Genotoxic effects in human peripheral lymphocytes from healthy individuals and head and neck cancer patients after treatment with hydrogen peroxide and pembrolizumab liposome

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N. M. A. Bobtina

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Nagah Mohmed A. BOBTINA

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Abstract

Nagah Mohmed A. Bobtina

Genotoxic effects in human peripheral lymphocytes from healthy individuals and head and neck cancer patients after treatment with hydrogen peroxide and pembrolizumab liposome

Keywords: Pembrolizumab; naked and liposome forms; DNA damage; genotoxic; genoprotective, human lymphocytes; head and neck cancer; healthy individuals

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. It has commonly been associated with exposure to tobacco-derived carcinogens and alcohol consumption. Pembrolizumab has shown to be effective in the treatment of many types of cancers such as melanoma, non-small cell lung cancer, due to its antiproliferative, immunoregulatory properties.

The aim of this study was to investigate the effects of naked Pembrolizumab and Pembrolizumab liposome on the level of DNA damage, gene, and protein expressions in peripheral lymphocytes from HNC patients and compared to the healthy individuals by using the Comet and micronucleus assays. Western blotting and real-time polymerase chain reaction were performed to assess the potential of improving the repair mechanisms after treatment with naked Pembrolizumab and Pembrolizumab liposome. According to the results, Comet assay and micronucleus assay showed a significantly decreased DNA damage in the lymphocytes from HNC patients after being treated with naked Pembrolizumab and pembrolizumab liposome. Furthermore, the results have shown that naked Pembrolizumab and pembrolizumab liposomes (10 µg/ml) greatly decreased the oxidative stress produced by H_2O_2.

Both forms of pembrolizumab have also demonstrated improving the repair mechanisms in lymphocytes from HNC patients by modulating the expression of P53, P21, and Bcl-2 at mRNA and protein levels. This study suggested that Pembrolizumab naked and liposome could have an antioxidant role alongside other actions in the treatment of HNSCC. However, further studies on Cancer cell lines and *in vivo* observation are required to validate the anticancer potential of pembrolizumab naked with liposome in HNC.

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List of Abbreviations

•OOH, Hydroxyl Radicals (•OH),
1,2-Dioleoyl-Sn-Glycero-3-Phsphoethanolamine (DOPE)
1,2-Dipalmitoyl-Sn-Glycero-3-Phospho-Rac-(1-Glycerol) Sodium Salt (DPPG)
Active Pharmaceutical Ingredients (Apis)
Adenine (A)
American Joint Committee On Cancer (AJCC)
Apurinic/Apyrimidinic (AP)
Base-Excision Repair (BER)
BCL-2 Homology (BH)
Binucleated (Binc)
Binucleated Cells (Binc)
Binucleated Cells (Binc)
Bovine Serum Albumin (BSA)
Butylated Hydroxyl Toluene (BHT)
Carbonate Radicals (CO3•)
CDKN1A (P21)
Cell Counting Kit-8 (CCK-8)
Central (Sequence-Specific DNA Binding (DBD)
Classical Hodgkin Lymphoma (Chl)
Colorectal Cancer (CRC)
Convention Liposomes (CL)
Cycle Threshold (CT)
Cyclin-Dependent Kinase 2 (CDK2)
Cyclin-Dependent Kinase Inhibitor (CDKI)
Cyclin-Dependent Kinase Inhibitor (CKI)
Cyclin-Dependent Kinase Inhibitor 1 (CKI)
Cytochalasin B (Cyto B)
Cytokinesis Block Micronucleus Assay (CBMN)
Cytokinesis-Block Micronucleus (CBMN)
Cytosine (C)
Deoxyribonucleic Acid (DNA)
Dichloromethane (DCM)
Dimethyl Sulfoxide (DMSO)
DNA Binding Domain (DBD)
DNA Damage Response (DDR)
DNA Synthesis Phase (S Phase)
DNA-Binding Domain (DBD)
Double Strand Breaks (Dsbs)
Double-Stranded Breaks (Dsbs)
Dynamic Light Scattering (DLS)
Enhanced Chemiluminescence (ECL)
Environmental Protection Agency (EPA)
Epidermal Growth Factor Receptor (EGFR)
Epstein–Barr Virus (EBV)
Ether Injection Vesicles (EIV)
Fetal Bovine Serum (FBS)
Foetal Bovine Serum (FBS)
French Press Vesicles (FPV)
Gain-Of-Function (GOF)
Gap 1 (G1)
Gap 1 Phase (G1 Phase)
Gap 2 (G2)
Gap 2 Phase (G2 Phase)

Guanine (G)
Head And Neck Cancer (HNC)
Head And Neck Cancers (HNC)
Head And Neck Malignancies (HNSCC)
Head And Neck Squamous Cell Carcinoma (HNSCC)
Head And Neck Squamous Cell Carcinomas (HNSCC)
Head And Neck Squamous Cell Carcinomas (Hnsccs)
Heat-Shock Protein 90 (HSP90)
Histone Deacetylases (HDAC)
Human Epidermal Growth Factor Receptors (HER)
Human Papillomavirus [HPV]
Human Papillomaviruses (HPV)
Hydrogen Peroxide (H2O2
Hydrogen Peroxide (H_2O_2)
Immuno-Liposomes (IL)
Immunoliposomes (Ilps)
Insulin Receptor (IR),
International Agency For Research On Cancer (IARC)
Large Unilamellar Vesicles (LUV)
Long Circulating Liposomes (LCL)
Mammalian Target Of Rapamycin (Mtor)
Merkel Cell Carcinoma (MCC)
Messenger RNA Expression (Mrna)
Micronuclei (MN)
Micronuclei (MN)
Micronuclei (Mni)
Micronuclei (Mni)
Micronucleus (MN)
Micronucleus FISH (Fluorescence In Situ Hybridisation)
Mismatch Repair (MMR)
Mitochondrial Membrane (MOM)
Mitochondrial Outer Membrane Permeability (MOMP)
Mitomycin (MMC)
Mitomycin C (0.4µm MMC)
Mitomycin C (MMC)
Mitosis (M)
Mitotic Phase (M Phase)
Mononucleated (Mononc)
Mononucleated Cells (Mononc)
Multialmeller Vesicles (MLV)
Multinucleated (Multinc)
Multinucleated (Multinc)
Multinucleated Cells (Multinc)
Negative Control (NC)
No Significant (Ns)
Non-Small Cell Lung Cancer (NSCLC)
Non-Small-Cell Lung Cancer (NSCLC)
Normal Melting Point (NMP)
Normal Melting Point Agarose (NMP)
Nuclear Bud (NBUD)
Nuclear Buds (Nbuds)
Nuclear Buds (Nbuds)
Nuclear Division Index (NDI)
Nuclear Division Index (NDI)
Nuclear Factor-Kappa B (NF-Kb)
Nucleoplasm Bridges (Npbs)

Nucleoplasmic Bridge (NPB)
Nucleoplasmic Bridges (NPBS)
Nucleoplasmic Bridges (NPBS)
Nucleotide-Excision Repair (NER)
Oligomerization Domain (OD)
Olive Tail Moment (OTM)
One-Way Analysis Of Variance (ANOVA)
Overall Survival (OS)
PD Ligand 1 (PD-L1)
Pembrolizumab (Keytruda)
Phosphatidylinositol 3-Kinase (PIK3CA)
Phytohaemagglutinin (PHA)
Platelet-Derived Growth Factor Receptor (PDGFR)
Polymerase Chain Reaction (PCR)
Polymerase Chain Reaction (PCR)
Positive Control (PC)
Posttranslational Modifications (Ptms)
Primary Mediastinal Large B-Cell Lymphoma (PMLBCL)
Programmed Cell Death (PCD)
Programmed Cell Death 1 (PD-1)
Programmed Death Ligand-1 (PD-L1)
Progression-Free Survival (PFS)
Proliferating Cell Nuclear Antigen (PCNA)
Proline-Rich Domain (PRD)
Pyridyloxbutyl T (POB-T)
Quantitative PCR (Qpcr)
Quantitative Reverse Transcription PCR (Qrt-PCR)
Quantitative Reverse Transcription PCR (Qrt-PCR)
Quantitative Reverse Transcription Polymerase Chain Reaction RT-PCR (Qrt-PCR)
Reactive Oxygen Species (ROS)
Reactive Oxygen Species' (ROS)
Real-Time Polymerase Chain Reaction (Qpcr)
Recurrent Or Metastatic HNSCC (R/M HNSCC)
Regulatory Domain (RD)
Regulatory T Cells (Treg)
Renal Cell Carcinoma (RCC
Repair Enzyme Poly (ADP-Ribose) Polymerase (PARP)
Reverse Phase Evaporation Vesicles (REV)
Reverse Transcription PCR (RT-PCR)
Single-Cell Gel Electrophoresis (SCGE)
Single-Strand Breaks (Ssbs)
Small Unilamellar Vesicles (SUV)
Sodium Dodecyl Sulphate-Polyacrylamide Gel (SDS- PAGE)
Squamous Cell Carcinoma (SCC)
Squamous Cell Carcinoma Of The Head And Neck, The EGFR Is Overexpressed (SCCHN)
Superoxide (O2●−)
Superoxide (O2•)
Synthesis (S)
The Battle Against Cancer
The Micronucleus Assay (MN)
Thymine (T)
Transition Temperature (TC)
Translation (TLS)
Transmission Electron Microscopy (TEM)
Tumour Proportion Score (TPS)
Tumour-Node-Metastasis (TNM)

Ultraviolet Radiation (UV)
Union For International Cancer Control (UICC)
Union For International Cancer Control (UICC)
Vascular Endothelial Growth Factor (VEGF)
Western Blot (WB)
Western Blotting (WB)

CHAPTER ONE: Introduction

1 Introduction

1.1 Cancer causes and development

Cancer presents an ongoing mortality challenge faced by all populations around the world. Rather than responding appropriately to the signals that control normal cell behavior, cancer cells grow and divide in an uncontrolled manner, invading normal tissues and organs and eventually spreading throughout the body. The generalized loss of growth control exhibited by cancer cells is the net result of accumulated abnormalities in multiple cell regulatory systems and is reflected in several aspects of cell behavior that distinguish cancer cells from their normal counterparts. (Bray et al. 2018; Cancer Research UK 2021a). Cells in the emergent neoplasm go through a variety of genetic and epigenetic alterations that ultimately alter gene activities and phenotypes, as a result, allow cancer to develop (Bhattacharya et al. 2018; Guma 2019). It occurs annually in more than 14.1 million people throughout the world (Ahmadi and Shadboorestan 2016). Cancer can occur in all cell types via the development of tumour to malignant tumour; however, carcinomas can only originate from epithelial cells (Yuan et al. 2019; Pham et al. 2018; Zhou et al. 2021). Common examples of cancers that develop due to tobacco inhalation are lung, breast, and ovarian forms, whereas radiation is more associated with skin cancer. Other problems that can result in cancer diagnosis include lifestyle and diet, as well as family history stress (Olusanya et al. 2018).

The lymphatic system is integral to metastatic cancer cell diffusion to organs beyond the primary tumour site. Nevertheless, there remain numerous challenges to the development of advanced techniques for not only identifying micro metastasis but also

providing prognoses and treatment. Furthermore, there are no FDA-approved drugs that target tumour lymphangiogenesis, despite the consequences of metastasis through the lymphatic system (Yukuyama et al. 2020)

Despite a considerable body of research, these processes are still poorly understood at the molecular level. The spread of cancerous cells begins when they enter first the lymphatic system and then the blood vessels, eventually reaching the bloodstream by a mechanism where cells first loose cadherin and break cells' wall (Cancer Research UK 2021a). Once they have spread to healthy tissue, they accumulate and grow, generating secondary tumours as part of the metastasisprocess (Callens et al. 2021). Deaths from cancer tend to be due to secondary tumours rather than the primary one, with metastasized cancer being the main cause of morbidity and mortality, accounting for around 90% of cancer fatalities (Şen et al. 2021). Tumour cells at secondary malignant sites can occur in one of three states, namely passive solitary cells, active pre-angiogenic cells, or micro metastasized cells (van Zijl et al. 2011; Łukaszewski et al.2017).

When the cell becomes incapable of fixing damaged genes, additional problems will be encountered. These can include cancerous cells spreading to other body parts, expanding at an increasing rate and also becoming immune to treatment (Seyfried and Shelton 2010). Such cells can also develop a resistance to signals indicating they should self-destruct, meaning that apoptosis will have no effect. Blood vessels and lymphatic streams can enable malignant neoplastic disease cells to circulate around the body, meaning that the problem spreads. With this occurring, metastasis can accelerate and result in a secondary tumour developing. Thosediagnosed with cancer tend to receive treatment based on anticancer agents, which can perform by utilizing chemicals to destroy cancer cells outright, or by generating apoptosis so that the

cancer cells respond to instructions to self-destruct (Gogoi et al.2016). A wide range of behaviors and issues can lead to a cancer diagnosis, with smoking and alcoholism being two obvious causes, but consuming processed meat can also be harmful, as can exposure to radiation, environmental factors and other lifestyle choices (Vineis and Fecht 2018).

Regarding those cancers typically linked to smoking and alcohol, they most commonly develop in the head and neck, and frequently result in oral cavity, hypopharynx, oropharynx, and voice box diseases. Indeed, research shows that three-quarters of head and neck cancers (HNC) can be explained by alcohol consumption or smoking (Dhull et al. 2018a).

Additional aspects that can result in gene mutations contributing to quick health decline could be biological and environmental aspect in nature. Holland et al. (2011) showed how exposure to environmental genotoxicants in Polish cities resulted in children being diagnosed with chromosomal damage, as well as a significant micronuclei (MNi) transformation identified in the peripheral blood lymphocytes.

An alteration in any of three particular types of genes such as tumour suppressor genes, protooncogenes, and DNA repair genes that regulate cell cycle, will cause tumourigenesis (Vogelstein and Kinzler 2004; Chow 2010; Llinas-Arias and Esteller 2017; Vogelstein and Kinzler 2004).

To achieve a robust cancer treatment, it is vital for healthcare experts to appreciate how it emerges and develops within the human body. Therefore, by researching how genetic damage progresses in specific cancers, medical professionals can be better informed and equipped to handle the task of caring for and treating the disease. In comparison to the typical healthy cells found in the human body (Cancer Research UK 2020; Miller et al. 2015).

1.1 Head and Neck cancer

Head and neck cancers are derived from the mucosal epithelium in the oral cavity, pharynx and larynx and are known collectively as head and neck squamous cell carcinoma (HNSCC). Oral cavity and larynx cancers are generally associated with tobacco consumption, alcohol abuse or both, whereas pharynx cancers are increasingly attributed to infection with human papillomavirus (HPV), primarily HPV-16. Thus, HNSCC can be separated into HPV-negative and HPV-positive HNSCC. Despite evidence of histological progression from cellular atypia through various degrees of dysplasia, ultimately leading to invasive HNSCC, most patients are diagnosed with late-stage HNSCC without a clinically evident antecedent pre-malignant lesion. As Mehra et al. (2016) confirm over half a million head and neck squamous cell carcinoma (HNSCC) are identified in one year alone (Mehra et al. 2018). Furthermore, Webber et al, (2019) recognize that head and neck forms represent one of the biggest global health challenges caused by cancer, being fatal in more than 350,000 cases (Webber et al. 2019). As tumours, HNSCC are identified as heterogeneous, typically diagnosed in the larynx and hypopharynx, upper aerodigestive tract, oropharynx, and oral cavity (Argiris et al. 2018). Types of HNC are mentioned below as well in figure 1.1A

1.1.1 Squamous-cell carcinoma

Squamous cell carcinoma is the sixth most common malignancy worldwide (Jemal et al. 2010). The anatomical complexity of the head and neck region (The anatomy of the head and neck is complex because so many different functional structures are located close to each other. Paired organs include the tonsils, parotid glands, other major salivary glands, maxillary and frontal sinuses, and the nasal cavities), along with

complications of the patterns of tumour invasion, gives rise to difficulties in the treatment of HNSCC (Kareemaghay and Tavassoli 2019). Squamous-cell carcinoma impacts the squamous cell, which is a type of epithelial cell found in mucous and also skin membranes. Researchers estimate that more than nine out of ten HNSCC cases can be attributed to this form (Johnson et al. 2020b).

1.1.1 Adenocarcinoma

Adenocarcinoma is a type of head and neck non-squamous cell carcinoma. Surgery is considered a radical treatment for head and neck adenocarcinoma, and postoperative radiation therapy remains the treatment of choice in locally advanced cases (Lund et al. 2012). Epithelial tissue is impacted by adenocarcinoma, resulting in common types identified by Haines (2013) as Salivary gland carcinoma, Neuroendocrine carcinoma and Sino nasal carcinoma (Haines 2013).

1.2 Location of Cancer

1.2.1 Mouth

The oral cavity is the location of many forms of squamous cell cancer, which can end up forming in the tongue, the hard palate, the floor of the mouth, the inner lip and also the gums (Mihai et al. 2019).

1.2.2 Nasopharynx

Nasopharyngeal cancer is located in the nasopharynx, which consists of the upper pharynx area and also the nasal cavities (National Cancer Institute 2021).

1.2.3 Throat cancer

Emerging in the throat (or oropharynx), oropharyngeal squamous cell carcinomas affect the central throat area, which can mean the tongue's base, the soft palate, and the tonsils (Bron et al. 2019).

1.2.4 Hypopharynx cancer

The lower part of the throat, the hypopharynx is made up of the posterior pharyngeal wall, the post-cricoid anterior wall section, and the pyriform sinuses (Petersen et al. 2018).

1.2.5 Laryngeal cancer

It is in the voice box where laryngeal cancer takes hold, in some cases affecting the vocal folds (glottis cancer) and in others the tissues positioned above or beneath them – known as subglottic and supraglottic. See Figure 1.1 to pinpoint where the various regions affected by HNSCC are located in the body (National Cancer Institute 2021).

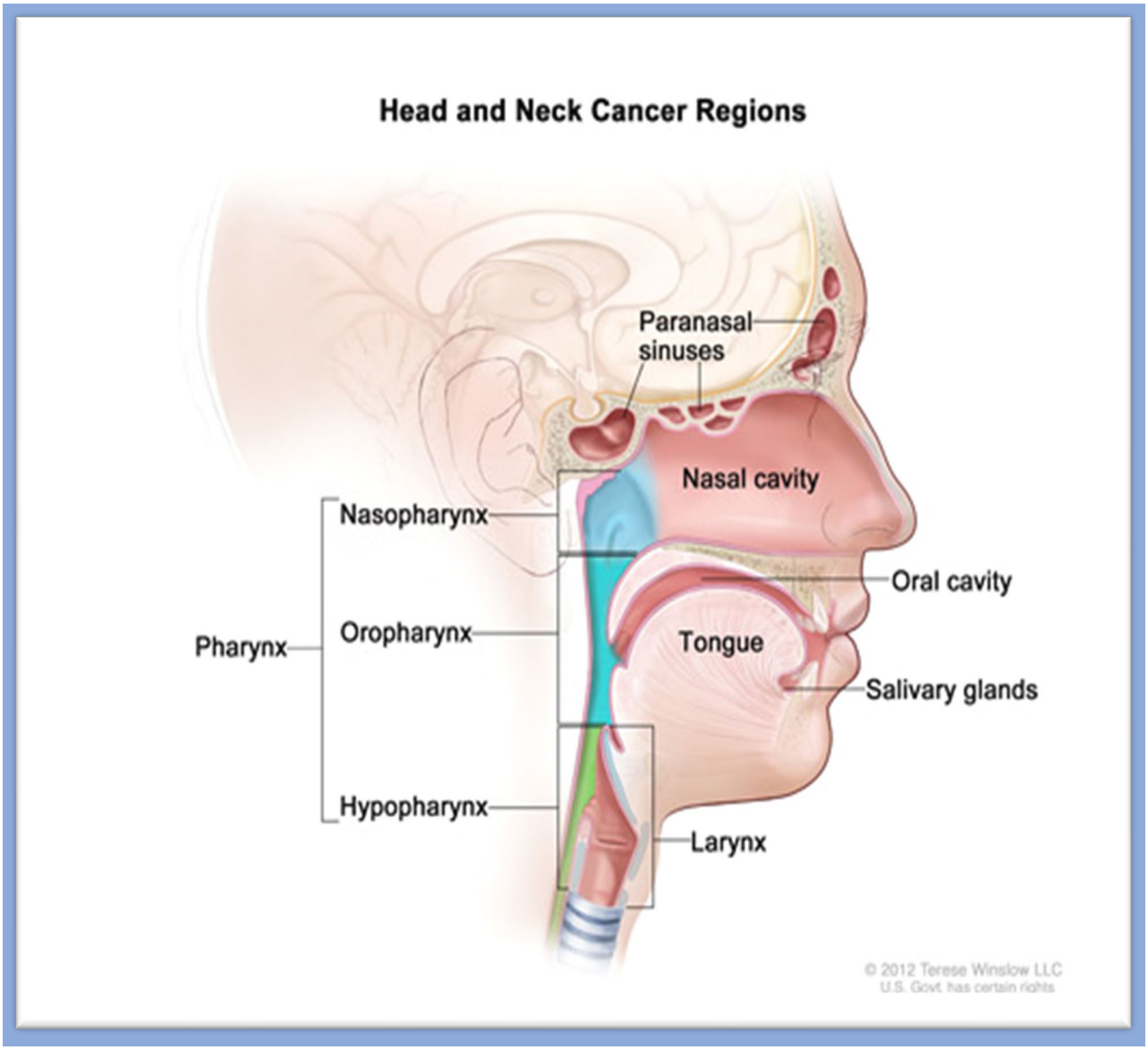


Figure 1-1 A labelled image of the regions that may be impacted by HNSCC (National Cancer Institute 2021).

1.3 Disease Statistics

According to the American Cancer Society (2017), more than 65,000 individuals in total were predicted to receive a cancer diagnosis (American Cancer Society 2017).Worldwide, HNC accounts for more than 650,000 cases and 330,000 deaths annually (Vigneswaran and Williams 2014). In the United Kingdom, HNSCC cancers are considered to be the eighth most common type of cancer, making up three percent of diagnosed cases. Indeed, as far as men are concerned, HNSCC has the fourth

most common case among all cancers (Cancer Research UK 2018). Figures taken in 2015 show that 8,400 cases in total were confirmed. In contrast, HNSCC cancer is only the 13th most frequently reported type found in women, equaling 3,700 confirmed cases in 2015. In addition, rates of diagnosis are similarly higher among the older populace, with the most vulnerable group being aged between 70 and 74 years old. The most common HNSCC cancer type is oral, with oral squamous cell carcinoma cases making up almost nine out of ten of all cases (Liu et al. 2019).

1.2 Risk factors

There are many risk factors for cancer. These can be lifestyle factors, such as smoking and alcohol or processed meat consumption, environmental factors, including exposure to radiation, e.g. gamma or x-ray radiation, asbestos, formaldehyde, and health-related factors such as the Epstein-Barr virus which are associated with a high risk of HNC (Dhull et al. 2018a).

1.2.1 Alcohol and tobacco

The evidence provided by the body of research, asserts that smoking is a risk factor for a minimum of 16 cancer types (ASH 2017). Specifically, in the UK, smoking has been identified as the cause in 64% of laryngeal cancer cases, 37% of pharyngeal cancer cases, 25% of nasopharyngeal cancer cases, and 17% of oral cavity cancer cases (Cancer Research UK 2021a). More generally, in the UK smoking has been linked to 65% of oral cavity (lip, tongue and mouth) and pharynx (throat) cancers, and the research has also shown that 91% of all oral cavity and pharynx cancers are preventable (Hashim et al. 2019). Rare cases such as salivary gland malignancies represent only around 5% of head and neck malignancies, and while benign salivary gland neoplasms occur more frequently than salivary gland malignancies, the gland of origin determines the proportional frequency of the malignancies (Son et al. 2018).

Tobacco smoke commonly contains various harmful compounds, including nicotine, ammonia, formaldehyde, carbon monoxide, carbon dioxide, cadmium, tar, acetone, hydroxyquinone, benzopyrene, and nitrogen oxide (Yamaguchi 2019). It also contains the major constituent crotonaldehyde, an α, β-unsaturated carbonyl compound that can cause substantial eye, skin, and respiratory irritation. Furthermore, acrolein reacts primarily with deoxyguanosine in the smoker's DNA, producing two pairs of regioisomeric 1,N^2-propanodeoxyguanosine adducts: (6*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8,-tetrahydro-6-hydroxypyrimido[1,2-*a*]purine-10(3H)one (α-Acr-dGuo) and (8*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8,-tetrahydro-8-hydroxypyrimido[1,2-*a*]purine-10(3H)one (γ-Acr-dGuo). and (8*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8,-tetrahydro-8-hydroxypyrimido[1,2-*a*]purine-10(3H)one (γ-Acr-dGuo). According to the Environmental Protection Agency (EPA) based on data from animal studies and genotoxicity, crotonaldehyde is a potential human carcinogen (group C) (Bagchi et al. 2018).

Tobacco smoke contains several inactive carcinogenic compounds; once activated by P-450 cytochrome enzymes, these consequently directly bind to DNA to create DNA adducts. As reactive metabolites can bind to macromolecules, including DNA and proteins, they disturb their functioning, which has several adverse health impacts (Hecht 2012). In particular, nicotine has an immunosuppressive influence on the immune response because it contains immunosuppressive agent that can modulate innate and adaptive immune responses (Sopori, 2002; Cui and Li, 2010) through interacting with nAChRs on the surface of immune cells, including macrophages, T and B lymphocytes (Cloez I, Changeux P 2007). Recently, considerable work has been done to show that α7 nAChR, one type of nAChRs, plays a crucial role in nicotine's anti-inflammatory effects. The activation of α7 nAChR by nicotine in murine

macrophages interacted with Jak2 and then induced the phosphorylation of STAT3, which subsequently inhibited the transcription of pro-inflammatory cytokines (Feifei Q et al 2017) as well as the production of reactive oxygen species(ROS) which can damage the DNA of the epithelial cells of the larynx, bronchi, and lungs (Yamaguchi 2019). Specifically, tobacco smoke directly impacts the DNA due to DNA miscoding. As a result, DNA polymerase enzymes are unable to function correctly, leading to permanent genetic mutations (Lu et al. 2021).

In vivo experiments have suggested that pol replicates POB-T, bypassing lesions in the major grooves. This replication causes around 50% of T→A mutation. The evidence also to some extent explains the role played by human polymerases, suggesting that tobacco takes on various glycosidic orientations within the pol as well as at active sites (Bhutani et al. 2021). This T→A mutation has been found to possess the mutational signature that has previously been linked with human cancers due to tobacco smoking (Wilson et al. 2019).

Numerous studies have revealed a direct link between childhood cancer and second-hand tobacco smoke exposure, both pre-natal and post-natal. There is also evidence that adult and childhood exposure to tobacco smoke may increase the risk of non-Hodgkin lymphoma, depending on the histological subtype (Diver et al. 2014). Meanwhile, a large-scale case-controlled study in the US showed that passive exposure to smoke in childhood plays a role in the aetiology of HNSCC. This exposure was more significantly linked to oropharyngeal cancer (a subtype of HNSCC that is usually related to a human papillomavirus [HPV] infection) than to non-oropharyngeal HNSCC (Troy et al. 2013). HNSCC represents a significant global health burden and establishing a diagnostic or recurring test to monitor the disease would be very useful (Chow 2020). Head and neck anatomical regions, as demonstrated by the distribution

of P53 mutations in relation to their sub sites. Laryngeal and oropharyngeal tumours primarily show mutations in exon 6 and

There is considerable biological diversity among the tumours found in the various head exon 7, with the most frequently encountered mutation occurring in codon 220. In addition, in HNSCC the most frequent P53 mutation is the Y220C mutation, which in the oropharynx is also a relative hotspot mutation (Van Kempen et al. 2015).

The IARC P53 mutation database presents that most P53 mutations impact exons 5-8, representing site-specific and DNA-binding domains. Meanwhile, mutations were detected in the N- and C-terminal regulatory domains in less than 2% of cases (Rossner Jr et al. 2009). As an example of underlying genetic disorders, patients with Fanconi anaemia (FA) is a rare recessive DNA repair disorder that is clinically characterized by congenital malformations, progressive bone marrow failure, and increased incidence of malignancies, especially acute myeloid leukaemia and squamous cell carcinomas of the head and neck (HNSCCs) and the anogenital regions, Fanconi anaemia were 500 to 1000 times more likely to develop HNSCC (Beddok et al. 2020).

Despite their increasing popularity as an alternative method to consume tobacco, there is no concrete evidence that e-cigarettes are safer than conventional cigarettes. Nevertheless, e-cigarette use is swiftly rising, with an estimated 15.5% of UK smokers reportedly taking up e-cigarettes in 2016, compared to 6.7% in 2012 and 2.7% in 2010 (Rawlinson et al. 2017).

The liquid used in e-cigarettes mainly consists of the so-called e-liquid constituents of nicotine, glycol and propylene glycol. However, impurities have been identified even

in pharmacopoeia-quality nicotine, and propylene glycol and glycerol have been shown to thermally decompose into eight different products (Margham et al. 2016).

While the constituents of e-cigarettes are often utilized in the food sector, it should not be assumed that they are entirely safe for repeated and long-term inhalation. Thus far, the FDA has not approved the use of any type of e-cigarette as a cessation aid. Furthermore, current users of e-cigarettes have been found to be two times more likely to be long-term tobacco smokers (Szukalska et al. 2020).

The suppression of the cellular antioxidant defence mechanism due to the exposure to the aerosol extracts of e-cigarettes substantially damages DNA. In addition, the vapour from e-cigarettes, regardless of whether it contains nicotine, has been proven to not only be cytotoxic to epithelial cells but also induce breaks in the DNA strands (Yu et al. 2016; Ganapathy et al. 2017).

Clearly, further research is still needed to explore the effects of e-cigarettes, especially whether they affect the user's risk of developing HNC (Szukalska et al. 2020).

1.3.1 Occupational carcinogen exposure

The most vulnerable location of occupationally acquired malignancy is HNC, which is increased by exposure to occupational carcinogens. Laryngeal carcinoma is caused by certain occupational exposures (National Cancer Institute 2021). Occupational exposures are thought to be responsible for 4–10% of all HNC incidences in Western industrial countries (Maier and Tisch 2000).

Nasopharyngeal carcinoma is linked to occupational exposure to wood dust. Certain occupational exposures, such as asbestos and synthetic fibres, have been linked to voice box cancer, although the evidence for this link is still inconclusive. Certain

occupations in the construction, metal, textile, ceramic, forestry, and food sectors may raise the risk of voice box cancer (Hayes et al. 1986; Hashim and Boffetta 2020).

In a study conducted by radio et al it was concluded that leather dust exposure for long term (more than 3 years) linked to an elevated risk of laryngeal cancer (Radoï et al. 2019). According to the data, perchloroethylene or trichloroethylene exposure may raise the incidence of HNSCC inwomen (Carton et al. 2017).

The data suggest that poor occupational socioeconomic status, position, and manual labour are linked to HNC risk, with smoking, drinking, and occupational exposures accounting for just a portion of the risk. In comparison to socioeconomic position and manual/non-manual employment, perceptional occupational psychosocial status (SIOPS) appears to be the biggest socioeconomic determinant (Conway et al. 2021).

1.3.2 Age

The risk of HNC is closely linked to age and gender. HNC mortality is significantly linked to age, with older persons having the greatest mortality rates. Among patients in the UK, more than a third of fatalities (36 %) occurred in persons aged 75 and over on average between 2016 and 2018. When compared to most malignancies, this is a smaller proportion of fatalities in older age groups (Heck et al. 2010).

Ageing is described as a gradual organic functional decline marked by a loss of homeostasis and an increase in the likelihood of disease and mortality (Liguori et al. 2018). One of the probable causes of ageing is DNA damage. According to previous research, there is a link between age and DNA damage. Smoking habit have been found to impact age-related DNA damage. Nonetheless, gender had no effect on this relationship (Soares et al. 2014).

The 2450 individuals with HNC were identified in the HNC centre between 2004 and 2018 in Germany (Hermanns et al 2021). The age group >70 years old saw the greatest increase in incidence rates. In addition, yearly incidence rates and mean age both increased significantly over time (Vahl et al. 2021).

1.3.3 Gender

Head and neck cancer is the 13th most frequent malignancy in women in the United Kingdom (representing 2 % of all new female cancer cases). It is the fourth most frequent cancer among men in the United Kingdom (4 % of all new male cancer cases) (Owonikoko et al. 2018; Argirion et al. 2019). Females account for 31% of HNC cases in the UK, whilemales account for 69% (Cancer Research UK 2021b). Between 2014 and 2035, thereis anticipated to be an additional 33 % rise in the cases of HNC (ASH 2017). A statistically significant difference has been found after taking into consideration a variety of demographic variables, including age, smoking history, alcohol usage, comorbidity, and tumour site. Women, on the other hand, were nearly twice as likely as likely as males to die from HNC (Goodwin 2018; Benchetrit et al. 2019).

1.3.4 Radiation exposure

The development of basal cell carcinoma and squamous cell carcinoma is influenced by the cumulative exposure of skin to UV radiation from the sun (Adalsteinsson et al. 2021). Many clinicians believe that sun exposure may be a risk factor for Merkel cell cancer since it frequently appears on sun-exposed parts of the head and neck (Villani et al. 2019).

People who spend a lot of time outside during peak sun hours are also at a higher risk (Guerra et al. 2020). The next biggest percentage of attributable cases (3.8 % in UK people) were from UV radiation and occupational hazards (Brown et al. 2018).

Due to high occupational UV exposure at the capillitium, face, lip, neck, dorsum of the hands, forearms outside, and outdoor workers exposed to high solar UV radiation have a two-fold increased risk of developing basal cell carcinoma compared to controls, regardless of phototype, tumour localization, or histological subtype. The BCC risk-doubling dosage was 6126 SED after adjusting for age, gender, and non-occupational UV exposure (Bauer et al. 2020).

Based on the limited data that is available, the International Agency for Research on Cancer (IARC) has categorised solar radiation as a likely cause of lip cancer. Radiation to the head and neck, whether for noncancerous or cancerous disorders, increases the chance of salivary gland cancer (Bozan et al. 2016).

