnosed while still localised (Smith and Glynn 2000). Squamous cell carcinoma appears chiefly in proximal bronchi and remains localised, exhibiting a strong association with smoking. Adenocarcinoma patients undergo metastasis whilst at an asymptomatic stage. Despite its association with smoking, a greater occurrence is found in non-smoking patients (Hirsch et al. 2008; Saito et al. 2017).

1.1.3 Stages of lung cancer

Staging is the process the location of cancer and evaluating its measurement (American Cancer Society 2015). The Union for International Cancer Control (UICC) used the Tumour-Node-Metastasis (TNM) classification system for staging types of cancers. This classification assesses the size of cancer, the degree of any penetration into other structures within the chest (T), the location of any lymph nodes which are affected by the tumour (N), and the spread of cancer from one part of the body to another, a process called metastasis (M) (Meaden et al. 2014). The aim of staging system is to help clinicians to put the treatment plan, determine the prognosis for the patient, evaluate the results of treatment, and facilitate the exchanging of information between many centres around the world (Sobin et al. 2009).

The 8th edition of the TNM classification for lung cancer was accepted by the UICC and the American Joint Committee on Cancer. It includes innovations in T,

and M components, with no changes in the N component for staging lung cancer, which is modified and summarised in Table 1.1 (El-Sherief et al. 2018; Rami-Porta and Eberhardt 2018).

After the diagnosis and accurate staging of lung cancer, it is crucial to determine the most suitable method for treatment. If lung cancer is at an early stage, and surgical extraction is feasible, the single most successful choice for a cure is surgery. Otherwise, approximately 70% of lung cancer patients present metastatic disease at the time of diagnosis, and therefore chemotherapy is the appropriate treatment (chemotherapy with radiation is used for stage III lung cancer) (Molina et al. 2008). Despite the improvements in palliative chemotherapy, patient survival is 8-10 months. However, specific molecular-targeted therapeutic agents have provided different treatment choices to extend lung cancer patients' survival period (Soo and Mok 2011) . People with advanced and metastatic NSCLC that responds to targeted therapies or checkpoint inhibitors now routinely survive for three or four years after diagnosis (Eisenstein 2020).

Table 1.1 TNM 8th Edition for lung cancer classification (El-Sherief et al. 2018; Rami-Porta and Eberhardt 2018).

Component of the classification	The proposed changes
T_x	Tumour in sputum/bronchial washing but not be determined by bronchoscopy and imaging.
T_0	No tumour
T_s	Carcinoma in situ
T1	≤3cm surrounded by visceral pleura/lung, without involving the main bronchus.
$T_{1a(mi)}$	Minimally invasive carcinoma
T_{1a}	≤1cm
T_{1b}	>1 but ≤2cm
T_{1c}	>2 but ≤3cm
T_2	Invasion of main bronchi without the involvement of carina.
T_{2a}	T2a >3 cm but ≤4 cm
T_{2b}	>4 cm but ≤5 cm
T_3	>5 cm but ≤7 cm
T_4	>7 cm
N_1	Refers to lung cancer spreading to 1 or more ipsilateral hilar, interlobar, lobar, and segmental and sub segmental lymph nodes.
N_2	Lung cancer spreading to ipsilateral mediastinal and subcarinal lymph nodes.
N_3	Lung cancer spreading to contralateral mediastinal, hilar, interlobar, lobar, segmental, subsegmental lymph nodes and to contralateral or ipsilateral low cervical, supraclavicular, sternal notch lymph nodes
M_1	Distant metastases
M_{1a}	The tumour in the contralateral lung, pericardial nodule, malignant effusion
M_{1b}	Single extra thoracic metastasis.
M_{1c}	Multiple extra thoracic metastases in one or several organs

1.1.4 Genetics of lung cancer

Lung cancer has been frequently associated with some genetic mutations which fall into three classifications: rare high-risk variants (risk of 10 or higher and prevalence of 1% or less), moderate-risk variants (risk of around 2–5 and prevalence of not more than 5%), and common low-risk variants (risk of between 1·1–1·5 and prevalence of more than 5%) (Brennan et al. 2011). Other sorts of gene variants are unlikely to exist due to evolutionary pressures (e.g. common high-risk variants), or are rare low-risk variants (undetectable by current study designs) (Birch et al. 2001). High-risk gene mutations that show some association with lung cancer include genetic mutation such as *p53* gene, which is involved with Li-Fraumeni syndrome, and retinoblastoma gene (RB1) (Olivier et al. 2003), In spite of the lung cancer frequency in carriers of TP53 mutation, is only slightly higher than that of the general population, the lung cancer could develop at an early age (Brennan et al. 2011).

Additionally, a meta-analysis proposed that lung cancer occurrence increases with a family history of lung carcinoma. It is more likely to happen if multiple family members are affected and if it had been diagnosed at a younger age (Matakidou et al. 2005). However, 5-10% of all cancers are considered to be caused due to genetic defects (Cho et al. 2018). Some non-smokers have lung cancer, congenital defect could be a possible reason for the disease (Mendoza et al. 2019).

The single nucleotide polymorphisms (SNPs) in some members of cytochrome P-450 (CYP), glutathione S-transferase (GST) and N-acetyltransferase (NAT) gene families which have been found to influence detoxification of tobacco smoke components associated with increased risk of lung cancer (Ezzeldin et al.

2017).For example, elevated CYP1A1 expression is associated with pulmonary PAH related DNA adduction and increased lung cancer risk (Moorthy et al. 2015). The faulty DNA repair mechanism will end up with a change in DNA and make people more susceptible to lung cancer, which has also been reported *in vivo* studies on lung cancer cells compared to controls (Breuer et al. 2005; Torgovnick and Schumacher 2015).

Furthermore, different oncogenes and tumour suppressor genes have been correlated to lung cancer such as RAS genes, including K-RAS (Osada and Takahashi 2002). Activating mutations in the K-RAS oncogene are the most prevalent in lung adenocarcinoma, which happened in 25–40% of cases (Riely et al. 2008). Epidermal growth factor receptor (EGFR) overexpression in lung cancer was detected in 43–89% of NSCLC cases (Zhou et al. 2007). B-RAF mutations were observed in 6–8% of NSCLC patients (Lin et al. 2014). HER2 mutations have been observed in 2–4% of NSCLC patients (Mar et al. 2015), and the mutations in the TP53 gene have occurred in 50% of NSCLC patients, particularly in squamous cell carcinoma. Lung cancer patients with tobacco smoke have a higher risk for TP53 gene mutations than lung cancer patients who never smoked (Kanwal et al. 2017).

1.1.5 Biological or target therapies in NSCLC

An increase in the information about cancer biology has enhanced the development of new biological therapies that focus on many components of the tumour (Zugazagoitia et al. 2017). The first molecular target for cancer therapy was EGFR (Soo and Mok 2011). The EGFR is activated in patients of NSCLC

and causes over-expression of proteins, gene mutation, and elevates the gene copy number (Shepherd et al. 2005; Sharma et al. 2007).

The epidermal growth factor is a component of the human epidermal growth factor receptor (HER) family and is comprised of three constituents: an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (Chen et al. 2012). The signalling pathway for EGFR plays a fundamental role in the development of lung cancer. This is through the binding of ligands such as epidermal growth factor, transforming growth factor-alpha, epiregulin and amphiregulin to the HER family receptors. EGFR triggers receptor dimerization and phosphorylation of tyrosine residues in the kinase domain. Consequently, a number of cell signalling pathways are activated, including the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3’ kinase (PI3K)–AKT pathway, and the STAT pathway (Ciardiello and Tortora 2008). These pathways contribute to elevated cell proliferation, the suppression of apoptosis, enhanced invasion, and metastasis, as presented in Figure 1.1 (Cataldo et al. 2011).

The gefitinib and erlotinib are specific small molecule EGFR tyrosine kinase inhibitors (TKIs) (Asai et al. 2015), which prevent adenosine triphosphate binding to the tyrosine kinase domain of EGFR. This results in inhibition of autophosphorylation and blocks the downstream cascade signalling (Roengvoraphoj et al. 2013). These features could be used for the treatment of metastatic non–small-cell lung cancer (Burotto et al. 2015).

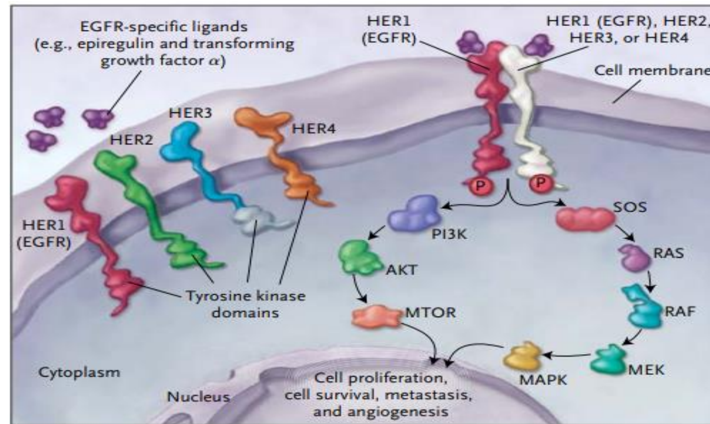

Figure 1.1 EGFR signalling pathways.

The figure shows four members of HER family receptors and all these receptors have tyrosine kinase domain. On the right side of the figure, the ligands bind to HER family receptors to induce dimerization and cause phosphorylation of the tyrosine part of the EGFR kinase domain. This results in phosphorylation of many intracellular signaling cascades, such as RAS–RAF–MEK–ERK, and PI3K–AKT pathways, enhanced cellular proliferation, angiogenesis, and metastases adapted from (Cataldo et al. 2011).

1.2 Interferons (INFs)

In 1957, Issacs and Lindenmann described interferons as soluble factors generated by cells infected by a virus. INF factors could create uninfected cultures resistant to another virus (Isaacs and Lindenmann 2015). So the interferons are named due to their ability to interfere in viral replication, but now the knowledge of interferons has been significantly broadened, and interferons are considered cytokines and interleukins (De Andrea et al. 2002). Interferons provide defence against bacteria, viruses, mitogens and cancerous cells. The interferons are divided into three major types: INF type I, II and III (Mahajan and

Kaur 2015), which are characterised by their genetic loci, sequence identity, cell of origin, and type of receptors and signalling molecules as presented in Table 1.2 (Saha et al. 2010; Ivashkiv and Donlin 2014; Wack et al. 2015).

The IFNs type I contains 17 distinct proteins, includes IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. The structural genes for INFs type I are located in chromosome 9. All IFN type I bind with the same cell surface receptor IFNAR, including IFNAR1 and IFNAR2 chains. The key producer for IFN-α and IFN-ω are hematopoietic cells, whereas the fibroblasts are the main source for IFN-β and produced by macrophage under proper induction (Pfeffer et al. 1998; Khan and SpringerLink 2016).

The major potent natural signal to produce the IFNs type I is the microbial challenge (i.e., viral and bacterial infections or microbial nucleic acids exposure), and the main biological action of the IFNs type I are preventing the viral replication, preventing the cell replication, elevate the lytic potential of natural killer cells, and modulate the expression of major histocompatibility complex (MHC) molecule by increasing the expression of MHC class 1 and decrease the expression of MHC class II molecules (Ferreira et al. 2018). Besides the role of IFNs type I as the first line against the viral infections, it also plays a main role in immunosurveillance of cancer cells (Dunn et al. 2006; Di Franco et al. 2017).

IFNs type II includes IFN-γ. It is different in structure from type I IFNs, which bind to a different receptor and is encoded by a separate chromosomal locus in human chromosome12 and produced by T helper cell type 1 (Th1) lymphocytes, cytotoxic lymphocytes, and NK cells (Bach et al. 1997; Muñoz-Carrillo et al. 2018). The IFN-III includes IFNλ1 (IL-29), IFNλ2 (IL-28A), and IFNλ3 (IL-28B), which have the same functions as IFN-I, except that they are produced mainly by

epithelial cells (Hermant and Michiels 2014). IFNλ-1 and IFNλ -2 regulate the expression of IFNs, being structurally and functionally like them through sharing beta chain but with less intensity (Fitzpatrick 2009), while IFNλ-3 induces an antiviral response in cells through signal transducer and activator of transcription, mediated by STAT1 and STAT2 (Carson IV and Kunkel 2017).

Table 1.2 Comparison of type I, type II, and type III IFN production and signalling (Saha et al. 2010; Ivashkiv and Donlin 2014; Wack et al. 2015).

Properties	**Type I IFN**	**Type II IFN**	**Type III IF**
Types	IFN-α, IFN-β, IFN-κ, IFN-ε, IFN-ω	Only IFN-γ	IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and IFN-λ4
IFN producing cells	All nucleated cells (leukocytes, fibroblasts, and endothelial cells)	T cells, B cells, NK cells, NKT cell, APCs	Epithelial cells
IFN responding cells	All nucleated cells	All nucleated cells	Lung, intestine, and liver epithelial cells
Stimuli	PAMPs and DAMPs	IL-12, IL-15, IL-18 and PAMPs	PAMPs and DAMPs
IFN receptor	IFNAR1 and IFNAR2	IFNγR1 and IFN-γR2	IFN-λ R1 and IL10Rβ
Signaling molecules	TYK2, JAK1, STATs, CRKL and IRS	JAK1, JAK2, STAT1 and STAT3	TYK2, JAK1, STAT1, STAT2, and IRF9
Transcription factor binding sites	GAS, ISRE	ISRE, GAS	ISRE
Function	Antiviral, antitumor antiproliferative and immunomodulatory	Antiviral, antitumor Antiproliferative and immunoregulation	Antiviral

1.2.1 IFN-γ (Interferon-gamma)

IFN-γ is the only member of the type II class of interferons to have a dimerized soluble molecule which is consisted of 143 amino acids. It is produced through a non-covalent bond of two 17kDA polypeptide subunits (Parhi et al. 2015).

After multiple N-glycosylation, both subunits associate in an antiparallel manner during synthesis, forming a mature 50 kDa molecule. It is also a

pleiotropic cytokine, which has been the central coordinators to virtually all immune cells' function and both adaptive and innate immune responses (Miller et al. 2009b; Ferreira et al. 2018).

It is produced mainly by Th1 CD4+ T cells, CD8+ T cells and natural killer (NK) cells. Studies indicate that other cells include B cells, NKT cells, and professional antigen-presenting cells (APCs), which may also produce IFN-γ. This secretion is controlled primarily by IL-12 and IL-18 both cytokines are produced by APCs (Mojic et al. 2017).

In regards to biological activities, the IFN-γ induces B-cell differentiation toward plasma cells immunoglobulin (Ig)-G-production (Vazquez et al. 2015). Furthermore, IFN-γ increases MHC molecules' expression in antigen-presenting cells, which is necessary at a later stage of the response. This cytokine forms an antiviral state by modulating the differentiation and maturation of B and T cells, by coordinating the transition from innate to adaptive immunity through promoting complement activation, increasing cytotoxic activity of T cells and differentiation Th1 cell for the clearance of infectious pathogens (Schroder et al. 2004; Carson IV and Kunkel 2017). Several well-known antiviral functions of IFN-γ mostly lack a specific antiviral mechanism. For example, IFN-γ is a potent inducer of indoleamine 2,3-dioxygenase (IDO) and nitric oxide synthase (NOS). This results in the depletion of tryptophan and production of nitric oxide (NO) due to IDO and NOS expression, expressing strong antiviral effects (Kang et al. 2018; Kosmidis et al. 2018).

IFN-γ induces intensive phagocytosis and pinocytosis on macrophages, triggering antimicrobial potential in these cells (Khan et al. 2016).

Also, IFN-γ works as a cell growth inhibitor and has the ability to induce apoptosis (Green et al. 2017).

IFN-γ executes roles as antiviral, immunoregulatory, and anti-tumour properties , via interactions with specific cell-surface receptors. This involves four transmembrane polypeptides receptor complexes: two ligand-binding chains (IFN-γR1) and two signal-transducing chains (IFN-γR2), which are required for signal transduction (Castro et al. 2018). The IFN-γR1 chain is expressed on B, T, and myeloid cells; on the other hand, the IFN-γR2 chain is highly expressed on B and myeloid cells but slightly expressed on T cells (Bernabei et al. 2001). Also, the expression of IFN-γR2 determines if the cell that IFN-γ stimulates undergoes proliferation or apoptosis. When the expression of IFN-γR2 is elevated, it will cause a switch from a proliferative to an apoptotic outcome following IFN-γ stimulation. In some CD4+ Th1 cells where the low IFN-γ R2 chain expression reduces the response to IFN-γ by the stimulated MHC class1 expression and cell proliferation, which unables the stimulation of the apoptotic pathway (Castro et al. 2018).

Janus kinase (Jak)-STAT1 pathway is the most critical transduction pathway stimulated by IFN-γ. This interaction results in IFN-γ presenting an anti-proliferative effect on different cell types, both pathways mentioned above have demonstrated their potential in inhibiting tumour progressions and eliminating pathogen-infected cells (Seif et al. 2017).This is done by binding two IFN-γ R2 subunits with two IFN-γ-bound IFN-γ R1 chains (Melmed et al. 2015). Once the association of such a receptor complex is induced, Jak1 and Jak2 are activativated and phosphorylate Tyr440 of IFN-γ R1 , forming a docking site for STAT1. Activation of STAT1 occurs via phosphorylation at

Tyr701, leading to homodimerization of STAT1 and nuclear translocation. The STAT1 dimers interact with specific DNA sequences called IFN-γ-activated sequences (GAS), present in the promoter regions of IFN stimulated genes and responsible for regulating their transcription (Krause et al. 2006; Kulling et al. 2018). The entirety of these mechanisms are presented in Figure 1.2 (Zaidi and Merlino 2011).

The transcriptional activity of STAT1 is stimulated through kinases. These kinases are mitogen-activated protein kinase (MAPK), protein kinase C(PKC), and phosphoinositide 3-kinase (PI3K)/AKT, which phosphorylates STAT1 in the transactivation domain (Zaidi and Merlino 2011).

The mechanism of (STAT1) pathway plays a positive role by regulating the expression of genes, encoding several antiproliferative and proapoptotic molecules such as IFN regulatory factor 1 (IRF-1). However, if IRF-1 is inhibited, the INF-γ activates the proliferative signals. On the other hand, when the IRF-1 is elevated, the INF-γ stimulates apoptotic signals (Bernabei et al. 2001; Rettino and Clarke 2013).

In mice, those with deficient IFN-γ spontaneously develop lung epithelial malignancies and lymphoma. Also, the mice with a deficient IFN-γ receptor and STAT1 manifest tumour growth following chemical carcinogen treatment, which emphasizes the ability of IFN-γ to act as an anticancer agent (Gao et al. 2018). Furthermore, preliminary research indicates that IFN-γ, given as a single agent, has a measurable effect as an antitumor agent in advanced NSCLC (Prior et al. 1999). Recent studies demonstrated that high doses of IFN-γ could induce apoptosis in NSCLC cell-lines,such as A549 and H460, by activating JAK-STAT1-caspase signaling. Western blot analyses results showed that STAT1

induced transcription and synthesis of caspase 3 and caspase 7, which initiated apoptotic processes in tumour cells (Song et al. 2019) .Furthermore, IFN-γ can selectively induce apoptosis in stem-like colon cancer cells through JAK-STAT1-IRF1 signaling in a dose-dependent manner. Specific sensitization to IFN-γ treatment is the consequence of higher expression of IFNGR on stem cell surface in comparison to other colon cancer cells (Ni et al. 2013) .Kundu et al. 2017 demonestrated that precise neutralization of cytokine from IL-12 family, namely p40 monomer, induces IL-12-IFN-γ signaling cascade in prostate cancer both in *vitro* and *in vivo*, which leads to tumor regression and cancer cells death. They found that anti-p40 antibody treatment significantly elevated the expression of apoptosis-related genes such as caspase 3, caspase 7, caspase 8, caspase 9, BAD, BID, cytochrome C, BAK, and p53 (Kundu et al. 2017).Consistently, in NSCLC cells lines, H1975, HCC827, and H1437, IFN-γ induced programmed cell death through the activation of caspases downstream of JAK-STAT1 signaling pathway (Hao and Tang 2018).Similar results have been reported in melanoma cell the activation of caspase 3 was IFN-γ/IRF3/ISG54 dependent (Guinn et al. 2017). However, IFN-γ signaling in tumor cells directly activates apoptotic processes, but non-specificity of IFNG/IFNGR interaction increases the chance for side effects. Therefore, exploring exclusivity of the IFN-γ/tumor cells/apoptosis relationship aid in the discovery of new therapeutic targets for cancer treatment (Jorgovanovic et al. 2020).

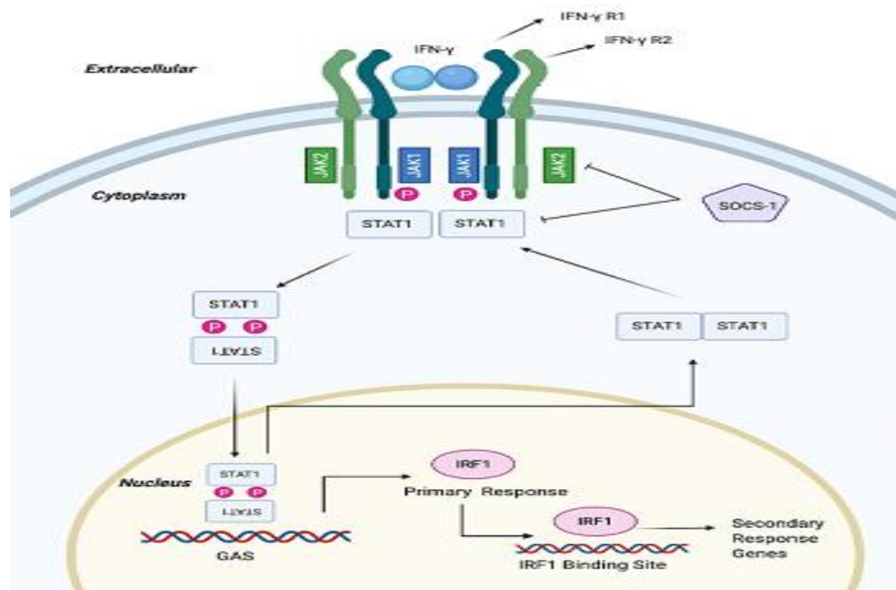

Figure 1.2 IFN- γ /JAK/STAT signaling pathway.

Binding of IFN-γ dimers to the extracellular domain of the IFN-γ R1 receptor subunit leads to engagement of the IFN-γ R2 subunit, which causes JAK1 and JAK2 to cross-phosphorylate each other and the receptor subunits. The parallel STAT1 homodimers are then recruited to the receptors, and their phosphorylation converts the homodimers into an anti-parallel configuration. The reoriented STAT1 homodimers translocate to the nucleus, where they bind to GAS sites on the primary response genes, including IRF1. IRF1 subsequently activates a large number of secondary response genes, which carry out a range of immunomodulatory functions. The suppressor of cytokine signaling (SOCS) proteins serve as the major negative regulators of the IFN-γ pathway by inhibiting the phosphorylation of JAKs and STAT1. Dephosphorylation and acetylation of STAT1 homodimers revert them to parallel configuration and cause their exit from the nucleus. adapted from (Zaidi and Merlino 2011).

1.3 Oxidative stress

Oxidative stress plays an important role in many disease states, including cancer (Liguori et al. 2018), and it has been revealed that oxidative mechanisms take part in the initiation, promotion and progression of carcinogenesis (Laviano et al. 2007). Oxidative DNA damage by reactive oxygen species (ROS) is also thought to contribute to carcinogenesis. Oxidative stress occurs from the imbalance between the production of reactive oxygen and nitrogen species (RONS) and antioxidant defences. It is well-known that increasing ROS levels can potentially cause damage to the cells' own DNA, proteins, and essential fatty acids, leading to oxidative DNA damage and lipid peroxidation (Naveenkumar et al. 2013). It

has been shown that oxidative DNA damage can demonstrate direct genotoxic effects such as single or double DNA strand breaks, chromosomal aberrations, the induction of transcription and signal transduction pathways and genomic instability, all being related to carcinogenesis (Filaire et al. 2013).

ROS are reactive molecules and free radicals derived from molecule of oxygen. Such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}HO$), they can be present at picomolar or micromolar concentrations in environmental systems (Parvez et al. 2018; Collin 2019).

ROS are commonly produced by photolysis, electron transfer or energy transfer reactions. Free superoxide and hydroxyl radicals can be generated by the univalent oxygen reduction pathway (Schmidt 2006). The $O_2^{\bullet-}$ is generated by the activity of enzymes such as NADPH oxidase and xanthine oxidase (Collin 2019). The $O_2^{\bullet-}$ is considered an essential form of free oxygen radicals. It can be in two forms, such as $O_2^{\bullet-}$ or hydroperoxyl radical (HO_2) at low pH, the most occurring form under the physiological pH is superoxide (Phaniendra et al. 2015). The $O_2^{\bullet-}$ react with another superoxide radical in a dismutation reaction, in which one radical is oxidized to oxygen and other is reduced to H_2O_2. (Zuo et al. 2015). Hydrogen peroxide (H_2O_2) is an active form, which is enzymatically generated from the superoxide radical and it can cause damage to the cell (Turrens 2003). A more effective and aggressive and highly reactive form of oxygen radical is the hydroxyl radical ($OH^{\bullet}$), which can be generated by the combination of ($O_2^{\bullet-}$) and H_2O_2 (Sakai et al. 2017). It causes severe biological damage once it reacts with all biological molecules; furthermore, it can damage all DNA components, including pyrimidine and purine bases (Pizzino et al. 2017).

1.4 DNA repair mechanisms

DNA damage can occur due to alteration in the chemical structure of DNA, such as a break in DNA strand, a missing base from a DNA backbone, or a chemically altered base such as 8-hydroxy-2-deoxyguanosine (8-OHdG) (Ciccia and Elledge 2010).

In human cells, it has been estimated near to 70,000 lesions per cell happens every day naturally or because of environmental factors such as radiation, UV light and other genotoxins. Therefore, a variety of repair mechanisms are important to maintain the DNA genome integrity and stability (Tubbs and Nussenzweig 2017).

The DNA repair process exists in eukaryotic and prokaryotic cells, and numerous associated proteins have been highly conserved throughout evolution. The cells use different mechanisms to determine and repair the various damage, which can happen to DNA, no matter if this damage has occurred due to the environment or inaccurate replication (Abbotts et al. 2014). The DNA plays a crucial role in protecting the genetic traits and plays an important role in cell division, it controls the DNA repair that is highly correlated to regulating the cell cycle (Clancy 2008). The checkpoint processes show that the DNA in cells are intact before the happening of DNA replication and cell division during the cell cycle. However, the failure of these checkpoints can contribute to an accumulation of DNA damage, which gives rise to mutations and many diseases such as cancer (Goodarzi and Jeggo 2013; Torgovnick and Schumacher 2015).

The cells have employed different types of repair mechanisms dependant on the type of DNA damage. The repair mechanisms generally include five major types: The Single-strand DNA repair include base excision repair (BER), mismatch

repair (MMR), nucleotide excision repair (NER), while the double-strand break repair includes homologous recombination (HR) and non-homologous end-joining (NHEJ) (Chatterjee and Walker 2017).

1.4.1 Single strand DNA repair

The repair mechanism for single-strand breaks (SSBs) involves one strand to be used as a template to guide the modification of the damaged strand by different excision repair mechanisms by replacing the damaged nucleotide with an undamaged complementary nucleotide that presents in the undamaged DNA strand. The BER is a type of excision repair process that repairs a single base on DNA that is damaged through different biochemical mechanisms (Herrington 2020). The damaged base is removed by a repair DNA glycosylase enzyme catalyse the N-glycosidic bond hydrolysis between the damaged base and deoxyribose sugar, resulting in an AP site (apurinic/apyrimidinic site). Then, the AP site is processed by an AP endonuclease and recognise the missing base. The DNA polymerase synthesises a new strand using the complementary strand as a template, which is sealed up by enzyme DNA ligase (Liu et al. 2007; Drohat and Maiti 2014). Commonly NER repairs DNA bulky adducts, arising from the damage caused by UV and alkylating agents (Chatterjee and Walker 2017).

Another type of repair mechanism is MMR which targets base-base mismatches and inaccurate insertion/deletion loops (IDLs) due to a consequence of base misincorporation and polymerase slippage of DNA during DNA synthesis and recombination (Jiricny 2013; Reyes et al. 2015).

1.4.2 Double strand DNA repair

Double-strand breaks (DSBs) are one of the most cytotoxic types of DNA lesion (Blackford and Jackson 2017). It was observed in studies that DSBs and cross-linkage joining both strands of DNA is irreparable because both strands cannot be used as a template for DNA repair and contribute to cell death. Furthermore, a failed normal repair system can cause deletions or chromosomal aberrations, which are linked with abnormal genomic syndromes and cancer progress (Dexheimer 2013). Subsequently, the repair of DSBs is important for cell survival, maintenance of genome stability and integrity (Srivastava and Raghavan 2015). There are two mechanisms for DSBs repair which include the homologous recombination repair (HR) and non-homologous end joining (NHEJ) (Pardo et al. 2009; Chang et al. 2017). These two-repair processes differ in their accuracy and the homologous DNA template that is required for DSBs repair. The HR is an error-free repair mechanism. It uses the genetic information that is presented in the identical sister chromatid or a homologous chromosome as a template to repair the damaged chromosome (Dexheimer 2013). Otherwise, the NHEJ is typically the simplest and fastest way to repair the DSBs by direct resealing of the broken ends (Lieber 2010), and it is the main DSB repair mechanism in mammalian cells, although it may occasionally contribute to loss of genetic information (Chang et al. 2017). Even though the NHEJ is being active in all cell cycle phases, it is relatively more important in the G1 phase. On the other hand, HR is limited to the late-S and G2 phases (Vítor et al. 2020).

1.5 Liposomal delivery systems for the anticancer drug

The considerable harmful side effects and cytotoxicity of highly potent drugs on normal tissues require the development of many targeted drug delivery systems to overcome this issue and improve the pharmacokinetics and result in selective distribution of the loaded agent (Alavi et al. 2017).

Liposomes were discovered in 1963 by Alee Bangham when the phospholipid films exposed to excess water and they formed lamellar structures that separate an aqueous medium from another. The ability to entrap compounds triggered initial applications of lipid vesicles as a drug delivery system and to open a window for drug targeting (Gregoriadis and Ryman 1971; Laouini et al. 2012).

Liposomes are artificial vesicles synthesized from naturally-derived phospholipids, with spherical, self-assembled shapes and their sizes typically range from tens of nanometres up to hundreds of micrometres depending on their preparation methods and final use (Kim 2016). They are made from non-toxic surfactants, cholesterols and membrane proteins. Liposomes have extensively been utilised as a drug delivery system and used for biomedical and biotechnological purposes (Pattni et al. 2015; Chandrawati et al. 2017).

Liposomes are used as drug delivery systems (DDSs) because of their many advantages such as versatile structures, non-toxic, non-immunogenic, biodegradable, increases poorly water-soluble drugs, increase the bioavailability, reduces the toxicity of a drug, elevates the circulation half-life of various active pharmaceutical ingredients (APIs) , effective against the multidrug resistance, prevents drug degradation and brings the drug toward the target (Liu and Boyd 2013). Because of their hydrophilic, hydrophobic and biocompatibility nature, liposomes play as biomimetic parts that capture different substances such as

drugs, DNA, proteins and other substances (Chandrawati and Caruso 2012; Li 2017). Nowadays, liposomes have been highly recognised in food industries as they have the ability to entrap with water-soluble, lipid-soluble and amphiphilic substances such as lutein, curcumin and β-carotene (Michelon et al. 2017).