1.3.5 Human papillomavirus (HPV) and Epstein-Barr virus (EBV) infections

A subgroup of head and neck malignancies (HNSCC)is caused by human papillomaviruses (HPV). HPVs are small, double-stranded DNA viruses that tend to infect cutaneous and mucosal epithelial tissues of the anogenital tract (Stanley 2012). Earlier investigations indicated that persistent infection with high-risk HPVs is critical for the development of invasive carcinomas (Al-Thawadi et al. 2020). The median age at diagnosis of HNCs is approximately 60 years, yet the incidence of these cancers in adults younger than 45 years has increased in recent years due to higher numbers of oropharyngeal cancers associated with oncogenic HPV (Mody et al. 2021). HPV is primarily responsible for roughly 10% of all HNSCCs and more than 50% of Tonsillar squamous cell carcinoma (SCC). Prevalence rates are influenced by a variety of

factors, including the geographical location in which patients reside, as well as their social and sexual activities (Hoffmann and Quabius 2021). In comparison to North America, Europe has exhibited a quicker growth in the proportion of HPV-positive cases during the previous decade (Mehanna et al. 2013).

On the other hand, Epstein–Barr virus (EBV), a human DNA oncogenic gamma-1 herpes virus affects around 90% of adults (Ahmed 2018). During EBV infection, cells express six EBV nuclear proteins (EBNA1, -2, -3A, -3B, -3C, and -LP), three latent membrane proteins (LMP1, -2A, and -2B), and multiple non-coding RNAs (EBERs and miRNAs) (Murata and Tsurumi 2014). EBV's key oncogenic protein LMP1 induces cell growth, decreases apoptosis, promotes cell motility and angiogenesis and is also known to express frequently in human oral cancer (Luo et al. 2021). Moreover, various EBV genes including latent genes contribute to the transformation of human B and some oral epithelial cells and are continually expressed in EBV-associated cancer cells, which links EBV with viral oncogenesis (Al Moustafa et al. 2009); for example, expression of EBNA1, LMP1, -2A genes characterize type II latency and is linked with Hodgkin's lymphoma and some carcinomas like gastric, nasopharyngeal and breast (Cyprian et al. 2018).

The miRNAs encoded by the EBV genome play a role in the post-transcriptional regulation of oncogenic activity. Furthermore, novel therapies, such as immune checkpoint inhibitors like anti-PD-1/anti-PD-L1, combined with therapeutic vaccines, may activate the immune response against EBV-associated NPC, resulting in enhanced survival and tumour treatment (Skafi et al. 2020; Fernandes et al. 2018).

1.3.6; Genetic alteration in HNSCC

According to a study conducted by pelton et al (2010) 39 mutations identified in 26 tumours, 36 P53 mutations were confirmed in 23 tumours through PCR-SSCP sequencing, whereby half influenced the p53 protein function putatively. Eight of these were located in the L2 domain, three impacted the LSH motif, and three were in the L3 domain. Another three were identified in other domains. In four samples, it was observed that the rare mutation codon 259 (GAC > GAA). Moreover, a correlation between p53 aberrations and a combination of the effects of alcohol consumption, smoking, and work history was evidenced. Finally, cases that had no family cancer history demonstrated P53 mutations more frequently (71%) than those whose family history included cancer (46%) (Peltonen et al. 2010).

According to another study conducted by Xiaohua Jiang et al 2019 The emerging genetic alterations that represent a novel therapeutic target in HNSCC. These include frequent mutations of several genes (*TP53*, *PIK3CA*, *CDKN2A*, *NOTCH1*, and *MET*) as well as copy number gain in EGFR, CCND1, and PIK3CA. Those genomic alterations have been analyzed in relevance of sensitivity to chemotherapy, targeted therapy, and ionizing radiation that could potentially guide the therapy. It is essential to determine whether proteins or pathways are involved in the targeted therapy of HNSCCs, aside from gene mutation. These genetic alterations may serve well as novel therapeutic candidates and predictive biomarkers for prognosis in HNSCC (Jiang x et al 2019).

1.4 Classification of Stages

Staging is the process of determining how much cancer is within the body (tumour size) and if it has spread (Simon et al. 2020). In clinical practice, the tumour-node-metastasis classification system, developed by the Union for International Cancer Control (UICC), is generally utilized to stage most types of cancer (Brierley et al. 2019). The TNM system classifies the overall stage of a patient's cancer based on three categories. The first category (T) relates to the primary tumour and its characteristics, precisely its size and how far it has penetrated other tissue structures. The second category (N) refers to the primary tumour's proximity to and impact on the regional lymph nodes. The third category (M) is based on to what degree cancer has spread to other body parts, i.e. metastasised (Hermanek et al. 2013).

The fourth edition of the TNM classification for HNC, developed by the American Academy of Otolaryngology-Head and Neck Surgery American Head and Neck Society, was adopted by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC). Although the N category remains unchanged, this updated classification provides a new system for staging HNC by integrating new developments in the T and M categories. This modified classification system is summarized in (Table 1.1,Table 1.2 and Table 1.3). Clinicians can use the TNM system to categorize tumours, including those in the head and neck region. This allows them to assess the status of the disease and give a prognosis, create a plan for disease management and treatment, and subsequently assess the progress of the treatment. Furthermore, the system allows relevant information to be globally disseminated among various cancer treatment centres (Deschler and Day 2008; Sobin 2009).

The staging process can draw on a large body of clinical information, including physical exams, radiography, intraoperative care, and pathological findings. However, biomarkers and molecular studies in HNC staging are not yet utilized beyond histopathologic analysis. The patients' TNM components are eventually summarised to provide a numerical status that describes cancer as Stage I, II, III or IV (Shah 2018).

Each stage can contain further subdivisions, denoted by an a, b, or c. For instance, T4a, staged as IVa, refers to a moderately advanced stage specific to the subsite yet still resectable. Meanwhile, T4b staged as IVb, is a very advanced stage and includes previous findings that showed that the disease is unresectable, such as prevertebral involvement, carotid artery encasement, or skull base involvement. T4c staged as IVc, relates to any occurrence of distant metastasis (Lydiatt et al. 2018).

Stages I and II generally refer to early-stage cancer, while Stages III and IV denote a more advanced disease stage. Importantly, if there is any metastatic cancer in the neck, the disease is classified as advanced, except for certain thyroid and nasopharynx cancers (Oza et al. 2015).

Table 1.1 The TNM staging for the Larynx, Oropharynx, Hypopharynx, Oral Cavity, Salivary glands, and Paranasal Sinuses (TNM) (Deschler and Day 2008)

Stage grouping	T	N	M
Stage 0	Tis	N0	M0
Stage i	T1	N0	M0
Stage ii	T2	N0	M0
Stage iii	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage iva	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
Stage ivb	Any t	N3	M
	T4b	Any n	M0
Stage ivc	Any t	Any n	M1

Table 1.2 Clinical Stage Grouping by T and N Status (Deschler and Day 2008)

Clinical Stage Grouping by T and N Status					
N	T1	T2	T3	T4a	T4b
N0	I	II	III	IV a	IV b
N1	III	III	III	IV a	IV b
N2	IV a	IV a	IV a	IV a	IV b
N3	IV b	IV b	IV b	IV b	IV b

Table 1.3 The stage grouping of regional lymph nodes (TNM) (Deschler and Day 2008)

Stage Grouping **This stage grouping is unique to regional lymph nodes**			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N1	M0
	T2	N0	M0
	T2	N1	M0
Stage III	T1	N2	M0
	T2	N2	M0
	T3	N0	M0
	T3	N1	M0
	T3	N2	M0
Stage IVA	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IVB	Any T	N3	M0
Stage IVC	Any T	Any N	M1

1.5 Biological or target therapies in HNSCC

Head and neck squamous cell carcinomas (HNSCCs) are aggressive, genetically complex, and difficult to treat groups of cancers. Surgery and radiotherapy represent the primary treatment options for most patients. However, the poor response to therapy and aggressive nature of HNSCCs is affected by the complex alterations observed in intracellular signalling pathways and the behaviour of the extracellular

microenvironment (Alsahafi et al. 2019). Therefore, in an attempt to develop more effective targeted therapies, research has focussed on the actions of the immune system and the epidermal growth factor receptor (EGFR) (Byeon et al. 2019).

The extracellular ligand-binding domain, the transmembrane domain, and the intracellular tyrosine kinase domain make up the EGFR family of human epidermal growth factor receptors (HER) (Chen et al. 2012). In the majority of instances of squamous cell carcinoma of the head neck, EGFR is overexpressed (SCCHN). A high level of EGFR expression is linked to a poor prognosis. Cetuximab is a chimeric human/mouse IgG1 antibody that binds to the EGFR with a high affinity (Nair et al. 2020). It is the only targeted medication that has been legally authorized by European and American regulatory bodies for the treatment of SCCHN in both locoregionally advanced stages of the illness as well as recurrent/metastatic disease in conjunction with radiation. The findings of studies using other EGFR-directed monoclonal antibodies, such as zalutumumab and panitumumab, were similar to those of trials using cetuximab (Specenier and Vermorken, 2012).

The EGFR signalling pathway is critical in head cancer due to the ligand binding to the HER receptor family, including the epidermal growth factor. In the kinase domain, EGFR initiates receptor dimerization and tyrosine residue phosphorylation. This activates several cell-signalling pathways, such as phosphatidylinositol 3-kinase (PIK3CA) (Zaryouh et al. 2022).

Other potential targets for treatment in SCCHN include the entire ErbB family (In humans, the family includes Her1 (EGFR, ErbB1), Her2 (Neu, ErbB2), Her3 (ErbB3), and Her4 (ErbB4), the vascular endothelial growth factor (VEGF) and its receptor (VEGFR), the insulin-like growth factor 1 receptor (IGF-1R), the insulin receptor (IR),

histone deacetylases (HDAC), the mammalian target of rapamycin (mTOR), the platelet-derived growth factor receptor (PDGFR), heat-shock protein 90 (HSP90), nuclear factor-kappa B (NF-κB), aurora A or B, and phosphatidylinositol 3-kinase (PIK3CA) (Kiseleva et al. 2018).

ErbB protein family signalling is vital for the development of appropriate therapies. The mice die following exposure to ErbB due to abnormal development of the peripheral nervous system (Chan et al. 2002). ErbB-1 and ErbB-2 are found in many human cancers, and their excessive signalling may be critical factors in the development and malignancy of these tumours. This feature highlights its potential for metastatic HNC treatment (Miller et al. 2019).

1.6 Reactive oxygen species (ROS)

Reactive oxygen species' (ROS) is an umbrella term for an array of derivatives of molecular oxygen that occur as a normal attribute of aerobic life. Elevated formation of the different ROS leads to molecular damage, denoted as 'oxidative distress (Sies and Jones 2020). ROS play an essential role to regulate normal cellular processes and genotoxicology, promoting programmed cell death (PCD) and many disease states, including cancer (Liguori et al. 2018; Arfin et al. 2021; Parvez et al. 2018; Collin 2019).

Superoxide (O2•–) , •OOH, hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2), and carbonate radicals (CO3•) are examples of reactive oxygen species (ROS) that may oxidize a range of compounds, including biomolecules (Canonica et al. 2005; Burns et al. 2012). In the absence of efficient repair mechanisms in cells, increased oxidative stress from ROS damages sensitive biological components such as DNA, membrane

lipids, and proteins, possibly contributing to deadly illnesses such as cancer (Naveenkumar et al. 2013; Perillo et al. 2020).

Photolysis and electron transfer or energy transfer reactions are the processes through which ROS are produced (Bartosz 2006). For example, reducing oxygen produces free superoxide and hydroxyl radicals (Gutteridge 1994), while superoxide radicals are also produced by enzyme activity, e.g., NADPH oxidase and xanthine oxidase (Collin 2019).

Superoxide is an essential free oxygen radical (Dohi et al. 2010; Phaniendra et al. 2015) and acts as an oxidant or a reductant. Superoxide can be an oxidant, a reductant, or both, depending on the coordination environment of the metal. The production of superoxide by the mitochondrial electron transport chain is the gateway to the low-flux signaling circuits of cells and tissues, it provides the connection to the high flux, energy-harvesting circuits of ATP production. Superoxide reacts with the metal-containing active site of SODs alternately as a reductant and then as an oxidant, yielding oxygen and H_2O_2. The steady-state level of O2•– is inversely related to [SOD] (Batinic-Haberle and Spasojevic 2019). Highly reactive, far more than any superoxide produced as part of internal biological processes, superoxide radicals produced by organic agents can severely damage DNA (Ighodaro and Akinloye 2018; Turrens 2003).

Finally, hydroxyl radicals are aggressive and effective oxygen radicals that can be produced by combining superoxide and hydrogen peroxide (Ben-Shaul et al. 2001; Sakai et al. 2017). ROS are the most reactive oxygen radicals and can cause considerable biological damage when in contact with biological molecules, with the components of DNA, suchas the pyrimidine and purine bases, also being susceptible

(Pizzino et al. 2017). Mitochondria is the most critical source of superoxide O2•– and hydrogen peroxide(H_2O_2) in cells. The increasing of these molecules cause human diseases, ageing and tumour progression (Ippolito et al. 2020; Mailloux 2020). Increased level of ROS in mitochondrial DNA for long term leads to the damage to Mitochondrial DNA, inhibiting mitochondrial functioning leads to the tumour progression and proliferation (Naveenkumar et al. 2013).

ROS are well recognized as mediators of DNA damage. For example, Ionizing Radiation (IR) induces DSBs through direct high-energy damage to the sugar backbone of DNA, but also through free radicals generated in cells- mostly •OH from water. Chemotherapeutics like doxorubicin and cisplatin increase ROS levels, which contributes to their genotoxicity. ROS have also been reported to directly induce other forms of DNA damage through oxidizing nucleoside bases (e.g. formation of 8-oxo guanine) It is clear that increasing ROS has effects on transcription pathways, stimulates DNAmutation and genomic instability, tumour development (Filaire et al. 2013; Perillo et al.2020). There is no doubt that it is important to understand the complex mechanism of redox biology is imperative to take control of cancer (Wang et al. 2021b).

1.7 Pembrolizumab (Keytruda)

Pembrolizumab (brand name Keytruda), is an immunotherapy works by helping immune system to kill cancer cells. It can be used for the treatment of multiple type of cancers such as non-small cell lung cancer (NSCLC), melanoma skin cancer, bladder cancer, Hodgkin lymphoma. Pembrolizumab's Structure is shown in the figure 1.2. Cancer is responsible for millions of fatalities annually and severely damages life quality for a far higher number of individuals. Cancer is a malignant neoplastic disease

with various risk indicators, including eating large quantities of processed meat, drinking alcohol, smoking tobacco, generally unhealthy lifestyles, and environmental inputs (Olusanya et al. 2018). Over the last 20 years, there has been a significant evolution in drugs to combat cancer, and researchers have been specifically focused on creating treatments that can directly target tumours and metastases whilst not damaging any healthy cells. Focusing anti-cancer treatments solely on the targeted tissues can make the treatment more effective and also lead to reductions in side effects (Medina et al. 2004).

Pembrolizumab, monoclonal immunoglobulin (Ig) G4 antibody directed against human cell surface receptor PD-1 antibodies responsible for programmed cell death 1 (PD-1) blockade (Hendriks and Besse 2018), was later developed and shown to be effective in patients with advanced melanoma (Robert et al. 2019), non-small cell lung cancer (NSCLC) (Paz-Ares et al. 2018; Mok et al. 2019) as well as renal cell (Rini et al. 2019) and urothelial carcinomas (Bellmunt et al. 2017). Durvalumab, avelumab, tremelimumab, and atezolizumab are PD-L1 inhibitors that are available on the market, however they are not licensed for the treatment of HNC (Desilets and Soulières 2020). However, severe side effects have been recorded regularly with the use of checkpoint inhibitors in clinical studies, most commonly in the form of autoimmune T-cell cytotoxicity (Dubey et al. 2020). The immunogenic potential of HNC is well documented. On HNSCC cells, high levels of PD-L1 expression have been seen, typically in conjunction with T cell infiltration in the tumour microenvironment (Yang et al. 2018). As a result, PD-1 and PD-L1 inhibitors for recurrent or metastatic HNSCC (R/M HNSCC) were investigated and eventually approved (Ran and Yang 2017). Checkpoint bypass in cancer cells is associated with cell death and loss of proliferative capacity and therefore is believed to contribute to the efficacy of DNA-

damaging therapies. Are cancer cell clones that bypass checkpoints invariably more sensitive to DNA damage than checkpoint-proficient cells in normal tissues (Chung et al,2010). There were some clinical studies of the effect of Pembrolizumab in HNC patients, which will be mentioned later.

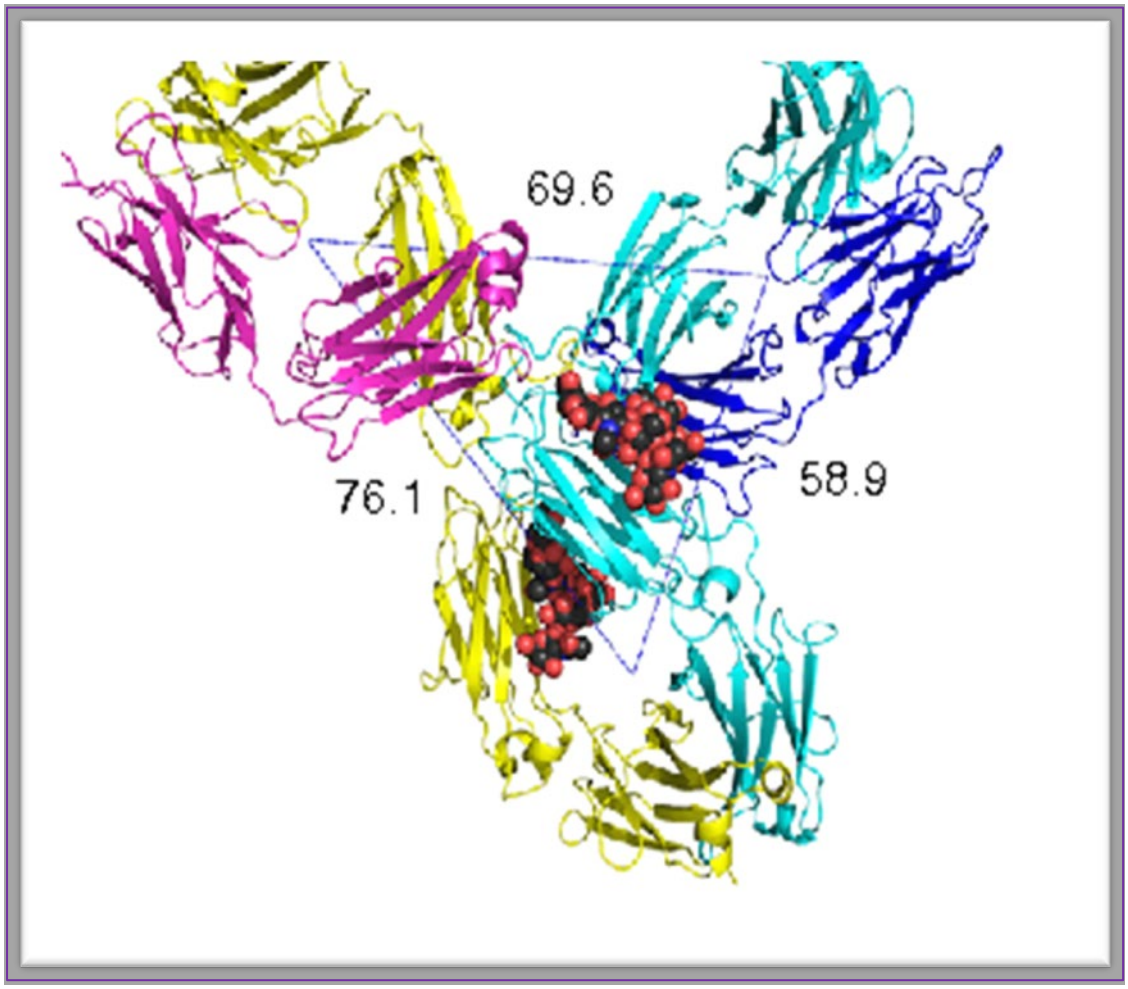


Figure 1-2 Protein structure of pembrolizumab**.** The diagram illustrates the heavy chains in yellow and cyan and the light chains in magenta and blue. carbon in black, oxygen in red, nitrogen in blue and shows the distance between three the domains of pembrolizumab (Drug Bank 2022).

1.7.1 Clinical pharmacology of pembrolizumab

Pembrolizumab is a humanised, IgG4-κ monoclonal antibody directed against the PD-1 receptor of T-lymphocytes with an approximate molecular weight of 149 kDa, it is administered at a dose of 200 mg intravenously every 3 weeks. In the advanced melanoma setting, there were no clinically significant differences between the 2 mg/kg and 200 mg dosing schemes in terms of safety or efficacy (Robert et al. 2014). Merck

developed the medication, which was originally tested in metastatic melanoma patients before receiving FDA clearance in September 2014 for this use (Desilets and Soulières 2020).

1.7.2 Pharmacokinetics

Pembrolizumab is an injectable medication that is quickly and totally accessible after perfusion, with just little effects from plasma protein binding. Pembrolizumab concentrations approach steady-state after 16 weeks of treatment on a 3-week cycle. Its steady-state distribution volume is confined to extracellular space. Pembrolizumab has a terminal half-life of approximately 22 days in terms of elimination, with minor fluctuations in clearance between first drug administration and steady state (Sureda et al. 2021; Ryman and Meibohm 2017), Drug interactions are not anticipated to be clinically important due to the fact that PD-1 inhibitors' catabolism is independent of drug-metabolizing enzymes (Desilets and Soulières 2020).

1.7.3 Programmed death pathway

Pembrolizumab binds to PD-1, a transmembrane protein implicated in immunological tolerance, with high affinity, blocking the interaction with its known ligands, PD-L1 and PD-L2 (or PD-L2). T-cell inactivation and a reduction in cytokine production are caused by PD-1 inhibition, which inhibits cancer cell death in the presence of a non-self-antigen. The PD-1 pathway is crucial for the maintenance of self-tolerance. In the presence of pro-inflammatory cytokines like IL-12 and IFN-gamma, PD-L1 cellular expression is usually elevated, emphasizing PD-L1's role as a physiologic brake on unopposed cytotoxic T-cell activity (Kinter et al. 2008). Cancer clones might improve their PD-L1 surface presentation under selection pressure, allowing them to evade immune surveillance (Chen et al. 2019).

It is widely accepted that T cell responses are essential for effective anti-tumour immunotherapy. However, T cell activities are controlled at multiple levels. These regulatory controls are necessary to prevent T cells from becoming hyperactivated, causing significant collateral damage to non-target tissue**.** unrestrained cytotoxic T-effector activity can cause collateral damage in response to anti-PD-1 treatment, which is commonly expressed as site-unrelated autoimmune side effects. In the fast-developing area of checkpoint inhibition, safety data analysis is thus essential to investigate the spectrum of treatment-related problems. (Feng et al. 2022).

When DNA damage happens in the cells. DNA damage repair mechanism regulates PD-1 gene expression. The DNA damage checkpoint route, neoantigen pathway, and cGAS-STING pathway can upregulate PD-1 expression. Single- or double-strand breaks in DNA trigger the ATM/ATR-CHK1 signalling pathway, which in turn stimulates the JAK-STAT-IRF pathway. IRF1 transcription factor binds to the PD-1 promoter to stimulate PD-1 expression. DDR abnormalities, such as DDR-related gene mutations, increase the TMB or MSI, and these newly synthesized peptides are displayed as HLA neoantigen complex on the cell surface. The T cell receptor recognizes the HLA-neoantigen combination and activates T cells. IFN secreted by activated T cells promotes the STAT-IRF pathway via IFN receptors, elevating PD-1 expression in additional tumour cells. DNA damage-induced cytoplasmic DNA fragments stimulate the cGAS-STING pathway. The cGAS-STING pathway boosts the production of transcription factors NF-B and IRF3 and promotes the release of IFN, which in turn activates the STAT-IRF pathway and promotes the overexpression of PD-1. DNA damage response and PD-1/PD-L1 pathway in figure1.3 (Zhang et al, 2021).

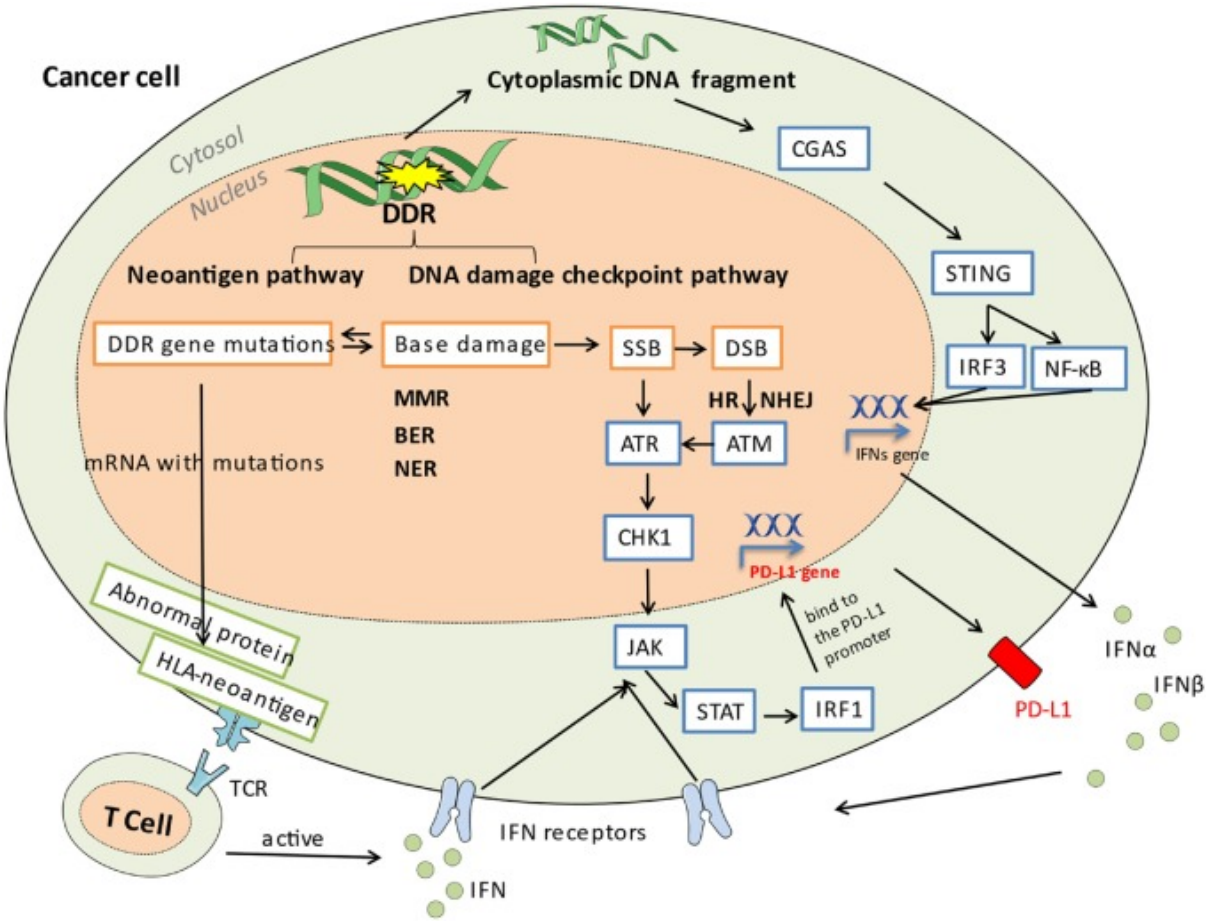


Figure 1-3 The PD-L1 expression and DNA damage repair pathway.a-PD-L1 expression could be upregulated by DNA checkpoint pathway, ATM/ATR-CHK1 signalling will be activated DNA damage b-The PD-L1 bind with to the transcription factor IRF1 to increase PD-L1expression. and bind with the T cell receptor and will activate T cells.c-IFN that released from T cells will stimulate the STAT-IRF pathway to upregulate PD-L1 expression in tumour cells. d- And the cGAS-STING pathway will activate the STAT-IRF pathway and lead to PD-L1 upregulation (Zhang et al ,2021).

1.7.4 Pembrolizumab outcomes in clinical trials

Toor et al (2017) investigated how pembrolizumab affects cancer cells and T-regulatory cells subsets. They found that the drug interacts with PD-ligand 1 (PD-L1) and programmed death-1 (PD-1) and plays an essential part in allowing the immune response to fight tumours in various ways, which includes the suppression of cell proliferation, stifling cytotoxic activity, encouraging tumour cell apoptosis, and producing more regulatory T cells (Treg) (Toor et al. 2018). As shown in figure 1.6.

In a previous study conducted by Seiwert et al (2015) suggested that pembrolizumab was more effective in comparison to cetuximab. Previously, the standard therapeutic choice was the only targeted head/neck cancer therapy that has gained approval. Additionally, responses were shown to be sustainable over a considerable period, which had not been seen before when treating head/neck carcinoma. In an *in vivo* study, the researchers reported that during the initial phase pembrolizumab, given in fortnightly doses of 10 mg/kg, achieved a 20% response rate for a patient cohort that had been given enrichment for PD ligand 1 (PD-L1) expression. The group that underwent testing in the first phase comprised 132 patients with no selection for PD-L1 expression; they were given a set dose of pembrolizumab at 200 mg/m^3 weeks for 24 months or up to the stage of either disease progression or unsustainable levels of toxicity (Seiwert et al. 2015).

The outcomes of this research found that 57% of patients experienced tumour shrinkage over the course of the research. The total objective response rate was 24.8% for HPV-positive patients and 26.3% for HPV-negative patients. Approximately 25% of the patients were regarded as enjoying stability, with disease control rates of around 50%. The research is referred to the results as "remarkable." Additionally, it was shown that the response was sustainable over a considerable period of time for 85% of patients. The research conclusion was that pembrolizumab was more tolerable for patients in comparison to aggressive chemotherapy/radiotherapy, with approximately 60% of patients suffering no negative side-effects. 15% of patients did report lethargy as a side-effect.

Mehra et al (2018) conducted a study where 196 patients were recruited outcomes demonstrated that 24 patients (13%) experienced side-effects. While there was no any

casualty after treatment with pembrolizumab. Some patients received 2 years of treatment and the responses were ongoing for more than 30 months; the durable anti-tumour activity and tolerable safety profile, observed with long-term follow-up, support the use of pembrolizumab as a treatment for recurrent/metastatic HNSCC. (Mehra et al. 2018).

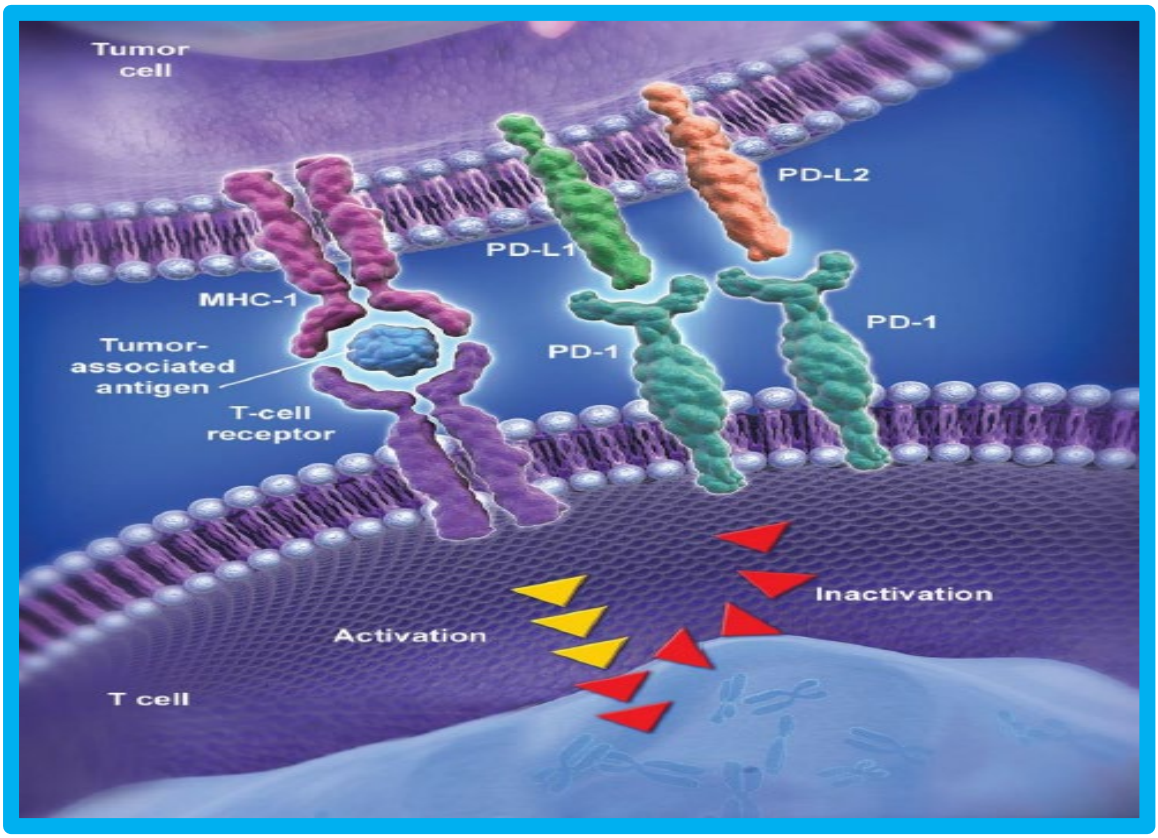


Figure 1-4 The programme cell death pathway. a-Inhibitory receptor expressed primarily on the surface of activated T cell. B-Binding of PD-1 to one of its ligands, PD-L1 or PD-L2, protects healthy cells from autoimmunity. Tumours can exploit this pathway to escape immune surveillance (Hsu et al, 2015).

1.8 Liposomes

Liposomes, the vesicles of phospholipid bilayer, can encapsulate both hydrophilic and lipophilic drugs and protect them from degradation. Liposomes have been extensively studied and continue to create intense interest in research since their discovery in the mid-1960s. Since then, liposomes have been considered to be the most successful

nanocarriers for drug deliver and have made their way to the market. Currently, a number of liposomal formulations are on the marker for cancer treatment and many more are in pipe line. Liposomes have revolutionized cancer therapy by their broad clinical applications. Liposomes overcome the limitations of conventional chemotherapy by improving the bioavailability and stability of the drug molecules and minimizing side effects by delivering drugs to targeted areas of the body. Following his groundbreaking research, greater attention has been paid to liposomes as representing a means of delivering drugs to targeted areas of the body (Shilakari et al. 2013).

A liposome is a biodegradable and essentially non-toxic vehicle capable of encapsulating either hydrophilic or hydrophobic material, and so can play an important role in carrying drugs when used in drug delivery systems (Medina et al. 2004). A liposome is a colloidal sphere comprising membrane proteins, sphingolipid long-chain fatty acids, cholesterol, and non-toxic surfactants. They are also capable of porting drug molecules through the vascular system (Yadav et al. 2017). A liposome is a microscopic entity, ranging in size between 50 nm and several microns (Poorahmary Kermany 2010). They totally encompass aqueous volumes inside membranes created from lipid bilayers. The inter-vivo performance of the agents the liposomes carry must be understood if we are to comprehensively evaluate the bioavailability of all treatments that liposomes encapsulate (Medina et al. 2004).

Research with a specific focus on the science and technology involved with liposomes is one of the most swiftly expanding scientific sectors; this type of research is making essential contributions to a number of areas, including our understanding of the functioning of biological membranes, drug delivery systems, and cosmetics (Hamed

2001). One challenge that arises from chemotherapy is that the agents used for the treatment can be damaging to healthy tissue as the active pharmaceutical ingredients (APIs) used in chemotherapy are highly toxic (Olusanya et al. 2018). Anti-cancer drugs may be encapsulated within liposomes, delivering highly focused treatment targeting malignant neoplastic disease (Hirai et al. 2013). This form of treatment has the potential to reduce a number of the cytotoxic side-effects anti-cancer drugs deliver to healthy tissue. This makes liposomes a key player in the battle against cancer (Olusanya et al. 2018). Researchers have experienced success in deploying liposomes for the treatment of cancer cells and the delivery of macro molecules to necrotic tumour areas, According to Gabio et al (1998) the pharmacokinetic properties of the liposomal drug, the vesicle size of the liposome carrier and the vascular permeability of individual tissues will determine the extravasation and biodistribution profile. The pharmacokinetics of polyethylene-glycol-(PEG)-liposomal doxorubicin are characterized by an extremely long circulating half-life, slow plasma clearance and reduced volume of distribution compared to free doxorubicin. These carrier systems show an improved extravasation profile with enhanced localization in tumours and superior therapeutic efficacy in comparison to doxorubicin in free form. These properties are the result of an optimized liposome composition and of a special drug-loading method which produces stable and long-circulating carriers. In clinical studies, doxorubicin encapsulated in PEG-coated liposomes shows a unique pharmacokinetic-toxicity profile and promising antitumour activity. (Gabizon et al. 1998). A variety of drug loads, charges, composition sizes and different molecule combinations can be used, e.g., plasmids, small drug molecule proteins, or nucleotides. Certain drugs have been developed as liposomes to improve their therapeutic qualities. Vesicular drug delivery mechanisms have included transferases, liposomes, and noisome.

There are a number of advantages to using liposomes for drug delivery. These include the fact that they cause inhibition of drug degradation (Cristiano et al. 2017) can cause reductions in a drug's toxic potential, can target specific areas, permit the administration of drugs using sustained release systems, and enhance the efficacy of drugs using solubility (Allen and Martin 2004). Due to new developments using liposomes, clinicians have managed to develop drug release systems that are both targeted and controlled and thus localized to disease areas (Olusanya et al. 2018). For effective cancer treatment, a tumour must either be shrunk or totally eliminated without causing damage to healthy tissue. If this is possible, patients will experience longer survival times and a better quality of life (Gogoi et al. 2016). Drugs designed to combat cancer have shown significant progression in the last 20 years. There are two ways in which liposomes can be used to target tumours, these being passive and active. Passive systems take advantage of the fact that tumours blood vessels have a tendency to leakage, which naturally accumulates liposomes from circulating blood within the tumour caused by internal fluid molecular movement. If passive approaches are used, when liposomes stay long enough in the circulation, they will boost the number of liposomes within the tumours tissue. If an accumulation of macromolecules builds up inside tumours tissue, this is referred to as enhanced permeability and retention (EPR). Most anti-cancer drugs based on liposomes currently commercially available leverage the EPR effect, focusing on prolonged liposome circulation times (Kiyohara et al. 1993). Another way in which passive targeting can be used is to indirectly target tumour tissues using factors to aggravate macrophages, stimulating those that exist inside liposomes, Liposomes passively accumulate in the target tissues through penetration retention effect. Vasculatures in tissues affected by the tumour, infection, and inflammation have higher permeability and larger pores relative

to the vasculatures in healthy tissues. Thus, drug-loaded liposomes in the range 60–150 nm extravasate from the vasculatures into the diseased tissues, and subsequently release their cargo at the desirable site of action. In addition, the retention of liposomes is enhanced in the tumour tissues due to poor lymphatic drainage. These properties have made drug-loaded liposomes superior to free drugs which unselectively distribute into both healthy and diseased tissues. Upon arrival of liposomes at the target site, drug releases through passive diffusion across the bilayer lipid. Released drug diffuses in the interstitial fluid of the target tissue and is subsequently up-taken by the target cells (Figure 1.3). Such techniques have particular efficacy when used in the treatment of metastasized tumours (Fouladi et al. 2017).

Active and passive approaches both need a substantial liposome accumulation within tissue macrophages after drugs have been incorporated into liposomes. One example of this is liposomes encapsulating lipophilic derivatives of muramyl dipeptide causing up-regulation of cytokine production, stimulating the human monocytes to increase their attack on tumours and build infection resistance. These forms of drugs are frequently employed when treating bone diseases (Arnold et al. 2005). Liposomes can also be employed with antibody-based approaches for active targeting of tumour tissue. This may be accomplished through the incorporation of particular antibodies in the liposome's surface in the shape of immunoliposomes (ILPs) ILPs have specificity to endometrial cells in the vasculature of tumours or cancer cells (Kunjachan et al. 2015; Medina et al. 2004). In vivo and in vitro investigations of the interactions between liposomes and cells have revealed that the primary interaction is either simple adsorption (by particular interactions with cell-surface components, electrostatic forces, or by non-specific weak hydrophobic) or endocytosis (by phagocytic cells of

the reticuloendothelial system, for example macrophages and neutrophils) (Akbarzadeh et al, 2013).

Fusion with the plasma cell membrane via insertion of the liposome's lipid bilayer into the plasma membrane, accompanied by simultaneous release of liposomal content into the cytoplasm, is extremely uncommon. The fourth interaction is the interchange of bilayer components, such as cholesterol, lipids, and membrane-bound compounds, with cell membrane components. It is frequently difficult to establish which mechanism is operating, and many mechanisms may operate simultaneously (Sharma et al, 2018)

ILPs are considerably more targetable towards the tumour location (in this instance, tumour tissue and endothelial cells in the lungs) than standard liposomes. (Maruyama et al. 1999; Olusanya et al. 2018). Other means of targeting have been created employing external triggers to circumvent this difficulty. Chemotherapy agents may be triggered for discharge inside the interstitial following accumulation within human tissues. This can be achieved using the EPR effect (Enhanced Permeability and Retention), or agent release may occur within the tumour vasculature employing liposomes that have been specially created to provide responses to external triggers, e.g., heat (Kunjachan et al. 2015). One example of this is the development of thermosensitive liposomes capable of systematic administration. Initial investigations into the *in-vivo* activity of drug-loaded liposomes in animal models took place by administering the anti-cancer drug cytosine arabinoside to mice that had previously been infected with L1210 leukemia. These experiments demonstrated that this approach was successful in extending the lifespan of the experimental subjects. Such approaches are now widely used as models facilitating investigations of a wide range of liposome properties and potential therapeutic applications (Mayhew et al. 1979).

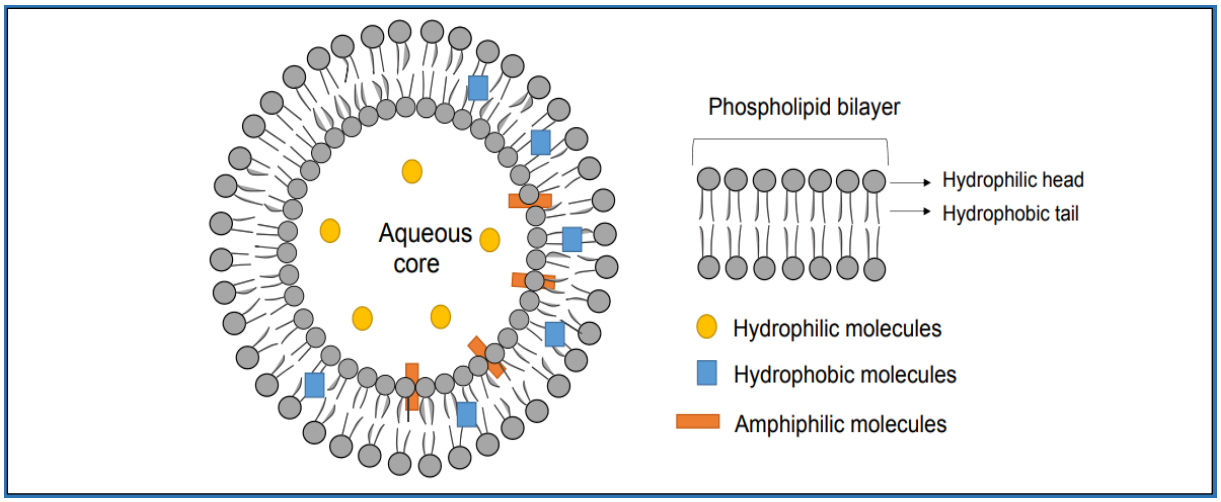


Figure 1-5 The general structure of liposome (Guimarães et al. 2021)

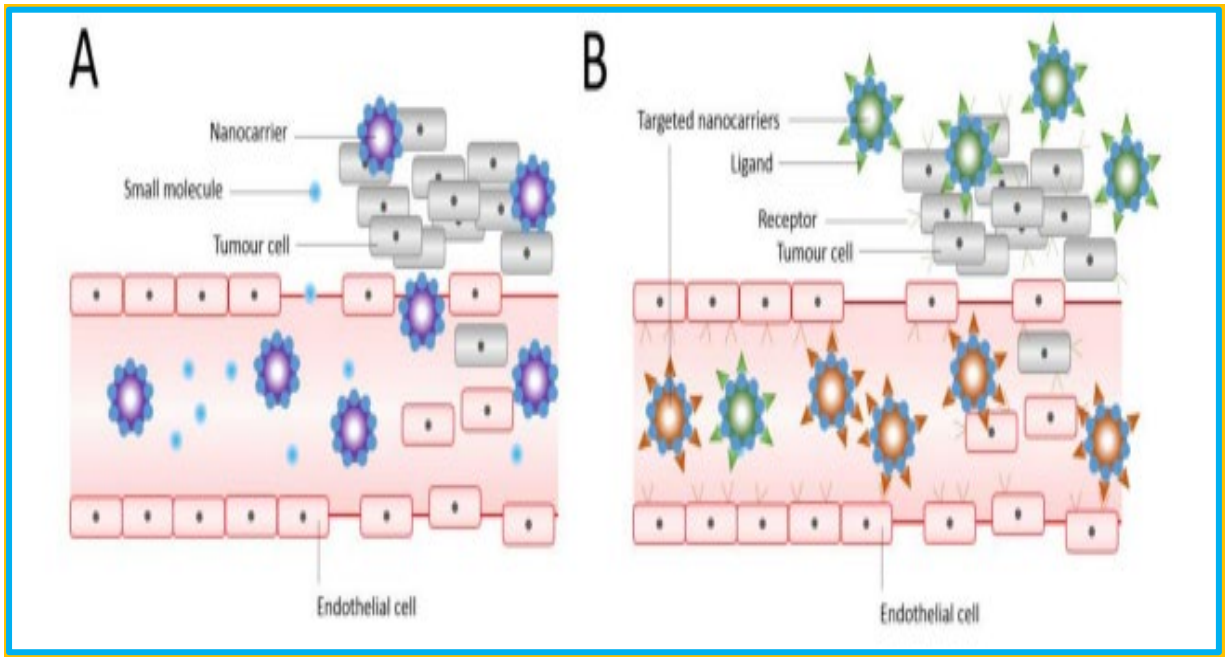


Figure 1-6 Passive transport (A) and active transport (B) of nanocarriers. through the endothelium cell of capillaries, depending on the size of nanocarriers and retention in and out the blood vessels of the tumour (Chenthamara et al. 2019).

1.8.1 Liposome classification

There are a number of ways in which we can classify liposomes in accordance with their size and how many bilayers they have, and on the basis of how they are composed, on the basis of how they are prepared (Swami et al. 2015; Durgavati et al. 2017)Table 1.4.

Table 1.4 Classification of Liposome (Durgavati et al. 2017)

No	Classification of Liposome depended on their size and how many bilayers	Based on how they are composed	Based on how they are prepared
1-	Multilamellar vesicles (MLV)	Convention liposomes (CL)	Reverse phase evaporation vesicles (REV)
2-	Large unilamellar vesicles (LUV)	Long circulating liposomes (LCL)	French press vesicles (FPV)
3-	Small unilamellar vesicles (SUV)	Immuno-liposomes (IL)	Ether injection vesicles (EIV)

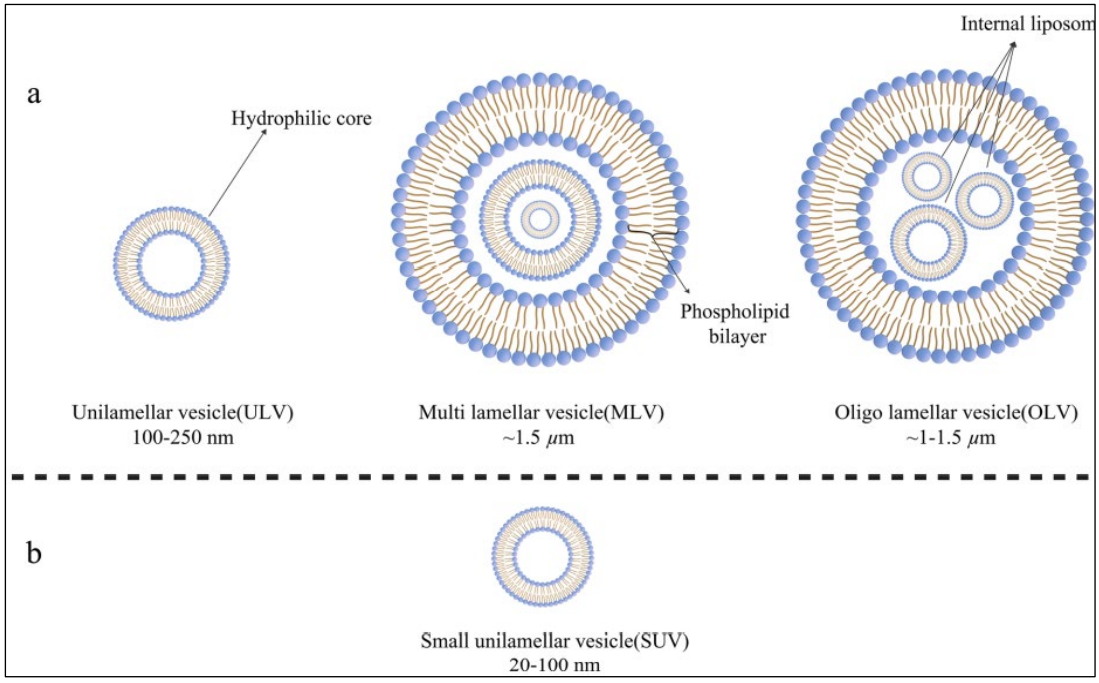


Figure 1-7 The types of liposomes depended on their size and structure composition (Rommasi and Esfandiari 2021)

1.8.2 Influences on formulation of liposomes

There are many elements that affect the formulation of liposomes. A brief description of these is offered below.

1.8.2.1 B.1 level of drug entrapment.

The level of drug entrapment and retention rates combined with other factors affecting these elements must be considered when designing drug delivery systems using liposomes as mediators.

1.8.2.2 B.2 level of drug entrapment.

B.2 drug entrapment can occur in either aqueous or lipid phases of liposomes. Aqueous entrapment involves aqueous volume being trapped within liposomes. The greater the aqueous capacity of the liposome, the more drug concentration that may be encapsulated. Lipid entrapment involves lipid soluble drugs being trapped between the liposome's lipid layers. Past research has demonstrated that this technique has an entrapment efficiency of up to 100%, no matter what type of liposome is used or what it is composed (Bhai 2012).

1.8.2.2.1 B.3

b.3 Liposome stability is an essential element to be considered vis-a-vis drug efficacy, and this may be influenced by a number of variables, including production processes, how it is stored, and how it is delivered. With stable liposomes the active molecule retains its chemical integrity and physical stability all through development and storage phases (Yadav et al. 2011).

1.8.2.3 b.3.1. Physical stability

Vesicle size distribution and morphology are both important parameters that have a direct influence on liposomes' physical security (Grit and Crommelin 1993). Lipid membranes are strengthened by cholesterol. Nevertheless, cholesterol concentrations must not represent more than 50% of liposome structures (Danaei et al. 2018).

1.8.2.4 b.3.2. Chemical stability

Phospholipids are unsaturated fatty acids that have chemical stability. They are susceptible to oxidation hydrolysis, which can make drugs less secure as time goes on. Liposomes must be given protection against light through the addition of antioxidants, e.g., butylated hydroxyl toluene (BHT) or alfa-tocopherol or by removing trace heavy metals employing EDTA. Such steps can block oxidation hydrolysis (Çağdaş et al. 2014; Mulla et al. 2019).

1.8.2.5 Liposome formation mechanisms

Liposomes are created from phospholipids (amphiphilic molecules having hydrophobic tails and hydrophilic heads). The hydrophilic element is primarily comprised of phosphoric acid bound with water-soluble molecules; the hydrophobic element comprises a pair of fatty acid chains each incorporating 10-24 carbon atoms and 0-6 double bonds (Durgavati et al. 2017). The way in which the lipids interact with each other and with water molecules in both hydrophilic and hydrophobic ways lead to stimulation of bilayer vesicle development which achieves thermodynamic equilibrium during the aqueous phase (Li et al. 2015a).

Liposomes have a specific transition temperature (TC), a point at which they undergo evolution from a gel into liquid crystals. The drugs they are transporting are released

at this point. Liposomal formation may only occur once the temperature is in excess of the TC (Durgavati et al. 2017). Pure liposomes have a TC of 41.4°C, whilst naturally-originating fats, e.g. lecithin, have a wider range of TC (Taylor and Morris 1995). The Krafft point of lecithin, representing the highest temperature at which liposomal formation can occur, is 58°C. Thus any temperature from 41.4°C to 58°C is suitable for liposomal formation, e.g., thermosensitive liposomes (Dölle et al. 2012). The size of the vesicles is dependent on concentration of phospholipids (Durgavati et al. 2017; Biswas et al. 2011).

1.9 DNA and Chromatin

The cell nucleus retains a replica of our genetic code, organised by chromosomes, which store inheritable information in DNA's long molecules (Alberts et al. 2003). Cellular DNA is constantly coming under assault from agents that can damage it, both external and internal. Tens of thousands of DNA lesions each day appear in all the human body's ~10^{13} cells (Williams and Schumacher 2016). The majority of such occasional lesions occur as a result of a physiological process, e.g. ROS compounds created in standard aerobic metabolism processes, redox-cycling actions that involve environmental toxicity, or Fenton reactions (describes the formation of hydroxide (OH^-) and hydroxyl radical by a reaction between Iron (II) (Fe^{2+}) and hydrogen peroxide (H_2O_2) caused by heavy metals (Moretton and Loizou 2020; Poetsch 2020). Agents that suppress antioxidant systems can drive oxidative pressure to toxic levels selectively in tumour cells, resulting in oxidative DNA damage to endanger cancer cell survival (Chang et al. 2020).

Damage to DNA can also occur when there are errors in DNA replication in the course of DNA synthesis; additionally, external factors, e.g., ionising radiation and genotoxic

chemicals, can also damage DNA (Tiwari and Wilson III 2019; Chang et al. 2020). If DNA damage is not repaired it may result in the chromosomes being rearranged and/or translocated, which may result in cell death/cancer. In order to survive, cells in mammals possess complex mechanisms for the detection and repair of various types of damage (Broustas and Lieberman 2014; Chatterjee and Walker 2017).

1.9.1 DNA Damage

Various forms of DNA damage occur, depending on the source causing the damage. Such as exogenous and endogenous damage including chemicals, radiation, free radicals, each causing different forms of damage (Srinivas et al. 2019). A simple type of endogenous DNA damage is caused in the DNA's surrounding aqueous environment, when the N-glycosidic bond between DNA bases is hydrolysed and thus the nucleobase is isolated from the deoxyribose which leaves apurinic/apyrimidinic (AP) site. Another frequent hydrolytic reaction is for the DNA bases that carry exocyclic amino groups to be deanimated. The lesion which most frequently arises is caused by cytocine-uracil conversion which ultimately causes mutations (Alberts et al. 2003).

DNA is subjected to chemical alterations caused by molecular oxygen in cellular metabolism processes. ROS (oxidising agents) can lead to impaired physiological function through cellular damage of DNA, proteins, lipids, and other macromolecules (Poetsch 2020), are not only generated by ionising radiations but also from cellular metabolism (Roy and Sil 2012). DNA may also be subjected to chemical alterations caused by molecular oxygen in the standard cellular metabolism processes. Some of the most essential molecules are reactive oxygen species (ROS), e.g. hydroxyl radicals, hydrogen peroxide, and superoxide (Birben et al. 2012; Held 2012).

ROS is responsible for over 100 forms of oxidative DNA adducts, including single-strand breaks (SSBs) double-stranded breaks (DSBs), chemical modification of bases/sugars, and cross-linking DNA proteins (Roedl 2013). ROS could cause gene and chromosome mutations through DNA double strand breaks (DSBs) it is considered as the worst form of DNA damage. DSBs, if did not repair correctly, it causes cell death, and instability of chromosome (Sharma et al. 2016), such as oncogenes that cause cancer development (Kaur et al. 2019).

External agents, including ionising radiation and ultraviolet radiation (UV), are capable of penetrating and damaging the human genome through the induction of a variety of DNA lesions, which includes producing cyclobutene pyrimidine dimers that, without repair or inaccurate repairs in the course of replication, may result in mutations. Adenine and guanine may be subjected to spontaneous deamination, producing hypoxanthine (adenine) and xanthene (guanine) (Broustas and Lieberman 2014).

Since DNA is such a crucial component, cells have evolved a number of repair processes to protect it from harm and to preserve the integrity and stability of our genome (Dexheimer 2013; Moretton and Loizou 2020). The cells activate the DNA repair response, which recognizes, identifies, and mediates repair processes by activating several pathways such apoptosis, cell-cycle arrest, and DNA repair by activated tumour suppressor genes such as p53 (Huang and Zhou 2020).

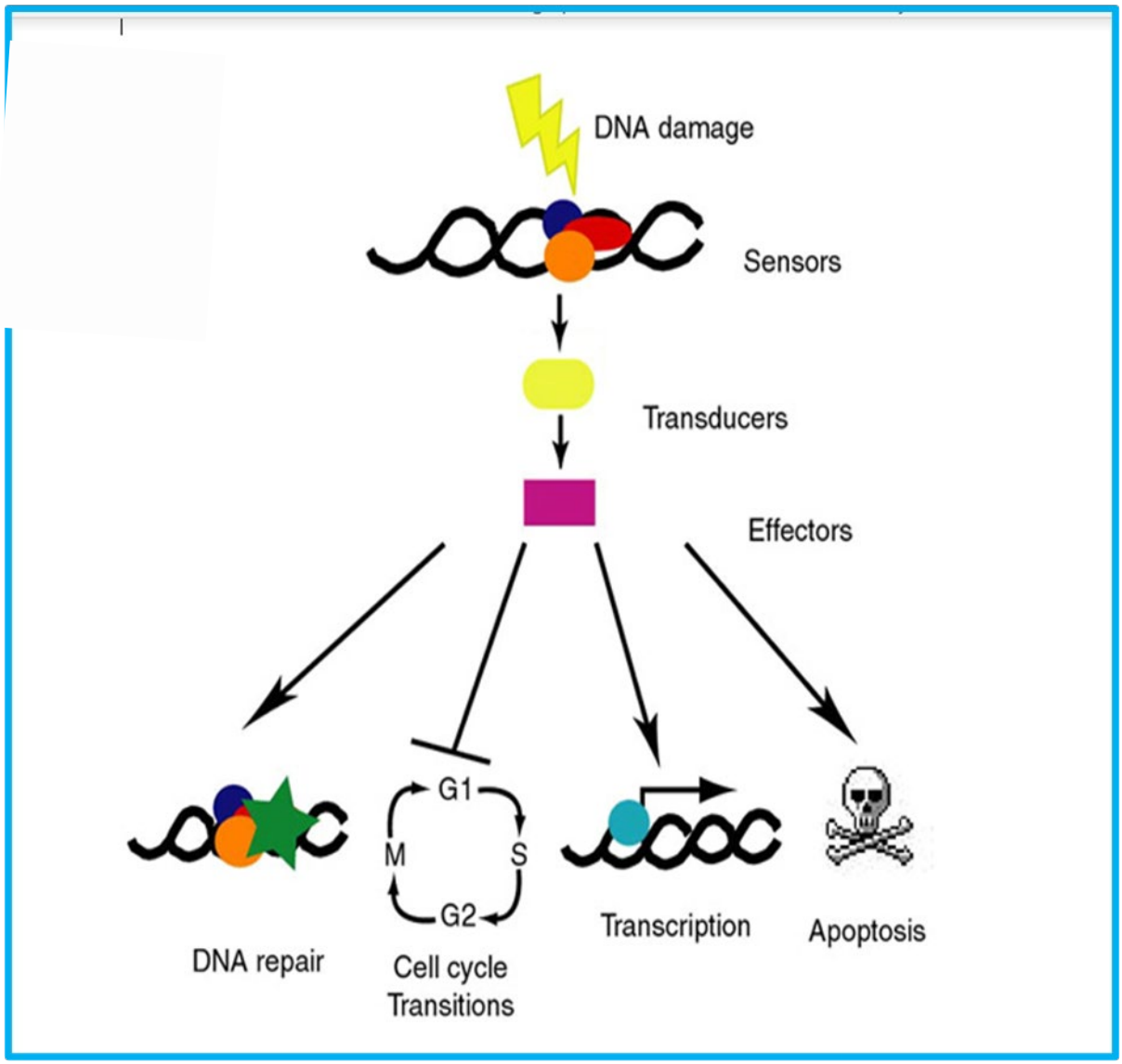


Figure 1-8 The pathway of endogenous and exogenous DNA damage (Exogenous: Cell metabolism, oxygen radical, replication error Chemical exposure, UV light exposure and endogenous: Radiation, chemotherapy, viral infection) (Jewish General Hospital 2017).

1.10 Tumour Suppressor Genes and Oncogenes

There are three primary classes of cancer genes, oncogenes, tumour suppressor genes, and DNA repair genes. Oncogenes have been recognised as genes that cause cancer, being responsible for activating cellular proliferation and promoting differentiation/cell growth without regulation (Lee and Muller 2010). Oncogenes are genes that encourage cells to multiply and divide. (Kaur et al. 2019). The majority of

oncogenes derive from an altered version of a gene called a proto-oncogene that has responsibility for standard cell growth/division, apoptosis and response of DNA damage. The majority of oncogenes represent a dominant mutation that encompasses rises in protein activities, increases in concentration of proteins, or chromosomal translocation, leads to the formation of several types of cancer (Armaghany et al. 2012; Ahmadi et al. 2021). Meanwhile, oncogenes, which are cancer-causing genes, induce cellular proliferation that instigates unregulated cell growth and differentiation (Ponder 2001; Wuputra et al. 2020). For example, proto-oncogene c-Myc is known as master regulator which transcribe about 15% of expressed genes, mRNA translation, cell-cycle regulation, proliferation, programmed cell death, and immune regulation. In addition, high level of c-MYC is significant sign of many human cancers and contributes to the cause of at least 40% of tumours (Ahmadi et al. 2021; Almajali et al. 2022).

In normal cells tumour suppressor genes regulates cell proliferation. Mutations of such genes cause losses of gene function, promoting carcinogenesis. These are generally dominant, and standard mutations may be represented through increases in protein activities, losses of regulation, increases in concentration of proteins, or chromosomal translocation, meaning that different cell types will be expressed by the gene (Lodish et al. 2000; Almajali et al. 2021).

Mutations to tumour suppressor genes lead to a loss of function. This significantly increases the risk of unregulated cell growth and has been established as an important contributor to cancer development (Harvey Lodish et al. 2000).

Moreover, there are also DNA repair genes whose function is to repair DNA damage. But if a cell whose DNA is damaged bypass cell cycle and form a protein, cells will

have less ability to repair themselves (Zhao et al. 2021). However, if mutations exist in these genes, the repair system fails, leading to the accumulation of successive mutations. Damaged DNA repair genes have been found in some cancers, such as bowel cancer (Li et al. 2021). DNA repair genes correct errors emerging during the cell duplication process prior to cell division. However, if mutations exist in these genes, the repair system fails, leading to the accumulation of successive mutations (Cortez 2019).

1.10.1 P53 tumour suppressor

P53 or cellular tumour antigen p53, is a tumour protein that is known as the Guardian of the genome (Toufektchan and Toledo 2018) p53 plays a prominent role as a facilitator of DNA repair by halting the cell cycle to allow time for the repair machineries to restore genome stability. In addition, p53 took on diverse roles to also directly impact the activity of various DNA-repair systems (Merlin et al. 2021). As a result, p53 is considered a tumour suppressor gene (Bourdon et al. 2005; Zhu et al. 2020).

The p53 tumour suppressor gene contributes to various cellular pathways as presented in Figure 1.8 (Boutelle and Attardi 2021).It plays a role in apoptosis, telomere erosion, metabolic disorders, DNA repair, cell senescence, and cell cycle arrest (Li et al. 2012a; Kim et al. 2015).

P53 protein has three functional domains; 1. Transactivation and proline rich domain, 2. Central DNA-binding domain (DBD), 3- Oligomerization domain (Raj and Attardi 2017).

Deactivating p53 mutations in cancer mainly arise in the DBD. The majority of these mutations impede the protein's capacity to attach to its target DNA regions, preventing transcriptional activation.

As a result, mutations in the DBD are loss of function mutations that are recessive. P53 molecules with DBD mutations dimerise with p53 and prevent it from activating transcription. As a result, DBD mutations have a strong detrimental impact on p53 function (Bell et al. 2002; Zhu et al. 2020).

Therefore, *P53* gene is the most often altered gene in human cancer (>50%), suggesting that it is defective (Giacomelli et al. 2018). In addition, the *P53* gene plays a critical function in cancer prevention (Surget et al. 2014) and is responsible for encoding proteins that attach to DNA and control gene expression to avoid genome mutations (Belyi et al. 2010).

The importance of p53 in suppressing cancer is evident, and it has been shown to be inactive in at least half of all human tumour cases that occur sporadically (Bieging et al. 2014; Zhu et al. 2020). The role played by p53 in ageing has also been demonstrated through several *in vivo* experiments. For instance, a truncated variant of p53 that promotes the activity of wild-type p53 was shown to retard the spontaneous development of cancerous cells in a transgenic mouse model (Moxley and Reisman 2021).

In human neural stem cells, the absence of p53 leads to genomic instability as well as metabolic change; this is likely because the centrosomes are amplified, which hinders cell division (Marin Navarro et al. 2020).

Meanwhile, a recent study has shown that depending on age, p53 expresses less in mesenchymal stem cells and neural progenitor cells (Wilson et al. 2010). In addition, at the organismal level, p53 efficiency has been shown to reduce with age. Overall, these findings indicate that age-related reductions in p53 expression may contribute to cancer development (Beck et al. 2020).

Similarly, Zhou and Myers (2016) showed that the most frequent somatic genomic alterations in HNSCC could be traced back to p53 mutations, underlining its crucial role in suppressing this disease. The authors further revealed that in clinical settings, p53 mutations correlate with increased tumour resistance to treatment as well as reduced survival times in HNSCC patients. Hence, the mutation status of p53 could serve as a molecular risk stratification factor to predict patients' clinical response. Beyond the decline in the functions of wild-type p53, compounded by the adverse impact on the wild-type p53 remnants, p53 mutations can also incorporate oncogenic functions that promote the development and progression of tumours (Zhou et al. 2016).

Moreover, cancer development can also be related to altered genes that participate in the p53 pathway. For example, a mutation in the G1 cell cycle checkpoint kinase destabilizes p53, resulting in cancer as those cells with damaged DNA begin proliferating (Bieging et al. 2014). The gene most often found to have mutations in relation to human cancer, p53 works to suppress the development of tumours by transcriptionally regulating the various target genes that contribute to some of the cellular processes (Aubrey et al. 2018).

P53 was initially thought to be a critical regulator in the response mechanisms to acute DNA damage (e.g. arresting the cell cycle and activating apoptosis) (Boutelle and Attardi 2021). However, research has recently found novel cell pathways and other transcriptional targets that occur downstream of p53 and which regulate metabolism, genomic integrity, stemness, redox biology, and non-cell-autonomous signalling, contributing to tumour suppression (Hafner et al. 2019).