Doxil® is the first liposomal pharmaceutical drug that was received by the USA food and drug administration (FDA) in 1995 for treatment of acquired immune deficiency syndrome (AIDS) related Kaposi's sarcoma (Pillai 2014). Meanwhile, they are used in anticancer drug including Daunorubicin (DNR), All-trans retinoic acid (ATRA), and Mitoxantrone (MXT) (Olusanya et al. 2018). There are currently approximately fifteen approved active liposomal drugs in the market and many others under clinical trials. Different administration routes were developed for these liposomal drug formulation, such as intravenous, intramuscular, epidural, and other administration routes have also been under clinical studies, such as dermal, transdermal, and oral route (Carugo et al. 2016).

Moreover, liposomes have been used in drug delivery systems for the treatment of different diseases. They are used as carriers of anti-cancer, anti-fungal ,antibiotic drugs, gene medicines ,anaesthetics and anti-inflammatory drugs (Hirai et al. 2013).

1.5.1 The structure and classifications of the liposomes

The liposome consists of an amphiphilic phospholipid, which includes a polar or hydrophilic head and a nonpolar or hydrophobic tail, as shown in Figure 1.3. The polar head consists of phosphoric acid bound in water-soluble substances, whereas the nonpolar tail consists of fatty acids with 10 to 24 carbon atoms (Bozzuto and Molinari 2015). The lipid will arrange it to phospholipids bilayer

under a suitable ratio of lipid to water and temperature, otherwise the micelle formulation form spontaneously and the energy must be added to the system to form the liposomes (Subramani and Ahmed 2012; Aulton and Taylor 2017).

Hydrophobic drugs are incorporated into the liposomes' bilayer membranes, which resemble drug uptake in the core of micelles. Also, the drug can be retained at different positions in liposomes, as shown in Figure 1.3 (Bardania et al. 2017).

Lipid e.g.Phosphatidylcholine (PC)

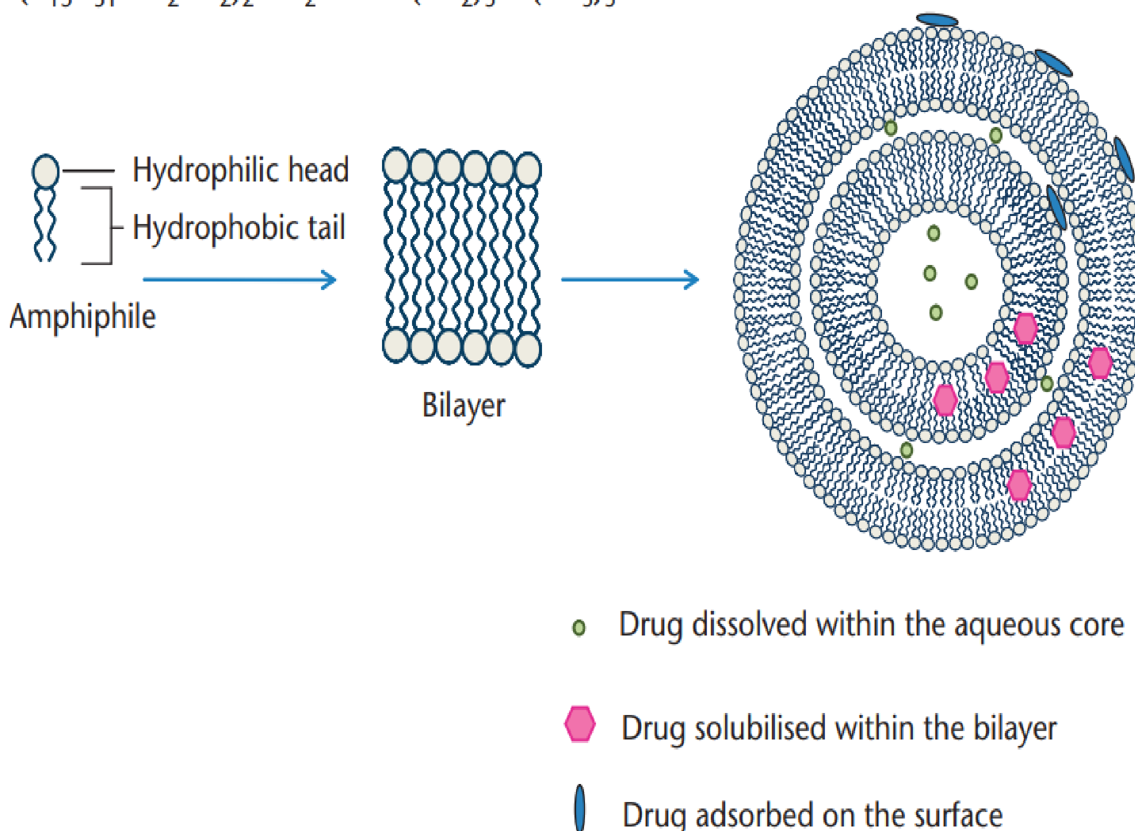

Figure 1.3 Diagram of lipids formation with drug retained in the aqueous core, within bilayer or adsorbed in liposome surface in the presence of electrostatic interactions (Aulton and Taylor 2017).

The liposomes are classified into different types based on their surface characteristics and their applications, including a conventional type consisting of negatively charged phospholipids (Laouini et al. 2012). Cationic liposomes with a

net positive charge considered for the delivery of the nucleic acid based therapies such as DNA and RNA with a negative charge and can electrostatically attract to cationic liposomes (Has and Sunthar 2019). The long-circulating liposomes are modified by coating the surface with the hydrophilic layer of glycoprotein, oligosaccharides and synthetic polymers to avoid the mononuclear detection of phagocyte cells (Sawant and Torchilin 2012). The Immunoliposomes are designed to target certain tissues through the addition of a specific antibody to the surface of the liposomes (Maeda et al. 2000; Kunjachan et al. 2015). Furthermore, the pH-sensitive liposomes which comprised neutral dioleoyl phosphatidylethanolamine (DOPE) and a weakly acidic cholesteryl hemisuccinate (CHEMS) in which they improve the intracellular delivery of different materials such as anti-tumour drugs, toxins, proteins and DNA (Paliwal et al. 2015).

Moreover, polymer polyethene glycol (PEG) is a particular type of polymer incorporated on liposomes surface through a cross-linked lipid (ie, PEG-distearoyl phosphatidyl ethanolamine [DSPE]) to prevent liposomes from being detected by the phagocyte system. These liposomes are called Stealth liposomes. The PEGylated vesicles can prolong the blood circulation of drug and improve the accumulation of liposomes at the site of action by improving the permeability effect (Noble et al. 2014).

The liposomes are synthesised in different sizes, specifically between 50 and 200 nm, to increase the enhanced permeability and retention (EPR) influence in tumour treatment (Kim 2016). Liposomes can be classified depending on the number of layers in the small unilamellar vesicles (SUV), which can easily be

prepared with homogeneous sizes between 20 and100 nm compared to other types and most widely used in pharmaceuticals. Large unilamellar vesicles (LUV) have a significant internal aqueous core size ranging between 100 to 1000 nm with a relatively large aqueous compartment compared to the SUV (Akbarzadeh et al. 2013). Giant unilamellar vesicles (GUV) with large dimensions which are near to cell size 1000 nm or larger than 1 μm (van Swaay and DeMello 2013). Oligolamellar vesicle consists between two to five concentric lamellae and their sizes are between 100–500 nm (Yoshimoto et al. 2013). Multilamellar vesicles (MLV) compose of many concentric bilayers with low aqueous solution volume and their sizes are less than 500 nm. Finally, multivesicular vesicles (MVV) which composed of smaller vesicles with similar sizes to MLV, as presented in Figure 1.4 (Mu et al. 2018).

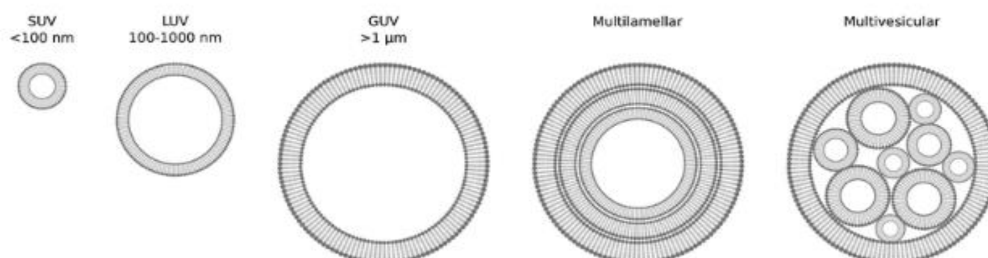

Figure 1.4 Different classification of liposomes based on their lamellarity and sizes (van Swaay and DeMello 2013).

1.5.2 Lipids used for liposomes preparation.

There are many types of lipids that are used in liposome formulations. These include phosphotadylcholine (zwitterionic), phosphatidic acid, phosphatidylglycerol (negatively charged), phosphatidylserine (negatively charged), and phosphatidylethanolamine (zwitterionic). Lipids with a positive charge (e.g., N-[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA)

and 1,2-dioleoyl-3-trimethylammoniopropane (DOTAP)) are specifically used for gene delivery by reacting with DNA and APIs with a negative charge (Munye et al. 2015).

Cholesterol is one of the most strategic constituents of liposomes. It decreases the permeability of liposomal bilayers and increases the stability of liposomes and efficiency of drug encapsulation (Ali et al. 2010).

1.5.3 Liposomes preparation methods

The importance of liposomes drove the efforts of developing a wide range of methods in which liposomes are synthesised, modified and manipulated in different ways (Zhao and Temelli 2015). The choice of the suitable method depends on different factors including the physicochemical properties of the loaded substance and liposome constituents, the concentration of the loaded drug and the toxicity, the medium that used for liposomes dispersion, the additional processes during the preparation or delivery of the liposomes, the size and the half-life needed for the successful application; reproducibility, and the costs, and applicability in regard to large scale manufacturing for clinical applications and good processing practice-relevant issues (Wagner and Vorauer-Uhl 2011).

One of the most commonly used methods for liposome preparation is the Bangham method or thin-film hydration. Briefly, it involves the dissolution of the lipid component in an organic solvent such as ethanol, methanol, dichloromethane, detergent solution or chloroform. Subsequently, evaporation

the solvent using rotary evaporator, and the dispersion of the lipid film in aqueous solution over a period of time (Liu and Loh 2019).

This method forms multilamellar liposomes with heterogeneous sizes between 1 to 5 μm diameter which are formed from spontaneously self-assembly of lipid in the bulk phase (Yu et al. 2009). However, hydration under hydrodynamic flow for long hours is employed for further size reduction or for conversion MLVs with heterogeneous size distribution. In addition, gentle hydration can produce GUVs (Patil and Jadhav 2014).

The thin-film hydration process needs a post-processing step such as sonication, extrusion, high-pressure homogenization, French press and freeze-thaw to control liposomes mean diameter and the polydispersity of liposomes then converting MLVs into SUVs or LUVs (Ong et al. 2016). Additionally, the introducing of freeze-thawing cycles after the hydration was reported to increase the encapsulation efficiency of the loaded drug (Nele et al. 2019).

1.5.4 The application of liposomes as drug delivery for proteins and peptide

In the last few decades, proteins and peptides (PPs) have shown a great role as therapeutic agents to treat various human diseases. Therefore, a variety of new PP drugs have been produced, which exhibit the advantages of high potency and specific therapeutic agents with low toxicity to normal tissue (Bruno et al. 2013). As consequence the great research efforts in both industrial laboratories and academic develop new tissue culture techniques to obtain PPs, on a commercial scale, which look like endogenous molecules and subsequently induce minimal immunological responses. They also produce the therapeutic agents produced

by the human body, including interferons, endorphins, enkephalins, and leutinizing hormone, which are important classifications of PPs (Sood and Panchagnula 2001).

There are currently different diseases correlated with PPs function like cancer, autoimmune diseases, infectious diseases, HIV, and others. Therefore, PPs-based drugs have obtained an important place in the development of medicines biotechnology because of their distinctive mode of action and more than 200 PPs as therapeutic modalities approved by the US FDA ,61 of these drug used in cancer therapy (Cryan 2005; Usmani et al. 2017).

The use of PPs as a therapeutic agent can be limited due to several factors such as the difficulty in a method of delivery, lack of an effective route. Hence, the various issues associated with PPs delivery include the following: high molecular weight and size of PPs decrease the permeability through the biological membranes on cells. They might be susceptible to enzymatic degradation, which may contribute to poor bioavailability (Tan et al. 2010). Also, due to proteins efficacious tertiary structure which can be lost under different physical and chemical conditions, causing degradation or denaturation and loss of biological activity (Borghouts et al. 2005). Furthermore, many of PPs have short biological half-life because of rapid clearance by proteolytic enzymes in liver and body tissues. The PP drugs are highly potent and have highly specific actions, so the accurate clinical dosing is off uttermost importance (Sood and Panchagnula 2001; Lu et al. 2006).

A carrier or novel delivery system must be introduced to improve the therapeutic activity of PPs. Therefore, among different novel drug delivery systems, liposomes were found to have the potential to overcome current limitations and

consider as next generation protein therapeutics due their ability to increase PPs solubility and provide controlled sustained release of PPs to decrease side-effects (Pisal et al. 2010). Additionally, liposomes can improve the bioavailability of PPs and have a high potential to promote PPs to target certain sites, particularly *in vivo* under some specific conditions, by EPR effect which referred to the increased permeability of the vasculature that supplies pathological tissues such as tumors (Tan et al. 2010). More particularly, solid tumors that undergo angiogenesis develop a discontinuous endothelium, with large fenestrations and activation of vascular permeability factors allowing molecules of up to approximately 4,000 kDa, or 500 nm, to enter the interstitial space (Nehoff et al. 2014).This will eanable the liposomes to extravasate and concentrate in the target site by passive targeting (Bozzuto and Molinari 2015) .

1.6 Methods to detect genotoxicity

Genotoxicity assays are methods usually used to detect the compounds that are able to react with DNA and induce genetic damage by different mechanisms. This interaction between the toxic agents and the DNA could result in SSB, DSB,and alkali labile sites (ALS), DNA cross-linking, loss of excision repair, point mutations, chromosomal aberrations and micronuclei formation (Dusinska et al. 2012; Tamokou and Kuete 2014).

The genetic material impairment could result in harmful consequences such as the establishment of diseases such as cancer, changed the heritable features, reduce reproductive capacity and elevate the mortality rate (Çavaş et al. 2014; Ershova et al. 2016). Therefore, there are a number of sophisticated methods

used for detecting the genotoxicity. The Comet assay single cell gel electrophoresis (SCGE) is one of the most common and useful tests for genotoxicity and has been extensively used in different studies performed in our laboratory (Anderson et al. 1997a; Anderson et al. 1997b; Najafzadeh et al. 2012a; Najafzadeh and Anderson 2016; Najafzadeh et al. 2016).

1.6.1 The Comet Assay

Comet assay is also known as SCGE or microgel electrophoresis (MGE), expressly due to the singular appearance of individual cell DNA migration patterns produced by this assay Figure 1.5 (Ahmed et al. 2017).

The Comet assay was developed in 1984 by (Ostling and Johanson 1984) for determining the gamma radiation-induced DNA damage in individual cells after exposure to electrophoresis under neutral pH conditions. This type of comet assay detects only DSB caused by the effects of radiation and radiomimetic agents (Andem et al. 2013). New version of Comet assay , the alkaline version with pH >13 was introduced in the year 1988. This type of Comet assay is most commonly used and detects DNA SSB, ALS and DNA cross-linking in individual cells (Singh et al. 1988).

The alkaline comet assay method is as follows: a cell suspension is spread in agarose on a microscope glass slide. Detergents and a high salt concentration are used to lyse cellular protein content and liberate the DNA. Thereafter, the slides are electrophoresed under alkaline conditions so that the DNA unwinds from sites of strand breakage. The DNA fragments induced by genotoxic agents migrate toward the anode. Then, the slides are washed with a neutralising buffer

and stained with a fluorescent DNA binding dye, such as ethidium bromide (EtBr) (Nandhakumar et al. 2011).

The shape, size of the comet, and the distribution of the DNA within the comet determines the extent of DNA damage. In order to quantify the extent of DNA damage, the Comet data may be scored by visual scoring or by computer automated image analysis systems. In general, cells with high DNA damage display elevated measurements of comet parameters. These may be presented as the tail length, % DNA in the tail and Olive tail moment (OTM; arbitrary unit, the fraction of DNA in the tail multiplied by the tail length) (Tice et al. 2000). The OTM is the prevailing parameter for the measurement of DNA damage, yet it is suggested that it is used together with % Tail DNA (Islam and Kabir 2019).

The Comet assay has been characterized by its sensitivity, rapidity, and cost-efficiency. It is also a relatively easy method for detecting genotoxic DNA damage in lymphocytes and sperm, as displayed in previous studies (Anderson et al. 1997a; Anderson et al. 1997b; Collins 2014). The Comet assay can also be used to detect *in vitro* and *in vivo* environmental genotoxins (Bajpayee et al. 2013). The Comet assay research mostly uses isolated lymphocytes as surrogate cells to predict human cancer due to different agents (Anderson et al. 2013). However, other cell types have also been employed; buccal epithelial cells, nasal epithelial cells, lens epithelium cells removed with cataracts, and biopsies extracted during surgery are all applicable examples (Collins et al. 2014).

The Comet assay has been used in numerous genotoxic studies, with these studies evaluating DNA damage caused by human exposure to genotoxic agents, such as occupational exposure, drug treatments and environmental pollution (Valverde and Rojas 2009). Other studies have used the Comet assay to exhibit

the associations between the high levels of DNA damage and many types of cancer such as breast cancer, oesophageal cancer, cervical cancer, Hodgkin's disease, among others (Collins et al. 2014).

The Comet assay has been used to assess the genotoxicity of chemicals, it features usage in human biomonitoring, epidemiology, and importantly in the measurement of DNA damage and repair (Azqueta et al. 2011; Collins 2014; Najafzadeh et al. 2016).

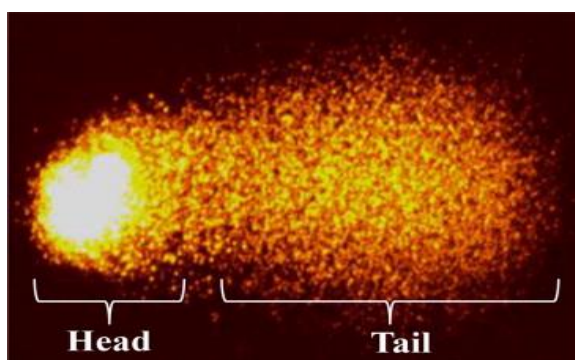

Figure 1.5 An image of a comet, exhibition parts of undamaged DNA nucleoid (head) and the damaged DNA streak (tail) (Beedanagari et al. 2014).

1.6.2 Comet repair assay

The repair of cellular DNA damage can be measured using two methods, whereby the first method involves the monitoring of cellular DNA repair by exposing cells to DNA damage-inducing agent and following a short interval (s) of time, the damage is measured (Collins 2004). Cells under treatment are usually exposed to chemicals, including H_2O_2 or ionising radiation. In this regard, the rate of repair is measured at certain time intervals, generally a short period of time after treatment. Lesions such as SSBs in all cells are predominantly measured in this way. Additionally, specific enzymes can be used to monitor lesions, such as

UV-induced pyrimidine dimmers (using NER) and oxidised bases (using the BER). According to Azqueta et al.(2013) , these enzymes distinguish the lesions and alter these lesions into strand breaks.

Due to the limitation of the standard alkaline comet assay, which detects strand breaks (SBs) and alkali-labile sites and due to the ability of many genotoxic agents to not directly induce SBs, but rather makes DNA adducts or base alterations, to avoid this limitation can be the use of DNA repair enzymes of bacterial origin such as formamidopyrimidine DNA glycosylase (FPG) and 8-oxoguanine glycosylase (OGG1), which a quantitative analysis of the comet can detect the increase in comet tail reflects the enzyme-sensitive lesion's presence (El-Zein et al. 2010; Azqueta et al. 2013).

The modified version of the comet assay is mostly used in determining extra lesions by incubation with the BER enzyme FPG, from Escherichia coli (e-coli). These lesions are referred to as net FPG-sensitive sites when the basal level of DNA strand breaks has been subtracted from the total range of lesions determined once incubated with the enzyme (Møller et al. 2017). The FPG is used to detect and remove 7,8-dihydro-8-oxoguanine (8-oxoguanine), other purine oxidation products, formamidopyrimidines (ring-opened adenine or guanine) and ring-opened N7 guanine adducts produced by alkylating agents (Speit et al. 2004; Azqueta et al. 2014).

The human 8-oxoguanin glycosylase (hOGG1) enzyme was proposed to be a proper replacement for FPG as it does not detect alkylated DNA bases in cultured cells (Cadet et al. 2017).

The hOGG1 is the main enzyme for repair the 8-Hydroxydeoxyguanosine (8-OH-Gua) in human cells. When the 8-OH-Gua residues present in the DNA, it

contributes to a GC → TA transversion unless it is repaired before DNA replication. As a result, the presence of 8-OH-Gua in DNA may cause mutagenesis and the level of 8-OH-Gua is generally used as a biomarker of DNA oxidative damage (Wozniak et al. 2009).

This assay has gone through a series of optimisation and validation studies in order to be widely accepted as a standard assay in determining genotoxicity (Raisuddin and Jha 2004; Godschalk et al. 2013).

1.6.3 Micronucleus assay (MN)

The study of DNA damage at the chromosomal level is paramount in the field of genetic toxicology because the chromosomal mutation plays a significant role in carcinogenesis. The MN assays are recognised methods for evaluating chromosome damage, as they are capable of reliably measuring both chromosome loss and chromosome breakage (Luzhna et al. 2013).

Micronucleus (MNis) are produced from whole chromosomes or chromosome fragments, which lag at anaphase during nuclear division, and they are not incorporated in the daughter nuclei (Fenech 2007; Sabharwal et al. 2015). MNis have been recognised as erythrocytes for over 100 years and have been called the Howell-Jolly bodies by haematologists. In 1959, the first instance of micronuclei induction occurred in root tips of Vicia faba after being exposed to gamma radiation (Evans et al. 1959). Boller and Schmid suggested the term micronucleus assay for the first time in 1970 (Schmid 1975). Micronuclei were then formed in erythrocytes using an *in vivo* assay, contrary to current studies where the micronucleus was formed via an *in vitro/ex vivo* approach (Heddle et al. 2010).

The exposure of cells to chemicals that result in the formation of micronuclei indicates that they are genotoxic and impact the chromosome structure or change segregation patterns. The numbers of micronuclei determine the toxic potential of a chemical (Fenech 2002; Hayashi 2016).

Micronuclei is expressible in bi-nucleated human or mammalian cells, following the utilisation of cytochalasin-B, a chemical that inhibits the assembly of a microfilament ring and blocks cytokinesis. Hence, the micronucleus assay is also referred to as the cytokinesis block micronucleus assay (CBMN) (Sommer et al. 2020). The scoring of MNi is limited in binucleated cells (BiNC). Scoring BiNC cells decreases the confounding effects caused by suboptimal cell division kinetics, increasing this assay sensitivity and reliability. The Micronucleus assay is a favourable approach compared to contending cytogenetic methods because it is inexpensive, quick, and easy for scoring chromosome aberrations (Fenech 2002). Chromosome breakage, chromosome loss, non-disjunction, DNA misrepair, necrosis, cytostasis, and apoptosis can also be identified by this method. Besides this, nucleoplasmic bridges (NPBs), biomarkers of DNA misrepair and telomere end fusion and Nuclear buds (NBUDs) are a biomarker of gene amplification can be measured using this method (Schmid 1975; Fenech 2000; Heddle et al. 2010) Figure 1.6.

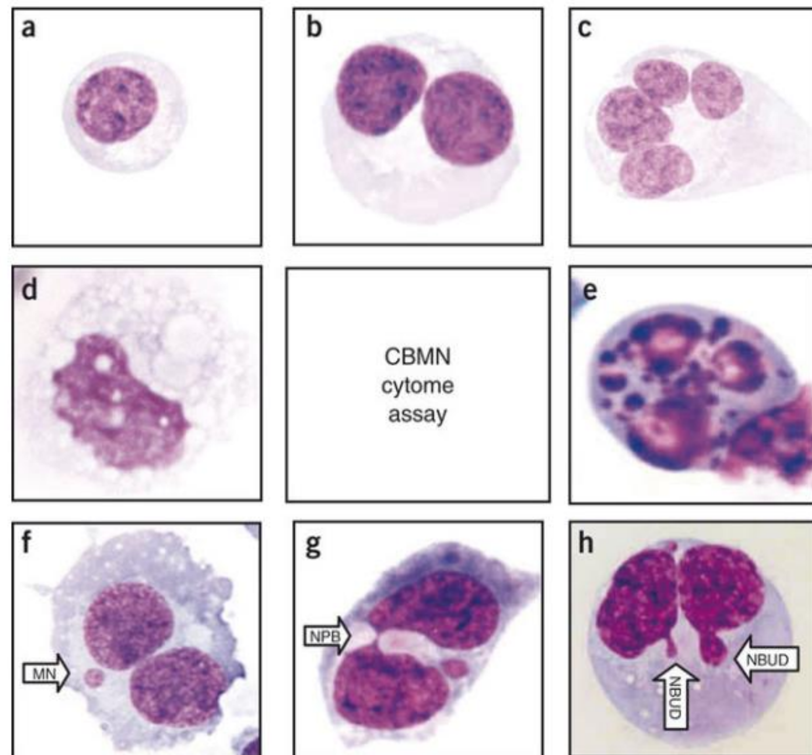

Figure 1.6 Photomicrographs of different types of cells scored in the CBMN assay. a) Mononucleated cell; (b) BN cell; (c) multinucleated cell; (d) necrotic cell; (e) late apoptotic cell; (f) BN cell containing one or more MNi; (g) BN containing an NPB (and a MN); (h) BN cell containing NBUD (Benassi-Evans et al. 2007; Fenech 2007).

1.7 Peripheral lymphocytes, as surrogate target cells in genotoxic studies

Lymphocytes are considered as the best choice to examine genome sensitivity; this is because the sub-populations of lymphocytes have long lives and can retain the genetic mutations induced by a mutagen for more than 40 years (Anderson et al. 2014).

Large numbers of lymphocytes circulate around the body in the blood which makes them easy target for genotoxins thus lymphocytes reflect an overall state of the organism (Albertini et al. 2000). The studies suggest that the cytokines and chemokines involved in tumour development impair lymphocyte functionality. Lymphocyte genomic damage is further enhanced when inflammatory

mechanisms directly affect cancer development by inducing mutations (Ben-Baruch 2006). Chemokines are involved in regulation of various cellular processes in WBCs (white blood cell) including lymphocytes via initiation of G proteins (Guanine nucleotide binding proteins) and downstream receptor kinases are important regulators of WBCs trafficking. Their participation in affecting the activities of other cell types and their expression in cancer development processes is well evident. Particular chemokines have been shown to be involved in recruitment of WBCs to tumour sites. This presents that the function and ability of the leukocytes including lymphocytes is connected to cancer development processes (Singh et al. 2011).

As lymphocytes form a major cellular part of the adaptive immune response, the DNA damage and repair biomarkers could potentially provide information about disease status and condition in other cell types. For instance, DSBs are the most deleterious lesions in DNA which if left unrepaired could cause mutations leading to cancer development. Their detection in lymphocytes could provide a track and extent of DNA damage and repair in other cell types (Jackson 2002).

The ability of lymphocytes to be stimulated once thawed from frozen conditions and repairing the induced damage make them better surrogate cells to be used in human monitoring and genotoxicity studies than other WBCs (Bausinger and Speit 2016) .

Therefore in current study we used the lymphocytes from lung cancer patients to investigate the effects of naked IFN-γ and IFN-γ liposome . The use of lymphocytes is supported by the World Health Organisation International Programme for Chemical Safety (WHO/IPCS), who reported that lymphocytes are suitable surrogate cells for the detection of cancer.

The IFN-γ liposome as DDS used in this study due to the limitation in use the naked IFN-γ such as rapid biodegradation and clearance, which leads to reduce it is efficacy. The liposomal form of IFN-γ prepared by thin film hydration method, and reversed-phase high-pressure liquid chromatography (RP-HPLC) assay was developed for quantification of encapsulating IFN-γ .

The both form of IFN-γ used to determine their effectiveness on DNA damage on healthy individuals and lung cancer patients by using the Comet , Comet repair and micronucleus assay.

In this study the H_2O_2 was used to induce the oxidative stress and increase the DNA damage in lymphocytes from healthy individuals and lung cancer patients to be used as the positive control (PC) and to investigate the *in vitro* protective role of naked IFN-γ and IFN-γ liposome .

After detecting the role of naked IFN-γ and IFN-γ liposome in DNA repair capacity and reducing the DNA damage in lymphocytes from healthy individuals and lung cancer patients by using the FPG and hOGG1, the p53, p21 and Bcl-2 genes and proteins levels were studied to confirm the role of naked IFN-γ and IFN-γ liposome in inducing the DNA repair in lymphocytes from lung cancer patients .

1.8 Aim of the thesis

IFN-γ plays a major role as an immunomodulatory, antiviral, antiproliferative, anticancer cytokine by inhibiting tumour cell growth and enhancing apoptosis. Numerous studies and publications have shown that IFN-γ has been utilised as an anti-tumour agent in certain types of cancer such as adult T cell leukemia (ATL)(Miller et al. 2009a) . However, these studies did not investigate the effect of IFN-γ at the DNA level on lymphocytes from lung cancer patients. Therefore,

the primary objective of this research was to study the DNA protective effect of naked interferon-gamma and interferon-gamma liposome on lymphocytes from lung cancer patients compared to healthy individuals.

The genotoxic and antioxidant effects of naked IFN-γ and IFN-γ liposome on DNA damage were detected by Comet and micronucleus assays in this study. The Comet assay was used in this study due to its sensitivity and simplicity for analysis of the genotoxicity of DNA in lymphocytes as presented in previous studies (Speit and Rothfuss 2012; Sykora et al. 2018). The Comet repair assay was also chosen for monitoring DNA damage repair by using DNA repair enzymes FPG and hOGG1.

Also, the effect of naked IFN-γ and IFN-γ liposome on the mitotic phases of the cell cycles was investigated by the micronucleus assay through the frequencies of the following biomarkers:

1 Micronuclei (MNi): the indicator for chromosomal breakage or loss of a whole chromosome
2 Nucleoplasmic bridges (NPBS): the indicator for DNA disrepair and telomere end–fusions
3 Nuclear buds (NBUDs): the Indicator of DNA amplification or DNA repair complexes.

Furthermore, the influence of naked IFN-γ and IFN-γ liposome on the expression of p53, p21 and Bcl-2 proteins was studied by Western blotting (WB). Finally, the role of both naked IFN-γ and liposome on *p53, p21* and *Bcl-2* gene expression in lymphocytes from lung cancer patients compared to those from healthy individuals were studied using Quantitative reverse transcription PCR (qRT-PCR)

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Chapter Two: Materials and Methods

2 Materials and Methods

2.1 Materials

The table below (Table 2.1) lists the material and reagents that were used to conduct this research study, along with their CAS number and supplier number.