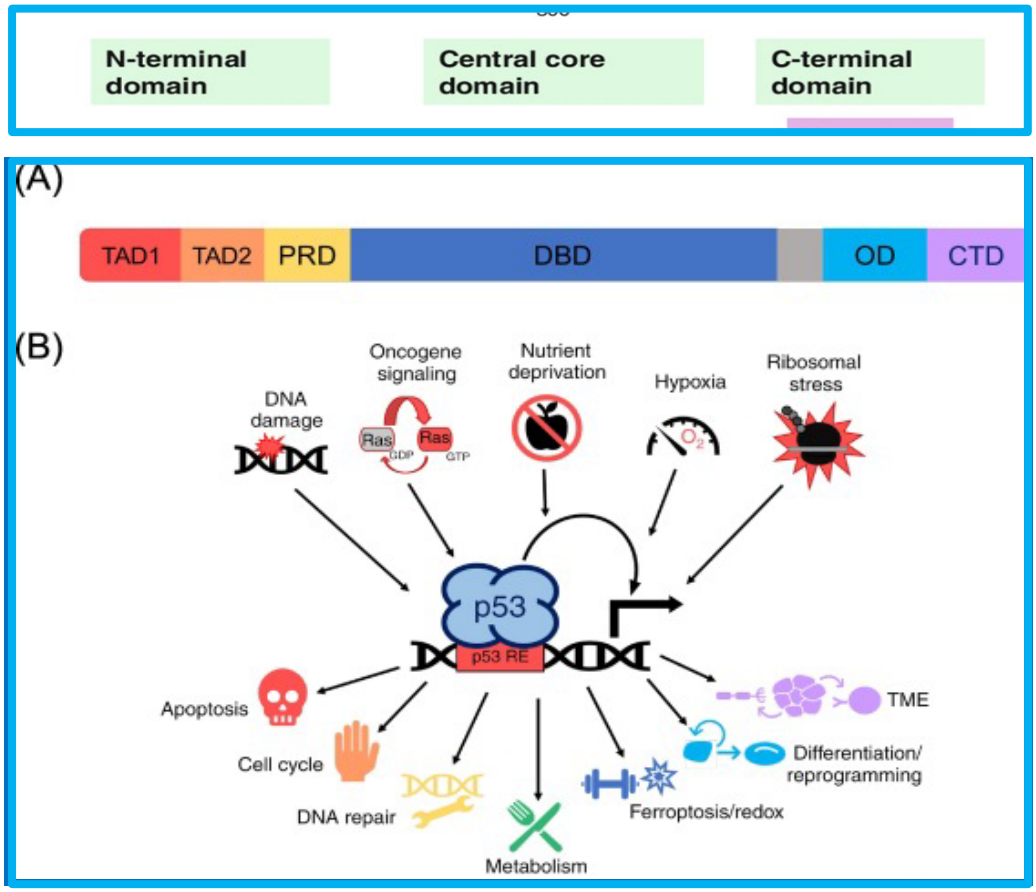


Figure 1-9 The structure and function of p53 (Boutelle and Attardi 2021). P53 protein consists of three regions are called: 1-N-terminal domains or Transcriptional Transactivation (TAD: Trans-activation domain and PRD: Proline-rich domain),2-Central core domain Central (Sequence-specific DNA binding (DBD)3-C-central domain or Negative Regulation (OD: Oligomerization domain and RD: Regulatory domain).

1.10.2 P21 cyclin-dependent kinase inhibitor 1

The cyclin-dependent kinase inhibitor (CKI) p21 is a cyclin-dependent kinase inhibitor 1, also referred to as CDK-interacting protein 1. It has the capacity to inhibit all cyclin/CDK complexes. Although it is mainly linked to CDK2 inhibition, p21 is also subject to p53 activity (Mijit et al. 2020). Therefore, it is thought to link the occurrence of damaged DNA with the arrest of the cell cycle (Abbas and Dutta 2009). Thereby regulating G1 and S phase cell cycle progression (Larrieu et al. 2010; Barr et al. 2017).

Numerous factors influence the level of control over the progression of the cell cycle. These include p21, which causes cell cycle arrest as a response to various stimuli.

Meanwhile, its nuclear localization contributes to its inhibitory effect on cell cycle progression (Karimian et al. 2016). Hence, p21 plays several roles in apoptosis, regulating the cell cycle, and gene transcription following damage to DNA. Research has demonstrated the key role played by p21 in carcinogenesis and tumour development. In terms of oncogenic function, it is primarily known for its above mentioned inhibition of apoptosis, as shown by the fact that tumorigenesis is reduced following radiation-based p21 knockdown (Xiao et al. 2020; Saleh et al. 2022).

A significant relationship between the expression of p21 and the depth of invasion, advanced TNM stage, and overall survival rate has been identified. Mechanisms either dependent on or independent of p53 can induce p21. The role of p21 in regulating transcription and modulating or inhibiting apoptosis occurs through direct interactions with proteins as well as on p21 subcellular localizations. Furthermore, through its interaction with the proliferating cell nuclear antigen (PCNA) derivative from the C-terminus in the cell-cycle checkpoint protein, p21 contributes to the repair of damaged DNA (He et al. 2020; Mansilla et al. 2020).

Kapranos et al. (2001) suggested that p21 activation is a key contributor to positive chemotherapy responses due to its functional promotion of apoptosis. Thus, it could be a predictive factor to provide improved prognoses for HNC patients in stage III (Kapranos et al. 2001).

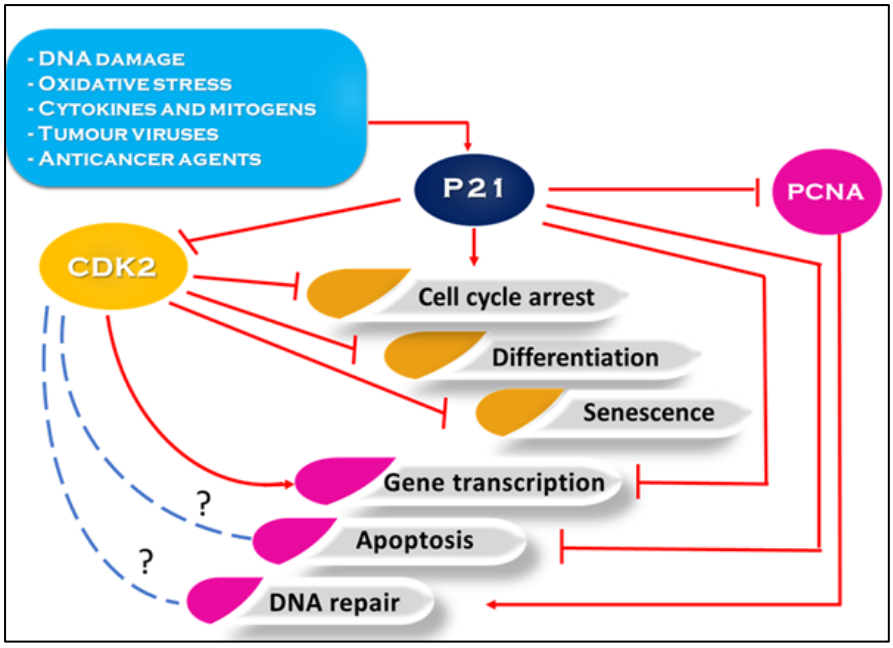


Figure 1-10 The rule of P21 in the cell (Abbas and Dutta 2009)

1.10.3 Bcl-2

The evasion apoptosis, i.e. programmed cell death, plays a crucial role in the onset and progression of tumour initiation. Although cancer cells contain the full apoptotic machinery, there are ways with which they can block the apoptotic process controlled by the BCL-2 family of proteins (Hafezi and Rahmani 2021), which are a set of evolutionarily conserved proteins with BCL-2 homology (BH) domains. These proteins exist in a variety of intracellular compartments, including the nucleus, endoplasmic reticulum, peroxisomes, and Golgi apparatus (Popgeorgiev et al. 2018).

BCL-2 family proteins notably regulate apoptosis at the mitochondrial level (Kale et al. 2018). Moreover, they also participate in regulating insulin production (Luciani et al. 2013) and metabolic activity in addition to the production of ROS (Aharoni-Simon et al. 2016).

Localized on the mitochondrion's outer membrane, BCL-2 family proteins are crucial to cellular survival, particularly as they regulate pro-apoptotic protein activity. Pro-

apoptotic proteins, such as BAX and BAK, are activated by BH3-only proteins and usually work on the mitochondrial membrane, ensuring permeabilization and the release of the apoptosis cascade signals cytochrome C and ROS. These pro-apoptotic proteins are, in turn, blocked by BCL-2 and BCL-Xl (Hardwick and Soane 2013).

Several cancers, such as breast and prostate cancer, melanoma, lung cancer, and chronic lymphocytic leukaemia, have been linked to damage to the BCL-2 gene. Damaged BCL-2 genes have also been suggested to lead to cancer treatment resistance (Campbell and Leung 2021) and there is also evidence indicating they may potentially play a role in schizophrenia and autoimmune disorders. Whether BCL-2 protein family staining appears in biopsies can under certain circumstances, determine a patient's relapse likelihood and inform their prognosis (Leong et al. 2003).

The presence of pro-survival members of the BCL-2 family proteins can foster tumour development and increase its resistance to treatment. Specifically, heightened levels of such anti-apoptotic proteins have been found to cause resistance to radiotherapy and chemotherapy, leading to worsened clinical outcomes (Zerp et al. 2015). Anti-apoptotic members of the BCL-2 family proteins are often over expressed in cases of HNSCC (Guy et al. 2021).

The BCL-2 gene is generally considered an apoptosis suppressor gene. Through the selection and maintenance of long-life cells and blocking cells in the cell cycle's G0 phase, the over expression of anti-apoptotic proteins in cancer cells can hamper or postpone the start of apoptosis (Vishnu et al. 2021). To inhibit BCL-family pro-survival molecules in patients with HNSCC, it is necessary to target both BCL-XL and MCL-1, with HNSCC displaying synergistic co-inhibition (Ow et al. 2019).

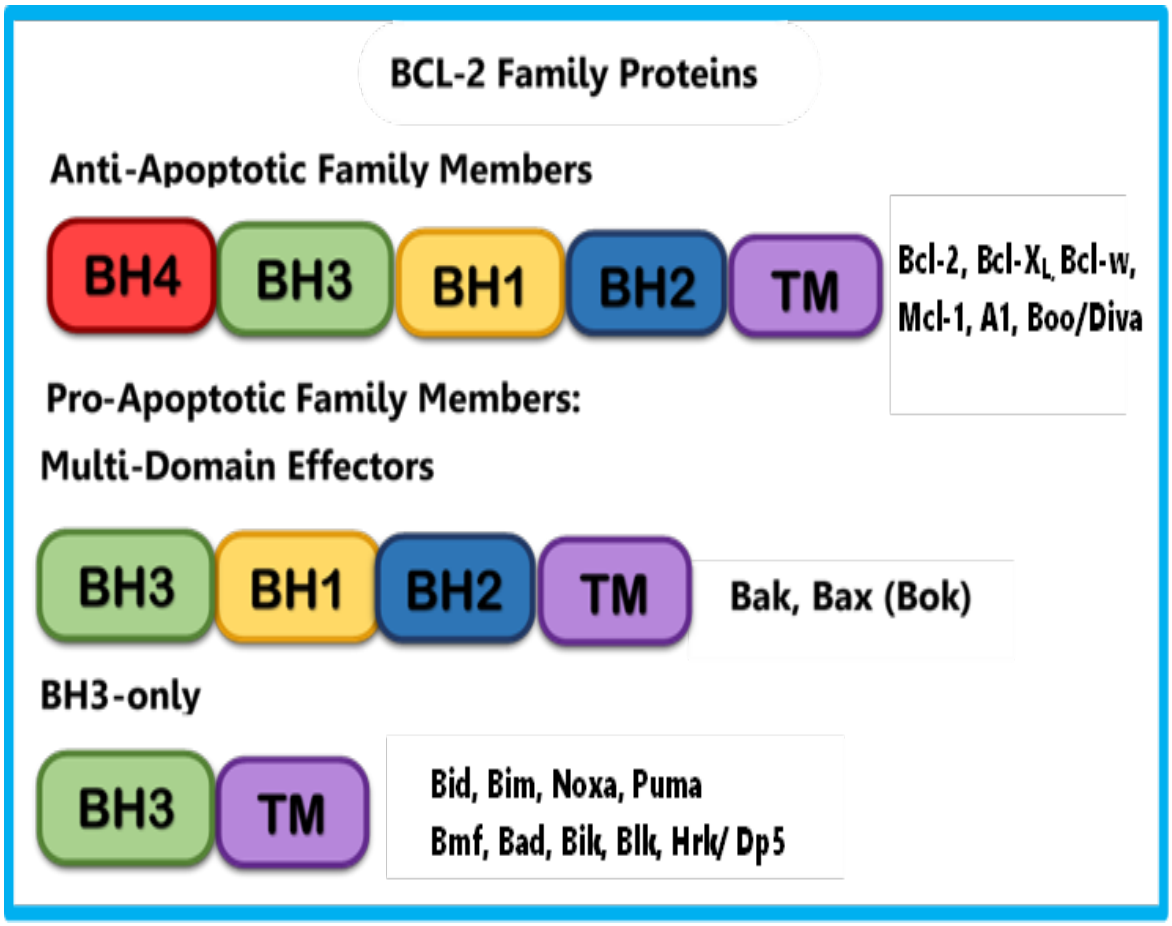


Figure 1-11 Bcl-2 Family protein (Parsons and Green 2010)

1.10.4 Cell cycle control and cancer

Although it has been generally held that the cell components are equally distributed in the daughter cells following cell division, this may not always be the case. Evidence has shown that there can be an asymmetric distribution of the proteins, organelles and membrane compartments, and indeed even the DNA, once the cell division process has completed (Neumüller and Knoblich 2009).

The cell cycle comprises four phases. In the M (mitosis) phase, which has a short span, first nuclear division and then cytoplasmic division (cytokinesis) occur. In the G1 (Gap 1) phase, which is the longest phase of cellular growth, certain cells undergo

further differentiation; other cells do not leave this phase and instead enter the G0 (resting) phase. Next, during the S (Synthesis) phase DNA synthesis occurs, involving genome replication and DNA-damage-related checkpoints. Finally, the G2 (Gap 2) phase consists of rapid cell growth before the next M phase; this involves G2/M checkpoints (Figure 1.11) (Qi and Zhang 2020). The cyclin-dependent kinases (CdKs) and their activation/inhibition regulate and control the machinery of the cell cycle (Otto and Sicinski 2017). Growth factor action, in turn, activates these CdKs (Ertosun et al. 2016).

The pacing and accuracy of genome replication are controlled by the CDK–RB–E2F axis, which comprises the main transcriptional cell cycle machinery. As a result, it is crucial for the accuracy of the genetic material as it is passed through the cell division cycle. The eight unique E2F genes that encode the transcriptional activators and repressors are the effectors of the CDK–RB–E2F axis. During the cell cycle, the transcriptional process of the E2F genes is controlled by transcriptional and translational regulation as well as protein degradation, post-translational modification, co-factor binding, and subcellular localization. Almost all cancers evidence changes in at least one main component of this axis, i.e. CDKs, CDK inhibitors, cyclins, and the RB protein family, triggering uncontrolled proliferation due to the increased oncogenic E2F activity. The following work explores E2F proteins and their activities, with a focus on the novel and atypical members of the E2F family in addition to E2F protein functioning, both redundant and specific. It further examines the cancer-promoting nature of E2F transcriptional target misexpression as well as the existing and developing therapies that act on this particular oncogenic pathway. In particular, the emphasis is on checkpoint mechanisms that verify that a phase has ended before the

next one can begin (Figure 1.11) shows the cell cycle and the relevant checkpoints (Schnerch et al. 2012; Kent and Leone 2019).

Under normal circumstances, cell growth is strictly regulated; however, disturbances to the cell growth process can instigate uncontrolled cellular growth, also known as neoplasms or tumours (Liu et al. 2020). Tumours can be benign (limited to an area) or metastatic (entering other body regions) (Van Calster et al. 2014). Cancer cells, whether due to chromosomal abnormalities or exposure to carcinogens are clonal (Hill et al. 2021)

The transition from a normal cell to a tumour cell takes place when a cell mutates and subsequently circumvents the checkpoints in the cell cycle, including in the G1/S phases (during replication), G2/M phases (the P53 or PRb DNA damage checkpoint) and the mitotic spindle (during the formation of the spindle). These particular checkpoints tend to reveal the defects of malignant cells (Hanahan and Weinberg 2011; Benedict et al. 2018).

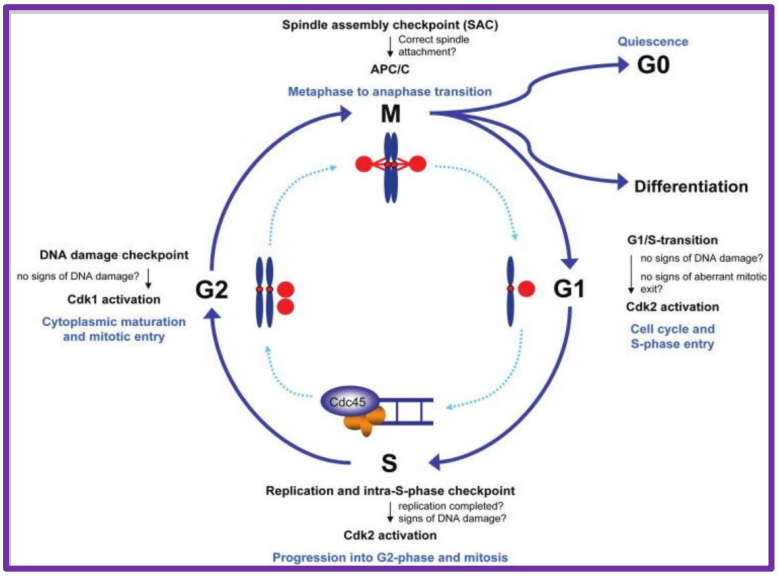


Figure 1-12 The cell cycle, checkpoint, and four phases -G1phase, S-phase, G2-phase, and M-phase in human cells during mitoses division (Schnerch et al. 2012).

1.11 Genotoxicity:

Genotoxicity refers to the ability of harmful substances to damage genetic information in cells. Being exposed to chemical and biological agents can result in genomic instabilities and/or epigenetic alterations, which translate into a variety of diseases, including cancer. The comet assay is widely used *in vitro* and *in vivo* genotoxicity testing. It measures DNA strand breaks and alkali-labile sites in virtually any eukaryotic cell including cells isolated from tissues. As well as micronucleus assay to test genotoxicity and detection of MNi in the cytoplasm during cell division (anaphase stage).

1.11.1 Comet Analysis

1.11.1.1 Development and principles of the Comet analysis

Firstly, Ostling and Johanson (1984) came up with a micro gel electrophoresis procedure for assessing DNA damage at a single-cell level. However, its general use has been limited due to neutral assay conditions (Ostling and Johanson 1984). Then, Singh et al (1988) have applied the same kind of method to discover the damage in DNA in single cells using alkaline conditions (pH >13) (Singh et al. 1988). The word comet identifies the singular appearance of individual cell DNA migration patterns developed by this assay. This development massively enhanced the sensitivity of the assay for determining genotoxic substances. Furthermore, due to the presentation of the assay at pH>13, its applications have considerably increased in different research areas including, genetic toxicology, DNA repair processes, and human and environmental biomonitoring (Ladeira et al. 2019).

Importantly, the Comet assay has been well-known, a highly demanded tool because of its attractive characteristics, such as its speed, simplicity, inexpensiveness, and

higher sensitivity (Milić et al. 2019). It can detect modest levels of damage with a smaller sample size (10,000 cells). Studies can be carried out using relatively modest doses of the drugs. Cells fixed in agarose were lysed to liberate DNA on a microscope slide, resulting in nucleoids, which are composed of supercoiled DNA rings coupled to a nuclear matrix. Electrophoresis towards the anode at pH >13 produces comet-like structures (Cordelli et al. 2021). After that, the alkali is neutralized, and the DNA is stained with ethidium bromide. Finally, it was discovered under a fluorescent microscope that longer intense tails caused more damage, while shorter tails caused less damage (Azqueta et al. 2019).

1.11.1.2 **The comet assay and *g*enotoxicity assessment**

According to a systematic review, single-cell gel electrophoresis (SCGE) or Comet analysis *in vivo* and *in vitro* is the third most commonly used technique for genotoxicity evaluation of herbal extracts (Sponchiado et al. 2016). It has been utilized in humans to access the exposure of individuals to genotoxic substances owing to numerous factors such as lifestyle and environment to determine the damage in genetics. Łukaszewski et al. (2017) used it for DNA repair investigations and environmental biomonitoring (Łukaszewski et al. 2017; Gopalan et al. 2011). Cancer susceptibility is determined by a number of factors, including the genome's sensitivity (Collins 2004).

Because lymphocytes have long lifetimes and may keep genetic changes produced by a mutagen for more than 40 years, they are regarded a good instrument for studying genome sensitivity (Anderson et al. 2014).

Furthermore, lymphocytes are easy to get for sample and circulate in great amounts in the blood, making it easier for genotoxins to target them (Albertini et al. 2000).

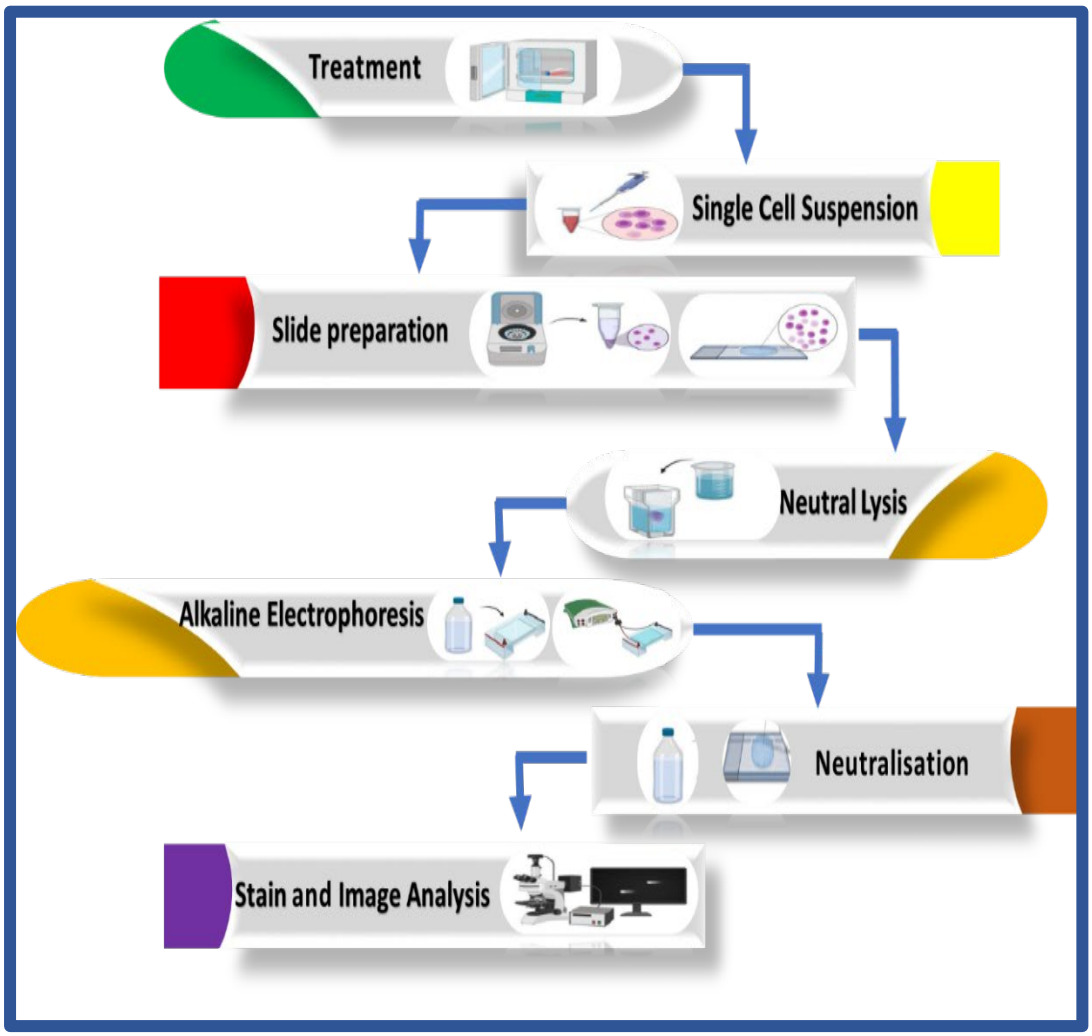


Figure 1-13 The principles steps of the Comet analysis (Gyori et al. 2014)

1.11.2 The micronucleus assay (MN)

Micronuclei have known by various descriptions in the past. It has assumed that micronuclei develop from acentric fragments extended from two daughter nuclei at the later phase of mitosis as those assemblies constantly found after cells exposed to radiation (Kirsch-Volders et al. 2003). Evans et al (1959) found micronuclei as a biomarker of cytogenetic damage in the Vicia faba roots when comparing the

effectiveness of gamma rays to that of neutrons (Evans et al. 1959), However, Countryman and Heddle developed the micronucleus test in human cells in 1976 (Countryman and Heddle 1976). However, due to its limitations, Michael Fenech discovered significant problems in this assay in its current form. He improved the approach by including a cytokinesis blocking step to prevent cells from completing cellular division after nuclear division was completed using the cytochalasin-B (Fenech 2009). As a result, the cytokinesis block micronucleus assay is also known as the micronucleus assay (CBMN). The MN assay has long been regarded as a very reliable, fast, and appropriate approach for determining a broad range of DNA damage at the basal level, particularly for mutagenicity testing (Hovhannisyan 2010). To determine cytogenetic damage, scoring has been developed. The CBMN approach is now the most extensively used method for determining MNi frequency in human cells. MNi, distinct DNA biomarkers present in binucleated cells (i.e. the nuclear buds and nucleoplasmic bridges), and cells suffering death, either by apoptosis or necrosis, may all be scored using CBMN. Various investigations have found a relationship between MNi, nuclear buds, and nucleoplasmic bridges, indicating that these genetic instability indicators are mechanically connected (Gashi et al. 2018).

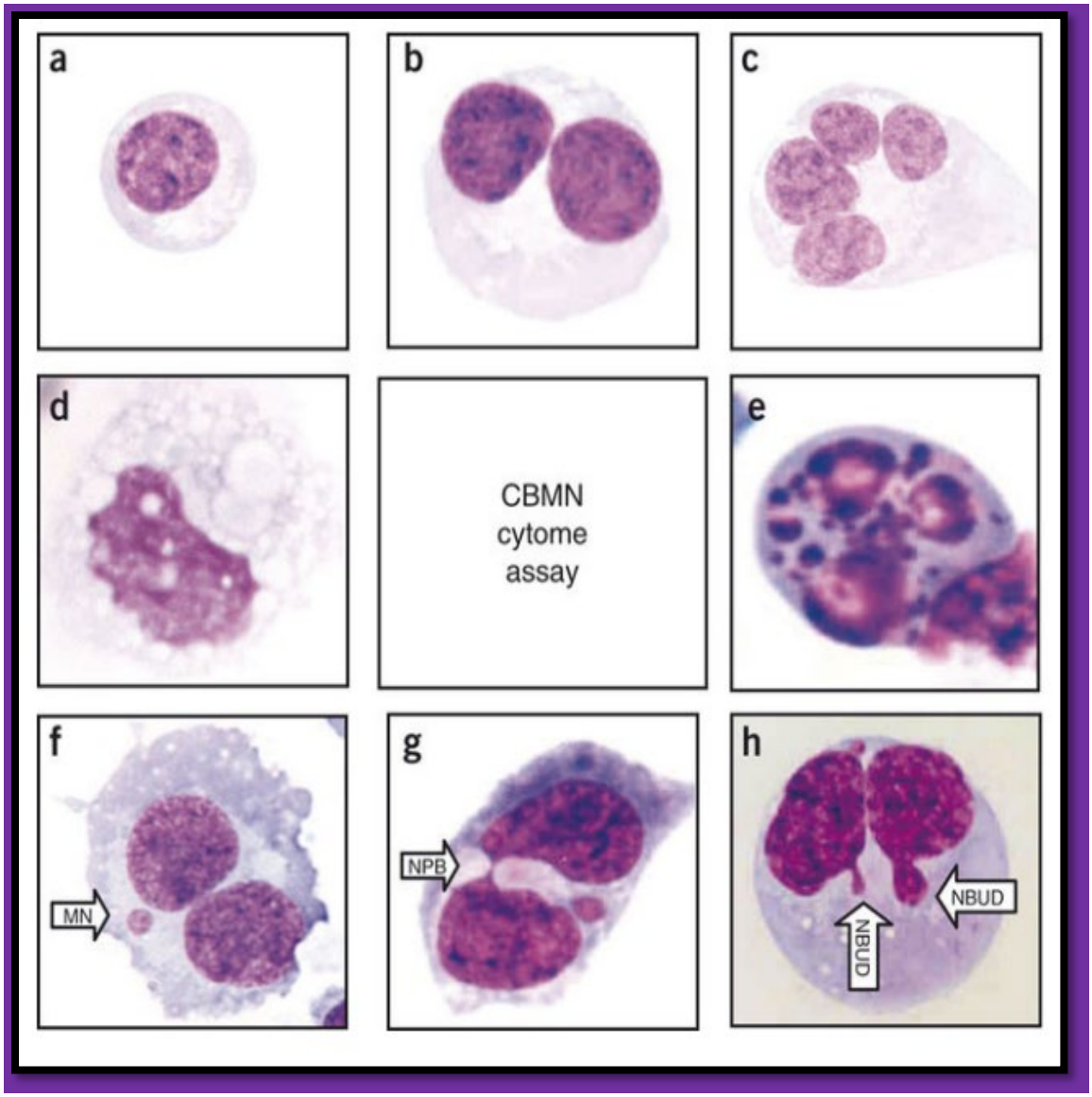


Figure 1-14 Photomicrographs of different types of cells scored in the CBMN assay. a) Mononucleated cell; (b) BN cell; (c) multinucleated cell; (d) necrotic cell; (e) late apoptotic cell; (f) BN cell containing one or more MNi; (g) BN containing an NPB (and a MN); (h) BN cell containing NBUDs (Benassi et al. 2007; Fenech 2007) .

1.11.3 Polymerase chain reaction PCR

An innovation that marked a turning point in molecular biology and the medical sciences, polymerase chain reaction (PCR) is a technique used in molecular biology that exploits the ability of cells to replicate strands of DNA. The aim hereby is to amplify a small strand of sample DNA until an analyzable quantity is reached (Ngô and Van

de Voorde 2014). PCR was developed in 1984 by the American biochemist Kary Mullis, patented in 1985 (Kadri 2019).

Due to its time, cost-effectiveness, accurate as well as ease of use, PCR soon emerged as one of the most important methods in the in molecular genetics and biotechnology. It has also gained significance in disciplines as varied as forensic sciences, agriculture, anthropology and evolutionary genetics, medical diagnostics, and molecular epidemiology (Singh et al. 2014; Jobling et al. 2019).

1.11.3.1 Real time-PCR or quantitative PCR (qPCR)

Higuchi et al. (1992) first developed quantitative real-time PCR (qPCR) (Devi et al. 2019). This process represented a significant advance in biology fields that could be employed in a variety of molecular techniques, such as the analysis of nucleic acids peripheral blood. It has been significantly contributed in the current level of human knowledge in many fields of scientific research (Rodríguez-Lázaro and Hernández 2013; Kralik and Ricchi 2017). The qPCR process uses fluorescent dye, such as SYBR Green, which stains the double-stranded DNA, enabling the amplification of the target DNA to be measured specific time at the end of each PCR cycle. During the magnification's log-linear phase, the fluorescence level rises to a measurable point known as the cycle threshold (CT) (López-Sanmartín et al. 2019). The qPCR amplification results comprise the standard curves of log concentration against the CT, plotted using serial dilutions of a known magnitude of standard DNA (Kralik and Ricchi 2017). Hence, using this standard curve, the amount of DNA, or complementary DNA (cDNA), in an unknown sample can be identified based on the CT value (Singh et al. 2014).

This process was further developed by combining the amplification and detection stages into a single step, thereby removing the need for the targeted DNA to undergo post-amplification processing (Mackay 2004; Lorsch 2013).

The qPCR is a highly sensitive, accurate, and fast real-time method for measuring how far the amplification process has advance and the concentration of DNA species in the reaction (Kim et al. 2018b; Kang 2019), via either fluorescent materials or oligonucleotides with fluorescent dyes whose intensity reflects the amount of formed target DNA (Nishi et al. 2015). Different polymerases can be used in the various qPCR reactions depending on the requirements, e.g., high-precision, “hot-start” or high/fast activity polymerases. After nucleotide linked together through DNA polymerase, the PCR product formed (Jalali et al. 2017). The qPCR machines enable these reactions by using a thermic cycler for DNA amplification, optics to stimulate fluorophores and capture the generated fluorescence, and specialized software for the quantitative data analysis. The qPCR presents a distinct advantage in that the contamination by conventional PCR products, e.g., cDNA and RNA, is low (Singh et al. 2014).

Nevertheless, qPCR also has disadvantages, such as the high cost of its reagents. Furthermore, it is a highly sensitive method, could be easily result in contamination making understanding its experimental design crucial to achieving accurate results (Hu 2016). Regardless, qPCR is the most commonly used PCR technique in diagnostic applications, primarily because of its sensitivity and ease of control important considerations in diagnostic use. qPCR has the ability to precisely quantify and isolate a target gene (Narrandes and Xu 2018). qPCR could be used to target a single cell, to be allowing for any given DNA combination to be targeted. Furthermore, qPCR tends to be favoured over conventional PCR because it can amplify short DNA fragments, enables fluorescence detection, facilitates the detection of small quantities

of amplified products, and displays increased tolerance to materials that might influence the DNA purification. Moreover, as qPCR machines are integrated units, there is less chance of post-PCR contamination, which can cause false-positive results. Finally, qPCR does not use the gel electrophoresis step, meaning it is easier to automate and offers fast throughout (Boonham et al. 2016).

1.11.3.2 RT-PCR/qPCR combined

Reverse transcription PCR (RT-PCR) was discovered 1970 and laid the foundation development of RT-PCR (used in molecular cloning) (Chrysostomou et al. 2021). Reverse transcription PCR (RT-PCR) is a technique that amplifies RNA to enable a qualitative assessment by first converting the RNA into cDNA. Meanwhile, to quantitatively measure messenger RNA expression (mRNA), RT-PCR and qPCR can be used together in a combined technique known as quantitative RT-PCR (qRT-PCR) or RT-qPCR (Singh et al. 2014). These techniques and their applications have developed life science research and clinical diagnosis.