Table 2.1 Chemicals and equipment used with the suppliers as well as the CAS numbers.

Chemicals and reagents	Company / Distributor	CAS Number
Interferon gamma	Sigma –Aldrich, UK	n/a
Low melting point agarose	Invitrogen, UK	39346-81-1
Normal melting point	Invitrogen, UK	9012-36-6
Dimethyl sulfoxide (DMSO)	Sigma –Aldrich, UK	67-68-5
EDTA ($Na_2EDTA.2H_2O$)	Sigma –Aldrich, UK	6381-92-6
Ethidium bromide	Sigma –Aldrich, UK	n/a
Phosphate buffered	Sigma –Aldrich, UK	P4417
Sodium chloride (NaCl)	Sigma –Aldrich, UK	7647-14-5
Sodium hydroxide (NaOH)	Sigma –Aldrich, UK	1310-73-2
Triton X-100	Sigma –Aldrich, UK	9002-93-1
Trizma base	Sigma –Aldrich, UK	77-86-1
RPMI-1640 medium	Sigma –Aldrich, UK	n/a
Distilled water(dH_2O)	n/a	n/a
Eppendorf ® tubes (1ml)	Sigma –Aldrich, UK	Z666548
Cover glass	VWR	n/a
Superfrost microscope slides	Thermo Fisher Scientific	n/a
ErgoOne® Starter Pack 4	Starlab,UK	n/a
Pipette tips	VWR	n/a
Black box	Sigma-Aldrich, UK	n/a
Lithium heparin tube 9ml	Griener Bio-One, Austria	n/a
Safety blood collection set	Griener Bio-One, Austria	n/a
Foetal bovine serum	Sigma-Aldrich, UK	n/a
2-Mercaptoethanol	Sigma-Aldrich, UK	60-24-2
Trypan blue	Sigma –Aldrich, UK	72-57-1
Potassium chloride	Sigma –Aldrich, UK	7447-40-7
penicillin-streptomycin	Sigma –Aldrich, UK	n/a

Glacial Acetic Acid	Fisher Scientific, UK	64-19-7
Mitomycin C	Sigma –Aldrich, UK	50-07-7
Phytohaemagglutinin	Sigma –Aldrich, UK	9008-97-3
Sodium phosphate diabasic	VWR, UK	7558-79-4
Sodium phosphate	VWR, UK	7558-80-7
DPX Mountant	Sigma –Aldrich, UK	n/a
Ethanol	Sigma –Aldrich, UK	64-17-5
Giemsa stain	VWR, UK	51811-82-6
Cell counting kit - 8 (CCK8)	Sigma –Aldrich, UK	n/a
96 well plates	VWR, UK	n/a
Falcon tubes	BD, Swindon, UK	n/a
SLS lab basics pipette filler	Scientific laboratory	n/a
Bradford Protein Assay Kit 1	Bio-Rad	n/a
Protease inhibitor	Sigma –Aldrich, UK	66701-25-5
Acrylamide 30%	Sigma –Aldrich, UK	79-06-1
Ammonium persulphate APS	Sigma –Aldrich, UK	7727-54-0
Recombinant Anti-Mutant	Abcam, UK	n/a
Anti-Bcl-2 antibody	Abcam, UK	n/a
Recombinant Anti-p21	Abcam, UK	n/a
Donkey Anti Rabbit IgG H&L	Abcam, UK	n/a
Bovine serum albumin	Sigma –Aldrich, UK	9048-46-8
Bromophenol blue	Sigma –Aldrich, UK	115-39-9
Western ECL Substrate, 200	Bio-Rad	n/a
SYBR® Green Supermix	Sigma –Aldrich, UK	n/a
iScript™ cDNA Synthesis Kit	Bio-Rad,UK	n/a
QIAamp® RNA Blood Mini	Qiagen	n/a
Hs_ACTB_1_SG QuantiTect	Qiagen	n/a
p53 primer	Qiagen	n/a
Hs_Bcl-2_1_SG QuantiTect	Qiagen	n/a
Hs_CDKN1A-va. 1-	Qiagen	n/a
Mini Gel Tank	Thermo Fisher Scientific	n/a
Bolt™ 4-12% Bis-Tris Plus	Thermo Fisher Scientific	n/a
20X Bolt™ MES SDS	Thermo Fisher Scientific	n/a
4X Bolt™ LDS Sample	Thermo Fisher Scientific	n/a
10X Bolt™ Sample Reducing	Thermo Fisher Scientific	n/a
RIPA Lysis & Extraction	Thermo Fisher Scientific	n/a
FPG FLARE™ assay kit	Trevigen	n/a
hOGG1 FLARE™ assay	Trevigen	n/a

2.2 Equipment

Table 2.2 below shows a list of the equipment and materials used and their manufacturers/distributors.

Table 2.2 Equipment and other materials

Equipment's and other Services	Company / Distributor
Amersham™ Hybond™ Polyvinylidene difluoride (PVDF) Blotting Membrane (0.45 µm x 150)	GE Healthcare Life Sciences, Germany
BioDrop™ Touch Duo Spectrophotometer	BioDrop Ltd, Cambridge, UK
Bolt™ Welcome Pack + iBlot™ 2 System	Thermo Fisher Scientific
BRAND® Filter Flask with lateral socket / Vacuum glass bottle	Sigma-Aldrich, UK
BRAND® Staining Trough / incubation box with tray	Sigma-Aldrich, UK
Centrifuge Mistral 3000	MSE, Albertville, USA
Centrifuge (biofuge 28 RS)	Heraeus, Sepatech, Germany
CCD camera	Hitachi KPMI/EK Monochrome,
Coplin jar	VWR, Lutterworth, UK
Corning® 15 mL centrifuge Tubes	Sigma-Aldrich, UK
Corning® cell culture flasks (25 cm^2)	Sigma-Aldrich, UK
Culture flasks (25&75 cm^3)	Corning Incorporated Costar®, NY, USA
Dry incubator (37° C) LKB BIOCHROM	Leec LTD, Nottingham, UK
Electrophoresis power supply	Consort (E861), Belgium
Electrophoresis tank (HU20)	Scie-Plas, Renfrewshire, UK
Falcon tubes	BD, Swindon, UK
Freezer -20° C	Sanyo, Ultra low, Japan
Freezer -80° C	Sanyo, Ultra low, Japan
Fluorescent microscope	Leica, Weztler, Germany
Fume cupboard	Milton, UK
Fume hood ray air	Maiche Aire, Bolton, UK
Ice maker (Scotsman AF 100)	Namur, Belgium
Incubator 37° C with 5% CO_2	Andor Technology Ltd, Belfast
Light microscope	Nikon, Japan
Komet 6 software	Kinetic Imaging, Nottingham, UK
Microcentrifuge MSE	GMI, Alberville, USA
Microplate reader	Dynex technology, Sussex, UK

Microscope (ortholux)	Leitz, Sturttgart, Germany
Mini Gel Tank	Thermo Fisher Scientific , UK
Pipettes	Gilson, Middleton, WI, USA
pH meter	Dunmow, UK
Power pack supply	Pharmacia LKB, Uppsala, Sweden
Water bath	Grant instruments, Cambridge, UK

2.3 Methods

2.3.1 Ethical approval

This study received approval from Leeds East Research Ethics Committee (REC number: 12/YH/0464), the University of Bradford Research Ethics Sub-Committee on Research in Human Subjects (Ref: 0405/8) and the Research Support and Governance office, Bradford Teaching Hospitals, NHS Foundation (Ref: RE DA 1202).

2.3.2 Volunteers recruitment

Each volunteer was given a participant information sheet consisting of two parts as follows (Appendix 1-3 used to collect the data). Part 1 provides an overview of the study aim and objective, thus providing participants with a voluntary opportunity to participate in the study by providing their blood sample. The subsequent part 2, provides the participants with more information on participation along with informed consent should they wish to proceed in taking part. Both data collected and the biological blood samples obtained are not associated with the donor, nor they can be traced or identified. The healthy volunteers (students and staff) from the University of Bradford were recruited from different ethnicities to participate in this study. The inclusion criteria for healthy volunteers include:

1) Being able to give the consent form.
2) Being in agreement with the storage of the blood sample at the University of Bradford
3) The participant age over 18 years old

While the exclusion criteria for healthy controls include:

1) The participants with anaemia (If the haemoglobin level is lower than the minimum normal range for the age and gender).
2) The participants who are not well enough to take part in the study.

The Lung cancer patients were recruited from Professor Badie Jacob and Dr Abid Aziz Clinics at Bradford Royal Infirmary (BRI) Hospital and Saint Luke's Hospital in Bradford, West Yorkshire.

Lung cancer patients inclusion criteria include:

1) Being able to give the consent form.
2) Being in agreement with storage of the blood sample at the University of Bradford
3) Having been diagnosed with lung cancer disease.

While the exclusion criteria for lung cancer patients include:

1) The participants with anaemia (If the haemoglobin level is lower than minimum normal range for the age and gender).

2) The participants who were on radiotherapy/ chemotherapy at the time of sample collection
3) The participants who are not well enough to take part in the study

The co-factors that were recorded for healthy volunteers and lung cancer patients are cigarette smoking, occupational risk exposure, diet, vitamins and antioxidant, alcohol intake and their medications.

Blood samples were collected by venepuncture after taking consent from healthy volunteers and lung cancer patients, as presented in Table 2.3 and Table 2.4.

Table 2.3 Brief information about healthy blood volunteers.

No	Age	Ethnicity	Gender	Smoking history	Family history
1	33	Caucasian	F	Non-smoker	-
2	34	Asian	M	Past smoker	-
3	20	Asian	F	Non-smoker	-
4	42	Asian	F	Non-smoker	-
5	21	British	F	Non-smoker	-
6	20	Asian	M	Non-smoker	-
7	21	Asian	M	Non-smoker	-
8	22	Asian	F	Non-smoker	-
9	21	British	F	Non-smoker	-
10	22	Asian	F	Non-smoker	-

11	24	Asian	F	Non-smoker	-
12	35	Caucasian	F	Non-smoker	-
13	37	Caucasian	M	Non-smoker	-
14	40	Asian	M	Non-smoker	-
15	39	African	F	Non-smoker	-
16	33	Asian	M	Non-smoker	-
17	39	Caucasian	M	Non-smoker	-
18	28	Caucasian	M	Non-smoker	-
19	35	Asian	Male	Non-smoker	-
20	48	Asian	Male	Non-smoker	-

Table 2.4 Brief information about the lung cancer patients.

No	Age	Ethnicity	Gender	Smoking history	Family history
1	75	Caucasian	M	Smoker	-
2	80	Asian	M	Past smoker	-
3	78	Caucasian	M	Past smoker	-
4	83	Caucasian	F	Past smoker	-
5	70	Caucasian	F	Smoker	-
6	73	Caucasian	M	Past smoker	-

7	63	Asian	F	Non-smoker	-
8	67	Caucasian	F	Non-smoker	-
9	45	Caucasian	F	Smoker	-
10	67	Caucasian	M	Past smoker	-
11	78	Asian	F	Past smoker	-
12	59	Caucasian	M	Past smoker	-
13	76	Caucasian	F	Past smoker	-
14	73	Caucasian	M	Non-smoker	-
15	68	Caucasian	M	-	-
16	72	Caucasian	M	Smoker	-
17	72	Caucasian	F	Past smoker	-
18	83	Caucasian	M	Past smoker	-
19	75	Caucasian	M	Past smoker	-
20	60	Asian	M	Smoker	-

2.4 Preparation of interferon gamma (IFN-γ)

The lyophilized powder was reconstituted in sterile double distilled water to form the stock solution of 1mg/ml . It was then diluted in RPMI medium with 10% foetal calf serum and stored at -20 ^{o}C.

2.5 Preparation and Characterization of Liposomes

Liposomes were prepared using the thin film rehydration method. 1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DPPG),1,2-dioleoyl-sn-glycero-3-phsphoethanolamine (DOPE) and cholesterol (4:4:2 molar ratio) were dissolved in Dichloromethane (DCM) and methanol (3:1 v/v). The solution was then transferred to a rotary evaporator with a 25ml round bottom flask. The organic solvent mixture was evaporated at 40 ˚C under vacuum for 6 hours to ensure no traces of the organic solvents were left.

The thin film was then hydrated by 10 ml of PBS solution of IFN-γ for 1 h at 60˚C using bath sonicator (150 W). The sample was then subjected to 4 repeated freeze-thaw cycles (freezing at -20˚C and thawing at 60˚C in the bath sonicator). The sample was centrifuged at 22,000 x *g* for 30 minutes at 4 °C for purification and removal of the non-encapsulated drug.

Average size and polydispersity index (PDI) of the liposome preparations were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS-90,Model ZEN3600 (Malvern Instruments, UK) . All measurements were performed in triplicate.

2.5.1 Determination of IFN-γ encapsulation efficiency

The IFN-γ encapsulation efficiency liposomes were determined by an indirect procedure based on the determination of uncoated free IFN-γ in the supernatant using RP-HPLC (Van Slooten et al. 2001; Haggag et al. 2019). Briefly, the RP-HPLC (Phenomenex- Luna® C18-5 column mm, 5 μm) with a flow rate of 1.0 ml per minutes, and UV detection (254 nm) was used. A mobile phase elution

gradient was used, composing two solvent mixtures solvent A: 0.1% trifluoroacetic acid (TFA) in acetonitrile; solvent B: 0.1% TFA in water. IFN-γ encapsulation inside the liposome was determined from the difference between the initial amount of IFN-γ added and the IFN-γ remaining in the supernatant after liposome fabrication. Each sample was performed in triplicate and loading of IFN-γ expressed as percentage encapsulation efficiency (%E. E.).

2.5.2 Transmission electron microscopy (TEM)

To visualise the particle surface morphology of the IFN-γ liposome the TEM Tecnai 12 (FEI Company, Netherlands) was used. Carbon-coated copper TEM grids were placed in the IFN-γ liposome suspension for 10 minutes. The grids were sequentially washed with distilled water. A drop of uranyl acetate was placed on the parafilm prepared in the petri dish. The grids were placed in uranyl acetate for 2 minutes followed by the visualization of the IFN-γ liposome shape and size by using the TEM.

2.6 Preparation of blood sample for the Comet assay

After consent forms were signed by the volunteers, the blood samples were collected in 9 ml lithium heparin-coated tubes were clearly labelled and anonymised. Then, samples were stored in the laboratory at room temperature up to 48 hours from the time of collection. Samples were diluted using the ratio of 1:1 with Roswell Park Memorial Institute (RPMI) 1640 Medium (RPMI-1640), and then mixed with 10% Dimethyl sulfoxide (DMSO). The diluted blood solution was aliquoted in 1.5 ml Eppendorf® tubes, which were closed tightly and freezing

at a controlled rate to a temperature less than −50°C and subsequent long-term storage at temperature below −130°C.

2.7 Lymphocyte isolation from whole blood.

Three millilitres of whole blood was diluted 1:1 with 0.9% saline and 6 ml of the total of this dilution was carefully layered on top of three ml of Lymphoprep (Axis-Shield, Norway) in 15 ml falcon tubes. The tubes were centrifuged for 20 minutes at 800 x *g*. Lymphocytes were harvested, washed with 10 ml saline and centrifuged again for 15 minutes at 500 x *g* at room temperature. Cells were re-suspended in RPMI 1640 Medium and used for the *in vitro* experiments.

2.8 Genotoxic effect induced by H_2O_2 .

This stage aims to determine the highest dose for H_2O_2 that causes DNA damage without toxicity to allow its use as a positive control (PC) on this research. The concentrations of H_2O_2 that were used; 25 μM, 50 μM, 75 μM and 100 μM. All the steps for the Comet assay were presented in sections 2.6 and 2.11**.** The OTM and %Tail DNA were measured to express the DNA damage displayed by Comet assay in the whole blood samples taken from healthy volunteers. To determine the optimum dose of H_2O_2 for maximum DNA damage without inducing apoptosis, the experiment was repeated five times and the mean of OTM and Tail DNA % was considered as the best dose positive control. Following statistical analysis by using analysed using the one-Way analysis of variance (ANOVA) with Dunnett's multiple comparisons test using GraphPad prism 8.1.2. The p values were considered significant at $P<0.05$(*), $p<0.01$(**), $p<0.001$(***) , 75 μM of H_2O_2 was

statistically the highest and the most significant concentration. Therefore, it was used in this project as a PC.

2.9 Cell viability

The viability of cells was detected after 24 hours of incubation of cells with different concentrations of treatments. Cells were then centrifuged at 160 x *g* for 3 minutes and mixed with 0.4 % of trypan blue stain (1:1 volume ratio). Ten microliters were taken from cells with 10 µl 0.4 % of trypan blue stain mixed well and left to settle for 30 seconds. It was then transferred to the Neubauer haemocytometer. If the cell membrane is compromised, then the trypan blue will penetrate the membrane and presenting a blue colour, which indicates cell death (Chan et al. 2020). Therefore, the percentage of cells that exclude the dye was counted by use of Neubauer haemocytometer. The concentrations of H_2O_2, blank liposome, naked IFN-γ and IFN-γ liposome with cell viability of ≥75% were used in all experiments (Henderson et al. 1998).

2.10 Cell counting kit-8 (CCK-8) (Cytotoxicity assay)

A convenient colourimetric assay was used to determine cytotoxicity and cell viability (Riss et al. 2016). It is based on the reduction of water soluble tetrazolium salt, WST-8 in cells to an orange color formazan dye, which is soluble in the cell culture media .The amount of WST-8 formazan , generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells (Cai et al. 2019). Isolated human lymphocytes from healthy individuals and lung cancer patients were placed in a 96-well plate at a concentration of $10x10^4$ cells

per well. The cells were treated with 75µM H_2O_2, blank liposome, naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml for 24 hours in a humidified incubator at 37 °C, 5% CO_2. Ten microliters of CCK-8 solution was added to each well of the plate, followed by incubation at 37 °C for 4 hours. Absorbance was measured at a wavelength of 450 nm using a microplate reader. The viabilities of the 3 freshly isolated lymphocytes in both groups were >75%.

2.11 Comet assay

2.11.1 Cell treatment

The stored frozen whole blood samples were allowed to thaw at room temperature. A hundred microliters of whole blood samples were incubated for 30 minutes at 37°C with different treatments to make the final volume of 1000µl with RPMI 1640 media (With sodium bicarbonate, without L-glutamine, liquid, sterile-filtered, suitable for cell culture). The treatments included a positive control of 75µM H_2O_2, blank liposome, naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml. To assess the effect of IFN-γ in a highly oxidising environment, 100U/ml of naked IFN-γ and IFN-γ liposome were treated in the presence of 75 mM H_2O_2. An untreated sample was used as a negative control (NC). Immediately after incubation, the tubes were centrifuged for 3 minutes at 160 x *g*. Then, 900 µl of the supernatant was discarded, leaving 100 µl of the pellet inside the 1.5 ml Eppendorf. Next, 0.5% low melting point agarose (LMP) was added and this mixture was re-suspended. Thereafter, 100µl of this suspension was added onto two duplicate and labelled slides, pre-coated with 1% normal melting point

agarose (NMP). The slides were covered with coverslips and were transferred onto a cold surface, ensuring they were kept cool for 5 minutes.

2.11.2 Cell lysis

The coverslips were removed at this stage, and the slides were immersed in a black box containing fresh cold lysis solution at 4°C, overnight. The lysis solution is composed of 200 ml lysis stock solution (2.5 M NaCl, 100mM EDTA, 10 mM Trizma base,10 % DMSO and 1% Triton X-100, pH 10). This solution consisted of a high salt concentration to breakdown protein – DNA bond without disrupting the DNA. Triton X-100 was used as a detergent, disrupting the cell membrane. DMSO was used as a scavenger for the free radicals generated from haemoglobin to prevent DNA damage.

2.11.3 DNA unwinding and electrophoresis

Then, the microscope slides were placed into a horizontal electrophoresis tank and incubated in 2000 ml of freshly prepared alkaline electrophoresis buffer. This buffer consisted of 70 ml of (10M NaOH and 200mM EDTA, pH >13), with 1930 ml of cold chilled distilled water, incubated for 30 minutes at a temperature of 4°C to facilitate DNA unwinding. Electrophoresis was then performed at 25 V, 300 mA (0.78 V/cm for the electrophoresis tank) for 30 minutes, using a compact power supply. The procedures were conducted at 4°C.

2.11.4 Neutralisation

The slides were transferred from the electrophoresis buffer and placed horizontally on a flat surface. They were covered with the neutralising buffer

(0.4M Trizma base, pH 7.5) for 5 minutes. This process was repeated twice to prevent further action of the alkaline buffer.

2.11.5 Slide staining and comet scoring

The slides were stained through the addition of 60µl of (20µg/ml) EtBr on each slide. These slides were covered with coverslips in a dark room with dim light. The EtBr was used in the Comet assay as it produces bright, fluorescent light which does not readily fade. The slides were coded before scoring; per slide, 100 nuclei were scored using a 20 X magnification fluorescent microscope. This was in connection with a CCD camera using Komet 6 software and Kinetic Imaging (Andor Technology Ltd, Belfast). Olive tail moment and % Tail DNA were used together to decrease variability in the results (Najafzadeh et al. 2016).

2.11.6 Statistical analysis evaluation

Statistical analysis was performed on healthy individuals and lung cancer patients, each having a sample size of 20 participants (n=20).

The final data were expressed as mean values with standard errors, provided for 100 cells scored on each slide. The results were analysed using the one-Way analysis of variance (ANOVA) with Dunnett's multiple comparisons test using GraphPad prism 8.1.2. The p values were considered significant at $P<0.05$(*), $p<0.01$(**), $p<0.001$(***).

2.12 The cytokinesis block micronucleus (CBMN) assay

The CBMN procedures were performed by using the protocol described by (Fenech 2007).

2.12.1 Collection of blood samples

Fresh whole blood samples in 9 ml lithium heparin-coated tubes were obtained from healthy individuals and lung cancer patients for the micronucleus assay, in accordance with the ethical approval presented in section2.3.1.

2.12.2 Preparation of basic culture medium

The basic culture medium was prepared under sterile conditions within the laminar flow hoods. 100 ml of basic culture medium, containing 15 % of foetal bovine serum, 1% of Penicillin-Streptomycin solutions , 25 mM HEPES and L-Glutamine with end concentrations of 15% and 1% added to RPMI-1640 medium aliquoted to 4.5 ml into T 25 cm^2 corning culture flasks. These were stored at -20°C, ready for future use.

2.12.3 Cell culture and treatment

The frozen T 25 cm^2 flasks which contained the basic culture medium were placed in the incubator at 37 °C with 5% CO_2 for 30 minutes. All procedures during the 72 hours must be under sterile conditions.

Five hundred microliters of whole blood were added to a T25 cm^2 Corning culture flask containing 4.5 ml RPMI-1640 medium supplemented with 1% of Penicillin-streptomycin, 15% Foetal bovine serum and 25 mM HEPES and L-Glutamine with end concentrations of 15% and 1%, respectively, followed by 130 µl of phythaemagglutinin (PHA) (2.5%). In the next 24 h, different treatments were added to the flasks, except for the first flask, it served as a negative control (NC),

50 µl of the blank liposome was added to the second flask. Two positive controls were used with 10 µl of mitomycin C (0.4 µM) used as the positive control 1, and 50 µl of 75µM H_2O_2 served as the positive control 2. Fifty microliters of 100U/ml naked IFN-γ and IFN-γ liposome were added to their respective flasks and the final two flasks contained 100U/ml naked IFN-γ, IFN-γ liposome with H_2O_2. Then, the flasks were transferred to the incubator at 37 °C with 5% CO_2 for 44 hours. The CBMN test preparations were performed according to (Fenech et al. 2003; Fenech 2007).

2.12.4 Cytokinesis block

After 44 hours of incubation, the flasks were transferred to the laminar flow hoods. 30 µl of 1mg/ml cytochalasin B (Cyto-B) were added into each flask to prevent cells from undergoing cytokinesis and inhibit the formation of actin filaments leading to the formation of binucleated cells (Fenech 2007).

2.12.5 The end of cell culture

At the end of 72 hours, the contents of the flasks being transferred into 15 ml falcon tubes and centrifuged at 107 x *g* for 8 minutes.

2.12.6 Cells hypotonic shock treatment

Following centrifugation, the supernatant for each tube was discarded, except for 500 µl, which was retained, using the vacuum pump. Then, 5 ml of 110 mM cold potassium chloride (KCl) was added gently to each tube while a vortex formed. The tubes were then stored for 15 minutes in the fridge at 4°C, and later centrifuged at 107 x *g* for 8 minutes. Afterwards, the supernatant was discarded

by a vacuum pump until 500 µl remained in the tubes. The solutions were re-suspended by patting the tubes gently.

2.12.7 Cell fixation

The fresh Carony's solution was prepared by mixing one part glacial acetic acid with three parts methanol. Then, 5 ml of this mixture was added to the 15 ml tubes, gently on vortex, followed by 3 drops of 38% formaldehyde using a Pasteur pipette. The Falcon tubes were then transferred to the centrifuge for 8 minutes at 107 x *g*. The supernatants were removed by the vacuum pump until 500 µl remained. The fixation was repeated twice without formaldehyde, and the tubes were stored overnight in a refrigerator at 4°C for the next day.

2.12.8 Slide preparation, staining and slides mounting

Following overnight incubation, the tubes were centrifuged at 107 x *g* for 8 minutes. Then, the supernatant was discarded by the vacuum pump until 100 µl remained. Depending on the pellet size, around 200-600 µl of fresh Carnoy's solution was added. Then, 20 µl of cell suspension was added twice onto the labelled slides, centre right and centre left and left overnight to dry.

After the slides were dried, the 200 ml of freshly prepared 5% Giemsa staining was added in serenson buffer (51ml of NaH_2PO_4 with 49 ml of Na_2HPO_4 and 100 dH_2O) and was filtered twice using filter papers. The slides were stained by freshly prepared Giemsa stain for 20 minutes and then rinsed with water for 2 minutes and left overnight to dry.

After that, the slides were mounted in dibutyl phthalate in xylene (DPX) on a heating block at 40°C, covered with coverslips and left overnight until the next day for scoring.

2.12.9 Slide scoring

One thousand cells were scored for each treatment group using a light microscope at 40 x magnification. The nuclear division index (NDI) is used to measure the proliferation status of the viable cells in culture, which indicates the cytotoxic effects and mitogenic response induced by the different compounds used.

If the viable cells have failed to divide during the cytokinesis-block, all cells will be mononucleated cells, and the NDI value will be 1.0. However, if viable cells complete one division, they will be binucleated. Here, the value of the NDI is 2.0. The NDI was calculated using the equation of $NDI = M1 + 2(M2) + 3(M3) / N$, where M1 = mononucleated cells, M2= binucleated cells, M3 = multinucleated cells and N = total number of viable cells scored. Micronuclei were scored from binucleated (BiNC) and mononucleated cells (MonoNC). Nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were only scored for BiNC (Fenech et al. 2003; Fenech 2007).

2.12.10 Statistical Analysis

The experiment was conducted on sample size (n=5) from different individuals with different treatments for each healthy individuals and lung cancer patients. The data were analysed by using one-way ANOVA, followed by Dunnett's post hoc test on GraphPad prism 8.1.2. All values are presented as the mean and

standard error of the mean (S.E.M) and plotted as a graph using GraphPad prism 8.1.2.

2.13 The enzyme-modified comet assay

A modification of the alkaline comet assay was used to determine DNA repair capacity in human lymphocytes from five healthy volunteers and five lung cancer patients using both Fpg and hOGG1 FLARE™ Assay Kit. The test agents included a positive control of 75μM H_2O_2, naked IFN-γ 100U/ml and IFN-γ liposome100U/ml; an untreated sample was used as a negative control. Scoring of cells and statistical analysis has been described in sections 2.11.5 and 2.11.6.

2.13.1 Fpg and hOGG1 modified comet assay

Super frosted microscopic slides were prepared. Each slide was pre-coated with 1% NMP. For each sample and control, the treated cells were mixed with molten LMP agarose (provided with the FLARE™ assay kit) at ratio of 1:10 (v/v) and placed on the slides. After 10 minutes of solidification on ice, the slides were immersed in a pre-chilled lysis solution (provided with the FLARE™ assay kit) and kept in a refrigerator at 4°C for 60 minutes. Then the slides were placed in flat surface and covered with the FLARE™ buffer, three times for 15 minutes. The slides were treated with 100μl of Fpg enzyme and the other slides were treated with hOGG1 (1:500 in REC dilution buffer). The enzyme was freshly diluted right before use. The untreated samples were treated with 100μl of REC dilution buffer only. The slides were incubated horizontally in a humidity chamber at 37 C for 45 minutes. All slides were then transferred to an electrophoresis solution of 1 L

(10M NaOH and 200mM EDTA, pH >13), with cold chilled distilled water, incubated for 30 minutes. Electrophoresis was then performed at 25 V, 300 mA (0.78 V/cm for the electrophoresis tank) for 30 minutes, using a compact power supply. The procedures were conducted at 4°C. After electrophoresis, the slides were rinsed gently three times with neutralization buffer (0.4M Trizma base, pH 7.5) for 5 minutes. Each slide was stained with ethidium bromide (20µg/ml) and covered with a coverslip. Slides were stored at 4°C in sealed boxes until analysis.

2.14 Quantitative reverse transcription PCR (qRT-PCR) method

2.14.1 Cell culture

Whole blood samples from lung cancer patients and healthy individuals were used to isolating the lymphocyte. Isolated lymphocytes suspension (2 x 10^6 cells/ml) were treated with 75µM H_2O_2, naked IFN-γ 100U/ml, IFN-γ liposome100U/ml and NC, following that the cells were incubated in 6-well plates for 24 hrs at 37°C in the presence of 5% CO_2.

2.14.2 RNA extraction from human lymphocytes

According to RNeasy Mini Kit (QIAGEN) manufacturer's instructions, the cells were transferred to Eppendorf Tubes® in a sterile laminar flow hoods, centrifuged at 400 x *g* for 3 minutes.

The supernatant was discarded and 350µl of the RLT buffer (10µl of B-mercaptoethanol per 1 ml buffer RLT) was added to the pellets and centrifuged for 2 minutes at maximum speed. The lysate was then added to a QIAshredder spin column in a 2 ml collection tube centrifuged at full speed for another 2 minutes. About 600µl of 70% ethanol was added to the lysate and mixed

carefully by pipetting. The pipetted sample, including any precipitate which may have formed, was transferred into a new QIAamp spin column placed in a 2ml collection tube and centrifuged for 15s at 8000 x *g*. The QIAamp spin column was transferred into a new 2 ml collection tube. Following this, the spin column membrane was washed with 700µl of RW1 Buffer and centrifuged for 15s at 8000 x *g*. The QIAamp spin column placed in a new 2 ml collection tube. In this step, the spin column was washed twice with 500µl RPE Buffer then centrifuged at 8000 x *g* for 3 minutes to avoid carrying over ethanol. Finally, the QIAamp spin column was transferred to a new 1.5ml microcentrifuge tube. RNA was eluted by pipetting 50µl of RNase-free water directly in the middle of QIAamp membrane by centrifugation for 1minute at 8000 x *g*. The purity of RNA were measured using BioDrop™ Touch Duo Spectrophotometer (BioDrop Ltd, Cambridge, UK) that measures UV light absorbance and calculates the 260/280 ratio.

The RNA A260/A280 ratio within 1.8-2.1 range was used for the cDNA synthesis. The RNA samples used for cDNA synthesis were finally held at -80ºC until qPCR.

2.14.3 Complementary DNA synthesis

To synthesise the cDNA from total RNA, it was carried out using the iScript cDNA Synthesis Kit (Bio-Rad) in 20µl reaction volumes was used by mixing the following components: 4ul of 5xiScript Reaction mix with 1ul of iScript Reverse Transcriptase, RNase-free water, and 1 µg of the thawed extracted RNA together in PCR tubes. PCR reactions were transferred to a Bio-Rad PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc. Hercules, CA, USA), and incubated for 5 minutes at 25˚C, reverse transcription for 20 minutes at 46˚C, RT inactivation

for 1 minute at 95°C and holding at 4°C for 10 minutes. The cDNA samples were stored at -20°C until used for the next step.

2.14.4 The quantitative real-time PCR method

After synthesising the cDNA, the real-time PCR reaction was set up in 96 PCR reaction plates and sealed using MicroAmp® Optical 8-Cap Strips.

The assays were performed in triplicate to reduce the variation in the result. Real-time PCR reaction total reaction volume was 20μl consisting of 2μl of primer for the target gene TP53, CDKN1A (p21), Bcl-2 and β-Actin, 10μl of SYBR® Green Supermix, 4μl of RNase/DNase-free water (Qiagen, UK) and 4μl of diluted cDNA. β-Actin was used as a housekeeping gene for the normalisation of the reaction. The 96 well plates were placed in a plate spinner to remove any bubbles present and settle the solution at the bottom of the plates. The plates were then placed in StepOnePlus Real-time PCR Detection System (Applied Biosystems, Warrington, UK), and the qRT-PCR thermal conditions were set up as follows: The denaturation at 95°C for 10 minutes then 40 cycles of denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1 minute. Finally, a melting curve of 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds.

2.14.5 Data analysis

The data were analysed using CFX Manager™ Software to determine the gene expression levels and normalised against the housekeeping gene/reference (β-Actin). The 2-ΔΔCT according to Livak and Schmittgen 2008 was used to determine differences in fold-change of genes expression between untreated

lymphocytes (control cells) from lung cancer patients and healthy volunteers, and treated lymphocytes with 75µM H_2O_2, naked IFN-γ 100U/ml, IFN-γ liposome 100U/ml (Schmittgen and Livak 2008).

2.14.6 Statistical analysis

Each experiment was repeated three times in three individuals and the results were presented as the mean ± SEM. The data analysis was performed using GraphPad Prism® software, version 8.4.0, with built-in two-way ANOVA and Tukey's multiple comparisons test.

2.15 Western blot assay

2.15.1 Cell culture

Peripheral blood (12 ml) from lung cancer patients and healthy volunteers were used to isolate the lymphocyte. The lymphocyte pellets with the concentration of $1x10^6$ cells were cultured in six-well plates containing RPMI 1640 medium with Glutamax-I, 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution under sterile conditions with the addition of PHA 2.5% final concentration. The cultures were incubated at 37°C in the presence of 5% CO_2 in the air for 72 hours. Additionally, 100U/ml of naked IFN-γ and IFN-γ liposome as a final concentration was added to the cultures after 24 hours of incubation time. The negative control was cultured without treatment.

2.15.2 Protein extraction

The culture medium was carefully removed from each well and transferred to Eppendorf Tubes® and centrifuged at 400 x *g* for 3 minutes.

The supernatant was discarded, and the lymphocyte pellets wash twice with cold PBS. Then the pellets were re-suspended in 100 µl of RIPA buffer with the addition of 2 µl of protease inhibitor cocktail to the cells, the cells were incubated for 30 minutes on ice.

Next, the cell suspension was briefly sonicated (~5 seconds) at 30 W followed by centrifugation at 14,243 x *g* for 30 minutes and the supernatant was collected to other labelled tubes. From each tube, 5 µl of extracted protein was taken for protein quantification by the Bradford assay and the rest of the cell lysates stored at -80°C.

2.15.3 Protein quantification

Total protein concentration was quantified by using a Bio-Rad Bradford assay kit. The assay is based on an absorbance shift in the dye coomassie (red colour) which changes and stabilizes into coomassie blue by binding protein. The characteristic blue colour has a maximum absorbance at 595 nm, which is proportional to the amount of bound dye, and thus to the concentration of protein present in the sample. In this assay, a range of different concentrations (0.125, 0.250, 0.5, 0.750, 1.00, 1.5 and 2 mg/mL) of bovine serum albumin was used to produce a standard curve. Five µl of each unknown sample, and standard, were pipetted into separate wells of 96 well plates in triplicate and 250µl of diluted Bio-Rad dye reagent. The mixtures were mixed on a shaker for 30 seconds and

incubated at room temperature for 5 minutes. The optical densities of Bovine serum albumin (BSA) standards and unknown samples were read at 595 nm.

2.15.4 Sodium dodecyl sulphate-polyacrylamide gel (SDS- PAGE)

Tris buffers (pH 6.8 & pH 8.8) were prepared for the resolving and stacking gels. The catalysts APS and TEMED and a final concentration of 10.4% SDS were added for polyacrylamide gel polymerization.

At the bottom, a resolving gel with a pH of 8.8 (5 ml ddH_2O, 6.67 ml 30% acrylamide, 4 ml 1.5 M Tris pH 8.8,160 µl 10% SDS, 160 µl 10% APS and 16 µl TEMED) was poured between 2 glass plates to a level of 1 cm below the teeth on the comb and 1 mL of dH_2O was added in the top of the resolving gel. The dH_2O was poured off after the gel had set. Stacking gel solution with a pH 6.8 (5.3 ml ddH_2O, 2 ml 30% acrylamide, 2.5 ml 1.5 M Tris pH 8.8,100 µl 10% SDS, 100 µl 10% APS and 10 µl TEMED) was then poured on top of the solidified separating gel to pack proteins together after loading and the comb was inserted. The gel was allowed to sit at room temperature for about 30 minutes. After that, the comb was removed. While gels were setting, the protein samples were prepared by mixing each sample with the 2X sample buffer (1:1) then heated in boiling water for 5 minutes. The Laemli buffer was used because it contains 2-mercaptoethanol that reduces the disulfide bonds. It contained SDS detergent, which denatures the proteins and gives each protein a negative charge; accordingly, each protein separates on a size base and not on a charging base. In addition, bromophenol blue is used because of the colour, making it easier to see the samples and glycerol to increase samples viscosity. Samples were then centrifuged for 10 seconds after boiling. The gels were immersed in

electrophoresis buffer (25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS) within the running tank and 30 μg of protein was loaded per well. On each gel, one lane contained 10 μl of protein ladder (Precision Plus Protein™ Dual Color Standards). Every sample was loaded carefully and slowly to prevent the sample from leaking out of the lane.

The Power PackTM Basic (Bio Rad, UK) was used to run the electrophoresis, the gel was run initially slowly through the polyacrylamide gel (50 Volts) for 30 minutes, giving clear and sharp bands, and then running at 100V (constant voltage) for 1hr 30minutes. The prestained protein ladder and the dye front were monitored to determine the end of the electrophoresis separation process. Low percentage gels separate larger proteins, whereas a higher percentage of gels separate smaller proteins better.

2.15.5 Transfer to membrane

After the electrophoresis, proteins were transferred to a blotting nitrocellulose membrane using the iBlot® Gel Transfer Device (Invitrogen) for 7 minutes at a constant voltage of 25V.

2.15.6 Blocking

After transfer, the nitrocellulose membranes were incubated with the blocking solution contained 5% (w/v) BSA in Tris-buffered saline containing Tween 20 (TBS-T)(150 mM NaCl, 20 mM Tris base and 0.1% (v/v) Tween 20, pH 7.4) for one hour at room temperature with gentle shaking. Membranes were washed 3 times for 10 minutes with TBS-T.

2.15.7 Primary antibody

A primary antibody was then added to the solution, which was able to bind to its specific protein. The blotting membranes were incubated overnight at 4ºC with shaking with the primary antibody. GAPDH rabbit monoclonal primary antibody was used as a loading control. A monoclonal antibody was used because it gives a better signal and lower background and has high specificity. The primary antibodies which include [GAPDH (1:10,000 dilution), p53 (1:1,000 dilution); P21 (1:1,000 dilution); BCL-2 (1:1000 dilution)] was diluted with TBS-T containing 5% (w/v) BSA. Membranes were then washed 3 times for 10 minutes with TBS-T.

2.15.8 Secondary antibody

A secondary antibody Donkey Anti-Rabbit IgG H&L (HRP) was added to find locations where the primary antibody bound. The secondary antibody was diluted 1:3000 with 5% (w/v) BSA in TBS-T solution. The membrane was incubated at room temperature with gentle agitation for one hour. The membrane was washed TBS-T 4 times for 15 minutes each.

2.15.9 Detection

The membrane was stained with enhanced chemiluminescence (ECL) kit solutions by incubating the membrane with the same amount of ECL solution 1 and 2 for 1minute at room temperature. Excess of detection reagent was removed, and the membrane was placed on GB Box (Gene flow, UK). Images were captured and saved to be analysed later.

2.15.10 Data Analysis

Relative expression of the protein was determined using image j software. GAPDH served as the protein loading control.

The data were presented as mean ± SEM of three independent experiments in isolated lymphocytes from 3 individuals from healthy and patient groups. To analyse the significance between the different treatment concentrations, the data were analysed by GraphPad Prism® software, version 8.4.0, with built-in two-way ANOVA followed by Dunnett's post hoc test for significant differences.

CHAPTER THREE: The Comet assay in peripheral blood lymphocytes after treatment with naked IFN- γ and IFN- γ liposome

3 Comet Assay

3.1 Introduction

IFN- γ is a pleiotropic cytokine with immunoregulatory roles and antiviral and anti-tumour properties. Several studies have been focused on examining the relationship between the level of IFN- γ intake and the treatment of different malignancies (Lin and Young 2012). The *in vitro* treatment of cancer cells with cytokines such as IFN- γ, contributes to apoptosis was reported by (Abdel-Messeih et al. 2017). Another illustration of the role of IFN- γ as an antitumor was presented by Fang et al. that IFN- γ can inhibit cell proliferation predominantly through Jak/STAT1 pathway (Fang et al. 2008). IFN-γ also can induce the expression of tumour suppressing factors such as Mig-1. The IFN-γ induced the monokine to attract activated T and NK cells and limited the metastasis in a mammary tumour model. Furthermore, GBP-1, the main product of IFN-γ signalling mechanism, significantly prevented the growth of highly malignant TS/A mammary carcinoma cells (Lin and Young 2013).

The most important assay used in *in vitro* and *in vivo* for genotoxicity testing is the Comet assay or single cell gel electrophoresis assay which is a technique

that was designed to measure DNA damage in individual cells including lymphocytes and sperm (Wu and Jones 2012; Langie et al. 2015a). Peripheral blood lymphocytes were used in previous studies as surrogate cells to examine the level of DNA damage (Anderson et al. 2014).

The Comet assay is a simple, versatile, visual, rapid, and sensitive test used extensively to assess the DNA damage, and DNA repair (Cortés-Gutiérrez et al. 2011).

The Comet assay is used to assess the DNA damage in lymphocytes by using two parameters OTM and % of Tail DNA (Intranuovo et al. 2018).

In these experiments, the effect of IFN-γ in both liposomal and naked forms was assessed for DNA damage in human blood lymphocyte cells. There were obtained from a healthy volunteer and lung cancer patients using the Comet assay. The classic genotoxic compound hydrogen peroxide (H_2O_2) (75 µM) was used as a positive control. The dose-response experiments were carried out to determine the optimal doses of naked IFN-γ and IFN-γ liposome used throughout the study. A fixed-dose of both forms 100U/ml of naked IFN-γ and 100U/ml of IFN-γ liposome.

3.2 Materials and methods

All chemicals and equipment used in the Comet assay are listed in Table 2.1 and Table 2.2 .The Comet assay methods are presented in chapter 2, section 2.6 and section 2.11.

3.3 Results

3.3.1 Encapsulation Efficiency determination of IFN-γ liposome

The encapsulation efficiency was calculated from the amount of free drug (IFN-γ) in the supernatant . The detection of the IFN-γ was carried out using RP-HPLC , and the retention time of IFN-γ was 1:50 minutes as shown in the chromatogram in Figure 3.1 and Figure 3.2

A calibration curve of the peak area of IFN-γ vs. concentration in the range of 0.25 to 10 µg/ml was produced (Table 3.1) . The regression equation of the line was y = 265838x - 97029 with a correlation coefficient $R^2 = 0.9897$ as presented in Figure 3.3 demonstrates the chromatogram of the supernatant of free drug (IFN-γ) . The peak area obtained from this figure was used in the calculation of the concentration of encapsulation efficiency based on the equations below. The encapsulation efficiency of IFN-γ was 72%. It was determined by using the equation generated by the calibration curve.

The initial concentration of IFN-γ was 1.38 µg/ml and the concentration of free drug (IFN-γ) = 0.38 µg/ml (based on the equation). So the concentration of encapsulated IFN-γ was 1µg/ml .

Encapsulation Efficeincy (%) = $\frac{Total\ \text{IFN}-\gamma - free\ \text{IFN}-\gamma}{Total\ \text{IFN}-\gamma} * 100$

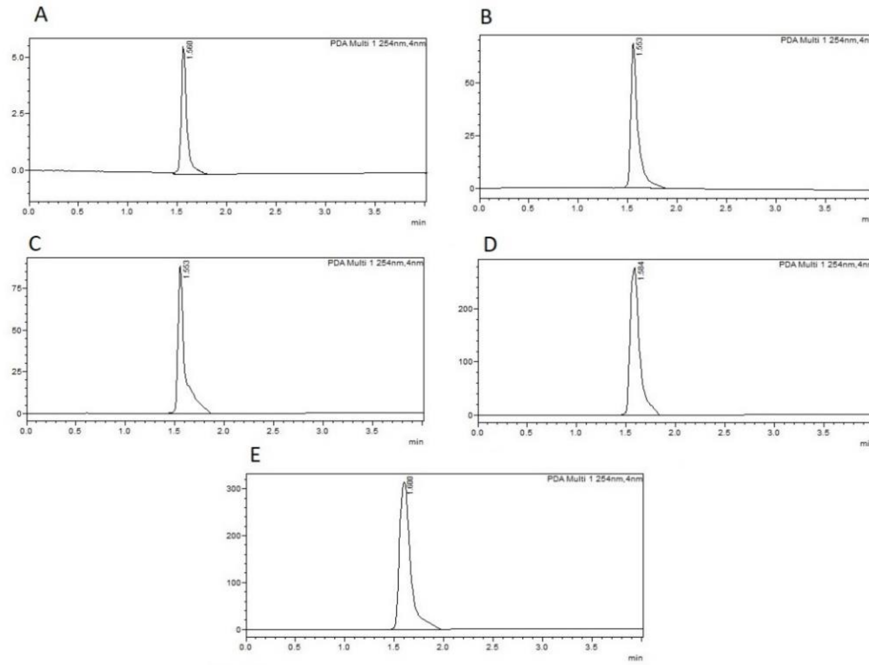


Figure 3.1 HPLC calibration curve chromatogram of IFN-γ with different concentrations, peak identified at 1.5 minutes (refer to Table 3.1 for concentrations)

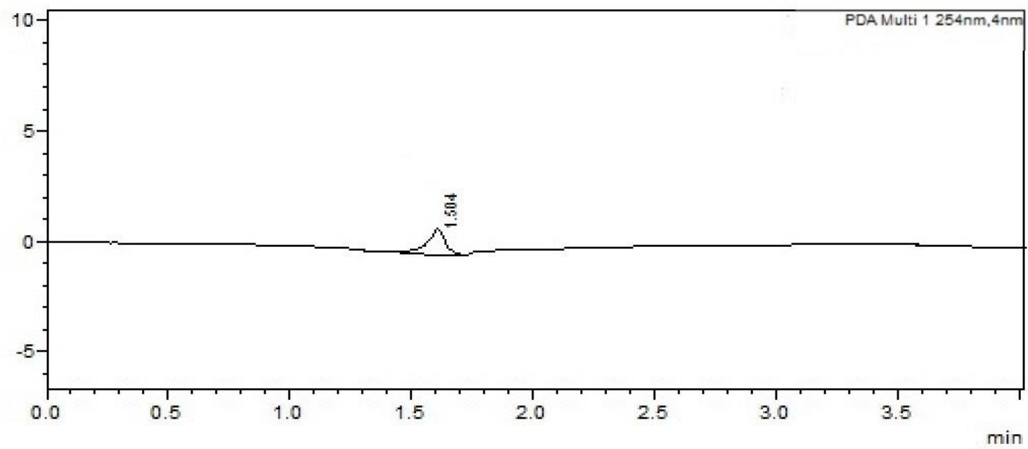


Figure 3.2 HPLC calibration curve chromatogram of free drug (IFN-γ), peak identified at 1.5 minutes

Table 3.1 The concentrations of IFN-γ with peak area

Chromatogram	Concentration ug/ml	Peak Area
A	0.25	23757
B	2	374962
C	5	1133943
D	6	1529361
E	10	2476851
Supernatant		6072

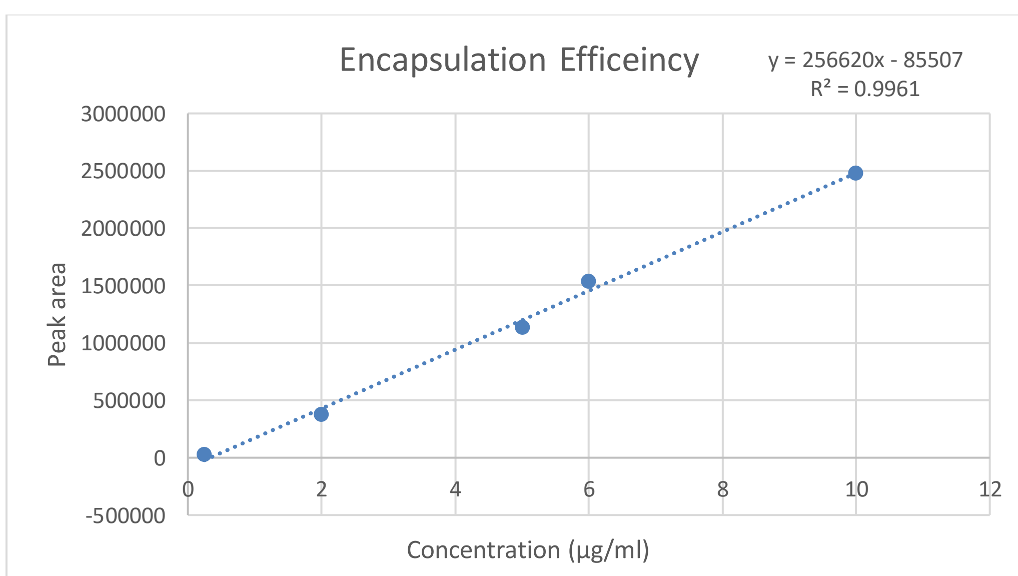


Figure 3.3 Standard calibration curve of IFN-γ

3.3.2 Particle size determination of IFN-γ liposome

The thin-film rehydration method was used to encapsulate IFN-γ due to its simplicity, practicability, reproducibility, and ability to produce small and uniform liposomes.

Figure 3.4 shows the Z-Average particle size was 146.9 nm with PDI 0.210. To ensure the liposome stability, it was stored in the refrigerator at 4°C and the particle size was measured. The results show non-significant increases in the particle size for ten days.

The surface morphology of the IFN-γ liposome was visualised by using transmission electron microscopy (TEM). TEM images represented uniform, smooth surface, spherical shape, homogenous, and no evidence of liposome aggregation (Figure 3.5).

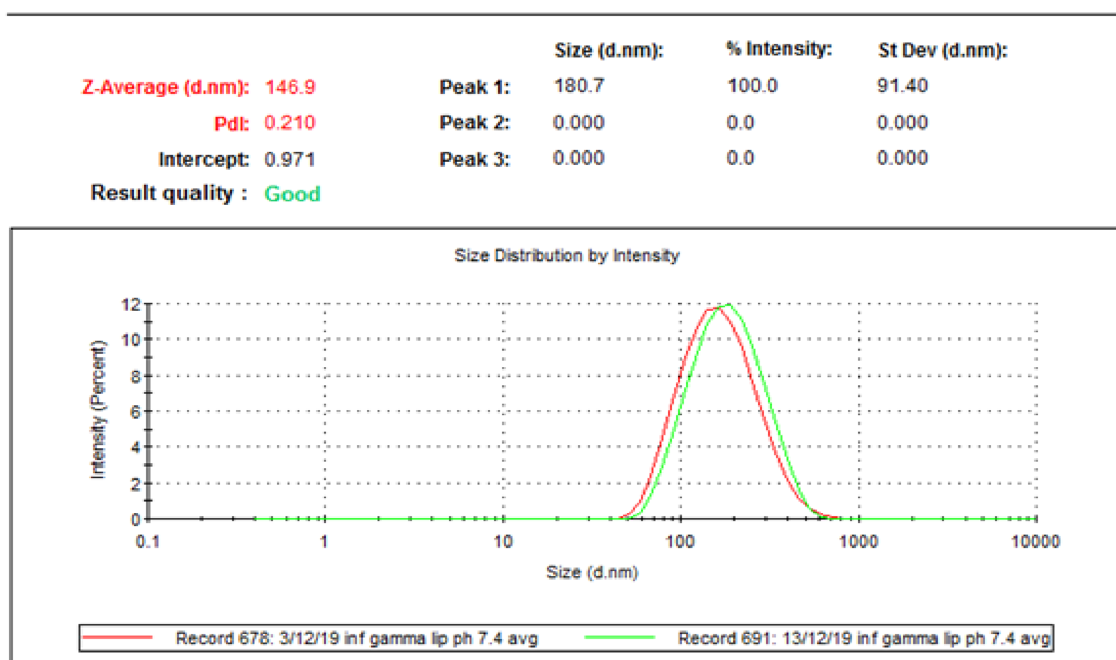


Figure 3.4 Measurement of particle size -distributon by DLS using Zetasizer Nano ZS-90 Model ZEN3600 (Malvern Instruments Ltd, UK).

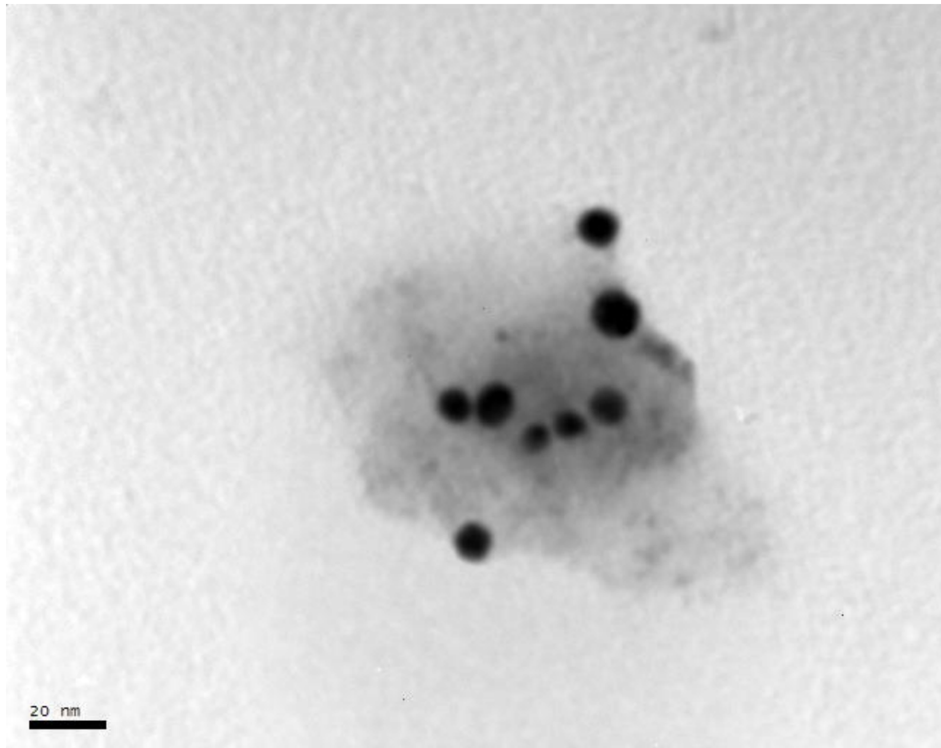


Figure 3.5 TEM image of IFN-γ liposome (200 k magnification).

3.3.3 Cell viability by trypan blue dye:

The viability of lymphocytes from 3 healthy individuals and 3 lung cancer patients was assessed after treatment with chemicals used in this study, between (75 - 85%) using trypan blue dye exclusion.

3.3.4 Cell counting kit-8 (CCK-8):

The viability of lymphocytes from 3 healthy individuals and 3 lung cancer patients treated with different treatment groups was also confirmed using the CCK-8 assay > 75 after 24 h treatment (Figure 3.6).

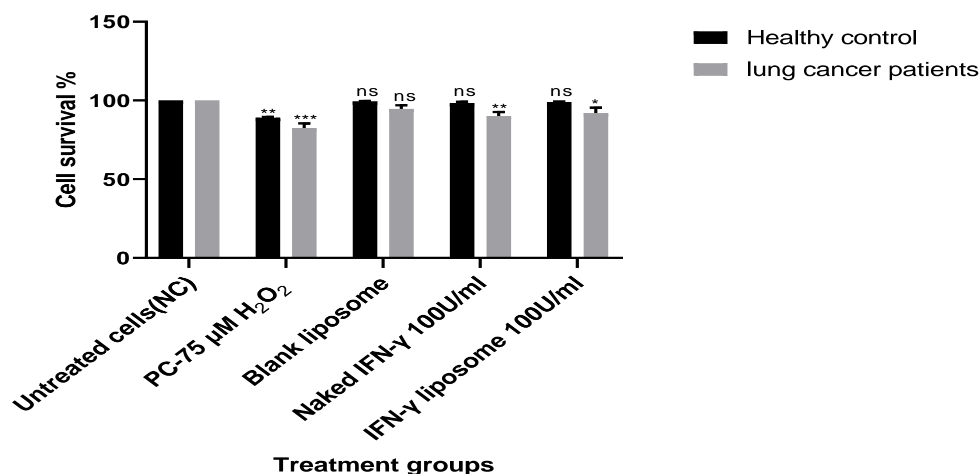


Figure 3.6 % survival in 10 × 10^4 cells of various test treatments in lymphocytes from healthy individuals and lung cancer patients using CCK8 assay. Error bars show mean ±SEM, n = 3. *p<0.05; ** p<0.01; *** p<0.001; ns = not significant . All the results for various treated groups were compared against the respective negative control group .The results were analysed using the one-Way ANOVA with Dunnett's multiple comparisons test using GraphPad prism 8.1.2

3.3.5 IFN-γ liposome and naked form concentration responses on lymphocytes DNA from healthy individuals.

Figure 3.7 and Figure 3.8 illustrates that the concentrations-response of 100U/ml naked IFN-γ, IFN-γ liposome and both forms with 75μM H_2O_2 on lymphocyte DNA obtained from 20 healthy individuals measuring % DNA tail and OTM. IFN-γ in both forms was clearly unaffected the lymphocytes from healthy individuals when measuring the two parameters the % DNA tail and OTM. Whereas the positive control showed high significance ***p ≤ 0.001.

Further reduction on lymphocyte DNA damage was shown with naked IFN-γ and IFN-γ liposome in the presence of 75 μM H_2O_2 compared with the PC (***P ≤ 0.001). Despite the encapsulation of the IFN-γ in liposomal formulation, the efficacy of the biological activity in reducing the DNA damage has not been significantly impacted. In fact the level of DNA damage is slightly lower in the liposomal formulation. (Figure 3.7 and Figure 3.8).

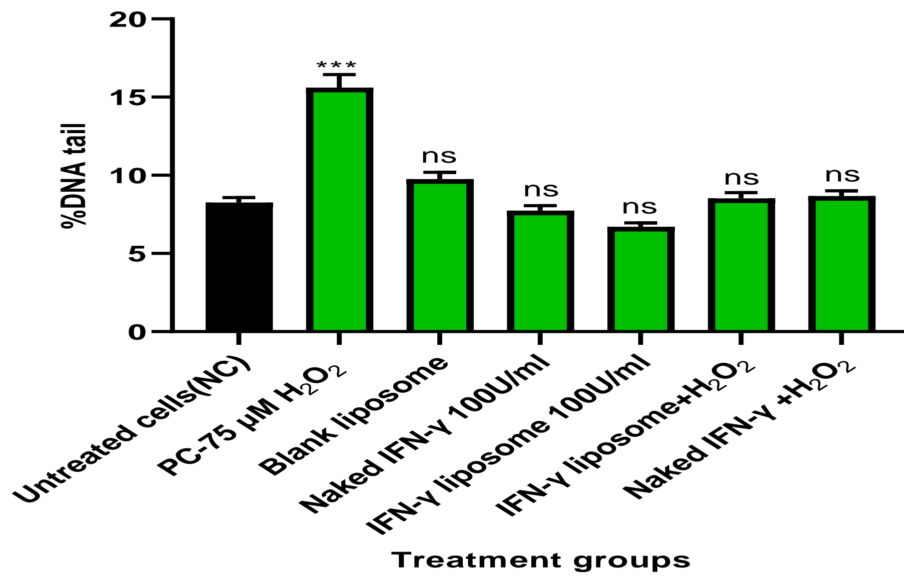


Figure 3.7 Effects of naked IFN-γ ,IFN-γ liposome and both form in the presence of 75 μM H_2O_2 in lymphocyte from healthy individuals by measuring the % DNA tail (n=20). Error bars show mean ±SEM, n = 3. *p<0.05; ** p<0.01; *** p<0.001; ns = not significant

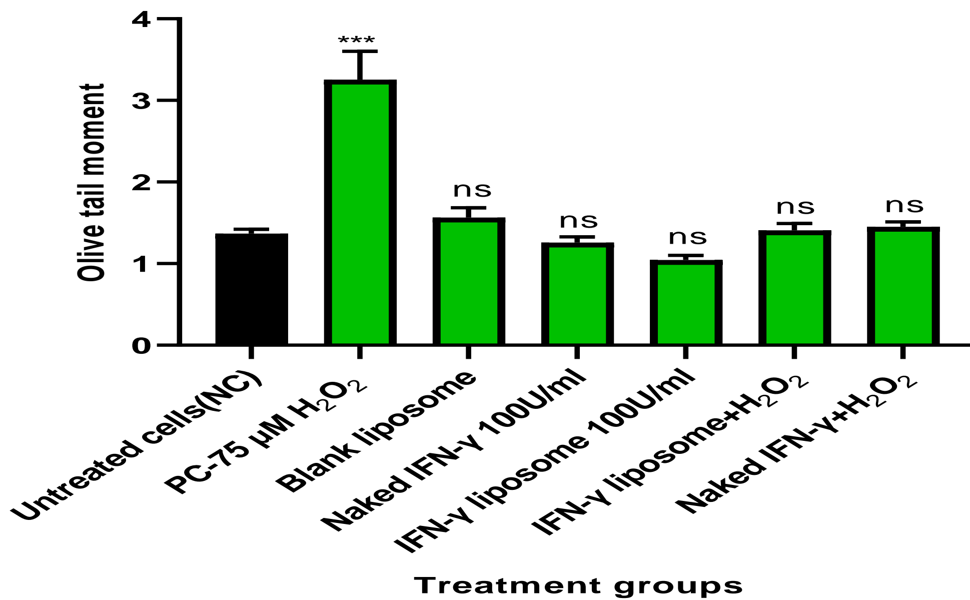


Figure 3.8 Effects of naked IFN-γ ,IFN-γ liposome and both form in the presence of 75 μM H_2O_2 in lymphocyte from healthy individuals by measuring OTM (n=20). Error bars show mean ±SEM, n = 3. *p<0.05; ** p<0.01; *** p<0.001; ns = not significant

3.3.6 IFN-γ liposome and naked form concentration responses on lymphocytes DNA from lung cancer patients.

The main aim of this study was to determine the effectiveness of IFN-γ as an anti-cancer treatment. Furthermore, this study attempted to determine their potential in reducing DNA damage among lung cancer patients cells.