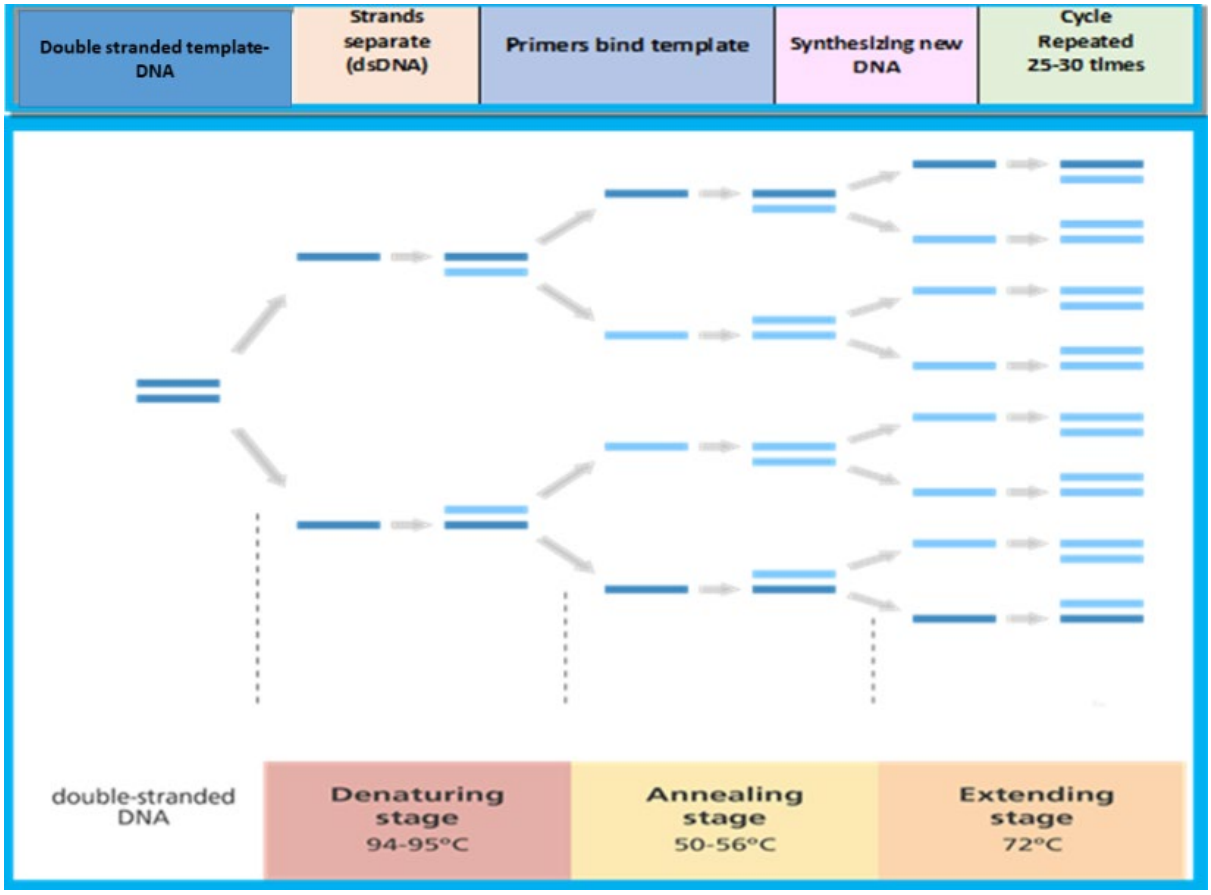


Figure 1-15 The principal stages of PCR (Sreejith et al. 2018)

1.12 Western Blot (WB)

The Western blot technique, also referred to as protein immunoblotting, is commonly utilized in the fields of biochemistry, immunogenetics, and cellular and molecular biology, among others. Specifically, it is a crucial method for detecting proteins and quantifying their expression (Mahmood and Yang 2012b).

Western blotting facilitates the identification of specific proteins from among complex mixes of proteins that are extracted from samples. The method comprises three steps; the first step uses gel electrophoresis to sort the proteins by size based on the type of protein and their molecular weight. The second step involves blotting onto a solid matrix, which generates a specific band for each of the investigated proteins. Finally,

appropriate primary and secondary antibodies are used to visualize or mark the target proteins (Hnasko and Hnasko 2015). As only specific antibodies bind to their corresponding proteins and any unbound antibodies are rinsed off, each protein should present a single band after the film has been developed. The band thickness hereby correlates with how much of that protein the sample contains; this can be determined based on a standard value. Use of high-resolution gel electrophoresis as well as strong specificity and increased sensitivity, this technique is able to detect protein concentrations as low as 1 ng (Akhtar 2019).

Western blot is the preferred technique in conducting immunoassays that investigate the expression of proteins because it offers good accuracy at a reasonable cost and it is simple to analyse and translate the results. It hereby enables the expression of proteins from different cells to be captured as well as the observation of specific protein responses following a given drug treatment (Kim et al. 2018a).

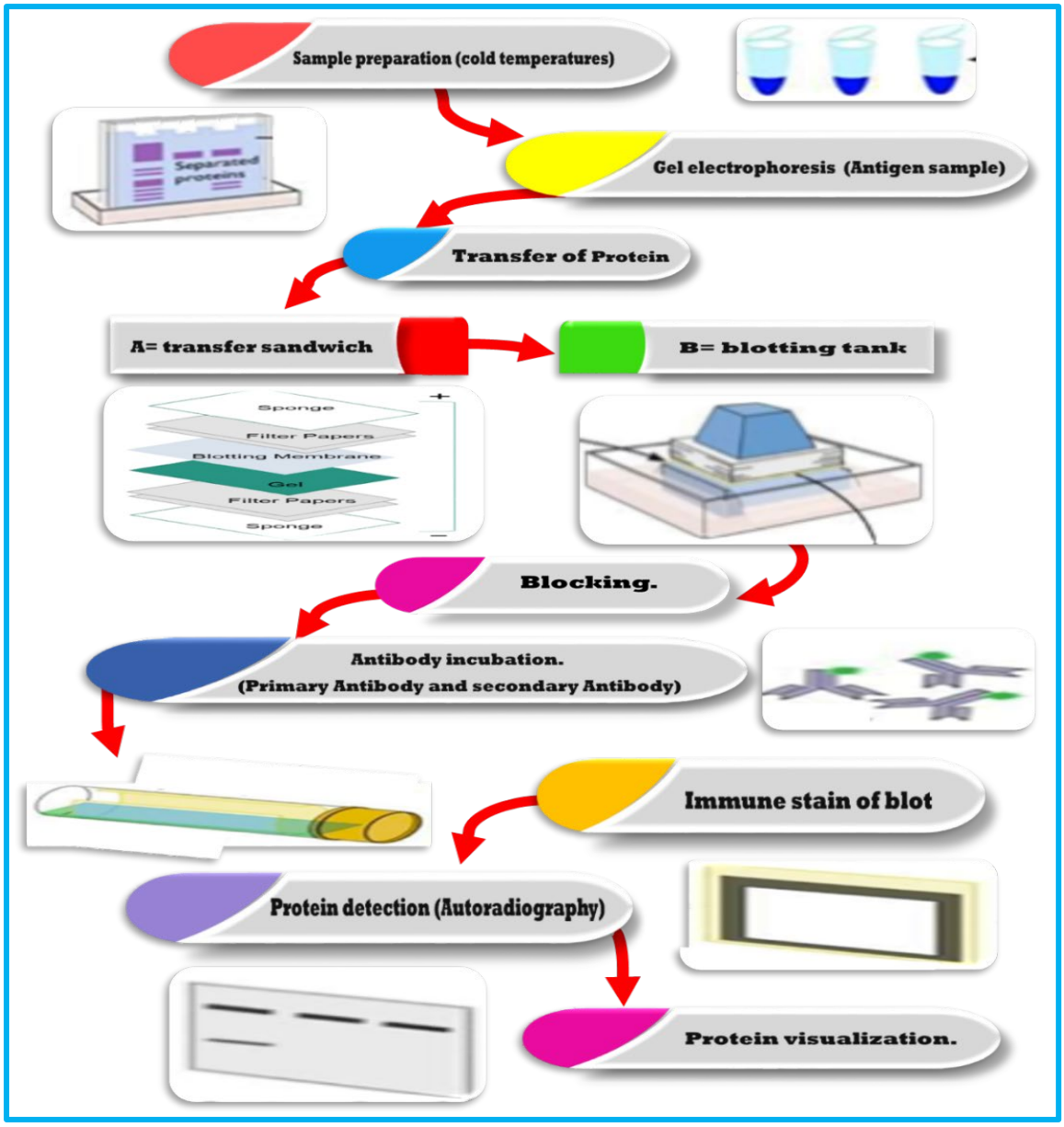


Figure 1-16 The principal steps of the Western blot analysis (Gwozdz and Dorey 2017)

1.13 Aims of the thesis

Pembrolizumab inhibits tumour cell proliferation and enhances apoptosis, making it an important immunomodulatory, antiviral, antiproliferative. Pembrolizumab has been used as an anti-tumour drug in a variety of cancers, including lymphoma and lung cancer, according to numerous trials and publications. However, there are no studies on the use of Pembrolizumab and liposome in head and neck cancer. The liposomes

have many advantages such as Increased stability of the encapsulated drug, and reduction in toxicity of the encapsulated agent site avoidance effect (avoid non-target tissues). Flexibility to couple with site-specific ligands to achieve targeting. It could be used as a nasal drop to treat nasal cavity cancer or as a cream to treat lip and skin cancer.

As a result, the study's main goal was to evaluate the DNA-protective effects of naked pembrolizumab and pembrolizumab liposome on lymphocytes from HNC patients to healthy people. The genotoxic and antioxidant effects of naked pembrolizumab and liposomal form on DNA damage were assessed using Comet and micronucleus assays in this study. The Comet test was utilized in this investigation because of its sensitivity and ease of use for analyzing DNA genotoxicity in lymphocytes, as shown in earlier studies on different types of malignancies.

The micronucleus assay was also used to assess the effect of naked pembrolizumab and pembrolizumab liposome on the mitotic phases of the cell cycle using the frequencies of the following biomarkers:

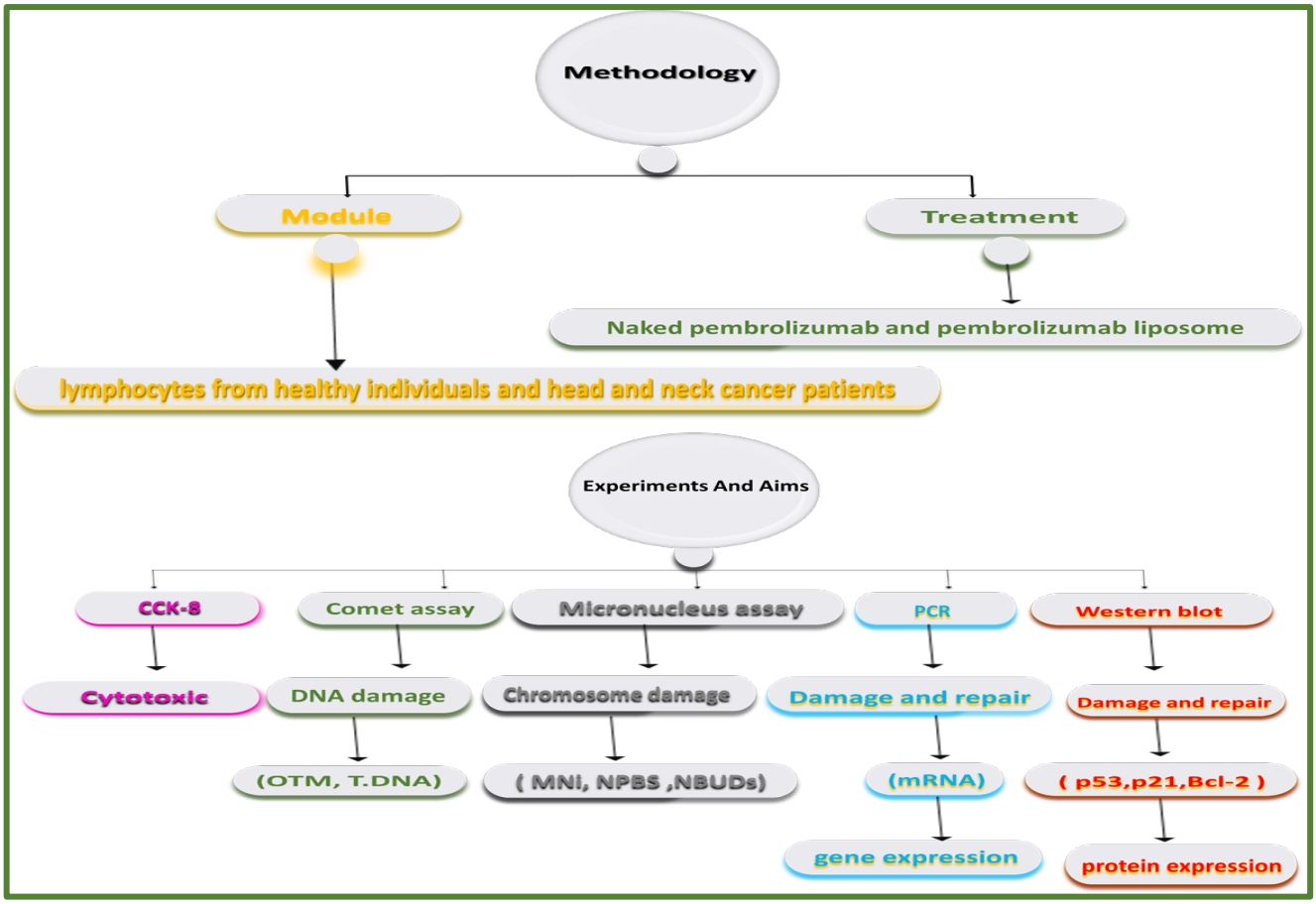
1.Micronuclei (MNi): the indicator for chromosomal breakage or loss of a whole chromosome

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.

Western blotting was used to investigate the effects of naked pembrolizumab and pembrolizumab liposome on the expression of p53, p21, and Bcl-2 proteins.

Finally, using quantitative reverse transcription PCR (qRT-PCR), the effect of naked pembrolizumab and liposome on p53, p21, and Bcl-2 gene expression in lymphocytes from HNC patients compared to healthy individuals was investigated.



- CHAPTER TWO: Materials and Methods

2 Materials and Methods

2.1 Materials

Table 2.1 lists the chemicals and equipment that were utilized in this study with the suppliers and the CAS numbers.

Chemicals and reagents	Company / Distributor	CAS Number
Pembrolizumab	Star Lab	1374853-91-4
Normal melting point	Invitrogen, UK	9012-36-6
Low melting point agarose	Invitrogen, UK	39346-81-1
Ethidium bromide	Sigma –Aldrich, UK	n/a
Dimethyl sulfoxide (DMSO)	Sigma –Aldrich, UK	67-68-5
EDTA ($Na_2EDTA.2H_2O$)	Sigma –Aldrich, UK	6381-92-6
Sodium hydroxide (NaOH)	Sigma –Aldrich, UK	1310-73-2
Phosphate buffered saline (PBS)	Sigma –Aldrich, UK	P4417
Sodium chloride (NaCl)	Sigma –Aldrich, UK	7647-14-5
RPMI-1640 medium	Sigma –Aldrich, UK	n/a
Triton X-100	Sigma –Aldrich, UK	9002-93-1
Trizma base	Sigma –Aldrich, UK	77-86-1
Cover glass	VWR	n/a
Distilled water(dH_2O)	n/a	n/a
Eppendorf ® tubes (1ml)	Sigma –Aldrich, UK	Z666548
Superfrost microscope	Thermo Fisher Scientific	n/a
Pipette tips	VWR	n/a
ErgoOne® Starter Pack 4	Starlab,UK	n/a
Safety blood collection set	Griener Bio-One, Austria	n/a
Black box	Sigma-Aldrich, UK	n/a
Lithium heparin tube 9ml	Griener Bio-One, Austria	n/a
Trypan blue	Sigma –Aldrich, UK	72-57-1
Foetal bovine serum	Sigma-Aldrich, UK	n/a
2-Mercaptoethanol	Sigma-Aldrich, UK	60-24-2
Mitomycin C	Sigma –Aldrich, UK	50-07-7
Potassium chloride	Sigma –Aldrich, UK	7447-40-7
penicillin-streptomycin	Sigma –Aldrich, UK	n/a
Glacial Acetic Acid	Fisher Scientific, UK	64-19-7
DPX Mountant	Sigma –Aldrich, UK	n/a
Phytohaemagglutinin	Sigma –Aldrich, UK	9008-97-3
Sodium phosphate	VWR, UK	7558-79-4
Sodium phosphate	VWR, UK	7558-80-7

96 well plates	VWR, 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
Acrylamide 30%	Sigma –Aldrich, UK	79-06-1
Falcon tubes	BD, Swindon, UK	n/a
Pipette filler	Scientific laboratory supplies	n/a
Bradford Protein Assay Kit	Bio-Rad	n/a
Protease inhibitor cocktail	Sigma –Aldrich, UK	66701-25-5
Ammonium persulphate	Sigma –Aldrich, UK	7727-54-0
Recombinant Anti-p21	Abcam, UK	n/a
Recombinant Anti-Mutant	Abcam, UK	n/a
Anti-Bcl-2 antibody	Abcam, UK	n/a
Bovine serum albumin	Sigma –Aldrich, UK	9048-46-8
Anti-Rabbit IgG H&L	Abcam, UK	n/a
Western ECL Substrate,	Bio-Rad	n/a
SYBR® Green Supermix	Sigma –Aldrich, UK	n/a
iScript™ cDNA Synthesis	Bio-Rad,UK	n/a
QIAamp® RNA Blood Mini	Qiagen	n/a
Primer (B-Actin)	Qiagen	n/a
p53 primer	Qiagen	n/a
Primer (Bcl-2)	Qiagen	n/a
Primer (P21)	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	Thermo Fisher Scientific	n/a
QIAamp RNA Blood Mini	Qiagen	52304
RIPA Lysis & Extraction	Thermo Fisher Scientific	n/a

2.2 Equipment

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

Equipment's and other Services	Company / Distributor
Amersham™ Hybond™ Polyvinylidene difluoride (PVDF) Blotting Membrane (0.45 µm x 150) mm)	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, Japan
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 (37º 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 -20º C	Sanyo, Ultra low, Japan
Freezer -80º 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 37º C with 5% CO_2	Andor Technology Ltd, Belfast Heraeus,
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

2.3 *Ethical approval*

Ethical approval was obtained for the execution of this research study. Blood samples were collected by venipuncture after taking consent from healthy non-smoking volunteers and HNC patients.

For this study, the following organisations have provided approval of ethics 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).

2.3.1 Blood samples for study

After informed permission from HNC patients and healthy individuals, all peripheral blood was taken in 9 mL lithium heparin-coated tubes that were properly labelled and anonymized. Venipuncture was used to acquire whole blood from 20 healthy individuals and 20 HNC patients (see Table 2.3 and Table 2.4). Samples were diluted in a 1:1 ratio of RPMI 1640 medium supplemented with 10% dimethyl sulphoxide (DMSO) and aliquoted into Eppendorf tubes before being frozen at a controlled pace to temperatures below 50°C and stored at temperatures below 130°C for long-term storage.

Table 2.3 Brief information about healthy samples

No.	Sample code	Age	Ethnicity	Gender	Smoking history
1	2711181	33	Caucasian	F	N
2	2711182	34	Caucasian	M	N
3	280119	20	Caucasian	F	N
4	1902191	39	Caucasian	F	N
5	220119	21	Caucasian	M	N
6	2901191	20	Caucasian	M	N

7	2901192	21	Caucasian	M	N
8	2901193	22	Caucasian	M	N
9	180219	21	Caucasian	F	N
10	1902193	22	Caucasian	F	N
11	090319	24	Asian	F	N
12	160419	35	Asian	F	N
13	010319	37	Asian	M	N
14	110619	40	Asian	M	N
15	211119-a1	45	Asian	F	N
16	211119-a2	35	Asian	M	N
17	211119-a3	40	Asian	F	N
18	211119-a4	35	Asian	F	N
19	211119-a5	37	Asian	M	N
20	211119-a6	39	Asian	M	N

Table 2.4 **Brief information about H&N patients' samples**

No.	Sample code	Age	Ethnicity	Gender	Smoking history
1	T.R300119	53	Caucasian	F	N
2	B.N310119	68	Caucasian	M	N
3	S.D270319	42	Caucasian	M	Y
4	T.P180419	69	Caucasian	M	Y
5	S.R240419	49	Caucasian	M	Y
6	S.R030519	58	Caucasian	M	Y
7	K.R170519	77	Caucasian	M	Y
8	K.D170519	40	Caucasian	F	N
9	G.D240519	57	Caucasian	M	Y
10	H.P250719	52	Caucasian	F	Y
11	R.P260719	68	Asian	F	Y
12	D.P260719	66	Asian	M	Y
13	G.M130919	65	Asian	F	N
14	P.A011019	63	Asian	M	N
15	S.M160120-a	41	Asian	F	N
16	W.K160120-b	73	Asian	F	N
17	L.T160120-c	91	Asian	F	N
18	M,C230120	52	Asian	F	N
19	E.W290120	73	Asian	F	N
20	F,W130220	58	Asian	M	Y

2.4 Methods

2.4.1 The preparation of the pembrolizumab

In the current study, the pembrolizumab was reconstituted in the sterile distilled water to prepare the stock solution. The 10 µg/ml of naked pembrolizumab and 10 µg/ml of liposome were used in this study.

2.4.1.1 The preparation of the pembrolizumab liposomes

Liposomes were prepared using the thin film rehydration process. In Dichloromethane (DCM) and methanol (3:1 v/v), 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DPPG), 1,2-dioleoyl-sn-glycero-3-phsphoethanolamine (DOPE), and cholesterol (2:2:1 molar ratio) were dissolved. After that, the mixture was placed in a rotary evaporator with a 25ml round bottom flask. To ensure that no residues of the organic solvents remained, the solution was evaporated at 40 °C under vacuum for 6 hours.

The thin film was then hydrated for 1 hour at 60°C using a bath sonicator and 10 ml of pembrolizumab PBS solution (150 W). The sample was subjected to four freeze-thaw cycles (freezing at -20°C and thawing at 60°C in a bath sonicator). The sample was then centrifuged at 22,000 x g for 30 minutes at 4 °C to remove the non-encapsulated medication and purify it.

Dynamic light scattering (DLS) was used to measure the polydispersity index (PDI) and average size of the pembrolizumab liposome preparations using a Zetasizer Nano ZS-90, Model ZEN3600 (Malvern Instruments, UK). All measurements were carried out three times.

2.4.1.2 Determination of pembrolizumab encapsulation efficiency

The encapsulation effectiveness of pembrolizumab liposomes was assessed using an indirect method based on RP-HPLC (available to use and is not expensive) analysis of uncoated free pembrolizumab in the supernatant (Van Slooten et al. 2001; Haggag et al. 2019). The RP-HPLC was employed with UV detection (254 nm) and a flow rate of 1.0 ml per minutes (Phenomenex- Luna® C18-5 column mm, 55 µm). A mobile phase elution 60 gradient was utilized, using two solvent mixtures: solvent A (0.1

percent TFA in acetonitrile) and solvent B (0.1 percent TFA in water). The difference between the original amount of pembrolizumab added and the pembrolizumab left in the supernatant after liposome production was used to evaluate pembrolizumab encapsulation inside the liposome. Each sample was conducted three times, with pembrolizumab loading indicated as a percentage of encapsulation efficiency (% E. E.).

2.4.2 Transmission electron microscopy (TEM)

The TEM Tecnai 12 (FEI Company, Netherlands) was utilized to visualize the surface morphology of the pembrolizumab liposome for 10 minutes, carbon-coated copper TEM grids were immersed in pembrolizumab liposome solution. Distilled water was used to clean the grids. On the parafilm created in the petri dish, a drop of uranyl acetate was inserted. The grids were immersed in uranyl acetate for 2 minutes before the TEM was used to examine the form and size of the pembrolizumab liposomes.

2.4.3 The determination of H_2O_2 concentration

The best concentration of H_2O_2 was determined using a dose-response curve to act as a positive control (PC) that causes DNA damage without toxicity for the current study. Four concentrations of H_2O_2 were considered in this step: 25 µM, 50 µM, 75 µM and 100 µM. To assess the optimum concentration without toxicity, 15 individual experiments were carried out. Statistical analysis of the results showed that 75 µM caused a significant level of damage without inducing any toxicity.

2.4.3.1 Lymphocyte isolation from whole blood for cell viability

Both blood and saline were mixed in a 1:1 ratio where the percentage of saline was 0.9%. Using a falcon tube precise amount of 6 ml of diluted blood was placed carefully on the top of a 3 ml lymphoprep. The tube was put into a centrifuge for 20 min at 600

x *g*. After the tube centrifuged using the topmost surface of lymphoprep, the white layer of lymphocytes was gained in the plasma interface of the specimen and washed with 10 ml saline in a universal tube.

Subsequently, the suspension was placed in the centrifuge again at RT, 375 x *g* for 15 min, where the supernatant treated with 2% virkon before being disposed of. The pellet was left to resuspend in 90 µl of foetal bovine serum (FBS) and 100 µl of dimethyl sulfoxide (DMSO). Lastly, it ensured that all tubes were firmly shut, mixed, and kept overnight at a temperature of -20 °C before being moved to a temperature of -80 °C for an extended storage period.

2.5 Cell viability using CCK-8 kit

Cytotoxicity and cell viability of the drug were determined using a highly sensitive colorimetric assay. Lymphocytes from healthy individuals and HNC patients were isolated and cultured in 96-well plates ($10x10^4$ cells/well). The cells were treated with 75µM H_2O_2, blank liposome, 10 µg/ml of naked pembrolizumab and 10 µg/ml pembrolizumab liposome were added to cells and incubated at 37 °C in the presence of 5% CO2 for 24 hours. Following incubation, 10 µl of CCK-8 solution (supplied with kit) were supplemented to each well and incubated for another 4 hours under the same laboratory conditions. Absorbance was read at 450 mm using a plate reader.

2.5.1 The Comet Assay

Using the Comet Assay protocol, the range of damage in DNA was described and implemented according to the rules of the International Workgroup on Genotoxicity Testing (Tice et al. 2000). Before use, the blood samples thawed for a few minutes at room temperature.

2.5.1.1 Preparation of agarose-coated slides

In order to prepare agarose with a normal melting point (NMP) of 1% ultra-pure agarose (1.25 g) was weighed and mixed with 125 ml of distilled water. The solution was thoroughly mixed and microwaved until it was brought to a boil and became a clear solution. Finally, slides were immersed into the agarose and were kept to dry up on a tray overnight, then stored for further use.

2.5.1.2 Cell treatment

Different volumes for various concentrations of treatments which included a positive control of 75μM H_2O_2, blank liposome, 10 μg/ml of naked pembrolizumab and 10 μg/ml of pembrolizumab liposomes. Furthermore 10 μg/ml of naked pembrolizumab and 10 μg/ml of pembrolizumab liposomes were treated in the presence of 75μM H_2O_2 to examine the impact of pembrolizumab in a highly oxidising environment. One hundred microliters of whole blood and the appropriate volume of RPMI medium were combined in Eppendorf tubes to form 1000 μl, which were then incubated at 37 °C for 30 minutes.

Following the incubation period, the tubes were centrifuged at 160 *x g* for 3 minutes, resulting in the separation of 900 μl of supernatant. Using a 0.5 % low melting point (LMP) agarose, the residual cell suspension was mixed with 100 μl. After thoroughly mixing the cell mixture, 100 μl was placed on labelled slides coated with 1% normal melting point agarose (NMP) and a glass coverslip was applied. The agarose was allowed to cool for 5 minutes on ice.

2.5.1.3 Lysis

The coverslips were removed after the agarose set and put in newly prepared lysis buffer (10% DMSO, 10 mM Trizma base, and 1% Triton X-100, 100 mM EDTA, 2.5 M NaCl, at pH 10) overnight at 4 °C.

2.5.1.4 Electrophoresis

The slides were stored flat on the gel electrophoresis tank the next day, when they were incubated for 30 minutes in cold alkaline electrophoresis solution (60ml 10M NaOH allowing alkaline PH >13 and 10 ml 200mM EDTA) in the fridge. After that, electrophoresis was performed for another 30 minutes at the same parameters of 300 mA current and 25 Volts.

2.5.1.5 Neutralisation

Using a neutralisation buffer (0.4M Tris, pH 7.5), the slides were cleansed by the process of washing three times for 5 minutes each.

2.5.1.6 Staining

The slides were stained with 60 µl of ethidium bromide (20µg/ml) to visualise DNA, and the coverslips were placed.

2.5.1.7 Scoring of Slides

To score the slides, a CCD camera and a computer system equipped fluorescence microscope was used. In the process, the entire 100 cells were arranged randomly for each slide. To be able to analyse data, the measurement of two parameters of the Comet assay was conducted. These included Olive tail moment (OTM) and % Tail DNA using Komet 6 software.

2.5.1.8 Statistical Analysis

Two procedures were used to evaluate the data and ascertain significance values. These were one-way analysis of variance (one-way ANOVA) and two-way ANOVA.

The statistical analysis performed on healthy individuals and HNC patients, each having a sample size of 20 participants. In this research, the significant values considered having the p values $P<0.05$(*), $p<0.01$(**), $p<0.001$(***). The statistical analysis was carried out via GraphPad Prism 8.

2.5.2 Micronucleus Assay

The micronucleus assay was based on the detection of the micronucleus formation, which is used as a diagnostic biomarker for chromosomal DNA damage and therefore monitored by staining of DNA and nuclear membranes followed by microscopic analysis. Fresh blood was taken in heparinized tubes from HNC patients and healthy participants and utilized for cell cultures.

2.5.2.1 Preparation of cell culture media

Using a laminar flow hood, all of the stages were carried out under aseptic conditions. Each 25 cm^3 culture flask received 4.5 ml of the culture media RPMI 1640 with L-Glutamine and 25mM HEPES (concentrations of 1% and 15%, respectively) ,1 percent penicillin-streptomycin, and 15% foetal bovine serum) and was refrigerated at -20 °C for subsequent use. After that, the flasks were incubated at a temperature of 37 °C (with 5% CO_2).

and 25 mM HEPES and L-Glutamine with end concentrations of 15% and 1%, respectively

2.5.2.2 Blood culture and treatment

The primary medium was created by mixing 130 µl of phytohaemagglutinin (PHA) with 350 µl of fresh blood in flasks. The materials were gently mixed before being incubated for 24 hours at 37 °C with 5% CO2.

Following the 24-hours, each chemical test was introduced to the cultures in a 50 µl amount. The negative control (NC) was plain medium, whereas the positive control1 (PC1) was 0.4 M mitomycin C and the positive control 2 was 75 M H_2O_2 (PC2). In their respective flasks, 50 microliters of the blank liposome, 10 µg/ml of naked pembrolizumab, and 10 µg/ml of pembrolizumab liposomes were added, and the last two flasks included 10 µg/ml of naked pembrolizumab and 10 µg/ml of pembrolizumab liposomes with H_2O_2. After another 20 hours of incubation, 30 µl of cytochalasin B (cyt-B) (1 mg/ml) was added to each culture to prevent cytokinesis. The flasks were left under the same experimental settings for incubation.

After 72 hours of incubation, the flasks were opened and the contents were transferred to falcon tubes. It was no longer necessary to maintain sterile conditions. The supernatant was collected from each culture through a vacuum pump after centrifugation at 107 *x g* for 8 minutes. This was repeated until only 0.5 mL of supernatant remained. It was then followed by the addition of 5 ml of cold 90 mM KCl, which was gently agitated on a vortex to give the cells the hypotonic shock, and then incubation at 4 °C for 15 minutes to allow the cells to relax. The tubes were centrifuged again to remove the supernatant until only 0.5 ml remained.

2.5.2.3 Fixation steps

Freshly prepared Carnoy's solution (1-part glacial acetic acid and three parts methanol) was slowly added to each tube while softly mixed on a vortex. Then, three

drops of 38% formaldehyde were supplemented. Again, the washing steps carried out to ensure a clean cell pellet. The fixation stage repeated two more times but without adding formaldehyde. Tubes were left overnight in the fridge.

2.5.2.4 Preparation and staining of slides

The next day, tubes centrifuged to obtain approximately 100 µl of cells suspension. The cell density and pellet size adjusted by supplementing about 200-600 µl of Carnoy's solution. A volume of 20 µl of this suspension was dropped on pre-labelled glass slides and left to dry up. Once dried, using Giemsa solution (5% Giemsa solution made in phosphate buffer at pH 6.8), the slides were stained for 20 minutes and carefully washed in water for a couple of minutes. A few drops of DPX mountant placed, coverslips were applied and dried overnight at room temperature.

2.5.2.5 Micronuclei (MN) Evaluation

To evaluate the frequency of MN, several 1000 cells were grouped for each test group using a light microscope according to the criteria described by (Fenech 2007), The nuclear division index (NDI) is derived by counting the number of MN in 1000 binucleated nuclei (BiNC).

(M1 + 2(M2) + 3(M3) / N = NDI

M1 represents mononucleated (MonoNC) cells, M2 represents binucleated (BiNC) cells, M3 represents multinucleated (MultiNC) cells, and N is the number of viable cells scored (i.e., 1000 cells per treatment group). The NDI is a key variable in the MN assay, which displays how a test chemical affects the cell cycle of living cells. Its value is normally in the range of 1.0 to 2.0. If the cells were able to complete more than one

nuclear division and the MultiNC number increased while the MonoNC number decreased, it may potentially be larger than 2.0.

2.5.2.6 Analytical Statistics

Each experiment was carried out at least 5 times in five different people from both the healthy and HNC groups. On GraphPad Prism 8, data were analyzed using one-way analysis of variance (ANOVA) and two-way ANOVA.

2.5.3 Quantitative Reverse Transcription PCR (RT-qPCR)

2.5.3.1 Cell culture:

Whole blood samples from HNC patients and healthy individuals were used to isolating the lymphocyte. The isolated lymphocytes 2 x 10^6 cells/ml from both groups were cultured in 6-well plates for 24 hours at 37°C in the presence of 5% CO_2 after being treated with 10 µg/ml of naked pembrolizumab and 10 µg/ml of pembrolizumab liposomes and NC.