The 100U/ml naked IFN-γ and liposome form, both significantly reduced the DNA damage in lymphocytes from lung cancer patients, compared to the untreated controls (***$p<0.001$) in % DNA tail and OTM (Figure 3.9 and Figure 3.10). However, when the cells were treated with naked IFN-γ and IFN-γ liposome with 75 μM of H_2O_2, a significant reduction was shown in DNA damage compared to the PC in % DNA tail and OTM (Figure 3.9 and Figure 3.10). On the other hand , the reduction in the DNA damage with IFN-γ liposome when co-administratd with 75 μM of H_2O_2 was less compared to the naked form (Figure 3.9).

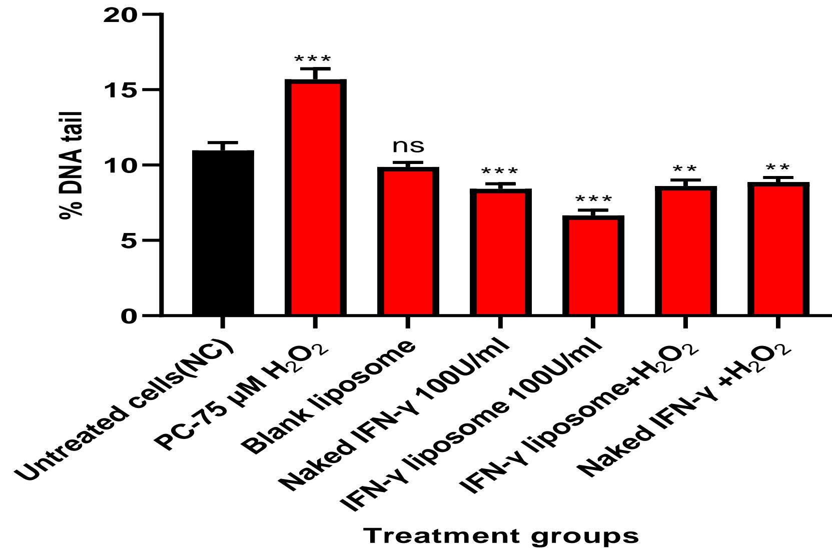


Figure 3.9 Effects of naked IFN-γ ,IFN-γ liposome and both form in the presence of 75 µM H_2O_2 in lymphocyte from lung cancer patients by measuring the % DNA tail(n=20). Error bars show mean ±SEM, n = 3. *p<0.05; ** p<0.01; *** p<0.001; ns = not significant

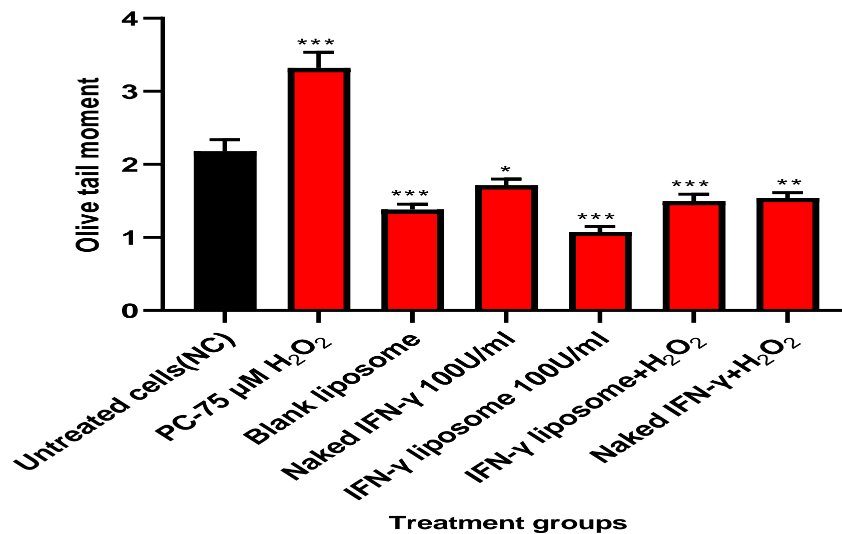


Figure 3.10 Effects of naked IFN-γ ,IFN-γ liposome and both form in the presence of 75 µM H_2O_2 in lymphocyte from lung cancer patients by measuring OTM (n=20). Error bars show mean ±SEM, n = 3. *p<0.05; ** p<0.01; *** p<0.001; ns = not significant

3.3.7 Effect of IFN-γ liposome & naked on DNA damage in lymphocytes from healthy compared to lung cancer patients using the Comet assay.

The data from the Comet assay results showed that lung cancer patient lymphocytes were more susceptible to DNA damage than healthy individuals, in % Tail DNA and OTM when compared with groups that received no treatment (***$p < 0.001$) as presented in Table 3.2, Figure 3.11 and Figure 3.12. A significant increase in % DNA tail and OTM in lymphocytes from healthy individuals and lung cancer patients after exposure to PC (75 µM H_2O_2) (***$p \leq 0.001$) (Figure 3.11 and Figure 3.12).

Lymphocytes obtained from 20 healthy individuals in Figure 3.11 and Figure 3.12 showed that 100U/ml of naked IFN-γ and IFN-γ liposome was clearly unaffected in lymphocyte cells when measuring % Tail DNA and OTM compared to untreated cells. In contrast, the H_2O_2-induced DNA damage was significantly reduced by 100U/ml naked IFN-γ and IFN-γ liposome in lymphocyte from healthy individuals compared to the PC (75µM H_2O_2) (***$p \leq 0.001$). In contrast, with IFN-γ liposome the reduction in the DNA damage was less compared to the naked IFN-γ, as presented in Table 3.2. Lymphocytes from lung cancer patients also showed a significant decrease in % tail DNA and OTM from 10.97% (% DNA tail) and 2.18 (OTM) compared to the untreated control groups to 8.42% (% tail DNA), and 1.38 (OTM), respectively, when cells were treated with naked IFN-γ. In contrast, the reduction in % DNA tail and OTM was more effective by using IFN-γ liposome, which showed a significant decrease in % DNA tail 6.65% and 1.07 (OTM). Furthermore, cells treated with naked IFN-γ and IFN-γ liposome co-

supplemented with 75 μM H_2O_2 showed a significant decrease in OTM and % DNA tail compared to the PC (75 μM H_2O_2) (***$p \leq 0.001$).

Table 3.2 The mean values of 20 experiments on blood samples from healthy individuals and lung cancer patients expressed as OTM and % DNA tail, including the statistical significance and SEM.

	Healthy individuals		Lung cancer patients	
	% DNA tail ± SEM	**Mean OTM ± SEM**	**% DNA tail ± SEM**	**Mean OTM ± SEM**
Untreated cells (NC)	8.26±0.32	1.37±0.05	10.97±0.52	2.18±0.15
PC-75 µM H_2O_2	15.61±0.83***	3.25±0.34***	15.69±0.71***	3.32±0.2***
Blank liposome	9.76±0.43 ns	1.57±0.12 ns	9.87±0.29 ns	1.71±0.08 ns
Naked IFN-γ 100U/ml	7.73±0.31 ns	1.26±0.06 ns	8.42±0.32**	1.38±0.07***
IFN-**γ** liposome 100U/m**l**	6.70±0.24 ns	1.04±0.05 ns	6.65±0.34***	1.07±0.07***
IFN-γ liposome+H_2O_2	8.53±0.36 ns	1.41±0.08 ns	8.61±0.38**	1.49±0.09**
Naked IFN-γ +H_2O_2	8.67±0.33 ns	1.45±0.06 ns	8.87±0.29*	1.54±0.06*

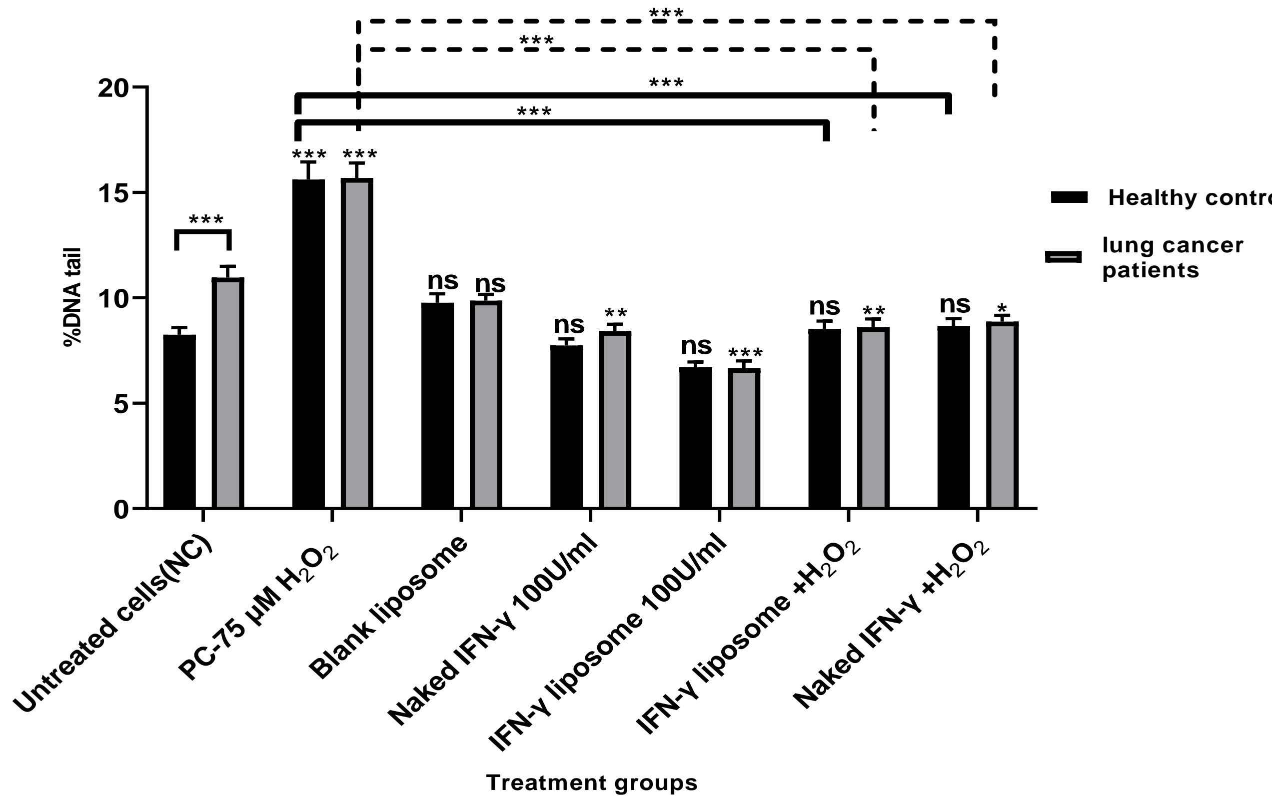


Figure 3.11 DNA damage measured as mean % DNA tail before and after treatment with naked and liposome form of IFN-γ in human lymphocytes from healthy individuals and lung cancer patients in the Comet assay. (n= 20 in each group). Error bars show mean ±SEM, n = 3. *p<0.05; ** p<0.01; *** p<0.001; ns = not significant

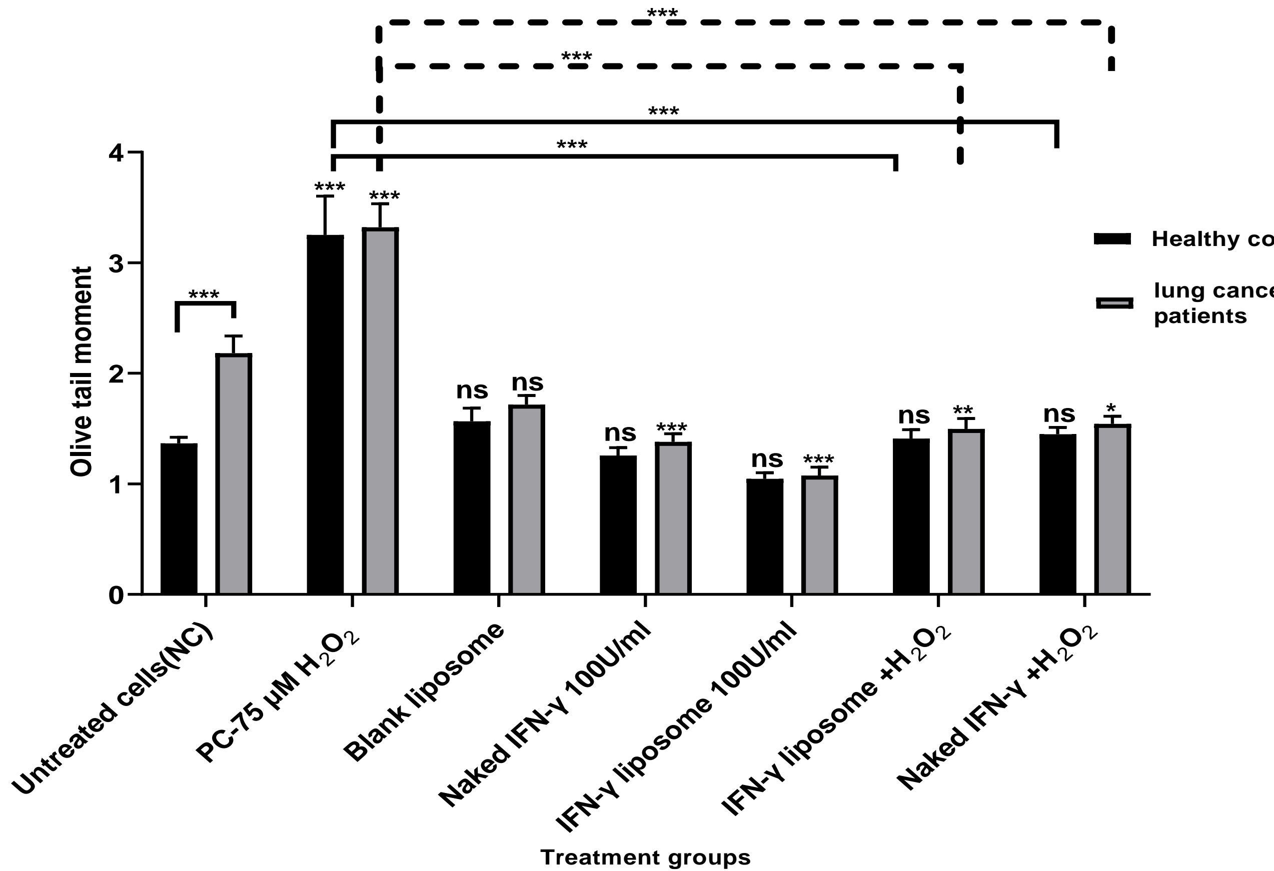


Figure 3.12 DNA damage measured as mean OTM before and after treatment with naked and liposome form of IFN-γ in human lymphocytes from healthy individuals and lung cancer patients in the Comet assay. (n= 20 in each group). Error bars show mean ±SEM, n = 3. *p<0.05; ** p<0.01; *** p<0.001; ns = not significant

3.4 Analysis of confounding factors

Confounding factors such as age, ethnicity, lifestyle (smoking, drinking and diet etc.), gender and environmental conditions are crucial to be taken into account when assessing DNA damage because these factors are potential contributors to DNA damage and, therefore, may cause genome toxicity (Fenech, 2007). Lifestyle variables (smoking and alcohol) are known as carcinogens. Therefore, these factors are particularly harmful and may increase an individual's vulnerability to cancer and other cardiovascular diseases. Consequently, we evaluated the influence of these elements on DNA damage levels in lymphocytes

from healthy individuals and cancer patients using the comet assay (Table 3.3). In the current study, we found no statistically significant differences between any of the groups and these factors have not shown any contribution towards the damage in our results. Healthy individuals have shown little DNA damage at basal levels compared to the patient group, which could be due to the disease conditions in patients. Overall, naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml forms have not induced any genotoxicity. In fact, both treatments have shown a trend of reduction in DNA damage compared to the untreated group and liposome form seemed to be more effective.

3.4.1 Age

The age between the healthy individual group and patient group varied greatly. Nevertheless, these were the only samples (especially from the patient group) present at the time of this study and were best possibly matched varies significantly between both groups, healthy individuals and cancer patients. Most healthy individuals were young compared to patients who were all above 50, so there may be some contribution to DNA damage in lymphocytes from the patient group observed at basal levels. Within our samples, the difference in DNA damage between untreated controls of both groups was highly significant (***$p < 0.001$). This could be due to the fact that one group contained cancer patients and the other group contained healthy individuals. However the difference could also be attributed to age as presented in Soares et al. (2014) a significant and positive association between age and DNA damage(Soares et al. 2014).

When the effect of age was determined using OTM values after treatment with naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml forms, no significant

difference was observed between different age groups due to the effect of both form of IFN-γ in reducing the DNA damage.

3.4.2 Gender

Gender made no influence on the level of DNA damage in lymphocytes in response to various treatments and also different study groups.

3.4.3 Smoking habits

There were no smokers in healthy volunteers, but there was a mix of individuals in the patients group. Therefore, when the effect of smoking was being analysed, the patient group was compared against the non-smoking control group. Due to smoking and the disease conditions in lung cancer patients, all patients have shown a higher level of DNA damage at basal levels. However, naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml forms tend to decrease this damage. The PC control, as expected, has induced significant DNA damage in all the groups.

3.4.4 Ethnicity

The results demonstrated no statistically significant differences between different ethnic groups of healthy and patient individuals.

Table 3.3 The means of Olive tail moment (OTM) in the peripheral blood lymphocytes of different various confounding factors in (A) healthy individuals, (B) lung cancer patients in the Comet assay after different treatments with the PC (H_2O_2)(75µM), naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml forms as well as the negative control of untreated lymphocytes (NC). Data show the mean ± SD. All the results for various treated groups were compared against the respective negative control group. Also, each confounding factor was compared against its control group. (***p < 0.001, **p<0.01 and ns=not significant).

Treatment Group and variable confounding factor		NC (Untreated)	PC (H2O2 75µM)	Naked IFN-γ 100U/ml	IFN-γ liposome 100U/ml
Age	Healthy individuals 20-50	1.37 ± 0.05	3.25± 0.34***	1.26± 0.12 ^{ns}	1.04± 0.5^{ns}
	Patients ≥50	2.18 ± 0.15	3.32± 0.2 ***	1.38± 0.8^{ns}	1.07± 0.07^{ns}
Smoking History	Non-smoking Healthy individuals	1.37 ± 0.05	3.25± 0.34***	1.26± 0.12 ^{ns}	1.04± 0.5^{ns}
	Smoking Patients	2.2 ± 0.1	5.3 ± 1.2 ***	1.7 ± 1.7 ^{ns}	1.6 ± 0.1 ^{ns}
	Ex-smoking Patients	2.0 ± 0.1	5.1 ± 2.1 ***	1.9 ± 0.2 ^{ns}	1.8 ± 0.7^{ns}
	Non-smoking patients	1.9 ± 0.05	4.8 ± 2.0 ***	1.5 ± 0.01^{ns}	1.2 ± 0.4 ^{ns}
Ethnicity	Caucasian Healthy individuals	0.8 ± 0.3	4.4 ± 0.3 ***	0.7 ± 0.7^{ns}	0.8 ± 0.1 ^{ns}
	Caucasian patients	1.3 ± 0.3	4.5 ± 0.02***	1.0 ± 1.2^{ns}	0.9± 0.5 ^{ns}
	Asian Healthy individuals	1.1 ± 0.5	3.8 ± 1.2***	1.0 ± 0.5 ^{ns}	0.8 ± 0.9^{ns}
	Asian patients	2.0 ± 1.1	4.7 ± 0.5***	1.9 ± 1.2 ^{ns}	1.7 ± 1.9 ^{ns}
Gender	Female Healthy individuals	1.5 ± 0.2	3.6 ±1.3**	1.3 ±0.4^{ns}	1.1 ± 0.5 ^{ns}
	Male Healthy individuals	1.2 ± 0.6	4.0 ± 1.3 ***	0.9 ±0.1^{ns}	0.7 ± 0.8 ^{ns}
	Female Patients	1.8 ± 0.6	5.6 ± 1.2***	1.3 ± 1.0^{ns}	1.0 ± 0.4^{ns}
	Male Patients	2.0 ± 1.2	5.0 ± 1.2**	1.7 ± 1.0^{ns}	1.5± 0.8^{ns}

3.5 Discussion

Liposomes have been reported as potential drug delivery system to target cancer cells. Liposomes with small vesicle size are able to escape the tumor vasculature and accumulate in the cells by passive targeting. However, IFN-γ had a short life span circulation, systemic toxicity and indiscriminating of the tumor and healthy tissues. So loaded IFN-γ liposomes were developed to improve its pharmacokinetic properties.

The film hydration method has been used to actively entrap IFN-γ into liposomes with relatively high efficiencies and small vesicle size 146.9 nm. The polydispersity index values of the obtained liposomes are 0.21 indicating narrow size distribution. These findings are in agreement with Sriwongsitanont and Ueno 2010, who showed that thin film rehydration method followed by freeze-thaw cycle can be employed for the production of homogeneously sized liposomes (Sriwongsitanont and Ueno 2010).

These small sized liposomes have the potential to penetrate tumor cell membranes and be taken up by the cells allowing efficient drug accumulation at the target site. The TEM of IFN-γ liposomes showed uniform and spherical-shaped liposomes with a smooth surface. Furthermore In this study, a sensitive and selective RP- HPLC method was used for quantification the concentration of encapsulated IFN-γ which was 1µg/ml. The liposomal formulation produced was shown to have 72% EE of IFN-γ.

This study investigated the efficacy of naked IFN-γ and IFN-γ liposome in human lymphocytes originating from groups of twenty healthy individuals and twenty lung cancer patients, using the Comet assay. Various *in vitro* and *in vivo* studies have shown the promising applications of IFN-γ in the field of

health, as a treatment displaying pleiotropic immunomodulatory, antiviral, antimicrobial, anti-neoplastic, pro-inflammatory activities (Bernabei et al. 2001; Regis et al. 2006; Lin and Young 2013). Furthermore, the potent inhibitory effects of IFN-γ in various tumour models, such as bladder carcinoma, colorectal cancer, ovarian cancer, and adult T cell leukaemia, human pancreatic carcinoma cells, and NSCLC have been evidenced (Prior et al. 1999; Zaidi and Merlino 2011).

None of the previous studies has focused on the effect of IFN-γ on DNA damage in human lymphocyte from lung cancer patients, using Comet assay.

The Comet assay was used in this study due to its sensitivity, simplicity, easy, time efficiency, and cost-effectiveness for assessing DNA integrity in cells (Gopalan et al. 2011).

Peripheral lymphocytes represent an excellent model and lymphocytes can be used as surrogate cells to assess genotoxicity and lymphocytes circulate in the bloodstream through the body consequently. They are vulnerable to endogenous and exogenous DNA damage by physio-chemical genotoxic insults (Najafzadeh et al. 2011).

Isolated and thawed lymphocytes obtained from healthy volunteers and lung cancer patients were treated *in vitro*, with 100U/ml naked IFN-γ and IFN-γ liposome individually for 30minutes at 37°C. H_2O_2 (75 µM) was used as PC. After treatment, the lymphocytes were scored for OTM and % DNA tail.

The Comet assay results showed that the treated lymphocytes from healthy individuals with naked IFN-γ and IFN-γ liposome did not induce significant DNA damage compared to the untreated cells. However, when the same concentration of IFN-γ in both forms were used on lung cancer patients lymphocytes, DNA

damage was reduced compared to the untreated cells. Still, the reduction of DNA damage by liposome form of IFN-γ slightly lesser than the naked IFN-γ (Figure 3.9 and Figure 3.10).

The Comet assay results showed that naked IFN-γ and liposome form has a protective effect against H_2O_2, which induces damage in DNA due to oxidative stress in healthy individuals and lung cancer patients lymphocytes. DNA damage significantly decreased compared to the PC.

Moreover in this study among the healthy individuals, there were no confounding effects on smoking history, since 100 % of them were non-smokers,but in lung cancer patients it was clear that the DNA damage increased due to the smoking habit .

Regarding the confounding effect of age on DNA damage between the healthy individuals group and patients group in this study the age varied greatly, due to the limitation of samples during that time but it is clear that the DNA damage in lung cancer patients with the age ≥50 is higher than the DNA damage in the age group between 20 and 50, and this is consistent with the previous study which presented that the DNA damage correlated with age (Soares et al. 2014). After treated lymphocytes from both groups with naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml forms, the difference wasn't statistically significant between different age groups due to the effect of both form of IFN-γ in reducing DNA damage.

CHAPTER FOUR: Anti genotoxic effects of naked IFN-γ and liposome treatments in lymphocytes using micronucleus assay

4 Micronucleus assay

4.1 Introduction

The naked IFN-γ and liposome results in the previous Chapter three showed the effectiveness of this compound to reduce DNA damage. This was clear, as seen by the significant decrease of % DNA tail and Olive tail moment using the Comet assay. Both IFN-γ B and liposome showed an ability to reduce DNA damage.

It is clear from this finding that Comet assay determines the effect of compounds at the genomic level. Therefore, another assay is needed, which can detect the compound effect at a cytogenetic level. So in this chapter, the main aim is to assess the impact of IFN-γ B and liposome on somatic cells (lymphocytes) using the cytokinesis-block micronucleus (CBMN) assay.

The normal cell cycle is an ordered sequence of processes that occur in a cell to proliferate into daughter cells and include mainly four stages: Gap1, or G1 stage, synthesis, or S stage, gap 2, or G2 stage, and mitotic stage or M phase. The cell cycle occurs over a 24 h period in eukaryotic cells (Bertoli et al. 2013). In the G1 phase, the cells are checked for any defect and progress into S phase by synthesising the protein required for DNA replication. In the S phase, chromosomes are duplicated to produce two identical copies of each chromosome. In G2 phase, all components are prepared for cell division and M phase. The M phase is a critical stage which constitutes to mitosis, and it is the

cycle in which the chromosomes are visibly condensed, this includes 5 sequences (prophase, prometaphase, metaphase, anaphase, and telophase) (Lumen Learning 2017).

Finally, this process followed by cytokinesis, in which the cell divides into two daughter cells. The two daughter cells can either start a new cycle or enter the G0 state (CNX 2019).

The CBMN assay uses a single dividing cell containing two nuclei known as binucleated cells (BiNC). Cyto-B inhibits the assembly of actin filaments network, which are very important in completing the cytokinesis stage, resulting in BiNC formation (Kanagaraj et al. 2017; Kirsch-Volders et al. 2018).

Chemicals and exogenous agents can have mutagenic consequences on cells and interfere with chromosome structure and separation during the cell cycle. Consequently, micronuclei (MNi) arise and originate from acentric or whole chromosomes that lag behind the anaphase stage of mitosis (Fenech et al. 2016; Sommer et al. 2020). MNi induction in BiNC cells indicates cytogenetic damage. MNi can also be found in mononucleated (MonoNC) cells and multinucleated (MultiNC) cells (Ali et al. 2014); However restricting the scoring of MNi in BiNC cells reduced the confounding effects happened by altered or suboptimal cell division kinetics, which is a main variable in the CBMN technique (Fenech 2006; Fenech 2007).

The CBMN assay was performed to evaluate MNi induction following exposure to 100U/ml naked IFN-γ and IFN-γ liposome and both forms with 75 μM H_2O_2. Mitomycin C (0.4 μM), and H_2O_2 (75 μM) were used as PCs. MNi, NBPs, and NBUDs were scored in BiNC up to 1000 cells. One thousand other cells were scored to calculate the percentages of each cell types (MonoNCs, BiNC, and

MultiNC), which were used to calculate the nuclear division index (NDI), to measure the rate of mitotic division as it reflects cytostatic effects. NDI, with the lowest possible value of 1, is mononucleated cell that have failed to divide. This is the case in all viable cells. However, viable cells with one nuclear division are binucleated ; therefore, they have an NDI value of 2. Cells with a substantial proportion of cells completing more than one nuclear division during the cytokinesis-block phase they will contain more than nuclei and will correspond with a higher NDI score (Ionescu et al. 2011; Al-Amili et al. 2013)

4.2 Materials and Methods

All materials and equipment used on this assay are presented in Table 2.1 and Table 2.2, and all methods for this technique described in chapter 2, section 2.12.

4.3 Results

4.3.1 Naked IFN-γ and IFN-γ liposome concentration-response on MNi induction in lymphocytes from healthy individuals

Figure 4.1 shows the concentration-response of both 100U/ml naked IFN-γ and IFN-γ liposome on lymphocytes from healthy individuals. Using the ANOVA test, both forms of IFN-γ showed no significant (ns) change in lymphocyte MNi frequency compared to the untreated cells. Whereas 75 µM H_2O_2 and 0.4 µM MMC (PC) showed a significant increase in MNi frequency ***$p \leq 0.001$ compared to the untreated cells.

On the other hand both forms naked IFN-γ and IFN-γ liposome with 75 µM H_2O_2 showed reducing in MNi frequency compared to the PC (75 µM H_2O_2).

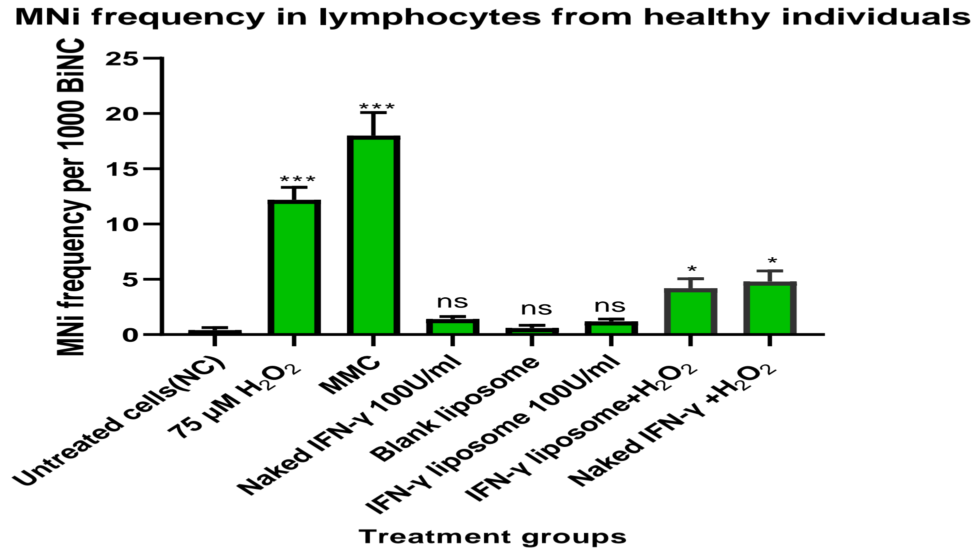


Figure 4.1 MNi frequency in lymphocytes from healthy individuals exposed to naked IFN-γ and IFN-γ liposome, including SEM and significance. (n=5).