2.5.3.2 RNA extraction from human lymphocytes

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

The supernatant was discarded, and 350 µl of RLT buffer, made by mixing 10 µl of B-mercaptoethanol with 1 ml RLT buffer, was added to the pellets and centrifuged at maximum speed for 2 minutes. The lysate was then placed in a 2 ml collection tube and centrifuged for 2 minutes at maximum speed using a QIAshredder spin column. A total of 600 µl of 70% ethanol was pipetted into the lysate and well stirred. The pipetted sample was put on a fresh QIAamp spin column and spun for 15 seconds at 8000 *x g* in a 2 ml collecting tube. The QIAamp spin column was inserted in a fresh 2

ml collection tube, rinsed with 700 l of RW1 Buffer, and centrifuged at 8000 x g for 15s. The QIAamp spin column was placed in a fresh 2 ml collection tube, rinsed twice with 500 µl RPE Buffer, and centrifuged for 3 minutes at 8000 *x g*. Finally, the QIAamp spin column was put in a fresh 1.5ml microcentrifuge tube, and 50l of RNase-free water was poured directly in the center of the QIAamp membrane and centrifuged at 8000 *x g* for 1 minute.

The BioDropTM Touch Duo Spectrophotometer (BioDrop Ltd, Cambridge, UK) detects UV light absorbance and computes the 260/280 ratio, which is used to determine RNA purity.

The cDNA was synthesized using an RNA A260/A280 ratio in the 1.8-2.1 range. The RNA samples used for cDNA synthesis were stored at -80 ° C

2.5.3.3 Complementary DNA synthesis

In 20 µl reaction volumes, the iScript cDNA Synthesis Kit (Bio-Rad) was used to synthesize cDNA from total RNA by combining the following components: In PCR tubes, combine 4 µl of 5xiScript Reaction mix, 1ul of iScript Reverse Transcriptase, and 1g of frozen extracted RNA with RNase-free water. The PCR reactions were 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 in a Bio-Rad PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The cDNA samples were kept at -20°C until they were employed in the next processes.

2.5.3.4 The quantitative real-time PCR method

The real-time PCR reaction was set up in 96 PCR reaction plates and sealed with MicroAmp® Optical 8-Cap Strips once the cDNA was synthesized.

The overall volume of the real-time PCR experiment was 20 µl, with 2 µl of primer for the target genes TP53, CDKN1A (p21), Bcl-2, and β-Actin, 10 µl of SYBR® Green Supermix, 2 µl of cDNA, and 6 µl of RNase/DNase-free water (Qiagen, UK). For the normalization of the reaction, β-actin was employed as a housekeeping gene. To eliminate any bubbles and settle the solution at the bottom of the plates, the 96 well plates were spun in a plate spinner. The plates were then put in the StepOnePlus Real-time PCR Detection System (Applied Biosystems, Warrington, UK) with the following qRT-PCR thermal conditions: Denaturation for 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing, and extension for 1 minute at 60°C. Finally, a melting curve of 95°C for 15 seconds, 60 °C for 15 seconds, and 95 °C for 15 seconds. To limit variance in the results, the tests were repeated three times.

2.5.3.5 Data analysis

The gene expression levels were determined using CFX Manager™ Software and normalized against the housekeeping gene/reference (β-Actin). The 2-ΔΔCT was utilized to assess differences in fold-change of gene expression between untreated cells from HNC patients and healthy volunteers and treated lymphocytes with 10 µg/ml naked pembrolizumab and 10 µg/ml pembrolizumab liposomes, according to (Schmittgen and Livak 2008).

2.5.3.6 Statistical analysis

Each experiment was carried out three times in three different individuals from both the healthy and HNC groups, and the data were given as the mean SEM. GraphPad Prism 8. software with built-in two-way ANOVA and Tukey's multiple comparisons test was used to analyse the data.

2.5.4 Western blot assay

2.5.4.1 Cell culture

The lymphocytes were isolated using peripheral blood (10 ml) from HNC patients and healthy participants. Under sterile circumstances, lymphocyte pellets containing $1x10^6$ cells were cultured in six-well plates with RPMI 1640 medium with Glutamax-I, 15% fetal bovine serum (FBS), and 1% penicillin-streptomycin solution. The cultures were also given 10 µg/ml of naked pembrolizumab and 10 µg/ml of pembrolizumab liposomes as a final concentration. For 24 hours, the cultures were incubated at 37°C in the presence of 5% CO2 in the air. The NC was cultured without any treatment.

2.5.4.2 Protein extraction

Each well's culture medium was transferred to Eppendorf Tubes® and centrifuged for 3 minutes at 400 *x g*.

The lymphocyte pellets were washed twice with cold PBS after the supernatant was discarded. The pellets were then re-suspended in 100 µl of RIPA buffer with the protease inhibitor cocktail added to the cells, which were then incubated on ice for 30 minutes.

The cell suspension was then centrifuged for 30 minutes at 14,243 *x g*, with the supernatant collected into additional labelled microcentrifuge tubes. 5 µl of extracted protein was collected from each tube for protein quantification using the Bradford assay, and the remaining cell lysates were kept at -80ºC.

2.5.4.3 Protein quantification

The total protein concentration was measured using a Bio-Rad Bradford assay kit. The test is based on an absorbance shift in the dye Coomassie (red colour) when it binds to the protein, which changes to Coomassie blue. The highest absorbance at 595 nm, which is proportional to the quantity of bound dye and consequently to the concentration of protein present in the sample, gives the distinctive blue colour. A standard curve was created using a variety of various quantities of bovine serum albumin (BSA) (0.125, 0.250, 0.5, 0.750, 1.00, 1.5, and 2 mg/mL). In triplicate, 5 µl of each unknown sample and standard were pipetted into independent wells of 96 well plates, followed by 250 µl of diluted Bio-Rad dye reagent. The plates were shaken for 30 seconds before being incubated for 5 minutes at room temperature. At 595 nm, the optical densities of BSA and unknown materials were measured.

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

For the resolving and stacking gels, pH 6.8 and pH 8.8 Tris buffers were used. For polyacrylamide gel polymerization, the catalysts APS and TEMED were used, as well as a final concentration of 10.4 % SDS.

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 µl10% APS and 16 µl TEMED) was used at the bottom and poured between two glass plates to a level of 1 cm below the teeth on the comb.

One millilitre dH_2O was added to the top of the resolving gel and poured between two glass plates to a level of 1 cm below the teeth on the comb. After the gel had set, the dH_2O was drained out. After loading, a pH 6.8 stacking gel solution (5.3 ml ddH2O, 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 poured on top of the solidified separating gel to pack proteins

together, and the comb was inserted. For around 45 minutes, the gel could be left at room temperature. The comb was then taken away. While the gels were setting, the protein samples were made by combining 30 µg of protein with 5 µL BoltTM LDS Sample Buffer (4X), 2 µL BoltTM Reducing Agent (10X), and deionized water to make a total volume of 20 µL, which was then boiled in boiling water for 5 minutes. After boiling, the samples were centrifuged for 10 seconds. The samples were added per well after the gels were soaked in electrophoresis buffer (25 mM Tris base, 192 mM glycine, and 0.1 % (w/v) SDS) in the running tank. One lane of protein ladder (Precision Plus Protein TM Dual Colour Standards) was included on each gel. To prevent the material from spilling out of the lane, each sample was loaded slowly and carefully.

The electrophoresis was performed using the Power Pack TM Basic (Bio Rad, UK), with the gel being passed gently through the polyacrylamide gel (50 Volts) for 30 minutes, yielding distinct and crisp bands, and then at 100V (continuous voltage) for 1hr 30 minutes. The end of the electrophoresis separation procedure was determined by monitoring the prestained protein ladder.

2.5.4.5 Transfer to membrane

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

2.5.4.6 Blocking

After transfer, the nitrocellulose membranes were incubated for one hour at room temperature with moderate shaking in a blocking solution comprising 5% (w/v) BSA in Tris-buffered saline containing Tween 20 (TBS-T) (150 mM NaCl, 20 mM Tris base,

and 0.1 percent (v/v) Tween 20, pH 7.4). TBS-T was used to wash the membranes three times for a total of 10 minutes.

2.5.4.7 Primary antibody

After that, a primary antibody was added to the solution that could attach to a specific protein. The primary antibody was incubated overnight at 4°C with shaking on the blotting membranes. As a loading control, GAPDH rabbit monoclonal primary antibody was employed. A monoclonal antibody was chosen because it produces a stronger signal, has a higher specificity, and has a smaller background. The primary antibodies were diluted in TBS-T containing 5% (w/v) BSA and included [GAPDH (1:1000 dilution), p53 (1: 1,000 dilution), P21 (1: 1,000 dilution), and BCL-2 (1: 1,000 dilution). TBS-T was used to wash the membranes three times for a total of 10 minutes.

2.5.4.8 Secondary antibody

Anti-Rabbit IgG H&L (HRP) was used as a secondary antibody to locate where the main antibody attached. In TBS-T solution, the secondary antibody was diluted 1:2000 with 2.5 percent (w/v) BSA. For one hour, the membrane was incubated at room temperature on a shaker. TBS-T was used to wash the membrane three times for 15 minutes each time.

2.5.4.9 Detection

The membrane was stained with enhanced chemiluminescence (ECL) kit solutions by incubating it for 1 minute at room temperature with the equal quantity of ECL solution 1 and 2. On GB Box, the membrane was installed (Gene flow, UK). Images were taken with the intention of analysing them afterwards.

2.5.4.10 Data Analysis

Image j software was used to calculate the protein's relative expression. Protein loading was controlled using GAPDH.

The results were provided as the mean ± SEM of three separate tests using isolated lymphocytes from three healthy individuals and three HNC patients. The data were analysed using GraphPad Prism® software 8, which included a built-in two-way ANOVA and Dunnett's post hoc test for significant differences, to determine the significance between the different treatment doses.

CHAPTER THREE: The Comet assay in naked pembrolizumab, and pembrolizumab liposome treated-lymphocytes

3 Comet Assay

3.1 Introduction

The programmed cell death protein 1 (PD1) is one of the checkpoints that regulates the immune response. Ligation of PD1 with its ligands PDL1 and PDL2 results in transduction of negative signals to T-cells. PD1 expression is an important mechanism contributing to the exhausted effector T-cell phenotype. The expression of PD1 on effector T-cells and PDL1 on neoplastic cells enables tumour cells to evade anti-tumour immunity. Blockade of PD1 is an important immunotherapeutic strategy for cancers. Pembrolizumab (Keytruda) is a humanized monoclonal anti-PD1 antibody that has been extensively investigated in numerous malignancies. In melanoma refractory to targeted therapy, pembrolizumab induced overall response rates (ORRs) of 21-34%. (Mehra et al. 2018; McCusker et al. 2020).

Unlike chemotherapy and radiation therapy, treatment with pembrolizumab represents a form of immunotherapy, meaning it functions alongside the patient's immune system to overcome the cancer. In addition, pembrolizumab has not affect T lymphocyte antigen (Toor et al. 2018). Pembrolizumab has been shown to reduce both the spread and invasion of lung cancer and skin cancer (melanoma or Merkel cell carcinoma) (Hamid et al. 2019; Robert et al. 2019), and it is used in the UK as a monotherapy for patients with NSCLC. It is the first line of therapy for patients with a tumour proportion score (TPS) of at least 50%, while for those with a TPS of at least 1%, it is a following line of therapy (Hui et al. 2017). In a study conducted by Barbara B et al. (2022) concluded Increased efficacy of pembrolizumab or pembrolizumab-chemotherapy with increasing PD-L1 expression in case of HNSCC. Although PD-L1 expression is informative, exploration of additional predictive biomarkers is needed for low PD-L1-expressing HNSCC.

In a previous study conducted by Huang et al. (2020) found a significant exponential reduction in the occurrence of non-small cell lung cancer for patients taking pembrolizumab. The average duration of survival for patients treated with pembrolizumab was currently 26.3 months, compared to the 14.2 months for patients receiving chemotherapy-based treatment. Meanwhile, the overall survival rate after 36 months is 43.7% for patients receiving pembrolizumab, which is significantly higher than the 24.9% 36-month survival rate for patients undergoing chemotherapy (Huang et al. 2020; Reck et al. 2021). Pembrolizumab plus chemotherapy had improved overall survival (OS) and progression-free survival (PFS) in patients with metastatic squamous NSCLC (Paz-Ares et al. 2020).

The last two decades have seen the development of techniques to detect and assess DNA damage. Where comet assay and the single-cell gel electrophoresis assay was

a method designed to identify and measure DNA damage occurring in individual cells, such as sperm cells and lymphocytes; the aim was to detect double-strand breaks (Azqueta and Collins 2013; Wu and Jones 2012).

The current experimental study examined the effect of the pembrolizumab alone and pembrolizumab liposome to assess their anti-tumour potential against H&N carcinoma by measuring the DNA damage as well as DNA integrity in human blood lymphocyte cells by using the Comet assay

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 2.5.1 .

3.2 Comet Assay Results

3.2.1 Encapsulation Efficiency determination of pembrolizumab liposome

The detection of the pembrolizumab was carried out using RP-HPLC, and the retention time of pembrolizumab was 1:5 minutes as shown in the chromatogram (Figure 3-1).To measure the encapsulation efficiency of the liposomes, they were centrifuged for 30 minutes at 324 g at 4 ℃.The Liposomes size was in nano-meter. Which requires a high speed in order to be able to separate the liposomes vesicle from the solution. The supernatant was then injected in the HPLC. The encapsulation efficiency was calculated from the amount of free drug pembrolizumab in the supernatant. A calibration curve of the peak area of pembrolizumab vs. concentration in the range of 10 µg/ml to 80 µg/ml was produced. The regression equation of the line was y = 900.88X+10172 with a correlation coefficient R^2 = 0.9949 as presented in Figure 1.1. The mobile phase used: Isocratic 70% Water 30% Acetonitrile and 0.1% Formic Acid at a flow rate of 1ml/min. The peak was detected at 1.5minutes.

The encapsulation efficiency was determined by measuring the free pembrolizumab after centrifuging the sample. Area under the peak for the sample was 16601. Therefore, the concentration was: Concentration= (16601-10172)/900.88 = 7.13 µg/ml

Total concentration= 1000 µg/ml

Encapsulated Liposomes= 1000 -7.13=992.87 µg/ml

Encapsulation Efficiency= $Total$ pembrolizumab $-free$ pembrolizumab $/Total$ pembrolizumab $*$ 100

(992.87/1000) *100% = 99.3%

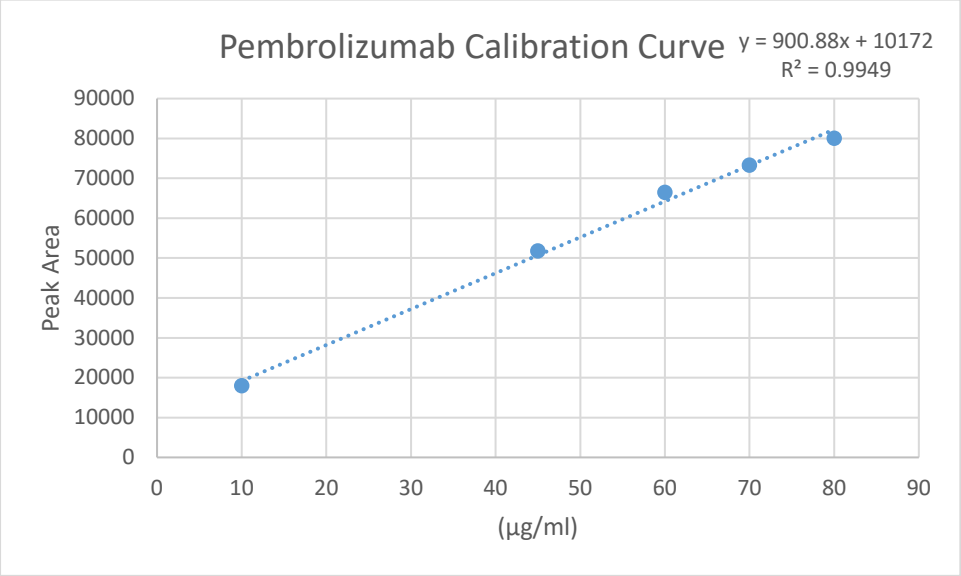


Figure 3-2 Standard calibration curve of pembrolizumab.

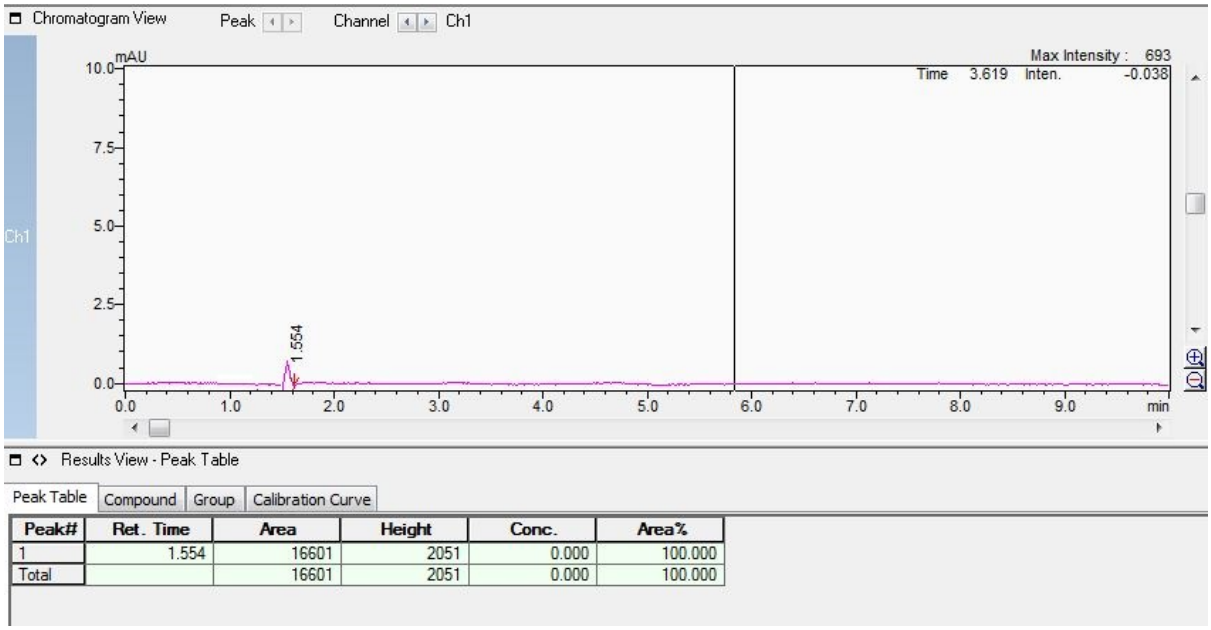


Figure 3-3 HPLC calibration curve chromatogram of free drug pembrolizumab peak identified at 1.5 minutes.

*Liposomes were prepared in PBS. Several Blanks were run before the injection of Pembrolizumab to make sure there was no interference in the peaks. Blanks were: Mobile phase, dH_2O, PBS, Methanol.

3.2.2 Particle size determination of pembrolizumab liposome

The thin-film rehydration method was used to encapsulate pembrolizumab due to its simplicity, practicability, reproducibility, and ability to produce small and uniform liposomes. Figure 3.3 shows the Z-Average particle size was 116.1 nm with PDI 0.227 (Standard error ± 5.0023). 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 pembrolizumab 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 3.4.

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 116.1	Peak 1:	148.4	98.8	86.00
PdI: 0.227	Peak 2:	4865	1.2	691.1
Intercept: 0.958	Peak 3:	0.000	0.0	0.000
Result quality : Good				

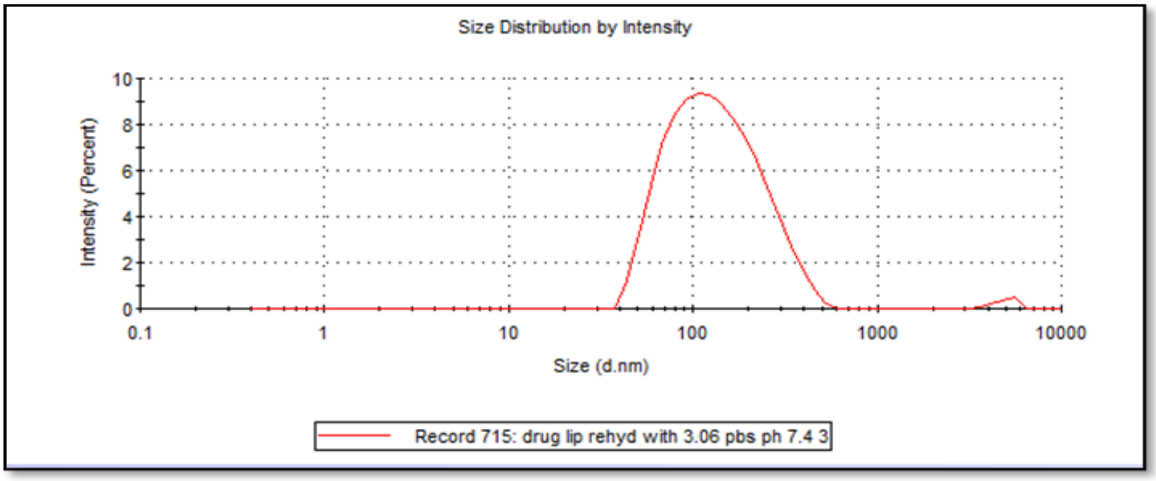


Figure 3-4 Measurement of particle size -distributon by DLS using Zetasizer Nano ZS-90 Model ZEN3600 (Malvern Instruments Ltd, UK).

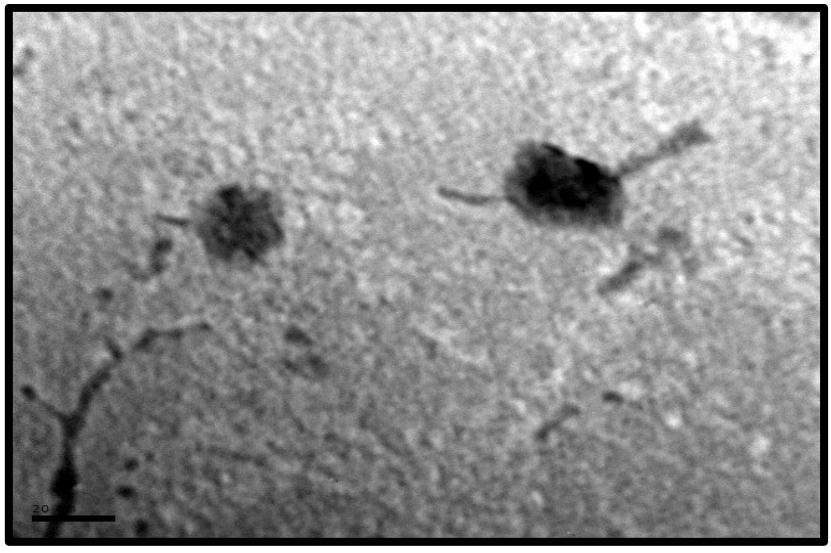


Figure 3-5 TEM image of pembrolizumab liposome (200 k magnification)

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

The viability of lymphocytes from 3 healthy individuals and 3 HNC patients treated with different treatment groups was also confirmed using the CCK-8 assay > 75% after 24 hours treatment Figure 3.5. % Cell survival = $Absorbance\ (Treated\ Cells)/Absorbance\ of\ Control\ (Medium\ only)$ x 100.

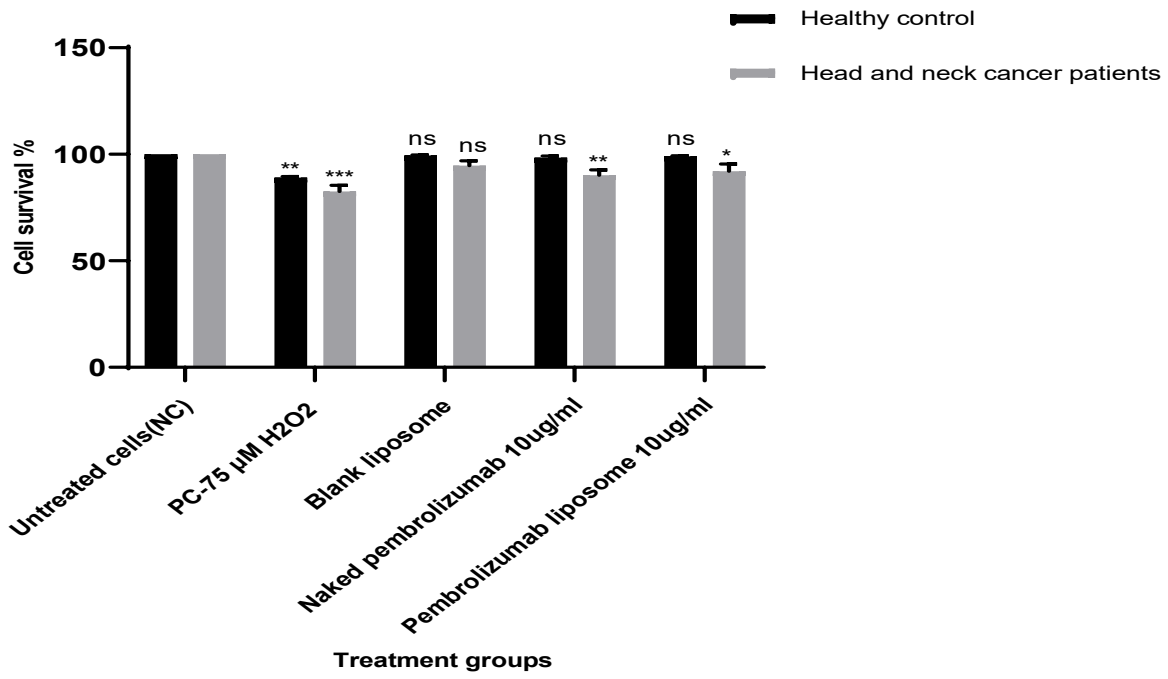


Figure 3-6 The % survival in 1 × 10^4 cells of various test treatments in lymphocytes from healthy individuals and HNC patients using CCK8 assay. Error bars show mean ±SEM, n=3. *p<0.05; ** p <0.01; ***p<0.001; ns = not significant. All the results for various treated groups were compared against the 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.

3.2.4 Comparison between the effect of naked and liposome pembrolizumab in lymphocytes from healthy individuals

Isolated lymphocytes from healthy individuals were treated with different treatment groups using the Comet assay to measuring the % DNA tail and OTM.

Results from % tail DNA showed that the positive control (PC) group 75 µM of H_2O_2 has caused a significant level of DNA damage however, blank liposome, naked pembrolizumab and pembrolizumab liposome groups have shown no significant difference compared to the untreated control.

Hydrogen peroxide 75 µM co-administrated with naked pembrolizumab and pembrolizumab liposome individually, has demonstrated a significant reduction in level of DNA damage compared to the PC group alone (***$p<0.001$).

Whereas pembrolizumab liposome has demonstrated better results. The results suggest that both forms of pembrolizumab do not cause DNA damage in healthy lymphocytes at basal levels. Figure 3.6 and Figure 3.7.

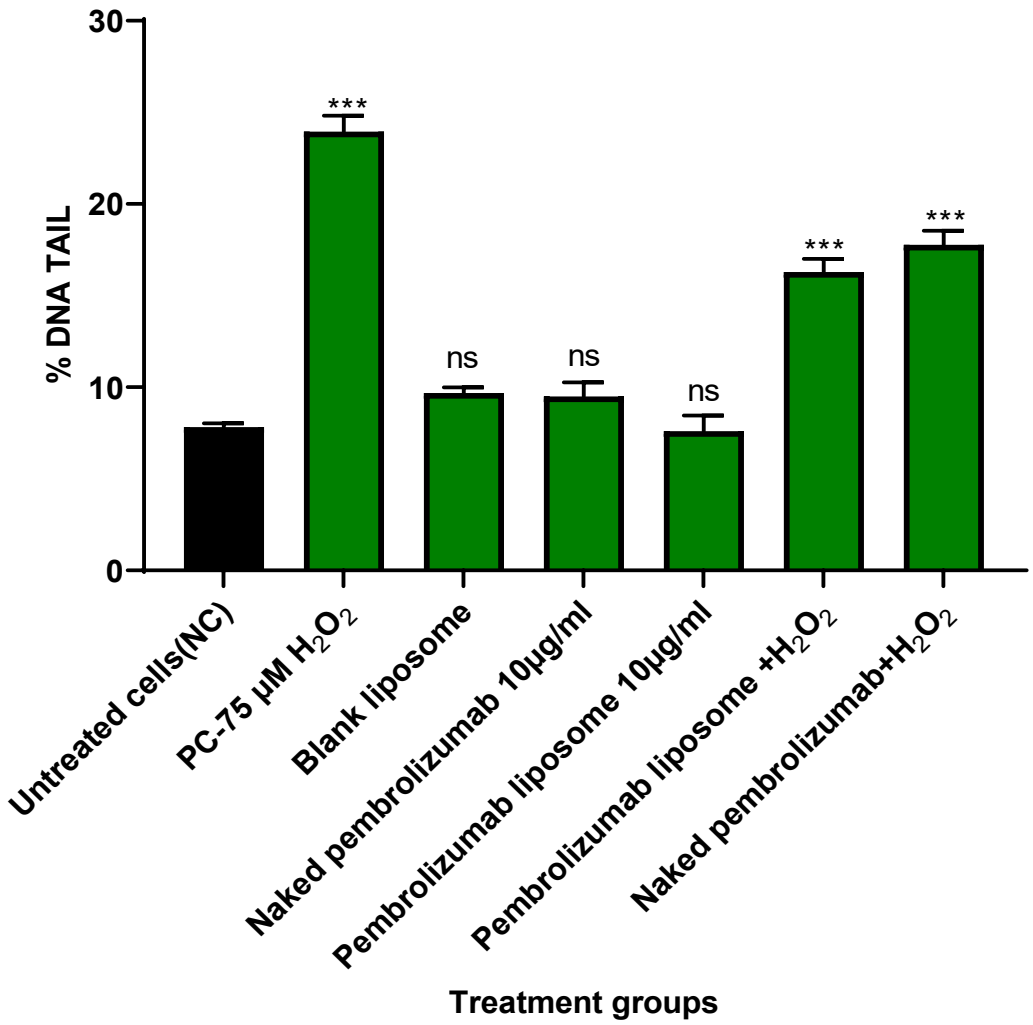


Figure 3-7 Showing the comparison between the effect of naked and liposome pembrolizumab in lymphocytes from healthy individuals using % tail DNA(n=20). Seven different treatment groups including an untreated lymphocyte group (NC), positive control (PC) 75µM H_2O_2, blank liposome, naked pembrolizumab 10µg/ml, pembrolizumab liposome 10µg/ml, naked pembrolizumab 10µg/ml +75µM H_2O_2, and liposome 10µg/ml +75µM H_2O_2. All treatment groups were compared against the negative control. The statistics show (ns=not significant, ***p<0.001).

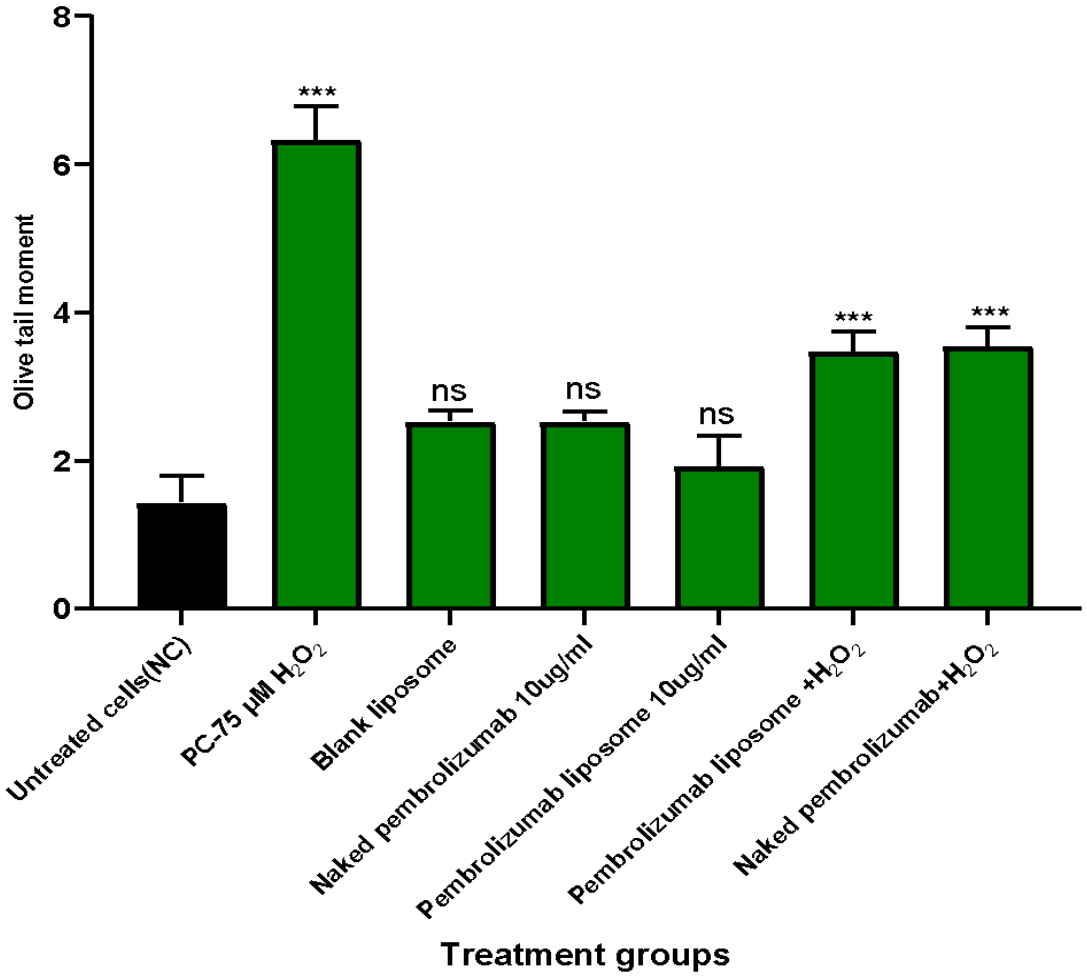


Figure 3-8 Showing the comparison between the effect of naked and liposome pembrolizumab in lymphocytes from healthy individuals using OTM. Seven different treatment groups including an untreated lymphocyte group (NC), positive control (PC) 75µM H_2O_2, blank liposome, naked pembrolizumab 10µg/ml, pembrolizumab liposome 10µg/ml, naked pembrolizumab 10µg/ml +75µM H_2O_2, and liposome 10µg/ml +75µM H_2O_2. All treatment groups were compared against the negative control. The statistics show (ns=not significant, ***p<0.001).