4.3.2 Naked IFN-γ and IFN-γ liposome concentration-response on MNi induction in lymphocytes from lung cancer patients

Figure 4.2 represents the concentration-response of 100U/ml naked IFN-γ and IFN-γ liposome in lymphocytes from lung cancer patients to induce MNi in BiNC. The graph shows that the IFN-γ in both forms significantly reduced the frequency of MNi in lymphocytes of lung cancer patients in vitro compared to untreated cells. But the IFN-γ liposome showed more reduction in the frequency of MNi in lymphocytes more than the naked form. Using the ANOVA test, results showed a significant decrease ***$p<0.001$ in MNi for IFN-γ liposome and ** $p<0.01$ for naked form, compared to the untreated cells.

Furthermore, 0.4 µM MMC and 75 µM H_2O_2 showed a significant increase in MNi ***$p \leq 0.001$ compared to the untreated cells**.** The reduction in MNi frequency by using the IFN-γ liposome with 75 µM H_2O_2 slightly lesser than the naked form 75 µM H_2O_2 compared to PC.

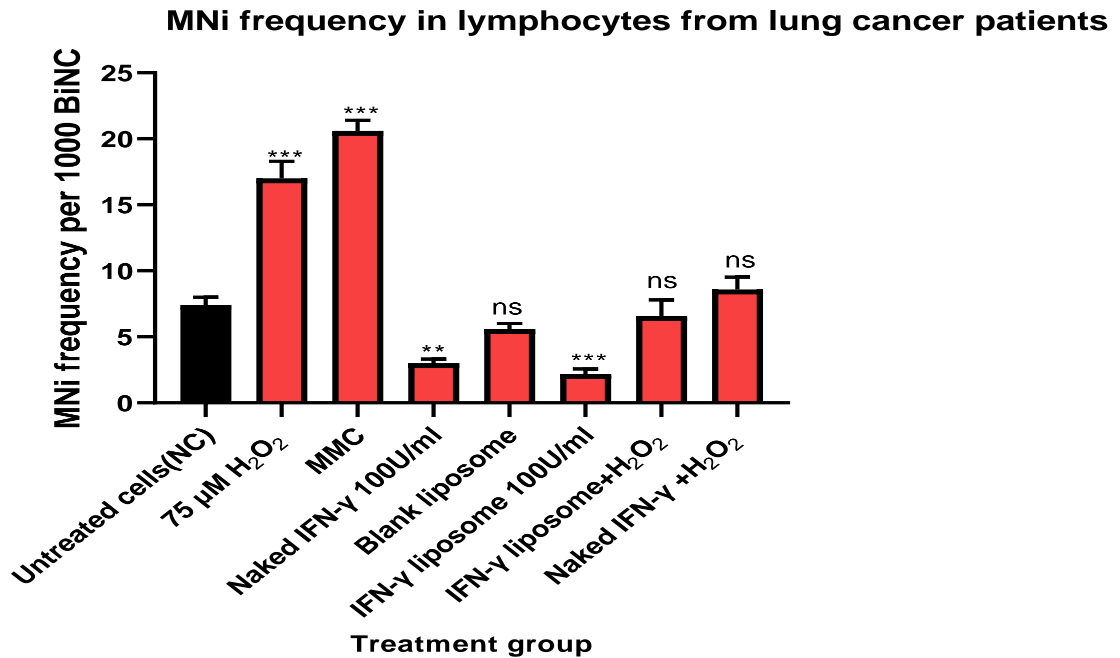


Figure 4.2 MNi frequency in lymphocytes from lung cancer patients exposed to naked IFN-γ and IFN-γ liposome, including SEM and significance. (n=5)

4.3.3 Comparing the effect of naked IFN-γ and liposomal form on MNi frequencies in lymphocytes from healthy volunteers and lung cancer patients

As presented in Figure 4.3 the lymphocytes from healthy individuals treated with 100U/ml of IFN-γB and IFN-γ liposome showed no effect in MNi frequency compared to untreated cells. The MMC and 75 µM H_2O_2 displayed a significant increase in the MNi of lymphocytes (***p<0.001), but 100U/ml of IFN-γB and IFN-γ liposome co-administered with H_2O_2 showed a significant decrease in MNi frequency of lymphocytes (*p<0.05) as shown in Figure 4.3.

It was evident that the 100U/ml of IFN-γ and IFN-γ liposome showed a significant reduction in the number of MNi in lymphocytes from lung cancer patients. Still, the reduction by IFN-γ liposome was more (***p<0.001), compared to untreated

cells. Furthermore, the MMC and 75 µM H_2O_2 showed a significant increase (***$p<0.001$) in the MNi number on lymphocytes compared to the untreated cells. Cytological scoring parameters are numerous and include biomarkers of cell mitotic division such as: mononucleated cells (MoNC), binucleated cells (BiNC), and multinucleated cells (MultiNC). From these parameters values, the NDI was calculated for healthy individuals and lung cancer patients. The mean values for the NDI for all treatment groups were within the normal range, as shown in Table 4.1.

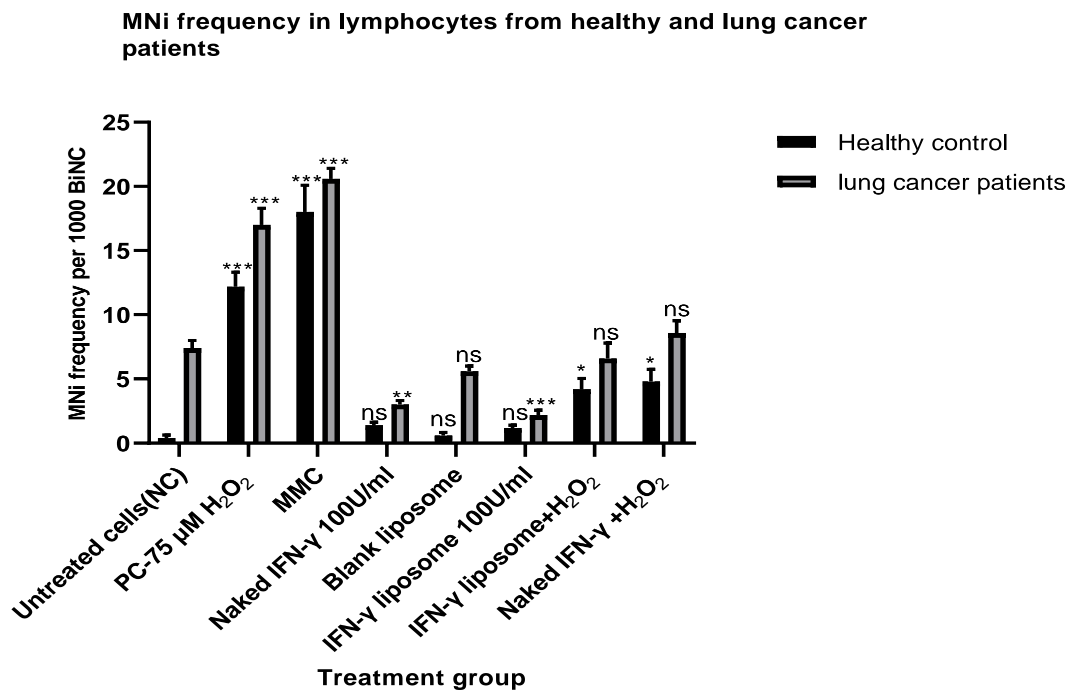


Figure 4.3 Comparison of MNi frequencies between healthy individuals and lung cancer patients, including SEM and significance. (n=5 in each group).

Table 4.1 The mean of different parameters for chromosomal damage in healthy individuals and lung cancer patients.

Subject	Treatment Group	Mean of NDI	Mean of % BiNC	Mean of % Multi	Mean per 1000 BiNC cells			Mean of % MNi in MoNC
					BiMNi	BiNPB	BiBuds	
Healthy volunteers	Untreated Lymphocytes	1.92±0.03	60.6±1.6	16.2±1.15	0.4±0.24	0	0	0
	75 µM H_2O_2	1.96±0.03	60.6±0.4	18.4±1.56	12.2±1.11	0.2±0.2	0	6.2±1.59
	0.4 µM MMC	1.88±0.06	59.2±2.4	15.6±3.81	18±2.09	0	0	8.4±1.8
	Naked IFN-γ	1.87±0.02	58.8±0.58	14.2±1.2	1.4±0.24	0	0	1.2±0.37
	Blank liposome	1.93±0.04	60.2±1.68	17±1.51	0.4±0.24	0	0	0
	IFN-γ liposome	1.98±0.03	61.6±0.4	19.4±1.4	1.2±0.2	0	0	0.6±0.4
	IFN-γ liposome+H_2O_2	1.96±0.02	59±2.28	19.8±2.72	4.2±0.86	0	0	3.2±1.28
	Naked IFN-γ +H_2O_2	1.92±0.07	59.6±1.91	17.6±4.45	4.8±0.96	0	0	4.4±1.43
Lung cancer patients	Untreated lymphocytes	1.90±0.03	58.4±2.2	16.4±1.43	7.4±0.6	0.2±0.2	0	3.4±0.4
	75 µM H_2O_2	1.89±0.03	56±2.46	17±2.07	17±1.3	0.6±0.4	0	6.4±1.2
	0.4 µM MMC	1.93±0.02	58±2.44	18±1.51	20.6±0.81	1.8±0.8	0.2+0.2	9±1.76
	Naked IFN-γ	1.97±0.03	59.6±0.5	19.2±1.59	3±0.31	0	0	3±0
	Blank liposome	1.91±0.02	60.8±0.86	15.2±1.42	5.6±0.4	0.2±0.2	0	3.2±0.48
	IFN-γ liposome	1.87±0.04	57.8±1.24	15.2±2.2	2.2±0.37	0	0	1.8±0.2
	IFN-γ liposome+H_2O_2	1.99±0.03	58.6±2.18	21±2.6	6.6±1.2	0	0	4±0.7
	Naked IFN-γ +H_2O_2	1.98±0.03	61±1.34	18.6±1.56	8.6±0.92	0	0	5±1.37

4.4 Discussion

The CBMN assay was used to investigate the influence of naked IFN-γ and liposome on the lymphocytes from healthy individuals and lung cancer patients at the chromosomal level. The CBMN assay has been a critical test in detecting different compounds genotoxicity by measuring micronuclei and other chromosomal abnormalities such as NPBs, a biomarker dicentric chromosomes, and by measuring NBUDs, which are a biomarker of gene amplification (Cho et al. 2020). MNis are cytoplasmic bodies generated as a consequence of disorder during the cell cycle division. Subsequently, when the centric fragments or whole chromosomes are incapable of travelling to opposite poles during anaphase, the nuclear envelope forms around the lagging chromosomes and fragments, which gradually take on the morphology of an interphase nucleus, which is smaller than the main nuclei in the cell (Fenech et al. 2011; Luzhna et al. 2013). In this assay, the dividing cell cytokinesis is inhibited with Cyto-B (Doherty et al. 2016).

In this study, amongst the different types of cells counted, specific consideration was made for the presence of MNis in the treatment groups since MNis are indicators of chromosomal breakage, loss, rearrangement, necrosis and apoptosis (Fenech 2007). Furthermore, MNis in binucleated cells only showed the damage after treatment, which decreased the probability of scoring the pre-existing damage (Li et al. 2012b). Mitomycin C (MMC) is recognized as an anti-tumour antibiotic, clastogenic, and genotoxic compound. Therefore, MMC served as a positive control in this study. MMC induced MNi production in binucleated cells more than mononucleated cells (Table 4.1). The percentage of BiNC after scoring 1000 cells was within the normal range in lymphocytes cultures for each individual. On the other hand, the percentage of mutliNC in cell culture was low,

thus elucidating that Cyto-B inhibited cell division after one cell cycle. Meanwhile, the NDI values were normal for all experiments (Table 4.1).

From this assay, the frequency of MNi in the lymphocytes from healthy individuals and lung cancer patients decreased when treated with IFN-γ and IFN-γ liposome compared to the NC in each group. In contrast, the IFN-γ liposome showed a more significant reduction in MNi frequency in lymphocytes.

Hydrogen peroxide (H_2O_2) was used in this assay and functioned as expected, inducing the MNi production on the lymphocytes from healthy individuals and lung cancer patients. The combination of naked IFN-γ and IFN-γ liposome with H_2O_2 reduced the frequency of MNi compared to H_2O_2 in patients lymphocytes. The study's most important finding was that the naked IFN-γ and IFN-γ liposome reduced the DNA damage effects at 100U/ml concentration in lung cancer patients lymphocytes .

CHAPTER FIVE: The Enzyme-Modified Comet Assay

5 Comet Repair Assay

5.1 Introduction

The comet assay is a rapid method used in genotoxicity studies both *in vitro* and *in vivo*. It has been recognised for its time efficiency, simplicity, sensitivity, and cost-effectiveness for analysing genotoxic DNA damage in sperm and lymphocytes, as shown in previous studies (Singh et al. 1988; Anderson et al. 1997a; Anderson et al. 2013) It is considered to be an indicator for assessing and detecting genotoxic damage (Gopalan et al. 2011).

Furthermore, the Comet assay has been used in human monitoring to investigate genetic damage with the object of measuring human exposure to genotoxic agents due to occupational exposure, drug treatments and environmental pollution (Gunasekarana et al. 2015b). It has also been deployed to assess DNA repair ability, radiation biology, environmental bio-monitoring, genetic toxicology and human epidemiology (Azqueta et al. 2014). Monitoring the repair of DNA is essential to assess cancer susceptibility (Gaivão and Sierra 2014). All cells possess repair pathways for various types of DNA damage to avoid permanent changes being made to the DNA (Feringa et al. 2018).

The alkaline comet assay detects DNA strand breaks (SBs) and alkali-labile sites (ALS). This assay is based on single nucleoids electrophoresis, giving a comet-like shape (Cortés-Gutiérrez et al. 2011). If nucleoids are digested with lesion-specific repair enzymes, different DNA lesions can be determined (Azqueta et al. 2014). The most commonly used repair enzymes (endonucleases) in the modified Comet assay are the bacterial enzymes formamidopyrimidine DNA

glycosylase (FPG) detects oxidized purines, formamidopyrimidines, ring-opened N7 guanine adducts produced by alkylating agents; and the human 8-oxoguanine-DNA-N-glycosylase 1 (hOGG1) detects oxidized purines and formamidopyrimidines. This technique was used to determine not only DNA lesions but also DNA repair from its very beginning (Møller et al. 2017; Muthusamy et al. 2017a).

In this chapter, the FPG and hOGG1 modified Comet assays were used to evaluate the ability of 100U/ml of naked IFN-γ and IFN-γ liposome to repair the DNA on lymphocytes from healthy individuals and lung cancer patients.

5.2 Materials and Methods

Reagents, materials, and equipment used in the enzyme-modified comet assay were listed in sections 2.1 and 2.2 , the method is described in section 2.13.

5.3 Results

5.3.1 The FPG and hOGG1 modified Comet assays on lymphocyte from healthy volunteers

Figure 5.1 and Figure 5.2 showed the concentration-response of 100U/ml of naked IFN-γ and IFN-γ liposome in the presence of FPG and hOGG1 enzymes in lymphocytes DNA from healthy volunteers using % DNA tail and OTM. Using the one-way ANOVA, It was clear from the graph in Figure 5.1 significant increases in DNA damage compared to untreated cells with FPG and hOGG1 enzymes in the presence of 75µm H_2O_2 (**p ≤0.01). However, both 100U/ml of naked IFN-γ and IFN-γ liposome with Fpg and hOGG1 enzymes showed no significant change in DNA damage compared to untreated cells .

The OTM in Figure 5.2 showed a significant increase in DNA damage compared to untreated cells by using FPG and hOGG1 in the presence of 75µm H_2O_2 were the p values respectively for both enzymes ***p ≤0.001, **p ≤0.01. The 100U/ml of naked IFN-γ and IFN-γ liposome with Fpg and hOGG1 enzymes presented no significant change in DNA damage compared to untreated cells.

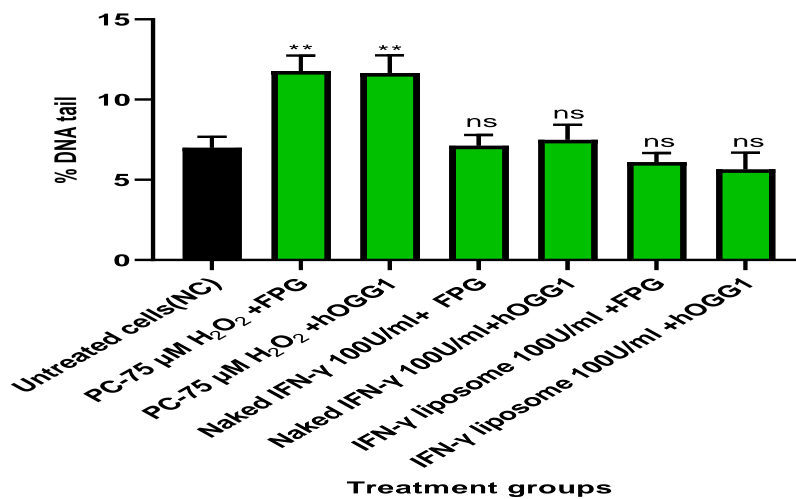


Figure 5.1 The FPG and hOGG1 modified Comet assays in the presence of naked IFN -γ, IFN -γ liposome and 75 µM H_2O_2 on lymphocyte DNA from healthy volunteers by measuring the %Tail DNA including SEM and significance. n=5

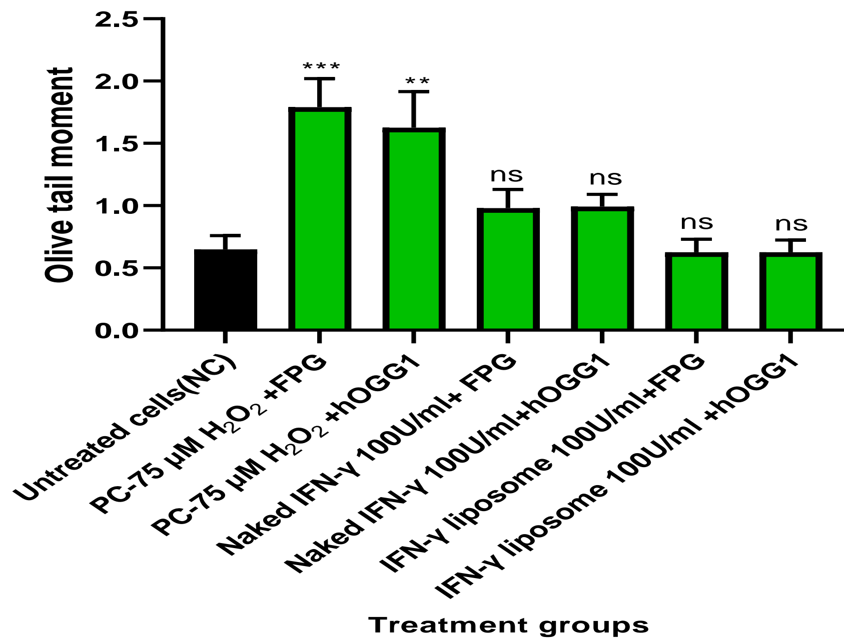


Figure 5.2 The FPG and hOGG1 modified Comet assays in the presence of naked IFN –γ, IFN –γ liposome and 75 μM H_2O_2 on lymphocyte DNA from healthy volunteers by measuring the OTM, including SEM and significance. n=5.

5.3.2 The FPG and hOGG1 modified Comet assays on lymphocyte from lung cancer patients.

Figure 5.3 and Figure 5.4 showed the concentration-response of 100U/ml of naked IFN-γ and IFN-γ liposome in the presence of FPG and hOGG1 on lymphocytes DNA from lung cancer patients using % Tail DNA and OTM.

Using the one-way ANOVA was clear from the chart for % DNA tail Figure 5.3 that the PC (75µm H_2O_2) with FPG and hOGG1 showed a significant increase in DNA damage compared to untreated cells ***$p \leq 0.001$. The 100U/ml of naked IFN-γ and IFN-γ liposome with Fpg and hOGG1 enzymes showed a significant decrease in DNA damage *$p \leq 0.05$ and **$p \leq 0.01$ respectively in lymphocytes from lung cancer patients compared to untreated cells.

The OTM in Figure 5.4 showed a significant increase in DNA damage compared to untreated cells by using FPG and hOGG1 in the presence of 75µm H_2O_2 (***p

≤ 0.001). However, the IFN-γ liposome with Fpg showed a significant reduction in DNA damage compared to untreated cells (**$p \le 0.01$). The hOGG1 enzymes with IFN-γ liposome also showed a significant reduction in DNA damage on lymphocyte from lung cancer patients compared to untreated cells (*$p \le 0.05$).

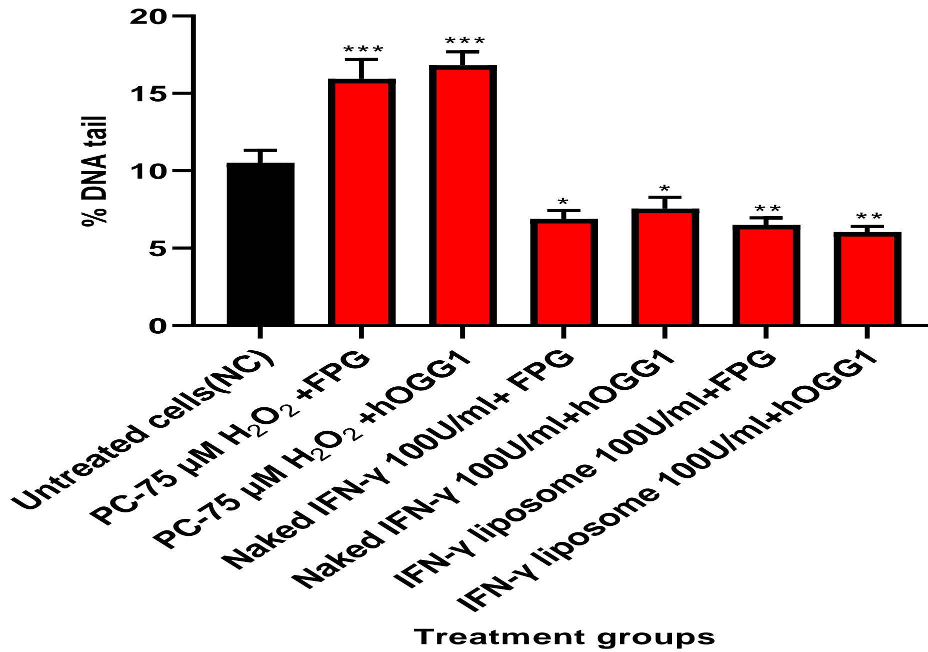


Figure 5.3 The FPG and hOGG1 modified Comet assays in the present of naked IFN –γ, IFN –γ liposome and 75 μM H_2O_2 on lymphocyte DNA from lung cancer patients by measuring the % DNA tail including, SEM and significance. n=5

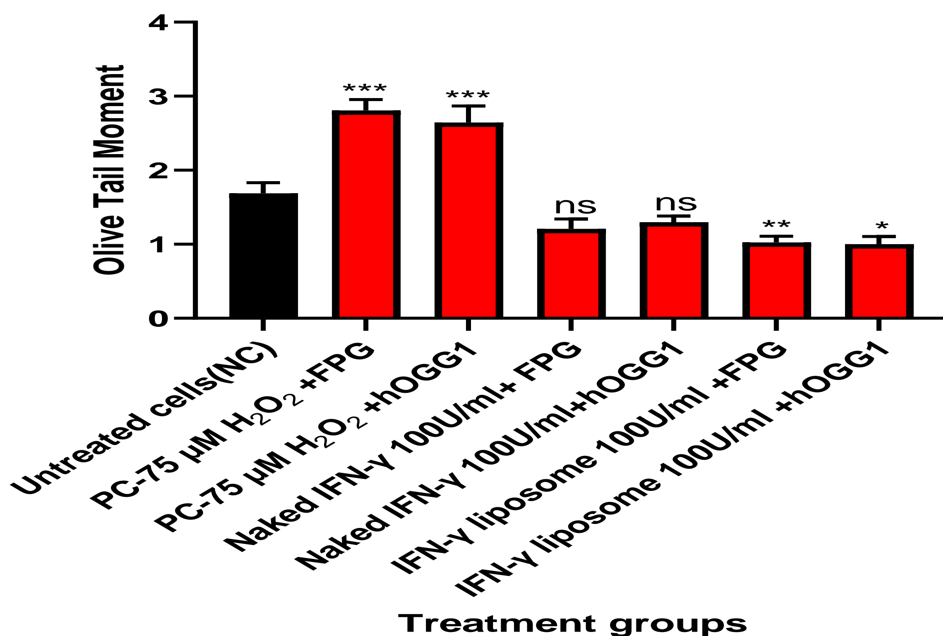


Figure 5.4 The FPG and hOGG1 modified Comet assays in the present of naked IFN –γ , IFN –γ liposome and 75 μM H_2O_2 on lymphocyte DNA from lung cancer patients by measuring the OTM, including SEM and significance. n=5

5.4 Discussion

Genomic instability is a major critical factor for tumour initiation and progression. The inability to maintain the genomic integrity can exhibit itself genetically on different diverse levels, ranging from simple changes on DNA sequence to structural and numerical chromosomal aberrations (Ferguson et al. 2015). Therefore, enhancing the DNA repair capacity is critical for maintaining cell cycle control and cell survival (Louka et al. 2015; Chatterjee and Walker 2017; Clementi et al. 2020).

Several lines of evidence have suggested that variation in the capacity of cells to repair DNA damage among individuals reflects individual genetic background variation (Nagel et al. 2014).

Different lymphocyte populations obtained from the same individuals have demonstrated a similar capability to repair DNA damage. The intra-individual

difference in DNA repair capacity (DRC) is statistically smaller than the difference among individuals. Therefore, measuring DNA repair by peripheral lymphocytes can reveal an individual's overall DNA repair capacity (Spitz et al. 2003; Nagel et al. 2014).

Many studies have shown that a defect in DNA repair capacity is strongly associated with an increased risk of developing breast cancer (Davis and Lin 2011) and Paz-Elizur et al. (2019) demonstrated that lung cancer patients had a significantly lower DNA repair capacity than was presented in healthy controls. Other studies reported that lower DNA repair capacity is an important factor for cervical carcinoma (Wei et al. 2005).

The Comet assay has been widely applied to assess the DNA damage in peripheral blood lymphocyte (PBL) and cancer cells and the characteristics of different DNA repair mechanisms with various DNA-damaging agents. Several studies on patients with different types of cancer showed an increased levels of basal DNA damage and low DNA repair capacity (Gunasekarana et al. 2015a).

The results of our study indicated that lymphocytes from lung cancer patients showed pronounced genomic instability that leads to an increased basal level of DNA damage and low DNA repair capacity compared to healthy individual lymphocytes (Figure 5.1 to Figure 5.4). Najafzadeh et al. (2012b) showed that peripheral lymphocytes from patients with malignant melanoma and colorectal cancer, or their precancerous states, were far more sensitive to genetic mutagenesis than were lymphocytes from control participants. A similar lack of DNA repair has been detected in some studies on lymphocytes from patients with lung, head and neck cancers (Flores-Obando et al. 2010; Jenkins et al. 2013).

The neutral and alkaline Comet modified to enzyme-linked assay (digestion with lesion-specific repair endonucleases) and prediction of the ability to repair oxidative DNA damage, which is becoming a widespread approach to human biomonitoring (Pu et al. 2015). These modified assays detect the genotoxic/carcinogenic potential of environmental chemicals and ultraviolet radiations (UVR) and could be useful in studying BER and NER mechanisms (Barnes et al. 2018). Modifications to the alkaline comet assay using lesion-specific endonucleases can detect DNA bases with oxidative damage and bulky DNA damages generate strand scission at that site (Muthusamy et al. 2017b).

The most used modification of the comet assay is the detection of extra lesions by incubation with formamidopyrimidine DNA glycosylase (Fpg), a BER enzyme from Escherichia coli (e-Coli) (Prakash et al. 2012). These lesions are referred to as net Fpg-sensitive sites when the basal level of DNA strand breaks has been subtracted from the total number of detected lesions after incubation with the enzyme (Møller et al. 2017). The Fpg-sensitive sites are widely accepted to encompass 8-oxo-7,8-dihydroguanine (8-oxoGua) and certain types of formamidopyrimidine nucleobases (such are produced as breakdown products of 8-oxoGua) (Davison 2016). The human 8-oxoguanine glycosylase (hOGG1) enzyme was suggested to be a suitable replacement for Fpg as it does not recognise alkylated DNA bases in cultured cells (Smith et al. 2006; Møller et al. 2017).

In this study the 75µm H_2O_2 used as the ROS-inducing agent on healthy individual and lung cancer pateint lymphocytes to measure differential sensitivity and variation in repair capacity. H_2O_2 undergoes a Fe (II)-mediated Fenton

reaction in the cell, resulting in the formation of the highly reactive hydroxyl radical and other reactive oxygen species which can induce strand breaks in the DNA. In our study the treatment of the cells with 75μm H_2O_2 reduced the DNA repair capacity and induce the DNA damage in both group but the DNA damage in lung cancer patients was higher than the healthy individuals. The level of DNA damage can be measured as %Tail DNA and OTM.

In this study, the naked IFN –γ and IFN –γ liposome have shown DNA repairing influence against DNA damage in human lymphocytes obtained from lung cancer patients and healthy individuals using hOGG-1 and Fpg comet modified assay compared to the untreated cells. But the IFN –γ liposome reduced the DNA damage more better than naked IFN –γ compared to the untreated cells .

The results of Comet, micronucleus and Comet repair assay were consistent and showed the ability of IFN –γ in both forms to reduce the DNA damage in lymphocyte in lung cancer patients and increase the repair capacity .

CHAPTER SIX: Effect of naked IFN –γ and liposome on major signal transduction pathways in isolated lymphocytes.

6 Introduction

6.1 Quantitaive reverse transcription polymerase chain reaction RT-PCR

DNA amplification is detected in many ways, one of which is real-time amplification through a technique called quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Maddocks and Jenkins 2017), which was invented by Kary Mullis in 1983 and rewarded chemistry Nobel Prize in 1993 for his intervention (Shampo and Kyle 2002). Fluorescence is detected as the fluorescence signal intensity is increased after each qPCR cycle; this amplification of DNA is then found in the amplified DNA quantity at the given time (López-Sanmartín et al. 2019). Initially, the fluorescence levels are too low to be detected; however, after each cycle, the fluorescence level increases to reach the detectable levels. The quantification cycle is the point by which target DNA absolute is determined in a given sample (Kralik and Ricchi 2017).