3.2.5 Comparison between the effect of naked and liposome pembrolizumab in lymphocytes from HNC patients.

Isolated lymphocytes from HNC patients were treated with different treatment groups using the Comet assay. Results from % tail DNA showed that the positive control (PC) group 75 µM of H_2O_2 has caused a significant level of DNA damage (***p<0.001). However, blank liposome and naked pembrolizumab have shown no significant difference compared to the untreated control. Pembrolizumab liposome (10µg/ml) has indicated a significant reduction in DNA damage comparable to the negative control in

% DNA tail and OTM (**p< 0.01); ***p<0.001) Respectively. Co-administrated of naked pembrolizumab and pembrolizumab liposome with 75 µM of H_2O_2, individually has also demonstrated a significant decrease in level of DNA damage compared to the PC group alone about (*p<0.05; ***p<0.001) in % DNA tail and OTM Respectively.

The results suggest that both forms of pembrolizumab do not cause DNA damage in lymphocytes from HNC patients at basal levels. In fact, pembrolizumab liposome has reduced this damage and also has been shown more H_2O_2 effective against the induced damage in patient group Figure 3.9.

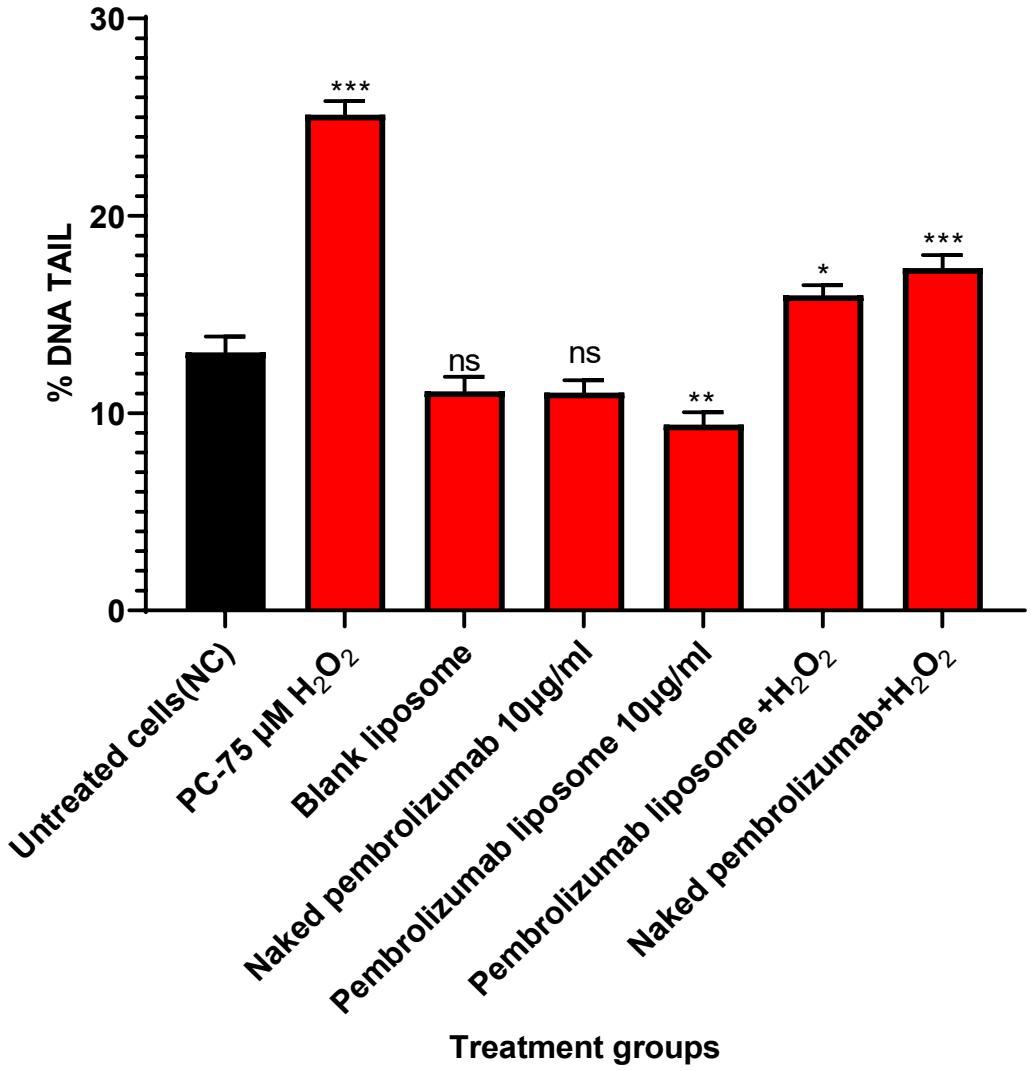


Figure 3-9 Showing the comparison between the effect of naked and liposome pembrolizumab in lymphocytes from HNC patients using % tail DNA(n=20). Seven different treatment groups including an untreated lymphocyte group (NC), positive control (PC) 75µM H_2O_2, blank liposome, naked pembrolizumab 10µg/ml, pembrolizumab liposome 10µg/ml, naked pembrolizumab 10µg/ml +75µM H_2O_2, and liposome 10µg/ml +75µM H_2O_2. All treatment groups were compared against the negative control. The statistics show (ns=not significant, ***p<0.001, **p=0.01, *p=0.05).

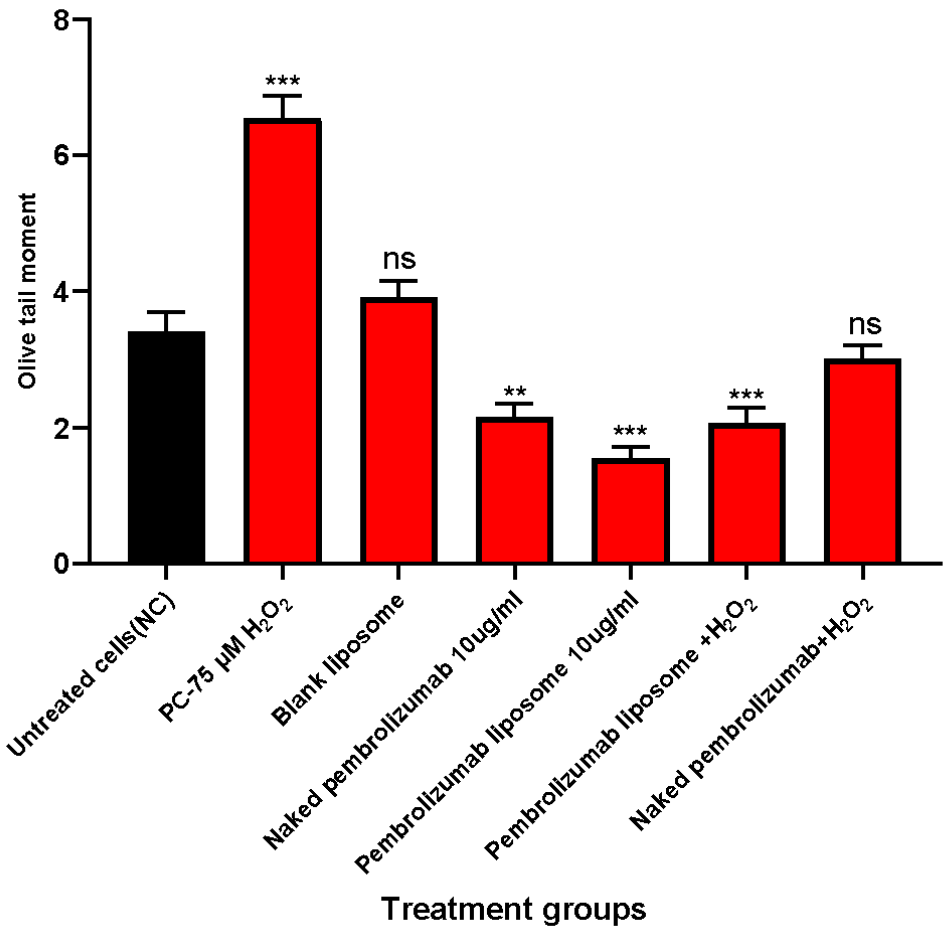


Figure 3-10 Showing the comparison between the effect of naked and liposome pembrolizumab in lymphocytes from HNC patients using OTM. Seven different treatment groups including an untreated lymphocyte group (NC), positive control (PC) 75µM H_2O_2, blank liposome, naked pembrolizumab 10µg/ml, pembrolizumab liposome 10µg/ml, naked pembrolizumab 10µg/ml +75µM H_2O_2, and liposome 10µg/ml +75µM H_2O_2. All treatment groups were compared against the negative control. The statistics show (ns=not significant, ***p<0.001, **p=0.01).

Table 3.1 The mean values of 20 experiments on blood samples from healthy individuals and HNC patients expressed as OTM and % DNA tail, including the statistical significance and SEM.

	Healthy individuals	**Healthy individuals**	**HNC patients**	**HNC patients**
	% DNA tail ± SEM	Mean OTM ± SEM	% DNA tail ± SEM	Mean OTM ± SEM
Untreated cells (NC)	7.826±0.19	1.44±0.34	13.07±0.78	3.417±0.27
PC-75 µM H_2O_2	23.95±0.85	6.335±0.44	25.13±0.67	6.55±0.32
Blank liposome	9.686±0.29	2.53±0.14	11.1±0.72	3.918±0.23
Naked pembrolizumab 10µg/ml	9.497±0.74	2.535±0.13	11.05±0.6	2.157±0.19
Pembrolizumab liposome10µg/ml	7.599±0.82	1.925±0.39	9.42±0.61	1.547±0.17
Pembrolizumab liposome +H_2O_2	16.29±0.69	3.479±0.25	15.97±0.5	2.07±0.21
Naked pembrolizumab+H_2O_2	17.77±0.74	3.549±0.25	17.35±0.64	3.019±0.18

3.2.6 Comparison between the effect of naked and pembrolizumab liposome in lymphocytes from healthy individuals versus those from HNC patients.

In vitro treatment of isolated lymphocytes from healthy individuals and HNC patients treated with different treatment groups has demonstrated similar results. Where very less damage in lymphocytes of healthy individuals compared to lymphocytes of cancer patients was observed, to healthy patient's lymphocytes compared to lymphocytes of cancer patients was observed. Untreated patient's lymphocytes have shown more basal DNA damage compared to the ones from healthy individuals in % Tail DNA and OTM (***$p<0.001$). Hydrogen per oxide (75 µM) has induced maximum level of damage in both investigative groups (***$p<0.001$).

Blank liposomes and naked pembrolizumab (10µg/ml) had minor effect on the DNA damage in lymphocytes from both group compared to pembrolizumab liposome (10µg/ml) demonstrated significant decrease in the DNA damage in % Tail DNA and OTM (**$p<0.01$, ***$p<0.001$) respectively.

In contrast, pembrolizumab liposome made more reduction of the DNA damage compared to the naked pembrolizumab, as presented in Table 3.1. Lymphocytes from head and neck patient cancer showed significant decrease in % tail DNA and OTM from 13.07% (% DNA tail) and 3.417 (OTM) compared to the untreated control groups and 11.05% (% tail DNA), and 2.157 (OTM), respectively, when patient cells were treated with naked pembrolizumab. In addition, pembrolizumab liposome showed significant decrease in % DNA tail and OTM 9.42% and 1.547 (OTM).

Pembrolizumab in both forms has significantly attenuated the adverse effects of H_2O_2 in both investigative groups, whereas pembrolizumab liposome has been proved more effective particularly in lymphocytes from patient group. Both parameters of the comet

assay; OTM and % tail DNA have shown similar results (Figure 3.10,Figure 3.11 and Table 3.2).

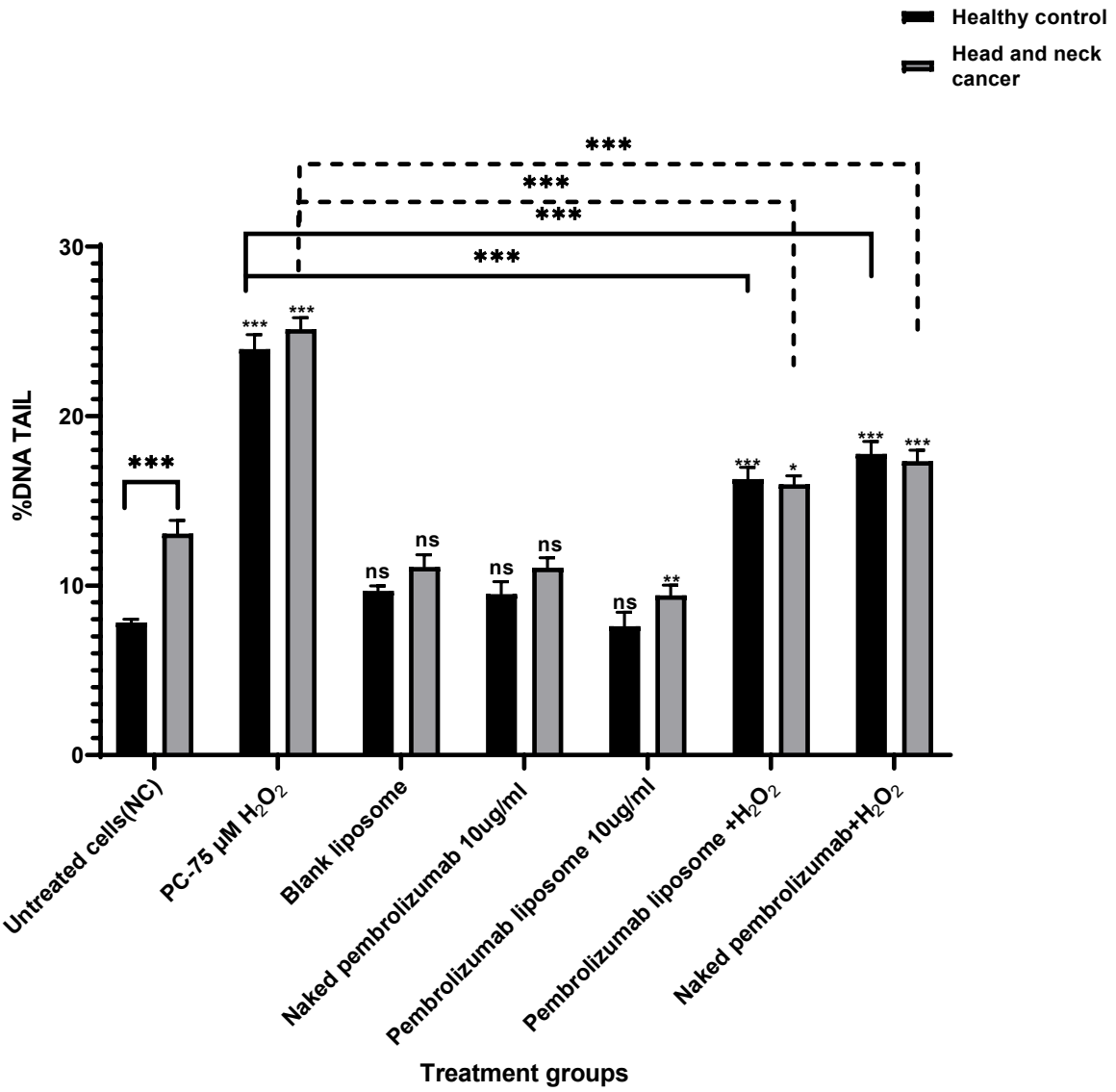


Figure 3-11 Showing the comparison between the effect of naked and liposome pembrolizumab in lymphocytes from HNC patients and healthy individuals (n=20) using % Tail DNA. Seven different treatment groups including an untreated lymphocyte group (NC), positive control (PC) 75µM H_2O_2, blank liposome, naked pembrolizumab 10µg/ml, pembrolizumab liposome 10µg/ml, naked pembrolizumab 10µg/ml +75µM H_2O_2, and liposome 10µg/ml +75µM H_2O_2. All treatment groups were compared against the negative control. Each treatment group from both groups was also compared against each other, represented by the horizontal lines on the graph and the statistics on top of these lines. The statistics show (ns=not significant, ***p<0.001, **p<0.01, *p<0.05).

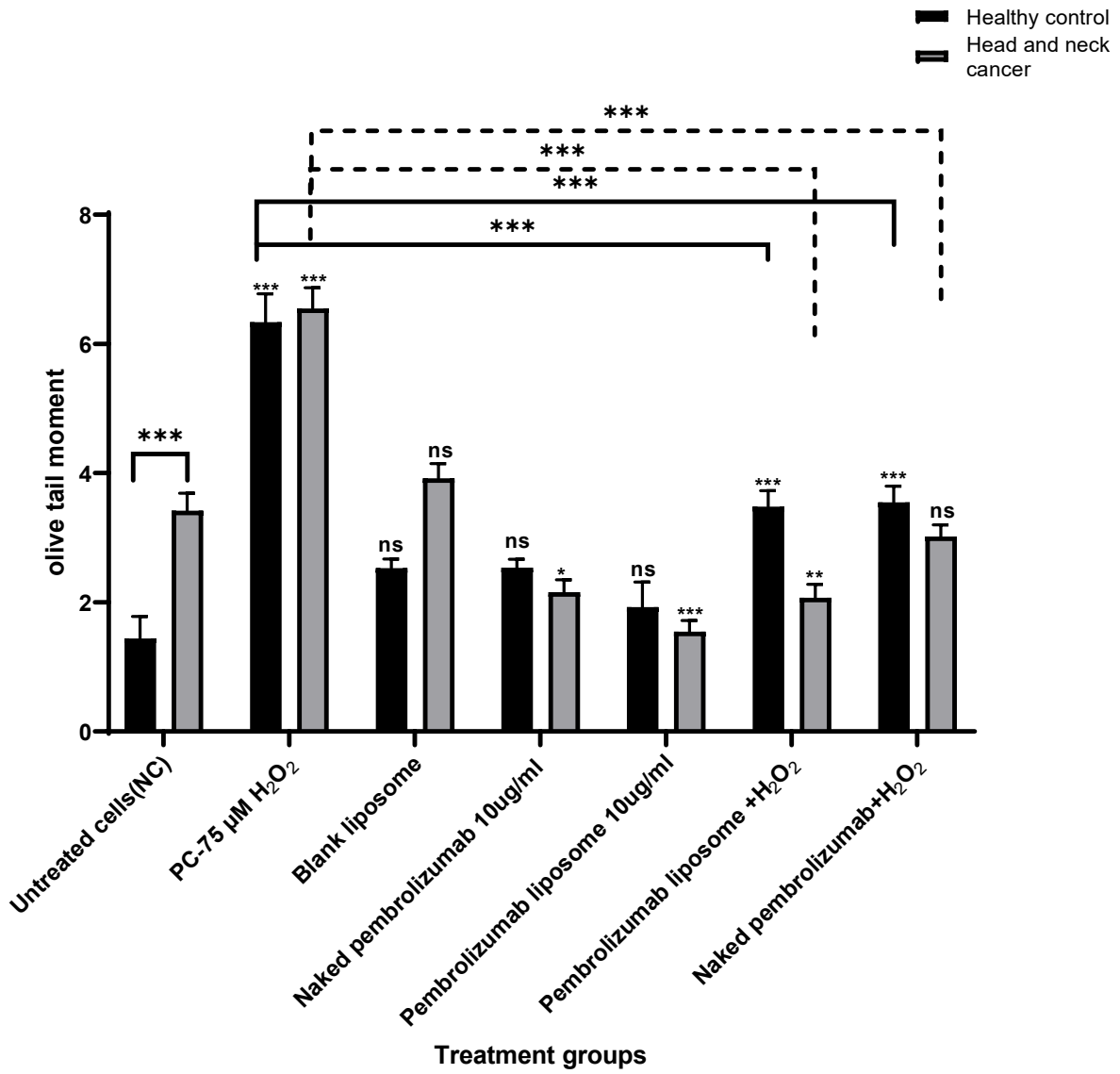


Figure 3-12 Showing the comparison between the effect of naked and liposome pembrolizumab in lymphocytes from HNC patients and healthy individuals using OTM. Seven different treatment groups including an untreated lymphocyte group (NC), positive control (PC) 75µM H_2O_2, blank liposome, naked pembrolizumab 10µg/ml, pembrolizumab liposome 10µg/ml, naked pembrolizumab 10µg/ml +75µM H_2O_2, and liposome 10µg/ml +75µM H_2O_2. All treatment groups were compared against the negative control. Each treatment group from both groups was also compared against each other, represented by the horizontal lines on the graph and the statistics on top of these lines. The statistics show (ns=not significant, ***p<0.001, **p<0.01, *p<0.05).

Table 3.2 The means of Olive tail moment (OTM) in the peripheral blood lymphocytes of different various confounding factors in (A) healthy individuals, (B) HNC patients in the Comet assay after different treatments with the PC (H_2O_2) (75µM), naked pembrolizumab 10 µg/ml and pembrolizumab liposome 10 µg/ml forms as well as the negative control of untreated lymphocytes (NC). Data show the mean ± SEM. 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 cell)	PC (H_2O_2 75µM)	Naked pembrolizumab 10 µg/ml	Pembrolizumab liposome 10 µg/ml
Age	Healthy individuals 20-45	1.44 ± 0.340	6.33 ± 0.44 ***	2.535 ± 0.13 ^{ns}	1.45 ± 0.39 ^{ns}
	Patients >40	3.417 ± 0.27	6.55 ± 0.32***	2.15 ± 0.19 ^{ns}	1.92 ± 0.17 ^{ns}
Smoking History	Non-smoking Healthy individuals	1.44 ± 0.340	6.33 ± 0.44 ***	2.53 ± 0.13 ^{ns}	1.45 ± 0.39 ^{ns}
	Non-smoking Patients	2.49± 0.1	6.61 ± 2.1 ***	1.81 ± 0.2 ^{ns}	1.46 ± 0.7^{ns}
	Smoking patients	4.17 ± 1.5	7.02 ± 2.0 ***	2.55 ± 1.1 ^{ns}	1.58 ± 0.4 ^{ns}
Ethnicity	Caucasian Healthy individuals	1.13 ± 0.3	6.15 ± 1.3 ***	2.46 ± 0.7 ^{ns}	1.63 ± 0.1 ^{ns}
	Caucasian patients	1.30 ± 0.3	6.5 ± 2.0***	2.52 ± 1.2^{ns}	1.7± 0.5 ^{ns}
	Asian Healthy individuals	1.023 ± 0.5	6.23 ± 1.2***	2.48 ± 0.5 ^{ns}	1.43 ± 0.9 ^{ns}
	Asian patients	1.37 ± 1.1	6.7 ± 0.5***	2.54 ± 1.2 ^{ns}	1.74 ± 1.9 ^{ns}
Gender	Female Healthy individuals	1.45 ± 0.2	3.6 ±1.3***	1.28 ±0.4^{ns}	1.09 ± 0.5 ^{ns}
	Male Healthy individuals	1.19 ± 0.6	3.95 ± 1.3 ***	1.87 ±0.1^{ns}	1.07 ± 0.8 ^{ns}
	Female Patients	1.78 ± 0.6	5.45 ± 1.2***	1.29 ± 1.0^{ns}	1.16 ± 0.4^{ns}
	Male Patients	1.9 ± 1.2	4.7 ± 1.4***	1.88 ± 1.0 ^{ns}	1.49 ± 0.8^{ns}

3.2.7 Confounding factors

Confounding factors such as age, gender, ethnicity, lifestyle (smoking, drinking, diet), and environmental conditions are crucial when assessing DNA damage. They are all potential contributors to DNA damage and may cause genome toxicity (Fenech 2007).

In this study, we evaluated the effect of pembrolizumab naked and pembrolizumab liposomes on the level of DNA damage in lymphocytes that taken from healthy and cancer patients by using the comet assay Table 3.2.

In the current study, the results suggest that both forms of pembrolizumab do not cause DNA damage in lymphocytes from HNC patients at basal levels compared with the negative group (NC). Both forms of pembrolizumab showed a significant decrease in oxidative stress of H_2O_2 in lymphocytes from HNC patients compared with the positive group (PC). Pembrolizumab liposome showed more effectiveness in reducing DNA damage.

3.2.7.1 Age

The age between the healthy individual group and patient group differed considerably.

At the time of study initiation, the best possible match samples available (especially from the patient group) varied in age significantly between the healthy subject group and the cancer subject group. Most healthy individuals were young compared with cancer patients who were all above 50. In the patient group, there may be some contribution to DNA damage in lymphocytes, observed at basal levels.

Within samples, the difference in DNA damage between the untreated controls of both groups (healthy and patient groups) was highly significant (***$p < 0.001$). This could be due to the appearance of cancer in the patient group. However, this difference could also be attributed to age of patients (Soares et al. 2014).

No significant difference was found on the DNA damage levels between different age groups after treated with naked pembrolizumab 10 µg/ml and pembrolizumab liposome 10 µg/ml forms to reduce the DNA damage.

3.2.7.2 Gender

Gender had no influence on the level of DNA damage in lymphocytes in response to various treatments, and in different study groups and these results are disagreeing with a previous study because the limited number of participated in this study (Hoang et al. 2012).

3.2.7.3 Smoking habits

There were no smokers in the healthy subject group; however, there was a mix of smokers and non-smokers in the patient group. To remove the artefact for this, when analysing the effect of smoking, the patient group was compared with the non-smoking control group. Due to smoking and the disease conditions in HNC cancer patients, all patients showed a higher level of DNA damage at basal levels (Cit-10), and these results are agreeing with a previous study (Beynon et al. 2018; Johnson et al. 2020a).

However, naked pembrolizumab 10 ug/ml and pembrolizumab liposome 10 µg/ml forms have a tendency to decline this damage. The PC control has produced significantly DNA damage in all groups as expected.

3.2.7.4 Ethnicity

There were no statistically significant ethnic differences between the healthy subjects and cancer patients.

3.3 Discussion

PD-1 blockade therapy has revalorized the treatment of recurrent (R/M HNSCC) cancer and, in comparison to chemotherapy alone, has been shown to have good overall response rates and enhanced survival outcomes (Desilets and Soulières 2020). Pembrolizumab has shown to be effective in the treatment of pembrolizumab -

refractory metastatic melanoma. It also has a generally good toxicity profile (Long et al. 2019).

Liposomes are considered as a potentially suitable potential drug delivery system that can target certain tumours. With their small vesicles, they can evade the vasculature of the tumour cells, accumulating within them via passive targeting. Due to its various attributes including short life in the circulatory system, systemic toxicity and the inability to distinguish between healthy cells and tumours, pembrolizumab has been loaded onto liposomes to enhance its pharmacokinetics.

In this study, the film hydration method was applied to entrap pembrolizumab into liposomes with high efficiencies in a small vesicle size of 116.1 nm. The polydispersity index values of liposomes that were obtained by the thin-film hydration method are 0.227 with narrow size distribution. These results are in agreement with Sriwongsitanont and Ueno 2010, who reported that the thin-film rehydration method followed by freeze-thaw cycle could be applied to the production of homogeneously sized liposomes (Sriwongsitanont and Ueno 2010).

Liposome formation depends upon the extent of location of the drug and its physiochemical characteristics and composition. For the deliverance of necessary drug molecules to the site of action, the lipid bilayers fuse with other bilayers of the cell (cell membrane) to release the liposomal content. The TEM showed that pembrolizumab liposomes have homogeneous spherical-shaped liposomes and a smooth surface.

Furthermore, RP- HPLC method has been used for quantification with a concentration of encapsulated pembrolizumab which was 10 µg/ml. It showed that the liposomal formulation has (99.3%) EE of pembrolizumab. In this study, the efficacy of naked

pembrolizumab and pembrolizumab liposome on lymphocytes in the human body was examined. The lymphocytes were taken from 20 HNC patients and 20 healthy individuals and were identified using the Comet assay.

Its strong inhibitory effect has also been evidenced on a number of tumour models, including breast cancer (Nanda et al. 2020; Ivashko and Kolesar 2016). Based on the findings of several in vivo studies, pembrolizumab together with PD-1 blockade offers new treatment approaches for recurrent or (R/M HNSCC) cancers, showing stronger response and survival rates than treatment based solely on chemotherapy (Desilets and Soulières 2020).

Thus far, no study has used Comet assay to examine how pembrolizumab influences DNA damage in the lymphocytes of HNC patients. The current work used Comet assay because it has a high sensitivity for assessing the integrity of cell DNA while also being time- and cost-efficient (Gopalan et al. 2011). Additionally to monitor the effects of antioxidants and DNA repair in maintaining genome stability.

The Comet assay has the ability to detect and evaluate the hazards and risks of occupational or environmental exposure to toxins. It can also identify diseases that are derived from oxidative stress, such as cardiovascular disease and diabetes, and inadequate nutrition. Finally, it enables clinicians to evaluate the efficacy of ongoing therapies and examine potential variation among individual patients concerning damage to their DNA that might be due to environmental or genetic factors. This test not only allows patients to receive tailored advice on how they can adjust their lifestyles and habits to improve their health status, but it also informs them on whether they are at risk of genotoxicity due to pollutants in the environment (Dusinska and Collins 2008).

Finally, the alkaline Comet assay offers significant benefits as it is one of the main screening methods when assessing drug-DNA interactions using in vitro experiments, especially in determining the mechanisms of action during new drug development (Afanasieva and Sivolob 2018).

Not only are peripheral lymphocytes a suitable model, but they can also serve as surrogate cells during genotoxicity assessment. Circulating throughout the bloodstream, lymphocytes are furthermore susceptible to DNA damage due to physiochemical genotoxic effects, both endogenous and exogenous (Najafzadeh et al. 2011).

This *in vitro* experiment involved lymphocytes that were frozen after being taken from the study participants, who included both healthy individuals and HNC patients. The lymphocytes were thawed and then treated with 10µg/ml naked and pembrolizumab liposome individually for 30 minutes at a temperature of 37°C. H_2O_2 (75 µM) was used as Positive Control (PC).

Following the treatment, we scored the lymphocytes regarding OTM and % DNA tail. Based on the Comet assay results, the lymphocytes taken from the healthy participants and treated with naked pembrolizumab and pembrolizumab liposome revealed no significant damage to the DNA in comparison to the untreated cells (NC). Both forms showed significantly reduced DNA damage when compared with the positive control(***$p<0.001$). However, the same concentration of pembrolizumab in both forms decreased the DNA damage in HNC patients' lymphocytes when compared to the untreated cells Figure 3.10 and Figure 3.11.

These results are consistent with those in (Kinter et al. 2008; Hsu et al ,2015; Zhang et al, 2020b; Han et al 2020) who report that pembrolizumab has the ability to bind

with receptors PD-L1 and PD-L2 on the surface of lymphocytes, followed by the inactivation of cells. This in turn causes a reduction in cytokine production by PD-1, which causes cancer cell death in the presence of a non-self-antigen and reduces DNA damage. Since not all PBMC express PD-1 it would be suggested to pre-screen patients for the presence or absence of the receptor prior to targeted therapy. The Comet assay results showed that naked and liposome pembrolizumab have a protecting influence against H_2O_2, which causes oxidative stress in healthy individuals' lymphocytes and HNC patients' lymphocytes. This result is consistent with the funding with Arnold et al, (2005) that liposome fusion within cell membrane of lymphocytes acts as an antibody and antioxidant against any damage to DNA. Yang et al,2016 reported the disclosure a new approach for direct drug administration into the cytoplasm of living cells *in vitro* and *in vivo* that used targeted membrane fusion between liposome and living cells. Where a pair of complementary coiled-coil lipopeptides were enclosed in the lipid bilayer of liposomes and cell membranes, resulting in the targeted membrane fusion and release of liposome-encapsulated cargo, and crossing of the membrane permeability barrier into the cell.

Furthermore, the result showed a significant decrease in DNA damage compared with PC. Both naked and liposome pembrolizumab treatments showed a significant decline in DNA damage in lymphocytes of HNC patients (***$p<0.001$, **$p=0.01$).

These results show that Naked pembrolizumab and liposome pembrolizumab have a protective influence against DNA in human lymphocytes obtained from healthy individuals and HNC patients. And they show the importance of pembrolizumab in liposome form in inducing DNA repair, especially with homogeneous surface of liposome and size of liposome. We can suggest that both forms of pembrolizumab will enhance of chemotherapy and cancer treatment and reduce the toxicity on the normal

cells; Moreover, reduce the oxidative stress that causes the DNA damage, impairment of chromosomes and mutation. (Azevedo et al ,2011) reported that DNA damage is assessed by single cell gel electrophoresis, in which the chromosomal DNA migration distance corresponds with the level of DNA damage, to identify background DNA damage and oxidative damage resulting from hydrogen peroxide

DNA repair was detected and measured as a decrease in comet tail length (OMT) with time following oxidative stress removal, a process adequately characterised by a tail length kinetics model. We further demonstrate that the antioxidant medicine (pembrolizumab) protects against DNA damage, as seen by a threefold decrease in the length of comet tails. The potential of assessing DNA damage and repair in lymphocytes has opened up new paths for investigating genotoxicity and DNA repair in the future by employing novel approaches to detect the cells' reaction and resistance to therapy.

This study further demonstrated that none of the healthy volunteers – 100% of whom did not smoke –presented confounding effects from smoking.

The age-related confounding effect on DNA damage among the healthy versus patient groups showed significant variation. While this was linked to the sample limitations during the experiment, the result presented that DNA damage in patients with HNC in age ≥50 group is higher than in (20-40) the healthy group. A previous study showed that damage to the DNA relates to age (Chen et al. 2020b).

Following the treatment of the lymphocytes of groups with naked and liposome pembrolizumab forms, statistically no significant differences were found among the different age groups. Because the possible match samples available (especially from the patient group) varied in age significantly between the healthy subject group and

the cancer subject group. Most healthy individuals were young compared with cancer patients who were all above 50. Because the healthy samples were obtained from student and our friends so that the most of doners were young, while the most of patient more 50 years.