This technique is very sensitive and rapid. As a result, it has a wide range of applications used by both researchers and clinicians for; gene cloning and sequencing, complex genomic studies, and disease diagnoses (Valones et al. 2009).

Polymerase Chain Reaction (PCR) can be applied to a wide range of DNA from different sources, such as; microbes, peripheral blood, saliva and hair (Garibyan

and Avashia 2013). During PCR, only a small amount of DNA is needed to generate a sufficient quantity of DNA to be analysed by conventional laboratory methods (Nolan et al. 2013).

PCR is also used for tumour analysis, as DNA can be isolated. Thus proto-oncogenes and tumour suppressor genes can be isolated and quantified. One of qPCR's key features is the ability to precisely quantify and isolate a target gene (Narrandes and Xu 2018). Further to this, qPCR can be specifically used to target a single cell, allowing for any given DNA combination to be targeted. This also extends to include proteins and mRNA (Ståhlberg and Kubista 2014).

To proceeds the PCR reaction a number of components are required, such as; DNA polymerase enzyme, nucleotides and primers. Individual nucleotides are linked together through DNA polymerase, which leads to the formation of the PCR product (Pray 2008; Clark et al. 2019). The DNA consists through the makeup of any combination of a nitrogenous base, Adenine (A), Cytosine (C), Guanine (G) and Thymine (T), which is used by DNA polymerase to generate the final PCR product (Jakubovska et al. 2018). It is through the use of primers specific DNA sequence/product can be amplified. The primers are short DNA fragments with a matching sequence to the target DNA, which is targeted and amplified as an extension point where DNA polymerase can build up (Garibyan and Avashia 2013). Thermal cycling is a common step used in PCR methods. This refers to a series of heating and cooling cycles; denaturation (where the duplex double-stranded DNA unwind and become ssDNA), annealing (primers specifically base-pair with ssDNA), and extension (DNA synthesis from primers). In the first step in PCR, the DNA denaturation step, where the temperature is extended to 94°C for a period ranging from 30 seconds to 2 minutes, will enable the DNA double helix

to unwind physically. The annealing step which proceeds this step which occurs at a temperature ranging between 45°C to 72°C for a period of 30 seconds. This allows for the two DNA strands to become two individual templates allowing for polymerase enzyme to target the DNA of interest specifically. The temperature is dependent on the sequence of the primers; the optimum temperature is determined experimentally, finally, in the extension step, which occurs at either 72 or 78 °C (Lorenz 2012).

p53 is a suppressor gene, encodes the p53 protein, which provides a key role in controlling the stress factor in human cells (Aubrey et al. 2018). The *p53* has a sophisticated signalling mechanism by sensing the excessive stress signals resulting from deregulated oncogene expression, such as telomere erosion, metabolic disorders or DNA damage. The p53 signalling, depending on a number of oncogenes and cellular states. *p53* induced apoptosis (self-cell death), perpetual, fugacious cell cycle arrest, metabolic equilibrium process and DNA repair (Li et al. 2012a). *p53* regulation depends on the accumulation of posttranslational modifications (PTMs), which triggers p53 through interaction with proteins, resulting in sub-cellular relocation and causing the genes associated with the signalling pathway be activated (Walerych et al. 2012).

Another interesting feature of *p53* is its ability to induce DNA repair pathways to minimise DNA damage (Williams and Schumacher 2016). The regulation of specific DNA repair is controlled via *p53*-mediated transcriptional genes depending on the type of DNA damage. The *p53* gene can induce the most important DNA repair genes, which are; BER, non-homologous end-joining and NER (Menon and Povirk 2014).

The first *p53* target gene to be identified was *Cdkn1a* (*p21*), a gene which encodes the cyclin-dependent kinase inhibitor, which plays a main role in cell cycle regulation. When it stimulated by *p53*, the p21WAF1/CIP1 binds to cyclin-dependent kinase 2 (CDK2), and causes the downregulation of CDK2 activity and G1 arrest (Bostwick and Cheng 2020). Furthermore, the *p21* plays a critical role in tumour suppression by regulating DNA replication, also DNA repair (Gorski et al. 2002; Hawes et al. 2009; Georgakilas et al. 2017).

6.2 Western blotting (WB)

Immunoblot, which is also referred to as Western blot (WB), is a highly effective quantitative and analytical technique used to identify specific proteins in a wide range of biological samples in tissue/cellular homogenates (Ghosh et al. 2014). This technique exposes useful information that is not available in other immunoassay methods. For this technique to be successful, the target protein must be present in a given sample. Results are produced in the form of bands. Band thickness compared to the control provides information on whether a protein has been overexpressed or downregulated. This information is useful, as it provides researchers with the genetic investigation of any changes made to a protein gene, such as partial deletion or duplication (Barresi 2011).

Furthermore, the WB method is a quick method allowing for medical diagnosis to be performed (Manole et al. 2018). WB can detect as little as 0.1ng of proteins due to its high-resolution gel electrophoresis, strong specificity and greater sensitivity (Gogia et al. 2017).

As explained above, the effectiveness of WB allows identifying of target protein even from a sample containing a mixture of different proteins. Protein separation

in a sample occurs through SDS-PAGE gel electrophoresis. These samples are then separated according to their molecular weight (Bendickson and Nilsen-Hamilton 2013). Gels used in electrophoresis are fragile, in order to overcome this issue, proteins are transferred onto a membrane, either on a polyvinylidene fluoride (PVDF) or nitrocellulose, which overcomes the fragility of the gels but maintains a copy of the gel patterns on them. This transfer occurs through electrical current (Mahmood and Yang 2012). Protein visualisation occurs through the application of a specific primary antibody that will bind to a protein of interest. The secondary antibody specifically binds to the primary antibody; this is then detected with a detection reagent (radioisotope, fluorophore and, enzyme). Proteins are visualised either directly in the membrane with the aid of an imaging system or through chemiluminescence on X-ray film (Bass et al. 2017; Manole et al. 2018).

Building on the previous results from; cell viability, Comet and micronucleus assays, we found that naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml forms do not induce cytotoxicity or genotoxicity in lymphocytes from lung cancer patients and those from healthy individuals. In fact, both forms of IFN-γ have indicated the DNA repairing effect in lymphocytes from lung cancer patients and by significantly attenuating hydrogen peroxide-induced DNA damage, and exhibited genoprotective effects in lymphocytes from lung cancer patients by significantly reducing the MNi induction determined by both the comet and MN assays. To investigate the molecular processes involved in mediating these effects, we studied the effects of naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml on gene expression levels of *p53, p21* and *Bcl-2*, at the mRNA level in isolated lymphocytes from healthy individuals and lung cancer patients and we

investigated the effect of both form of IFN-γ on *p53, p21* and *Bcl-2* proteins level in lymphocytes from lung cancer patients and compare them with those from healthy individuals using the quantitative PCR and western blotting techniques.

6.3 Materials and methods

All chemicals and equipment used in qRT-PCR and WB are listed in Table 2.1 and Table 2.2.The qRT-PCR method is described in section 2.14 and the WB assay method is described in section 2.15.

6.4 Results

6.4.1 Real-time qRT-PCR

6.4.1.1 Analysis of *p53*, *p21* and *Bcl-2* gene expression in lymphocytes from healthy individuals and lung cancer patients.

The qRT-PCR was conducted to analyse cell cycle regulatory (*p53* and *p21*) and apoptosis-related gene (*Bcl-2*). The results showed that naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml treatment do not significantly regulate the expression of these genes at mRNA level in lymphocytes from healthy individuals and H_2O_2 up-regulated the p53 significantly (***$p \leq 0.001$) as presented in Figure 6.1. However, in lymphocytes from lung cancer patients, their effect was the opposite. *p53* and *p21* both genes were significantly up-regulated in lymphocytes of the patient group when treated with naked IFN-γ $p<0.001$ and $p<0.05$, respectively. IFN-γ liposome form also significantly up-regulated the expression of *p53* (***$p \leq 0.001$) and p21 (***$p \leq 0.001$). In Figure 6.2 the expression of the anti-apoptotic gene, *Bcl-2,* was not significantly affected by naked IFN-γ and liposome forms. Furthermore, the H_2O_2 in Figure 6.2 showed significant up-

regulation in *p53*, *p21* (***$p \leq 0.001$) for both genes and down-regulation for *Bcl-2* (*$p \leq 0.05$)

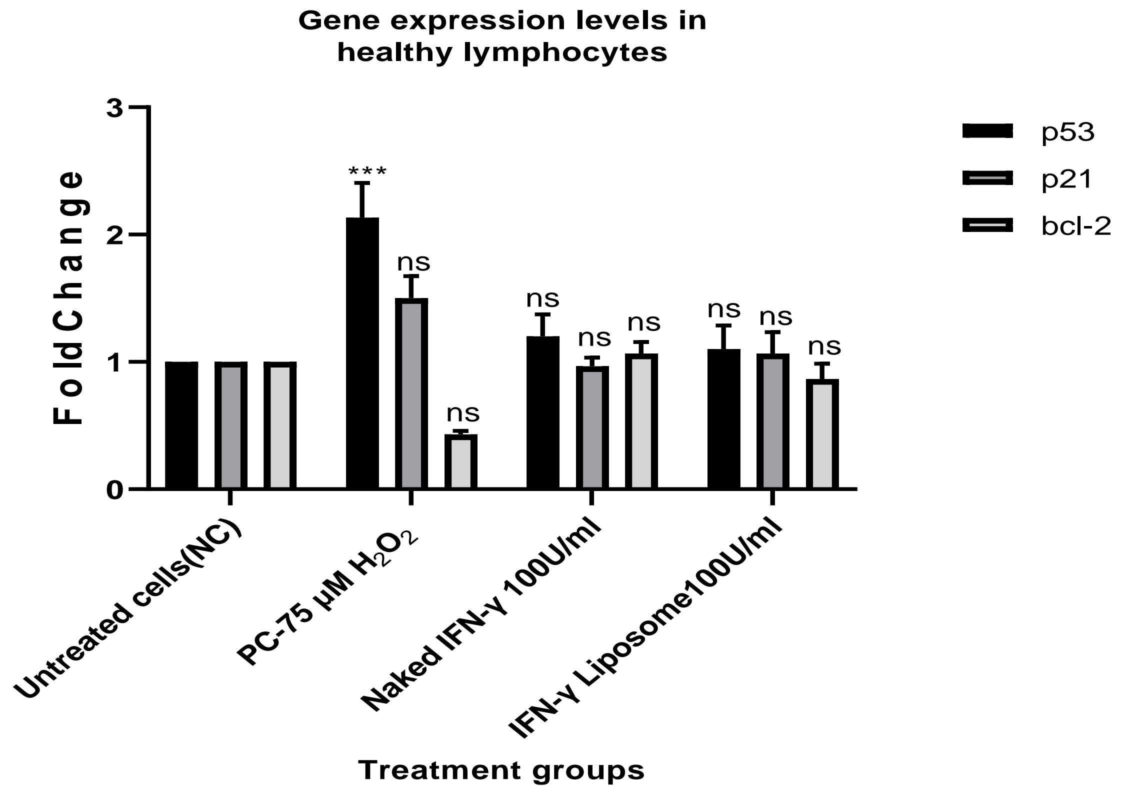


Figure 6.1 The effect of naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml on the gene expression levels of *p53, p21* and *Bcl-2* in lymphocytes from healthy individuals.

All data from the treatment groups were compared against the control group (C) and normalized against the internal home gene, β-actin. The experiment was repeated three times in three different individuals. The treatment groups included untreated cells (NC), H_2O_2 75µM as a positive control (PC) group, naked-γ 100U/ml and IFN-γ liposome 100U/ml. IFN-γ in both forms did not show any significant effect on *p53*, *p21* and *Bcl-2* expression. (ns=not significant, ***P<0.001)

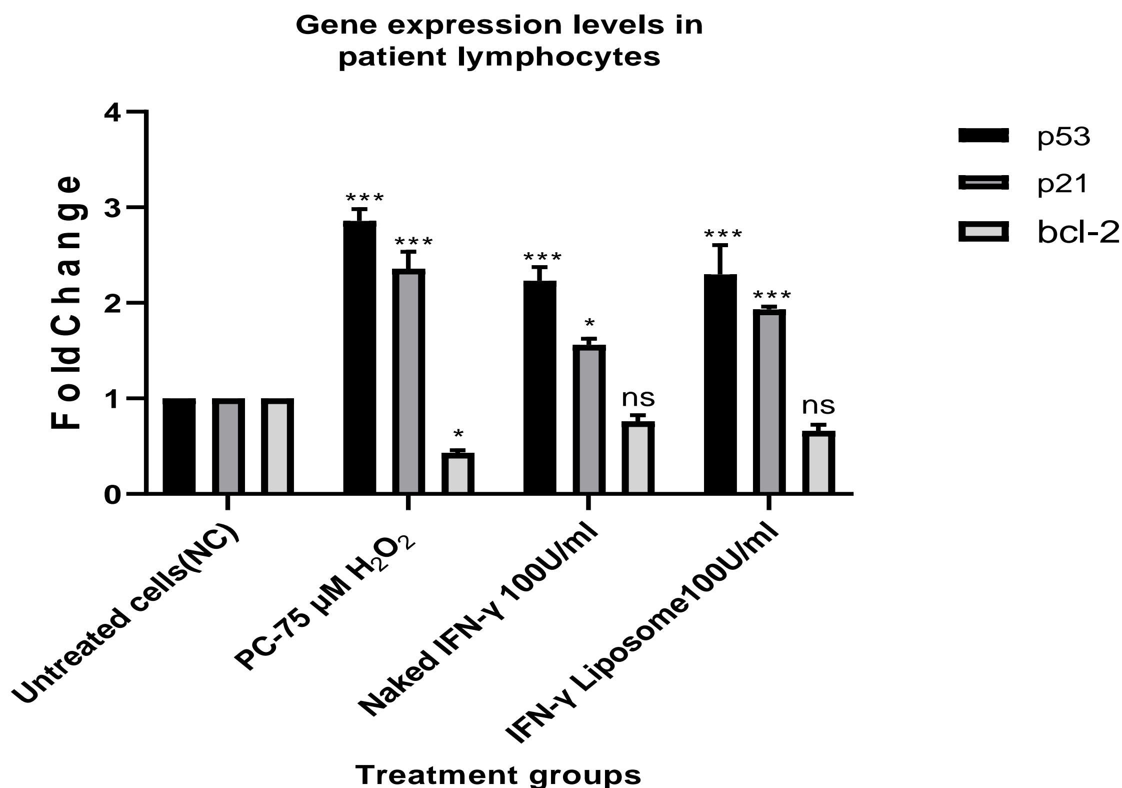


Figure 6.2 The effect of naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml on the gene expression levels of *p53, p21* and *Bcl-2* in lymphocytes from lung cancer patients.

All data from the treatment groups were compared against the control group (C) and normalised against the internal home gene, β-actin. The experiment was repeated three times in three different individuals. The treatment groups included untreated cells (NC), H_2O_2 75µM as a positive control (PC) group, naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml. IFN-γ in both forms did not show any significant effect on *Bcl-2* expression. However, the mRNA *p53* and *p21* expression were significantly up-regulated by both forms of IFN-γ (ns = not significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

6.4.2 Western blotting (WB)

6.4.2.1 The effect of naked IFN-γ and IFN-γ liposome on p53, p21 and Bcl-2 protein expression levels in isolated lymphocytes

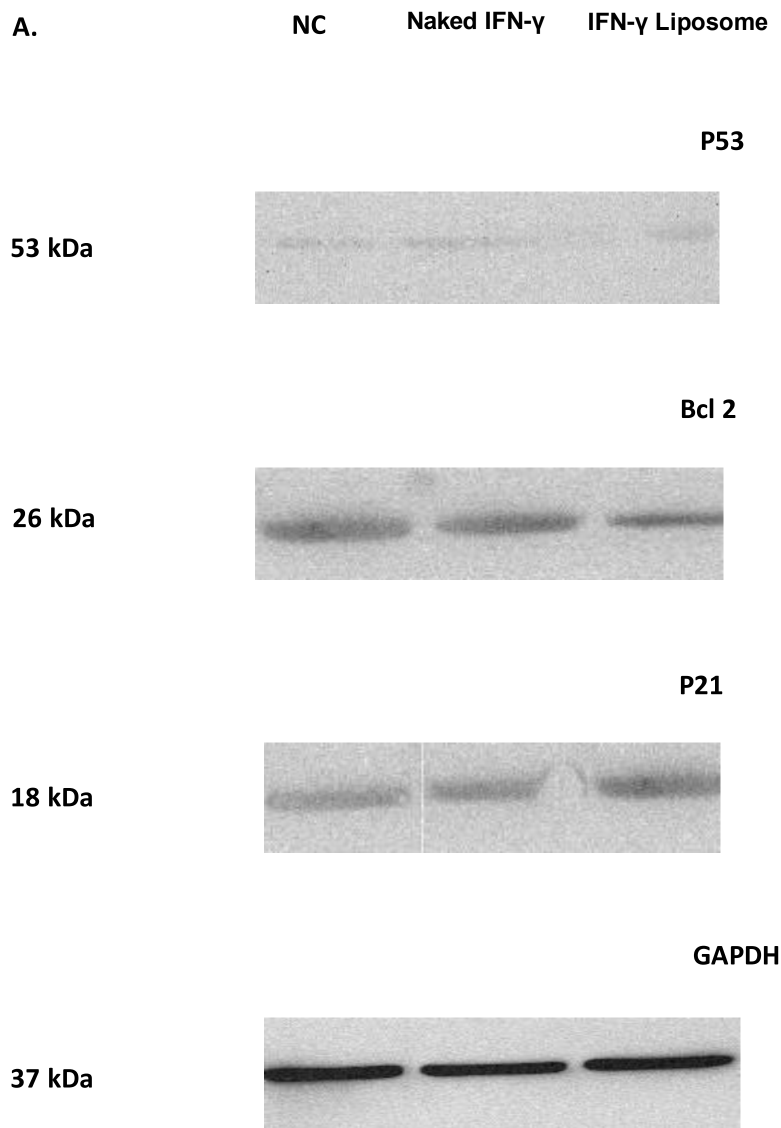
We piloted a series of WB experiments to investigate the protein expression levels of p53, p21 and Bcl-2 and validate the results we achieved from real-time PCR.

WB was used to investigate the effect of naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml on protein expression levels of main anti-apoptotic protein Bcl-2, tumour-suppressor p53 and cell cycle regulator p21 in lymphocytes from healthy individuals and lung cancer patients. p53 is a tumour-suppressor protein that possesses various attributes essential for maintaining homeostasis in our body's biological processes and activated in response to different types of stress to regulate the expression of genes to control proliferation and senescence, DNA repair, and cell death (Lacroix et al. 2020). Thus, we studied p53 protein to confirm this protein's role in naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml mediated effects in lymphocytes from lung cancer patients.

Analysis of the results showed that naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml had no significant effect on the expression of any of these proteins in healthy individuals' lymphocytes. And there was no difference observed in their regulation. However, there was statistically significant up-regulation observed in protein expression levels of p21 and p53 in lymphocytes from lung cancer patients. As shown in Figure 6.4 (A and B), the p53 have increased by 1.7 fold with naked IFN-γ 100U/ml and 1.8 fold with IFN-γ liposome 100U/ml in lymphocyte from lung cancer patients. However, the p21 also up-regulated to 1.8-

fold for naked IFN-γ 100U/ml and 1.7 for liposomal form compared to the control group.

These results indicated that naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml might suppress the adverse effects of lung cancer in lymphocytes from patients by stimulating the expression levels of tumour-suppressor protein p53 and p21, by triggering DNA damage response (DDR), bringing a protective and anti-tumour effect. Hence, could contribute to the survival pathway through repairing of the cells. Moreover, our WB analysis results were consistent with the gene expression results from the real-time PCR technique.


A.
NC
Naked IFN-γ
IFN-γ Liposome
P53
53 kDa
Bcl 2
26 kDa
P21
18 kDa
GAPDH
37 kDa

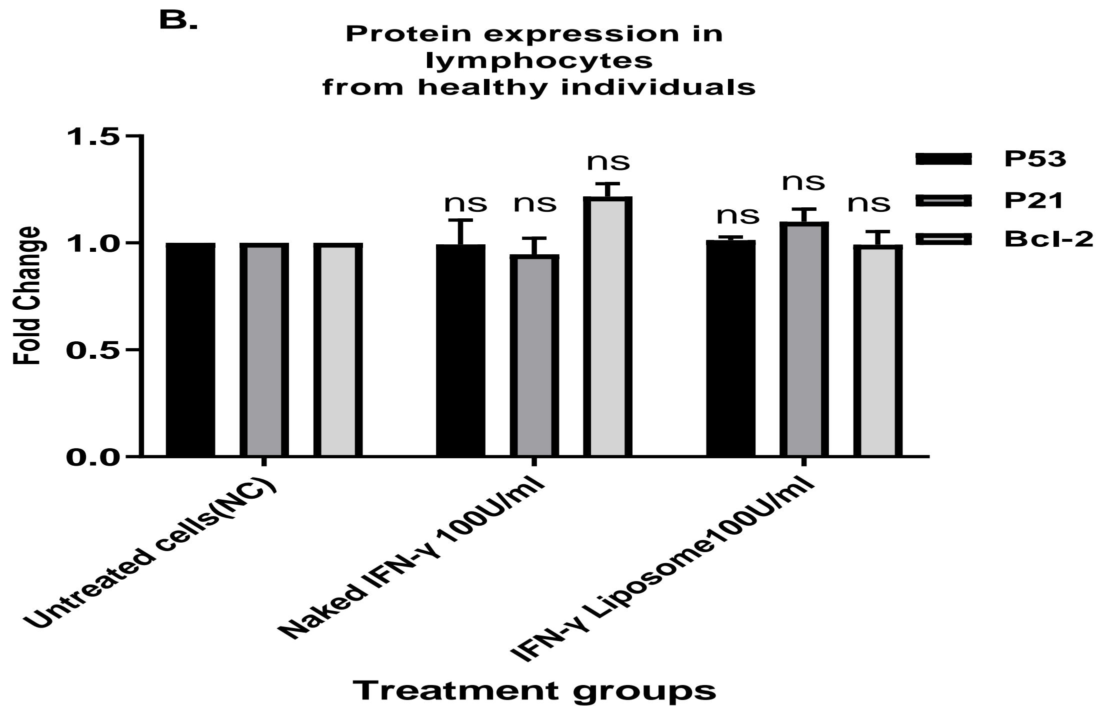
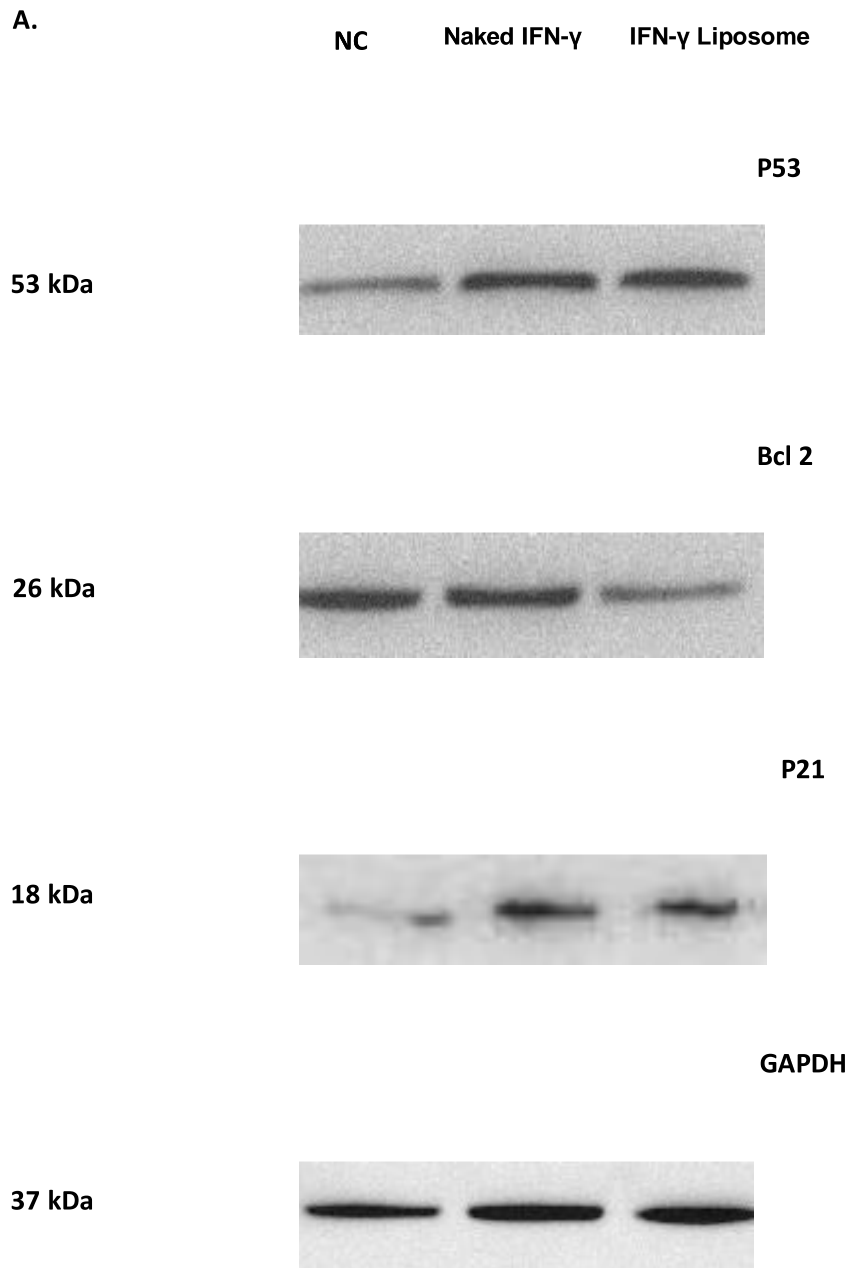


Figure 6.3 The effect of naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml on the protein expression levels of p53, p21 and Bcl-2 in lymphocytes from healthy individuals.

All data from the treatment groups were compared against the control group (C) and normalized against the internal home protein, GAPDH. The experiment was repeated three times in three different individuals. The treatment groups included untreated cells, negative control (NC), naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml. IFN-γ in both forms did not show any significant effect on Bcl-2, p53 and p21 protein expression. (ns=not significant), (A) Immunoblot analysis of the p53, p21 and Bcl-2 proteins in lymphocyte from healthy individuals treated with naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml. (B) Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean ± SEM of three experiments.


A.
NC
Naked IFN-γ
IFN-γ Liposome
P53
53 kDa
Bcl 2
26 kDa
P21
18 kDa
GAPDH
37 kDa

B.

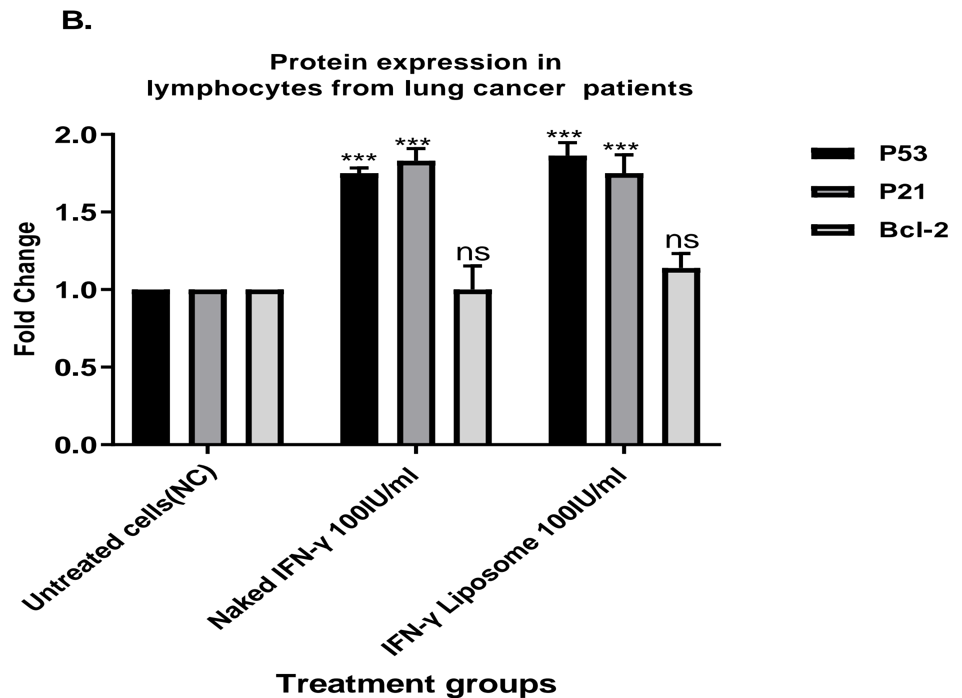


Figure 6.4 The effect of naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml on the protein expression levels of p53, p21 and Bcl-2 in lymphocytes from lung cancer patients.

All data from the treatment groups were compared against the control group (C) and normalised against the internal home protein, GAPDH. The experiment was repeated three times in three different individuals. The treatment groups included untreated negative control (NC), naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml. IFN-γ in both forms significantly up-regulated the protein expression levels of p53 and p21; however, the regulation of Bcl-2 expression was insignificant. (A) Immunoblot analysis of the p53, p21 and Bcl-2 proteins in lymphocyte from lung cancer patients treated with naked IFN-γ 100U/ml and IFN-γ liposome 100U/ml. (B) Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean ± SEM of three experiments. (ns = not significant, $^{***}P<0.001$).

6.5 Discussion

Genomic DNA is one of the most important molecules, and its stability is very important for appropriate function and existence of all living organisms. The DNA is exposed daily to different genotoxic chemicals and radiations that affect genome stability (Williams and Schumacher 2016). In healthy cells, cellular repair mechanisms effectively control the DNA damage, with only sporadic breaks or errors. The dysfunctional repair mechanisms may contribute to genetic or multifactorial disorders. This effect depends on if it happens in somatic or germline cells, which allows for making an alteration in the genome that comprises one of the major motivations in evolution (Chakarov et al. 2014).

Genomic instability is an enabling characteristic for most human cancers which caused from acquired defects in any one of six DNA repair pathways such as BER, NER, DNA MMR, HRR , NHEJ, and translesion DNA synthesis (TLS) (Li and Greenberg 2012; Langie et al. 2013; Broustas and Lieberman 2014). Furthermore, the genomic instability can be mediated through genetic or epigenetic alterations in oncogenes and tumour suppressor genes including DNA repair genes, BRCA1 and BRCA2, and TP53, which are mutated in various human cancers (Langie et al. 2015b). The lung cancer is one of the most important cancers that happens due to deficient DNA repair pathways (Wu et al. 2008; Broustas and Lieberman 2014) .

p53 is a tumour suppressor protein. It plays a key role as a facilitator of DNA repair by arresting the cell cycle to allow the repair mechanisms to restore

genome stability (Bieging et al. 2014; Aubrey et al. 2018). Consequently, it appears that the p53 is multitasking in providing a protective effect against cancer development by playing a fundamental role in the homeostasis between the damage and repair mechanisms to provide genome stability (Moulder et al. 2018). Moreover, it plays the main role to directly impact the activity of different DNA-repair mechanisms such as NER removes a variety of helix-distorting lesions such as typically induced by UV irradiation, whereas BER targets oxidative base modifications. MMR scans for nucleotides that have been erroneously inserted during replication. DNA DSBs that IR typically induces are resolved either by NHEJ or by HR (Williams and Schumacher 2016).

Without p53 normal function, DNA integrity could be compromised, causing high mutational rates (Rivlin et al. 2011). It has been well documented that p53 stimulated p21 interacts with a complex called proliferating cell nuclear antigen (PCNA), involved in inhibition of replicative DNA synthesis (Moldovan et al. 2007) and promoting the excision repair mechanism (Abbas and Dutta 2009).

Different studies confirmed various functions of p53 in mediated arrest in cell cycle to allow genome repairing and reducing the propagation of DNA damage, from that we understand that by increasing the p53 level, the cellular proliferation of cancer cells may be suppressed through DNA repair, cell cycle arrest and apoptosis (Kastan et al. 1992; Williams and Schumacher 2016).

Genetically compromised DNA repair mechanisms might contribute to high levels of DNA damage in lymphocytes (Hanahan and Weinberg, 2000, 2011) therefore; lymphocytes are chosen as model cells for the current study. Peripheral

lymphocytes represent an excellent model for examining the genome sensitivity (a factor of susceptibility to cancer) (Collins 2004).

Furthermore we studied the tumour-suppressor p53 gene at the gene level due to its ability to encode for proteins that prevent genome mutations by interacting with DNA and regulating gene expression.

In this study, our results represent that 24-hour treatment with both the liposome and naked forms of IFN-γ elevate the level of p53 in lymphocytes from lung cancer patients at mRNA and protein level (Figure 6.2 and Figure 6.4), suggesting that the IFN-γ may result in p53-mediated on cell cycle arrest and DNA repair in lung cancer patients, and this indicates that the protective effects caused by IFN-γ might be dependent on the tumour-suppression activity of the p53 gene.

Previous studies have demonstrated that the increased expression of p53 is usually accompanied by up-regulation of the p21 gene (Shih et al. 2007). A similar result was observed in our study the p21 was up-regulated in lymphocytes from lung cancer patients after treatment with IFN-γ liposome and naked forms in both mRNA and protein level (Figure 6.2 and Figure 6.4). On the other hand, the Bcl-2 was not significantly affected by naked IFN-γ and liposome on lymphocytes from lung cancer patients and healthy individuals level (Figure 6.2 and Figure 6.4).

However, the p53 and p21 expression at protein and mRNA level on lymphocytes from healthy individuals after treatment of the cells with IFN-γ liposome and naked form were barely detectable. Our results agree with a previous study where the p53 in normal cells is maintained at a low level by

the action of the MDM2 proto-oncogene (Midgley and Lane 1997; Rivlin et al. 2011).

Furthermore, the current study showed that the expression of p53 and p21 genes on lymphocytes from healthy and lung cancer patients was up-regulated by the stimulus of H_2O_2 and the Bcl-2 was down-regulated (Figure 6.1 and Figure 6.2), these results are consistent with a previous study where the H_2O_2 induced the apoptosis in H9C2 cells by an increase in p53 expression and decrease the Bcl-2 level (Zhang et al. 2013; Chang et al. 2016)

CHAPTER SEVEN: General discussion and future work

7 General discussion and future work

7.1 General Discussion

The current research study was piloted to determine the genoprotective effects of naked IFN-γ and IFN-γ liposome forms in lymphocytes from lung cancer patients compared to those from healthy individuals. Their effect on DNA damage and DNA damage repair capacity was measured using the comet assay and DNA repair enzymes FPG and OGG1, respectively. Moreover, the micronucleus assay was deployed to study the effects of naked IFN-γ and IFN-γ liposome on the mitotic phases of the cell cycle by assessing the frequencies of the main biomarkers of the assay, i.e. micronuclei (MNis), nucleoplasmic bridges (NPBS) and nuclear buds (NBUDs).

Also, the influence of naked IFN-γ and IFN-γ liposome on the expression of *p53*, *p21* and *Bcl-2* genes was studied both at m RNA and protein levels by Western blotting and qRT-PCR, correspondingly, in lymphocytes from lung cancer patients and healthy individuals.

Cancer presents a major threat to public health across the globe. Prevention of cancer is a promising strategy to overcome the dogma linked with a continually growing number of cancers worldwide (Bray and Soerjomataram 2015). The growth rate of cancer is alarming over time and currently, the main therapeutic strategies being used are chemotherapy and radiotherapy. Due to high amounts

of side effects and resistance to chemotherapy, there is a crucial need to develop novel drugs that could tackle these problems (Wang et al. 2019).

Various drugs have been shown to cause cytotoxicity in different cancer cell lines. However, the potential protective effects of IFN-γ on the lymphocytes from lung cancer are rarely reported. IFN-γ plays a major role as an immunomodulatory, antiviral, anti-proliferative, and anticancer cytokine by inhibiting tumour cell growth and enhancing apoptosis. Many studies and publications have shown promising health beneficial effects of IFN-γ as a treatment option presenting antiviral, antimicrobial, pleiotropic immunomodulatory and pro-inflammatory activities (Bernabei et al. 2001; Regis et al. 2006; Lin and Young 2013). It has been used in the clinical management in different types of cancer such as bladder carcinoma, colorectal cancer, ovarian cancer, and human pancreatic carcinoma cells, and NSCLC and it is approved as a treatment for adult T cell leukemia (ATL) in Japan (Prior et al. 1999; Zaidi and Merlino 2011). IFN-γ has long been associated with cytostatic/cytotoxic and antitumor functions (Zaidi and Merlino 2011).

Vesicle size and composition are critical parameters for determining the circulation half-life of liposomes. Size affects the degree of drug encapsulation in liposomes. The size, and properties of liposomes in an aqueous environment determine the potential applications of liposome systems as drug carriers. Liposomal formulations were therefore prepared to enhance the pharmacokinetics of IFN-γ and the impact on biological activities were investigated.

Liposomes were successfully prepared with a particle size of 146.9 nm and the PDI 0.210. The encapsulation efficiency was 72%.

It is important for cancer prevention studies to assess the underlying processes through which various drugs affect carcinogenesis. From this perspective, the Comet assay offers an excellent and reliable method for assessing genotoxicity and is a relatively easier, simpler, more sensitive and cheaper way to determine DNA damage and repair compared to other similar assays (Anderson et al. 2013). Therefore, we used it to study the DNA protective effect of naked IFN-γ and IFN-γ liposome in lymphocytes from lung cancer patients compared to those from healthy individuals.

Also, to determine the repair capability of lymphocytes using enzymes and modulating effects of IFN-γ on H_2O_2 -induced DNA damage.

Comet assay, along with the micronucleus assay, has been proposed by many studies to evaluate the mutagenicity and potential risk of cancer (Araldi et al. 2015). The MN assay can concurrently assess useful information about different chromosomal damage parameters such as chromosomal breakage and reshuffling (Ladeira et al. 2015). It has extensively been used as an accepted biomarker of *in vitro* and *in vivo* genome stability studies due to its beneficial attributes (Fenech 2002). In genotoxic studies, chromosomal damage caused by various genotoxic agents in peripheral lymphocytes has been widely assessed by determining the MN (Luzhna et al. 2013).

Therefore, we used the micronucleus assay to determine the effects of IFN-γ in lymphocytes from lung cancer patients to validate our findings from the Comet assay.

Peripheral lymphocytes were used as sample cells due to their ease of isolation and availability. They represent an excellent model of surrogate cells which can be used to assess genotoxicity. Hence, lymphocytes circulate all over the body, exposed to both endogenous and exogenous DNA damage by various physio-chemical genotoxic lesions (Najafzadeh et al. 2011).

We determined the cell survival and cytotoxicity of chemicals, using the trypan blue dye exclusion method and cell counting kit 8 (CCK8) assay, in lymphocytes from both healthy individuals and lung cancer patients. Treatment groups included were naked IFN-γ, IFN-γ liposome, untreated cells (NC) and hydrogen peroxide (75µM) was used as a positive control (PC). Analysis of results from trypan blue exclusion demonstrated that the cell viability remained within the range of 75%-85%. Results from CCK-8 assay also showed similar data where cell survival did not drop below 80% in lymphocytes from healthy individuals and lung cancer patients at any considered treatment group (Figure 3.6). Hydrogen Peroxide H_2O_2 (75µM) induced a significant level of cytotoxicity in both investigative groups. Naked IFN-γ and liposome also caused cytotoxicity in lung cancer patients to a significant level. However, the viability was more than 80% for all these groups. Therefore, in the present study, only non-cytotoxic doses with viability, more than 80% were used (Henderson et al. 1998), excluding any artefact activities due to toxicity.

The genotoxicity of the drug was also determined by employing the Comet assay. Indicated by the results only the optimal non-genotoxic concentrations determined by both factors (OTM and % tail DNA) of the Comet assay (100 U/ml for naked IFN-γ and IFN-γ liposome) were chosen for the current *in vitro* study in

lymphocytes from lung cancer patients compared to those from healthy individuals.

The Comet assay results have demonstrated that the chosen concentrations of naked IFN-γ and IFN-γ liposome (100 U/ml) were non-genotoxic, displaying no statistically significant DNA damage lymphocytes from healthy individuals at basal levels when compared to the control group. However, when the same concentration of IFN-γ in both forms was used in lymphocytes from lung cancer patients, DNA damage was significantly reduced in comparison to the untreated cells where the liposome form of IFN-γ was more effective in bringing this effect than the naked IFN-γ (Figure 3.9 and Figure 3.10) Patient group presented high levels of DNA damage at the baseline level throughout the *in vitro* study.

Lymphocytes from lung cancer patients and healthy individuals were also exposed to the stress inducer, H_2O_2 75 µM. The results showed a significant increase in the DNA damage compared to the untreated lymphocytes in both groups. However, this increase was significantly attenuated by supplementation of naked IFN-γ and liposome forms. The DNA damage analysed from the supplemented groups decreased to great levels compared to the H_2O_2 (PC) group. The liposome coated IFN-γ has been proven to be more effective against the H_2O_2-induced damage. These results propose a geno-protective and antioxidant potential of IFN-γ (100 U/ml).

Genomic DNA is a fundamental biomolecule; its stability and integrity are crucial for maintaining the proper functionality and existence of all living organisms. The DNA is vulnerable to various internal and external genotoxic insults daily, which cause adverse effects on genome stability (Williams and Schumacher 2016). In a healthy biological environment, cells are highly organised and equipped to carry

on a self-repairing mechanism when exposed to any form of cellular damage. However, compromised repair mechanism is not capable of doing its function properly and may contribute to mutations and multifactorial conditions (Chakarov et al. 2014).

Genomic variability causing mutations is a major factor for tumour instigation and development. An incapability to sustain the integrity of the genome can occur at different levels ranging from simple structural changes in DNA sequence to numerical chromosomal abnormalities (Ferguson et al. 2015). Therefore, enhancing the DNA repair capacity is crucial for the maintenance of homeostasis in living organisms. (Louka et al. 2015; Chatterjee and Walker 2017; Clementi et al. 2020).

Various research studies suggested that the differences found in the capacity to repair mechanisms among individuals depict their individuals' genetic backgrounds (Nagel et al. 2014), which can be determined overall using peripheral lymphocytes (Spitz et al. 2003; Nagel et al. 2014).

Many studies have shown that a defect in DNA repair capacity is strongly associated with an increased risk of many tumours such as breast cancer, lung cancer and cervical carcinoma (Davis and Lin 2011). We used hOGG-1 and Fpg comet modified assay to determine the repair capacity of lymphocytes from lung cancer patients and healthy individuals after treating those with naked IFN –γ and liposome forms.

Our results indicated that lymphocytes from lung cancer patients showed high levels of genomic instability that may have contributed to an increased level of basal DNA damage and a low DNA repair capacity compared to the lymphocytes from the healthy individual (Figure 5.1 and Figure 5.4)

In healthy lymphocytes, comet data showed a significant increase in DNA damage compared to untreated cells by using FPG and hOGG1 in the presence of 75Mm H_2O_2. In comparison, 100U/ml of naked IFN-γ and IFN-γ liposome with Fpg and hOGG1 enzymes had no significant effect (ns) compared to untreated cells. The positive control 75Mm H_2O_2 induced high levels of DNA damage as expected accessed in both OTM and % tail DNA and a significant decrease in DNA repair capacity in the patient group. Our data from OTM has shown that naked IFN –γ caused no significant effect on the DNA damage, however, IFN –γ liposome supplemented with enzymes, hOGG-1 and Fpg individually, has significantly reduced DNA damage and enhanced the repair capacity in lung cancer patients. Whereas, from the % tail DNA data, it is evident that both forms of IFN –γ separately supplemented with both enzymes have reduced the DNA damage to a significant level in lung cancer patients and also enhanced the repair capacity in patient lymphocytes ultimately protecting the cells from carcinogenesis and promote the survival of the cells.

The potential of IFN –γ in repairing mechanism in non-small cell lung cancer (NSCLS) plus other cancers is well-documented in that it works by stimulating DNA repair processes during the G2 phase of the cell cycle. It has also been reported to decrease the sister chromatin exchange (SCE) index in NSCLS patients, which is considered a clear biomarker of genome instability, DNA damage and repair (Baka et al. 2009).

The MN assay was used to investigate the influence of naked IFN-γ and liposome on the lymphocytes from healthy individuals and lung cancer patients. MN assay is considered an efficient tool to detect the genotoxicity of various genoprotective and genotoxic compounds at the chromosomal level by determining the different

parameters of this assay, i.e. micronuclei (indicators of chromosomal breakage, loss, rearrangement, necrosis and apoptosis (Fenech 2007), nucleoplasm bridges (biomarkers of di-centric chromosomes) and buds (biomarkers of gene amplification) (Cho et al. 2020).

In this study, the frequency of MNi was particularly determined in treatment groups amongst various types of cells counted (mono-nucleated, bi-nucleated and multi-nucleated cells). Additionally, MNi in binucleated cells and mono-nucleated cells were counted to assess the chromosomal damage after treatment and scoring the pre-existing damage, respectively (Li et al. 2012b). Mitomycin C (MMC), being a clastogenic and genotoxic compound, was used as a positive control in this study.

In the present study, our results from the micronucleus assay have shown that the levels of all the considered assay parameters such as MNi, NPBs and NPBs were higher in lung cancer patients at baseline level compared to those from healthy individuals.

MMC has induced a higher numbers of MNi in bi-nucleated cells than in mono-nucleated cells, confirming its genotoxic potential after the treatment (Table 4.1). The BiNC percentage per 1000 counted cells was calculated within the normal range (30-60%). However, the percentage of mutliNC observed was low, which means that the cell cycle inhibition was caused by Cyto-B mostly after one cell cycle.

Our micronucleus assay results were consistent with those from the Comet assay showing that naked IFN-γ (100 U/ml) and IFN-γ liposome (100 U/ml) treatment caused no statistically significant chromosomal damage at basal levels. Assessment of the assay results demonstrated that the frequency of MNi in BiNC

from healthy individuals and lung cancer patients significantly decreased when treated with naked IFN-γ and the liposome form compared to their respective control groups, but IFN-γ liposome was more effective owing to various advantages of liposomal-based drugs including better solubility, enhanced half-life, targeted delivery to the site, effective therapeutic index, and the capability to overcome chemo-resistance (Bozzuto and Molinari 2015; Senapati et al. 2018)

The frequencies of MNi in mono-nucleated healthy individuals' cells were very low, However, this was a higher in-patient group indicating pre-existing basal damage due to the disease condition. IFN-γ naked and liposome forms significantly reduced the MNi frequencies in MoNC from lung cancer patients compared to the NC, displaying its potential to decrease the basal damage in lymphocytes from lung cancer patients. Untreated lymphocytes from healthy individuals showed lower DNA damage than the untreated lymphocytes from lung cancer patients. This was observed throughout the *in vitro* study consistent with many past studies (Blasiak et al. 2004; Kontogianni et al. 2007; Stoyanova et al. 2010). The NDI values were normal for all experimental groups (1.8-2.2) (Table 4.1).

H_2O_2 (75μM) effects on genome stability of healthy individuals and lung cancer patients were also studied using MN assay for the initial time, to our knowledge. H_2O_2 treatment induced a significant increase in the formation of MNi in BiNC from both groups under investigations. However, MNi initiation was attenuated to statistically significant levels when H_2O_2 was co-supplemented with naked IFN-γ and IFN-γ liposome, individually. IFN-γ liposome form was more effective in bringing this effect than its naked form. These results confirmed that H_2O_2 treatment induced significant oxidative stress and damage (as expected) in

lymphocytes from healthy individuals and lung cancer patients. However, IFN-γ shows the potential to significantly inhibit the adverse effects of H_2O_2 by lowering the effect in geno-protective and antioxidant manner. Also, IFN-γ liposome form (100U/ml) has shown a better capacity to protect the lymphocytes from healthy individuals and lung cancer patients against H_2O_2-induced chromosomal damage. This could be possible due to the biocompatibility and enhanced cellular interaction of liposomes compared to their larger particles. The effective IFN-γ liposome results against H_2O_2-induced damage could be due to liposome's ability to prevent cell membrane alterations rather than its direct interaction with H_2O_2 (Trif and Craciunescu 2015).

Histone and non-histone proteins are very important for organising approximately 2 m lengthy DNAs in roughly 10 μm into the chromatin. The fitting of DNA into condensed chromatin is crucial for its functional properties and effective regulation with other molecular processes (Kinner et al. 2008). With respect to transcriptional mediation, the structural changes in chromatin have been extensively studied. Still, it is evident now that chromatin alterations play a key part in the DNA repair machines (Groth et al. 2007). Human cells are constantly vulnerable to different types of DNA damage or lesions which could be divided into two categories depending on the extent of their influences on genome stability. The first kind of lesions are simpler ones, including base damage and few single sugar backbone disruptions; these lesions could easily be repaired without affecting the genome integrity by deploying an error-free repair using a complementary DNA strand template with slight rearrangement of chromatin. However, the other kind is lethal to the cells and most dangerous types of DNA damage mainly includes DSBs. The compromised repair of these lesions could

bring deleterious effects and are a severe threat to genome stability and cellular survival (Podhorecka et al. 2010).

In the current study, our results from the Comet, modified enzyme repair Comet and micronucleus assays showed that naked IFN-γ and IFN-γ liposome forms (100 U/ml) do not induce DNA or chromosomal genotoxicity of any kind in lymphocytes from healthy individuals and lung cancer patients assessed at basal levels. In fact, IFN-γ enhanced the cellular repair capacity before entering replication, therefore helps to maintain genome integrity which can be used in cancer cells and leads to better prognosis affecting cancer cell replication and making tumors more sensitive to chemotherapy. These opens therapeutic windows to exploit more cancer vulnerabilities.

Our results were consistent with a previous study where IFN-γ induced early stimulation of PARP (the DNA repair enzyme) when the cells were exposed to pro-apoptotic agent (Saint Jean et al. 1999).

The cell cycle is a fundamental and highly regulated procedure accommodated by every individual eukaryotic organism, which permits cellular division and multiplication. The cell cycle regulation mainly depends on its checkpoints: the first one is found in G1-phase, where after progression to S phase, cells synthesize DNA and the latter comes at the G2/M end when cells undergo DNA repair just before mitosis division (Huang et al. 2015). *p53*, a tumour-suppressor gene exhibiting diverse functional properties, is considered as a critical regulator of transcription and the cell cycle (Darcy et al. 2008). The extent of DNA damage decides the destiny of a cell, whether it should experience cell cycle arrest at GI/arrest or subject to apoptosis. In case of little damage, p53 stimulates

G1/arrest signal initiating repair mechanism of cells before entering S-phase of the cell cycle. Still, in case of intense DNA damage, death signal is generated by p53 to cause apoptosis of cells. p53 is supposed to induce GI/arrest through mediation of the p21 expression (a cyclin dependent kinase inhibitor (CDKI)), ultimately controlling the passage of cells through further phases of cell cycle (Haupt and Haupt 2017). While p53 is a well-documented transcription regulatory factor, it is also involved in affecting important genes linked with apoptosis. p53 has been shown to down-regulate the expression of an anti-apoptotic protein, Bcl-2, meanwhile igniting the pro-apoptotic activities of Bax, activating the apoptotic pathway (Kuo et al. 2006). This differentially regulation of particular proteins confirms the transactivation activities of p53.

Most of the tumour cells exhibit this ability to evade apoptosis and cell cycle checkpoints, leading to uncontrolled cellular proliferation. Since p53 is the key regulator of healthy cellular growth, its modification or absence can lead to an unmonitored proliferation, aiding carcinogenesis and be proven lethal for cellular homeostasis. This can also cause high mutational frequency compromising the integrity of DNA. It is evident from research that p53 is the most often mutated gene in the majority of the tumours bringing harmful effects (Kong et al. 2012). Therefore, the introduction of p53 or raising its levels could potentially overcome the resistance of tumour cells to apoptosis and effectively regulate their proliferation. This could also help initiate repair pathways before mitosis preventing mutations (Shaw 1996).

Research confirms that p53 stimulated p21 promotes excision repair machinery (Abbas and Dutta 2009) by interacting with a complex called proliferating cell nuclear antigen (PCNA) (Moldovan et al. 2007). Thus, we investigated the role of

naked IFN-γ and liposome forms (100 U/ml) on the expression levels of P53 protein at post-translational and mRNA levels in lymphocytes from lung cancer patients compared to those from healthy individuals. We also investigated the effects on some of the P53 regulated genes; p21 and Bcl-2 while evaluating their regulation in lymphocytes from lung cancer patients and healthy individuals.

The protein and gene expression were analyzed using the WB analysis and the qRT-PCR study, respectively, in lymphocytes from lung cancer patients and healthy individuals. The 24-hour treatment with naked IFN-γ and liposome forms (100 U/ml) had no significant effect on p53 and p21 expression at protein and mRNA levels in lymphocytes from healthy individuals. These gene expressions were barely detectable. Our results are consistent with a previous study where the p53 in normal cells is maintained at a low level by the action of the MDM2 proto-oncogene (Midgley and Lane 1997; Rivlin et al. 2011).

However, determined from our results, p53 protein was significantly increased in lung cancer patients' lymphocytes with naked IFN-γ and liposome treatment (Figure 6.2), consistent with the results from PCR where the p53 gene significantly up-regulated in lymphocytes from lung cancer patients when treated with naked IFN-γ and liposome (100U/ml) suggesting that the IFN-γ may induce p53-mediated cell cycle arrest and DNA repair in lung cancer patients. A similar result was obtained related p21 expression where p21 was significantly up-regulated in lymphocytes from lung cancer patients after treatement with both forms of IFN-γ, liposome and naked at both mRNA and protein levels (Figure 6.2 and Figure 6.4), consistent with a previous study which demonstrated that the increased expression of p53 is usually accompanied with up-regulation of the p21 gene (Shih et al. 2007).

IFN-γ, liposome and naked forms have regulated the expression levels of these genes differentially in lymphocytes from healthy individuals compared to those from lung cancer patients. This might be due to variant effects of IFN-γ in lymphocytes from healthy individuals and lung cancer patient. The lymphocytes from patient groups could show increased sensitivity to IFN-γ than those from healthy individuals due to their compromised genome integrity and cancer condition.

However, naked and liposome forms of IFN-γ did not show any statistically significant effect on the expression level of Bcl-2 in lymphocytes from lung cancer patients and healthy individuals (Figure 6.2 and Figure 6.4). This suggests that IFN-γ does not affect apoptotic pathway rather could trigger p53-mediated repair mechanism inhibiting the uncontrolled proliferation of lymphocytes from lung cancer patients.

To conclude our findings, both forms of IFN-γ, naked and liposome (100U/ml) have demonstrated genoprotective, antioxidant and Increase the repair capacity in lymphocytes from lung cancer patients and healthy individuals. It exhibited strong antioxidant potential by protecting the cells against H_2O_2-induced DNA damage and stress in both investigative groups. IFN-γ in both forms has shown anti-carcinogenic effects in lymphocytes from lung cancer patients by triggering the p53 and p21-mediated cell arrest and repair processes.

In general, IFN-γ liposome (100U/ml) has shown slightly better protective effects compared to its larger particle counterpart, naked form at concentration of 100U/ml. This could be possible due to the enhanced solubility of liposome-coated IFN-γ in an aqueous medium stabilizing various therapeutic agents such as proteins and nucleotides (Moghimi and Agrawal 2005; Barenholz 2012; Cern

et al. 2012). Many hydrophilic and hydrophobic biomolecules can be encapsulated in liposomes and be protected from interactions with the external environment. Liposomes inhibit the metabolism of the drug before reaching the target cells plus protect the drug's interaction with healthy cells enhancing the therapeutic index of the drug. It has been well documented that the liposome form of any therapeutic drug shows enhanced pharmacokinetic properties and biodistribution than its free form. The morphology of liposomes is similar to that of the cellular membranes, making them an ideal drug-carrier system (Bozzuto and Molinari 2015).

Overall data from the present study proposes that IFN-γ could possibly protect and defend against lung cancer through cell cycle arrest of cancer cells and repair mechanisms and liposome can potentially be used as an alternative better drug delivery system in various conditions.

7.2 Future work

The results presented in this study showed that IFN- γ liposome reduced DNA damage *in vitro* in human lymphocytes from lung cancer patients. It also showed that IFN-γ had a protective effect on cells against DNA damage from oxidative stress caused by H_2O_2. The results also suggest that IFN- γ liposome decreased oxidative stress in lymphocytes from lung cancer patients in the presence of H_2O_2 in a concentration-dependent manner. Additional techniques needed to confirm these results, such as Micronucleus FISH (Fluorescence *in situ* hybridisation), allow determining the nature of chromosome damage lesions.

The IFN- γ liposome has shown regulating effect on p53, p21 and Bcl-2, but it requires more research on the regulation of repairing genes and protein such as

XRCC3, which classified as one of the DNA repair genes that encode for a protein that has an essential role in repairing the double-strand breaks, cross-link repair and genome stability.

Moreover, it has been reported that IFN- γ liposome reduced the oxidative stress of H_2O_2. This mechanism can be followed by treating human lymphocytes from lung cancer with antioxidants, such as vitamin E, to increase the effectiveness of IFN- γ liposome. Furthermore, this work could be repeated with lymphocytes from patients with different types of cancer and in lung cancer tissue to determine whether the results will be similar or different when compared against the results of lung cancer.

Genomic and chromosomal instability is considered a cancer enabling characteristic and a driving force behind tumor heterogeneity and evolution resulting in drug resistance and reduced clinical response rates. Due to our findings that IFN-γ enhanced the cellular repair capacity and increase the genome stability this represents opportunities for using IFN-γ on different type of cancer cells and check it is effectiveness to delay the onset of drug resistance.

Finally, the liposomal formulation requires further studies to investigate long term stability, loading levels and release kinetics. In addition, the potential for using liposomes for inhalation delivery could be investigated by studying the formation of aerosol size and drug deposition as well as bioavailability.

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Appendices

8.1 Appendix 1

Centre Number:

CONSENT FORM FOR PATIENTS

Title of Project: **Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints. (Version 3, 07- 07- 09)**

Reviewed by Leeds Central Research Ethics Committee (Podhorecka et al.) (REC reference number: 12/YH/0464)

1. I confirm that I have read and understood the information sheet (version 3, 19- 06-09) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust or the University of Bradford, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records

4. I agree that the sample I have given and the information gathered about me can be stored at the University of Bradford, as described in the attached information sheet.

5. I agree to take part in the above study.

Name of Patient **Date** **Signature**

8.2 Appendix 2

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

School of Life Sciences

DATA COLLECTION FORM
(To be completed by the Doctor)

STUDY TITLE: Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints
REVIEWED BY LEEDS East RESEARCH ETHICS COMMITTEE (REC)
(REC REFERENCE NUMBER: 12/YH/0464)

PATIENT NUMBER		DATE OF SAMPLE	
AGE			
SEX (PLEASE TICK)	M / F	CONSENT	Y / N
ETHNIC GROUP		INFORMATION SHEET	Y / N
OCCUPATION			

CURRENT SMOKER Y/N PAST SMOKER Y/N HOW MANY/MUCH PER DAY?
CIGARETTES CIGARS PIPE
ALCOHOL Y/N UNITS PER WEEK

DIET	WESTERN	ASIAN	OMNIVORE	VEGETARIAN	VEGAN

VITAMINS / ANTI-OXIDANTS (PLEASE LIST)

PRESCRIBED DRUG USE (PLEASE LIST)

RECREATIONAL DRUG USE Y/N

IF YES PLEASE LIST

MEDICAL **Asthma** **COPD** **others**

CANCER
Inflammatory disease

EXTENT		SITE		HISTOLOGY		SURGERY	

CANCER
Inflammatory disease
Pre cancerous state
OTHER MEDICAL CONDITIONS (PLEASE LIST)
Family history of cancer and Inflammatory disease
Chemotherapy or radiotherapy

MOST RECENT MEASURE

	RESULT	DATE		OTHERS	RESULT	DATE
WEIGHT						
HEIGHT						
BMI						

8.3 Appendix 3

School of Life Sciences

Participant Information Sheet for patients (Version 4, 28/01/2019).

Study title: Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints.

Reviewed by Leeds East Research Ethics Committee (Podhorecka et al.)

(REC reference number: 12/YH/0464)

Invitation to the research study

We should like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish and you will be allowed around 24 hours to consider this.

(Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study).

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you want to take part.

Part 1

What is the purpose of the study?

In this study blood cells will be treated in a test tube with very small chemical particles or UVA (Ultra Violet A light) to determine if patients with different diseases are more at risk after exposure compared to healthy individuals. For example, chemicals and UV (Ultra Violet) can break and damage the DNA of white blood cells. Further examination of this resulting damage may improve our knowledge of the cancers and other inflammatory diseases. The tests are not predictive for any kind of diseases and the test results will not impact on you or the healthy volunteers with whom you are compared. Blood samples for the study will be taken during the clinic visit in which you are recruited, if possible at the same time as the samples taken for your routine care.

A blood sample of around 2-4 teaspoons (20 ml) will be taken. Samples will be stored only until the end of the study (after 8 years) and used for studies of similar nature or to check original responses. The research is also used for some PhD programmes.

Why have I been invited

Because you are a patient at the Clinic and we should like to determine if these small chemical particles or UVA could be more harmful to you than to people without the disease.

Do I have to take part?

No, it is up to you to decide. We shall outline the study and go through this information sheet, which we shall then give to you. We shall ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

Part 2

What will happen to me if I take part?

Only a single blood sample will be taken for this research study. A brief questionnaire will need to be completed by the researchers.

Each individual will be given a coded study number so that your clinical data will be linked in an anonymous way with the research results.

The study tests are not predictive for you.

The data obtained will only be available to the research team and will **not** be returned to you. Responses will be compared only on group basis i.e. collective responses from patients with that individual disease compared to collective responses from people without that disease. Results could be published in the form of scientific papers. The work may benefit the medical and scientific community at large, but will not be of direct benefit to you as an individual. If, however, you would like more information, the appropriate consultant will be prepared to talk to you individually about study results.

The data will be stored until the study is completed at the end of 8 years.

People who cannot take part in the study.

People who are not well enough to take part will be excluded e.g. those with anaemia.

If you have any further questions, you could contact the research team:

Prof Badie Jacob, NHS Trust, St **Lukes Hospital**
Bradford Teaching Hospitals NHS Foundation Trust, BD5 0NA.

Telephone: 01274 542200

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