4 CHAPTER FOUR: Anti genotoxic effects of naked pembrolizumab and liposome treatments in peripheral lymphocytes using micronucleus assay

4 Micronucleus assay

4.1 Introduction

The experimental results for the drug pembrolizumab (both liposome and naked types) presented in the previous chapter demonstrated that these compounds have the potential to reduce DNA damage. This was evidenced by the significant OTM and % DNA tail decreases observed in the comet assay, which shows the compounds' effects at the genomic level. While both naked and liposome pembrolizumab were able to reduce DNA damage but the effect for the liposomes was stronger. However, using a comet assay only shows the effects of these compounds at the genomic level.

Based on these results, there is a need for a different technique, namely one that can examine the effects at the cytogenetic level. Therefore, the current chapter presents the results of the cytokinesis-block micronucleus (CBMN) micronucleus assay, which examines the effects of both compounds via somatic cells, i.e. lymphocytes. CBMN assay is easy method to score MN and get accurate information on nuclear bud (NBUDs) and nucleoplasmic bridge (NPB) formation, that could be used to determine DNA damage caused by environmental agents (Cho et al. 2020).

The cell growth cycle refers to a set of crucial processes that enable cells to proliferate into daughter cells through DNA replication and eventually reach apoptosis upon reaching senescence, i.e. undergo programmed cell death. In eukaryotic cells, this cycle occurs every 24 hours and comprises four stages. These are the gap 1 phase (G1 phase), the DNA synthesis phase (S phase), the gap 2 phase (G2 phase), and the mitotic phase (M phase) (Bertoli et al. 2013; Ansari et al. 2018; Gadre et al. 2020; Vermeulen et al. 2003; Kim et al. 2012).

This study used the CBMN assay to assess the induction of MNi following exposure to monoclonal antibody-PD-L1 (pembrolizumab; naked and liposome). The dose for naked and liposome pembrolizumab was 10 µg/ml, and 0.4 µM mitomycin C was used as a positive control 2. The occurrences of MNi, NBPs, and NBUDs were scored for up to 1000 BiNC, while 1000 other cells were scored to determine the percentages of each cell type's MonoNC, BiNC, and MultiNC. The three cell types' scoring proportions were subsequently used to estimate the nuclear division index (NDI), which reflects the cytostatic effects, allowing the mitotic division rate to be measured (Fenech 2007).

4.2 Materials and Methods

All materials and equipment used on this assay are presented in (Table 2.1and Table 2.2) and all methods for this technique described in chapter 2, section 2.5.2.

4.3 Results

Lymphocytes from HNC patients and healthy individuals were treated *in vitro* with various treatment groups to determine the frequency of micronuclei (MNi), a marker of DNA damage. The drug concentrations used for these experiments were the

optimum doses, chosen from the concentration response curve with no cytotoxicity induction.

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

The Figure 4.1 illustrated the concentration-response of both 10µg /ml naked pembrolizumab and pembrolizumab liposome from healthy individuals.

Using the ANOVA test, both forms of pembrolizumab showed no significant (ns) change in healthy lymphocyte MNi frequency compared to the untreated cells.

In contrast, 75 µM H_2O_2 and 0.4 µM MMC (PC) significantly increased MNi frequency ***$p \leq 0.001$ compared to the untreated cells.

Moreover, both forms of naked and liposome pembrolizumab with 75 µM H_2O_2 decreased MNi frequency compared to the PC (75 µM H_2O_2).

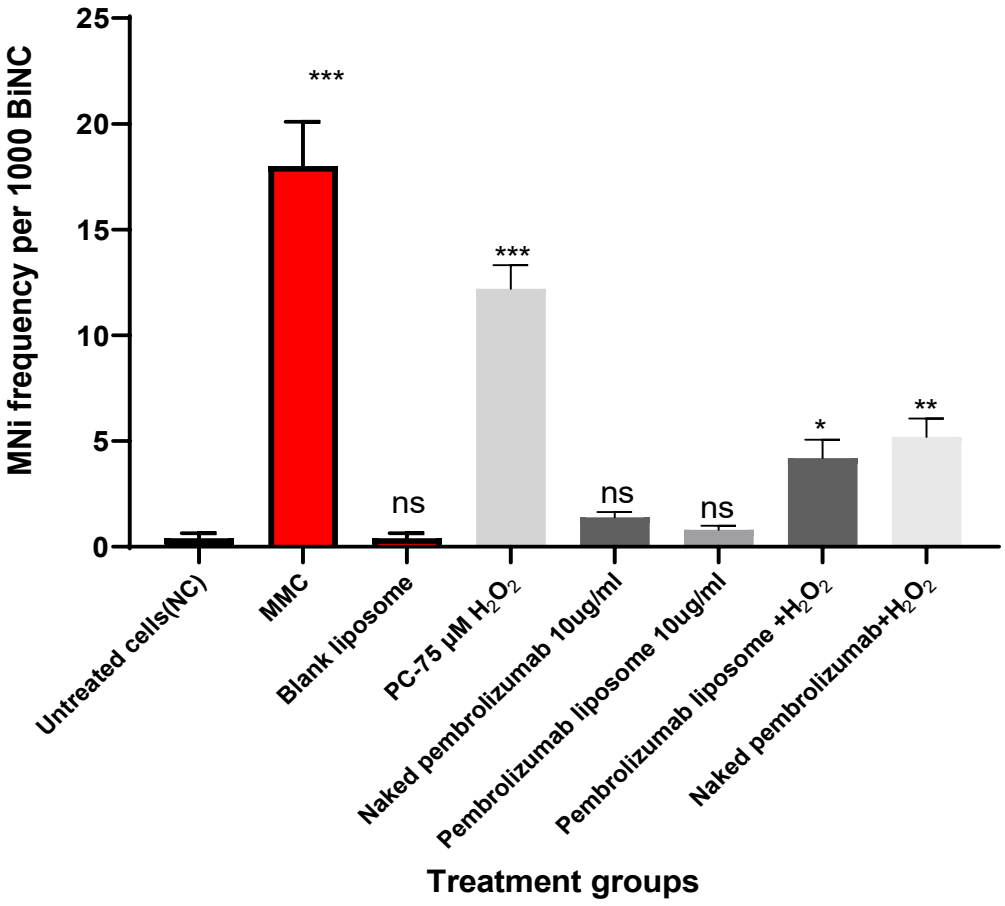


Figure 4-1 The average of BiNC MNi scored per 1000 cells from 5 healthy individual experiments. Data are expressed as means ±SEM. Eight treatment groups included the negative control, a positive control group (PC) 75µM H_2O_2 another positive control (Mitomycin C {0.4µM MMC}), naked pembrolizumab 10µM+75µM H_2O_2, pembrolizumab liposome 10µM+75µM H_2O_2, blank liposome, naked and liposome pembrolizumab. (*p<0.01, **p<0.001 ***p<0.0001, ns=not significant).

4.3.2 Naked pembrolizumab and pembrolizumab liposome concentration-response on MNi induction in lymphocytes from HNC patients

The Figure 4.2 represents the concentration-response of 10µg /ml naked and liposome pembrolizumab in lymphocytes from HNC patients to induce MNi in BiNC.

The graph shows that the pembrolizumab in both forms significantly reduced the frequency of MNi in lymphocytes of HNC *in vitro* compared with untreated cells. However, the pembrolizumab liposome showed a more significant reduction in the frequency of MNi in patients` lymphocytes than in the naked form.

By using the ANOVA test, results showed a significant decline ***p<0.001 in MNi for pembrolizumab liposome and ** p<0.01 for naked form, compared to the untreated cells. In addition, both 0.4 μM MMC and 75 μM H_2O_2 showed a significant reduction in MNi ***p ≤ 0.001 compared to the untreated cells.

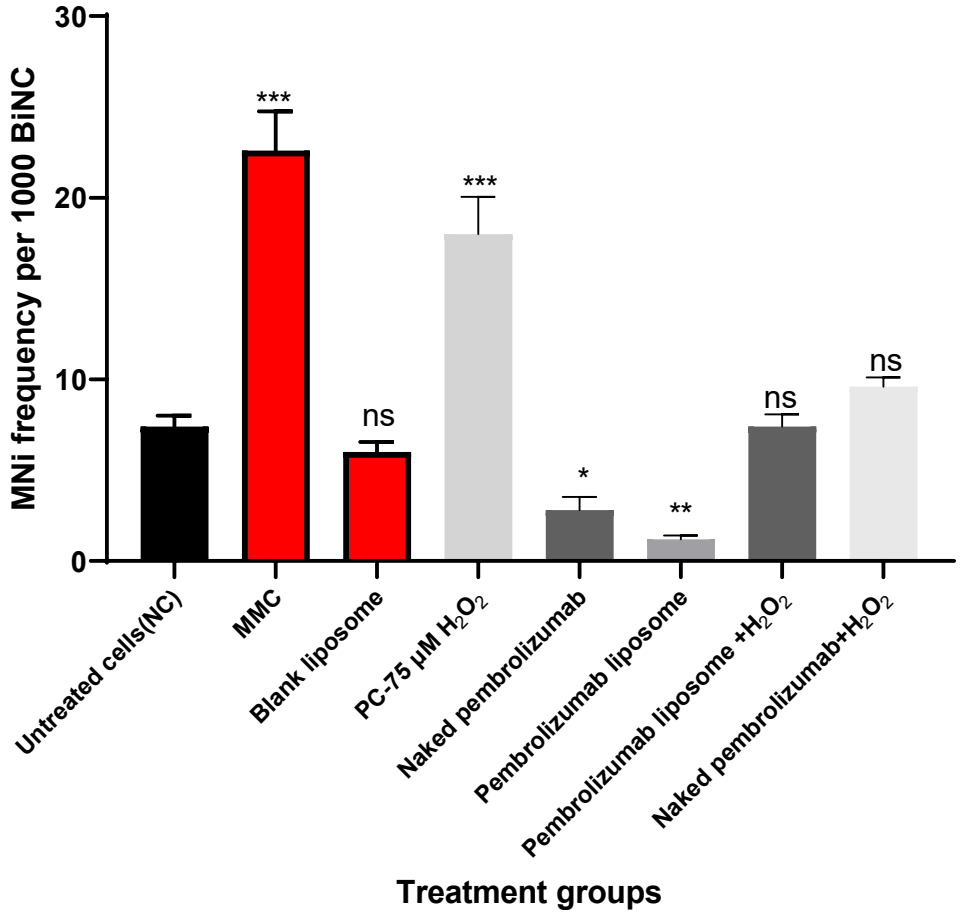


Figure 4-2 The average of BiNC MNi scored per 1000 cells from 5 HNC patient experiments. Data are expressed as means ± SEM. Eight treatment groups included the negative control, a positive control group (PC) 75μM H_2O_2 another positive control (Mitomycin C {0.4μM MMC}), naked pembrolizumab 10μM+75μM H_2O_2, pembrolizumab liposome 10μM+75μM H_2O_2, blank liposome, naked and liposome pembrolizumab. (*p<0.01, **p<0.001 ***p<0.0001, ns=not significant).

4.3.3 MNi in binucleated cells (BiNC)

Overall, the number of MNi in BiNC from HNC patients was way more significant than healthy individuals in respective untreated groups. Both forms of pembrolizumab naked and liposome have induced a non-significant increase in the number of MNi in

binucleated cells of healthy volunteers and a significant decrease in MNi frequency in binucleated from HNC patients when compared to their respective negative control groups. The positive control groups, 0.4 µM mitomycin (MMC) and 75 µM of H_2O_2 have induced a significant number of MNi in BiNC in both the healthy and patient groups as expected. However, co-administrated naked and liposome pembrolizumab effectively decreased the number of H_2O_2-induced MNi in BiNC of healthy individuals and patient groups. Regardless of the groups, naked pembrolizumab significantly decreased the MNi number in binucleated cells, whereas liposome pembrolizumab has demonstrated better effects in reducing the chromosomal damage at basal levels. Lastly, it was concluded that pembrolizumab 10 µg/ml was more effective in bringing this protective effect Figure 4.3.

4.3.4 Other determining factors of MN assay

The frequency of MNi in mononucleated cells from patients` lymphocytes was higher than those from healthy individuals. However, this frequency was significantly decreased upon treatment with pembrolizumab liposome (10 µg/ml) in the patient's group at a basal level. There was no significant effect on the induction of nuclear bridges and buds in binucleated cells of either group (Table 4.1).

Table 4.1 Showing various markers of chromosomal damage using micronucleus assay**.**

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	1.91±0.03	60.6±1.6	16.3 ±1.2	0.4±0.2*	0	0	0
	75 µM H_2O_2	1.96±0.03	60.6±0.4	18.4±1.56	12.2±1.1	0.2±0.2	0	6.2±1.6
	0.4 µM MMC	1.88±0.1	59.2±2.4	15.6±3.8	18±2.1	0	0	8.4±1.8
	Naked pembrolizumab	1.87±0.02	58.8±0.6	14.2±1.2	1.4±0.24	0	0	1.2±0.4
	Blank liposome	1.93±0.04	60.2±1.68	17±1.51	0.4±0.24	0	0	0
	Pembrolizumab liposome	1.98±0.03	61.6±0.4	19.6±1.4	0.8 ±0.2	0	0	0.6±0.4
	Pembrolizumab liposome+H_2O_2	1.96±0.01	59±2.3	19.9±2.7	4.2±0.9	0	0	3.4±1.3
	Naked pembrolizumab+ H_2O_2	1.92±0.1	59.6±1.9	17.6±4.5	5.2±0.9	0	0	4.6±1.4
HNC patients	Untreated lymphocytes	1.90±0.02	58.4±2.2	16.4±1.3	7.4±0.5*	0.2±0.2	0	3.4±0.5
	75 µM H_2O_2	1.89±0.02	56.2 ±2.3	18±1.8	18±1.8	0.80±0.3	0	6.4±0.9
	0.4 µM MMC	1.93±0.02	57.8±2.4	22±1.9	22.6±1.9	1.8±0.7	0.2+0.2	9.2 ±1.6
	Naked pembrolizumab	1.97±0.03	59.8±0.4	19.1±1.4	2.8 ±0.7	0	0	3.4 ±0.4
	Blank liposome	1.91±0.02	60.8±0.8	15.2±1.42	6.0 ±0.5	0.2±0.2	0	3.0 ±0.4
	Pembrolizumab liposome	1.87±0.04	57.8±1.2	15.2±2.2	1.2±0.2	0	0	1.8±0.2
	Pembrolizumab liposome+H_2O_2	1.99±0.03	58.6±2.18	21±2.6	7.4±0.6	0	0	4.0 ±0.7
	Naked pembrolizumab+ H_2O_2	1.98±0.03	61±1.3	18.6±1.6	9.6±0.5	0	0	5.2 ±1.3

* Significantly different between healthy cells BiMNi compered to patient cells when evaluated in per 1000 cell (p = o.ooo1)

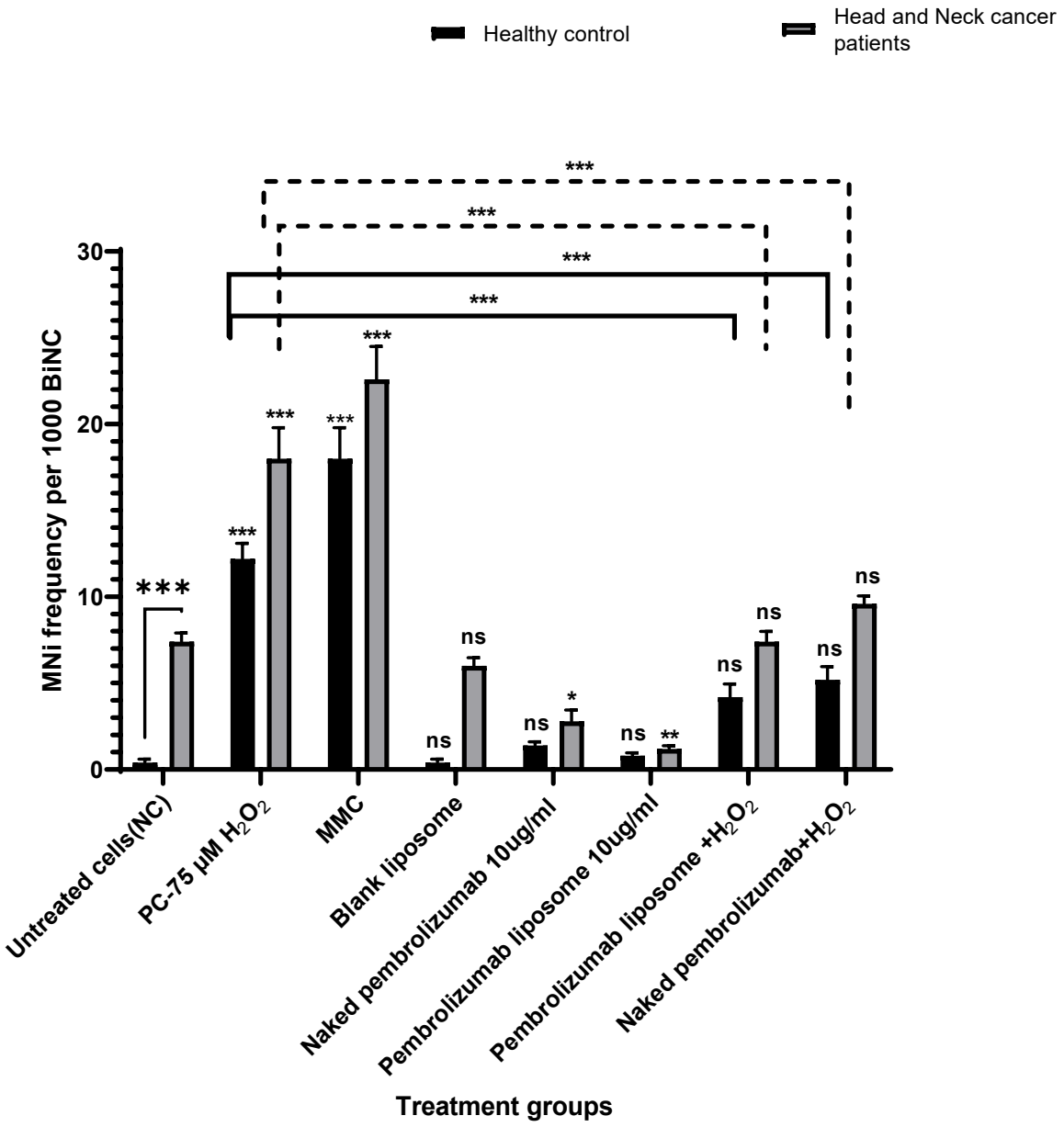


Figure 4-3 The average of BiNC MNi scored per 1000 cells from 5 individual experiments. Data are expressed as means ± SEM.Eight treatment groups included the negative control, a positive control group (PC) 75µM H_2O_2 another positive control (Mitomycin C {0.4µM MMC}), naked pembrolizumab 10µM+75µM H_2O_2, pembrolizumab liposome 10µM+75µM H_2O_2, blank liposome, naked and liposome pembrolizumab. Each treatment group from both groups were also compared against each other, represented by the horizontal lines on the graph and the statistics on top of these lines. (*p<0.01, **p<0.001 ***p<0.0001, ns=not significant).

4.4 Discussion

This study used a CBMN assay to examine how naked pembrolizumab and liposome affect lymphocytes at the chromosomal level using samples taken from either healthy people or HNC patients. This assay is crucial to the detection of the genotoxicity of various compounds. It achieves this by measuring the abnormalities of the micronuclei or other chromosomal components, including the presence of NPBs of dicentric chromosome biomarkers; it also measures the gene amplification biomarkers NBUDs (Cho et al. 2020; Fenech 2020). Disturbances during cell cycle division lead to cytoplasmic bodies known as MNis. As a result, a nuclear envelope forms fragments of centric – or even entire chromosomes – that cannot move to the opposing poles during the anaphase. Together, these slowly adopt a morphology unlike an interphase nucleus, i.e. not as large as the central cell nuclei (Fenech et al. 2011; Luzhna et al. 2013). In the assay, Cyto-B is used to inhibit cytokinesis in the dividing cell (Sommer et al. 2020).

The current study counted several different cell types, focusing on the occurrence of MNis in the treatment group, as these indicate chromosomal breakage, rearrangement, loss, apoptosis and necrosis (Fenech 2020). Moreover, the damage was only seen in the MNis contained in the binucleated cells after treatment, decreasing the likelihood that pre-existing damage was scored (Li et al. 2012b; Xiang et al. 2012).

Mitomycin C (MMC), an antibiotic, antitumour, genotoxic and clastogenic compound, was used as a positive control in this study. In binucleated cells, MMC is known to induce a higher production of MNi than is the case in mononucleated cells (Table 4.1). After 1000 cells were scored, the BiNC percentage was within the expected range for lymphocyte culture across the board. In contrast, there was a low percentage of

cell culture mutliNC, indicating that Cyto-B inhibits cell division after a single cell cycle. Finally, all experiments presented normal NDI values (Table 4.1)

Micronucleus assay showed that the frequency of MNi in the lymphocytes from healthy individuals and HNC patients declined when treated with naked pembrolizumab and liposome compared to the negative control (NC) in each group. In contrast, the pembrolizumab liposome showed a more significant decrease in MNi frequency in lymphocytes.

When using H_2O_2 in MN tests, the results showed the presence of the MNi on the lymphocytes from healthy individuals and HNC patients. The combination of naked pembrolizumab and pembrolizumab liposome with H_2O_2 reduction the frequency of MNi compared to H_2O_2 in patients' lymphocytes ($^{***}p<0.001$).

According to outcome, the study revealed that both the naked pembrolizumab and pembrolizumab liposome could reduce DNA damage in the lymphocytes of HNC patients. As already mentioned in a study conducted by Wookey and Grothey (2021) where pembrolizumab had a role in the prevention of many cancer types such as lung cancer and colorectal cancer (Wookey and Grothey 2021).

CHAPTER FIVE: The effectiveness of naked and liposome pembrolizumab on major signal transduction pathways in lymphocytes.

5 Quantitative reverse transcription polymerase chain reaction RT-PCR (qRT-PCR) and Western Blotting

5.1 Introduction

Real-time PCR enables the detection of brief sequences of DNA in real-time via the logarithmic amplification of specific nucleic acids and their subsequent detection using fluorescent probes. Theoretically, only a single copy of a DNA sequence is required, which following amplification, is identified using suitable probes and primers. There is a direct association between the initial amount of target DNA identified and the amount of product generated during each cycle (Zuo et al. 2010; Maddocks and Jenkins 2017). After the probe has adhered to the targeted DNA sequence, it cleaves and fluorescence is produced, which can be identified using a qPCR machine after each thermal cycle has been completed. Real-time PCR can be used to detect and quantify the presence of pathogens, such as parasites, viruses and other microbes, in sample media (Valasek and Repa 2005), as well as identify the DNA in human samples, such as saliva, blood and hair (Garibyan and Avashia 2013). It also allows rare diseases to be detected, particularly in the early stages, and can aid in the identification of gastric, breast, colorectal and nasopharynx cancers. Finally, it can also identify factors linked to neoplastic disease growth (Assumpção and da Silva 2016).

As it can be used to isolate DNA, PCR can be employed in the analysis of tumours. Specifically, it enables the isolation and quantification of tumour suppressor genes and proto-oncogenes, whereby qPCR in particular can accurately identify and quantify the target genes (Narrandes and Xu 2018). Moreover, it can target single cells to identify

specific combinations of DNA, including proteins and mRNA (Ståhlberg and Kubista 2014). Several components are needed for a PCR reaction, e.g. primers, nucleotides and DNA polymerase enzyme, with the latter linking the individual nucleotides (Clark et al. 2019) . The DNA polymerase eventually forms the final PCR product using the nucleobases, i.e. adenine (A), cytosine (C), guanine (G) and thymine (T). Finally, specific DNA sequences or products are amplified by the applied primers (Jakubovska et al. 2018).

Western blotting (WB) is a commonly used technique to quantitative and analytical of specific protein in cells and tissues (Ghosh et al. 2014). The method uses gel electrophoresis; whereby various proteins are separated according to their molecular weight. After being transferred to a PVDF/nitrocellulose membrane, these proteins generate respective bands. Subsequently, antibodies specific to the target proteins are incubated with the membrane (Liu et al. 2014). Any unlinked antibodies are then removed, leaving the remaining antibodies linked to the protein of interest. Imaging the membrane enables the respective bound antibodies to be identified. Any visible bands thus denote the proteins of interest, whereby the band thickness in conjunction with an established standard – demonstrates how much of the protein is present (Mahmood and Yang 2012a).

Band thickness provides great information of overexpressed or downregulated of protein when compared to the control (NC). That help researchers to investigate of any changes in genes such as deletion or duplication (Barresi 2011). WB is very sensitive and could detecting small protein (0.1ng) (Gogia et al. 2017) and a quick technique for medical diagnosis (Manole et al. 2018).

As it is very sensitive to stress inputs, the p53 tumour suppressor gene is uniquely able to prevent neoplastic changes and the growth of tumours (Aubrey et al. 2018). In addition, it ensures cellular homeostasis and the stability of the genome via a complex and diverse network of effector pathways and processes. Human cancers have a very high incidence of *p53* gene missense mutations, meaning the mutated p53 proteins no longer have the ability to suppress tumours; wild-type p53 proteins may experience trans-dominant repression by these mutants. By retaining the mutant protein types, tumour cells can have a selective advantage as they significantly alter the p53 pathway, leading to increased invasion and metastasis as well as chemo resistance (Lee et al. 2018).

Almost half the head and neck squamous cell carcinomas (HNSCC) cases, which occur in the mucous membranes of the mouth, throat and nose, have been found to have somatically mutated p53 genes. As most of these mutations involve changes to individual p53 amino acids, the protein's function is inhibited, triggering unregulated cell proliferation (Alvarado-Ortiz et al. 2021; Horn and Vousden 2007; Della-Fazia et al. 2020). Consequently, DNA damage accumulates in the cells, which experience uncontrolled division, leading to tumour development (Matthews et al. 2022).

P53 is critical in the development of cancer, particularly in its role as the main controller of intracellular functions, including cell growth and proliferation (Zhang et al. 2020a). As a result, p53 status and behaviour can determine the progression and prognosis in cases of cancer. Altered p53 alleles through loss, deactivation or, in particular, mutation have been detected in roughly half of the tumours in human patients (Alvarado-Ortiz et al. 2021). If tumour cells have mutant p53 alleles, they create mutant p53 proteins (mutp53), which subsequently influence the amino acids of the DNA binding domain (DBD). Not only does this cause normal tumour suppressor functions

to be lost, but it also gives these mutant proteins the ability to provide the tumour cells with important gain-of-function (GOF) activities (Hanahan and Weinberg 2011; Benedict et al. 2018; (Feroz and Sheikh 2020). These activities affect the molecules and pathways that are understood to be key to the development and progression of tumours (Hanahan and Weinberg 2011; Benedict et al. 2018). Evidence from genomic research on mouse models demonstrates that weak p53 function instigates the formation of tumours while restoring this function rapidly and effectively induces the retraction of in situ tumours that have already developed. This underlines the need to design chemotherapy techniques that can restore the function of p53 (Chow 2020).

While the effects of these GOF activities have not yet been fully elucidated, it is known that tumours demonstrating these activities are significantly driven by them in terms of migration, proliferation, immune evasion, and metabolic reprogramming (Duffy et al. 2020).

Cdkn1a (p21), encoding the cyclin-dependent kinase inhibitor, was the first target p53 gene to be identified; it is key to the regulation of the cell cycle. Activated by p53, p21WAF1/CIP1 binds to cyclin-dependent kinase 2 (CDK2), leading to CDK2 activity down-regulation and the arrest of G1 (Cheng et al. 2020). Meanwhile, through its role in controlling DNA replication and DNA repair, p21 is also crucial to the suppression of tumours (Georgakilas et al. 2017).

The role of the BCL-2 family in apoptotic regulation is typically described as the anti-apoptotic and pro-apoptotic BH3-only members existing in a state of competitive flux to influence the activation of the pore-forming executioners (Warren et al. 2019). The ratio of pro- to anti-apoptotic subfamily members present in a cell can be altered by a number of signalling pathways, effectively relaying information on cellular stress, such

as available nutrients, DNA damage, and protein processing (Hetz and Glimcher 2008). Once the executioners are activated, the molecules come together to form pores in the outer mitochondrial membrane (MOM) and thus trigger mitochondrial outer membrane permeability (MOMP), and therefore apoptosis (Suhaili et al. 2017). Studies that have assessed the significance of the transmembrane domain on the capacity for BCL-2 to regulate apoptosis and p53 concluded that the domain was vital for the efficiency of BCL-2 in these roles (Laulier and Lopez 2012).

This study explored p21, p53 and Bcl-2 protein expression in lymphocyte cells taken from HNC patients, who received either naked or liposome pembrolizumab, and healthy individuals. The study used RT-qPCR and western blotting to assess how far naked and liposome pembrolizumab affected the expression of the p21, p53 and Bcl-2 proteins.

5.2 Materials and methods

All chemicals and equipment used in qRT-PCR and western blotting are listed in Table 2.1 and Table 2.2. The RT-qPCR and wester blotting is described in section 2.5.3 & 2.5.4.

5.3 RT-qPCR Results

Pembrolizumab activates the P53 signalling pathway independent of DNA damage in lymphocytes.

Our results from the Comet, CCK-8, and micronucleus assays, demonstrated that various concentrations of pembrolizumab do not induce statistically significant genotoxicity in lymphocytes from HNC patients and in those from healthy volunteers. We determined the optimal dose of pembrolizumab (10 µg/ml) to be used for

subsequent studies. We then determined the effect of pembrolizumab liposome (10 µg/ml) compared to the naked form at the same concentration. Compared to the naked form, results piloted more effective and protective effects of liposome form using the Comet and micronucleus assays.

To understand the rationale behind this effect we further studied some key factors involved in the cell cycle regulation and apoptotic pathways. We determined the effects of pembrolizumab on gene expression levels of *P53*, *P21* and *Bcl-2*, in lymphocytes from HNC patients and from healthy volunteers at the mRNA level. The RT-qPCR technique was used to analyse the expression of these proteins. The results from the RT-qPCR data analysis demonstrated that both forms of pembrolizumab at a same concentration of 10 µg/ml, up-regulated the expression of P53 in lymphocytes from healthy individuals, however the difference was not significant. There seemed no effect on the regulation of *P21* in healthy lymphocytes. On the other hand, pembrolizumab naked and liposome affect the expression of Bcl-2 differentially in these cells. Naked pembrolizumab had no effect on its regulation but liposome form significantly down-regulated the Bcl-2 expression (*$p<0.05$) which is major anti-apoptotic protein involved in our intrinsic apoptosis pathway.

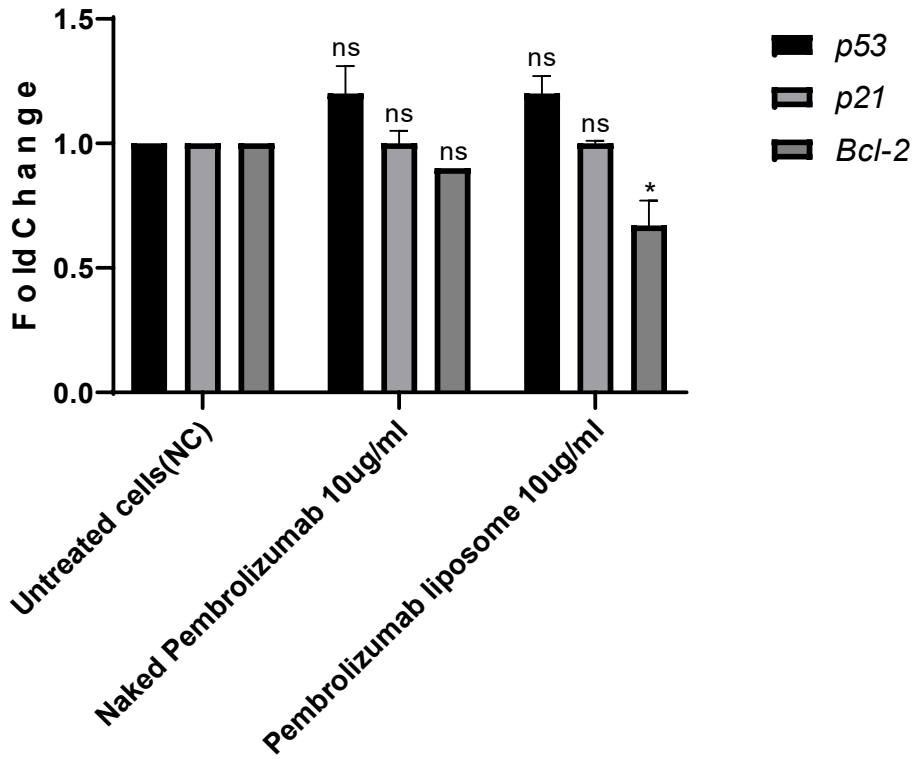


Figure 5-1 levels of Bcl-2, P21 and P53 in lymphocytes from healthy individuals(n=3). The data were compared against the control group (C) and normalised against the house gene, GAPDH. Three groups included a control group, naked pembrolizumab 10 µg/ml and pembrolizumab liposome 10 µg/ml. There was no significant effect seen on *p53* and *p21* expressions; however, liposome drug significantly down-regulated the gene expression of *Bcl-2*. (ns=not significant, *p<0.05).

5.3.1 Pembrolizumab activates intrinsic apoptotic pathway dependent on *p53* and *p21* in lymphocytes from HNC patients

The statistical analysis of RT-qPCR results in lymphocytes from HNC patients has shown a significantly different and substantial pattern in expression levels of these proteins. The expression of the tumour suppressor gene, *p53*, was significantly up-regulated particularly after pembrolizumab liposome treatment by 3 plus fold. The expression level of the cell cycle regulatory gene, *p21*, was also significantly up-regulated. However, the expression of the anti-apoptotic gene, *Bcl-2*, was significantly down-regulated by 0.2-fold, indicating the apoptotic potential of pembrolizumab in lymphocytes from HNC patients might be dependent on expression levels of *p53* and

p21 genes. Pembrolizumab liposome seemed to cause more significant effects than the naked form in vitro experiments.

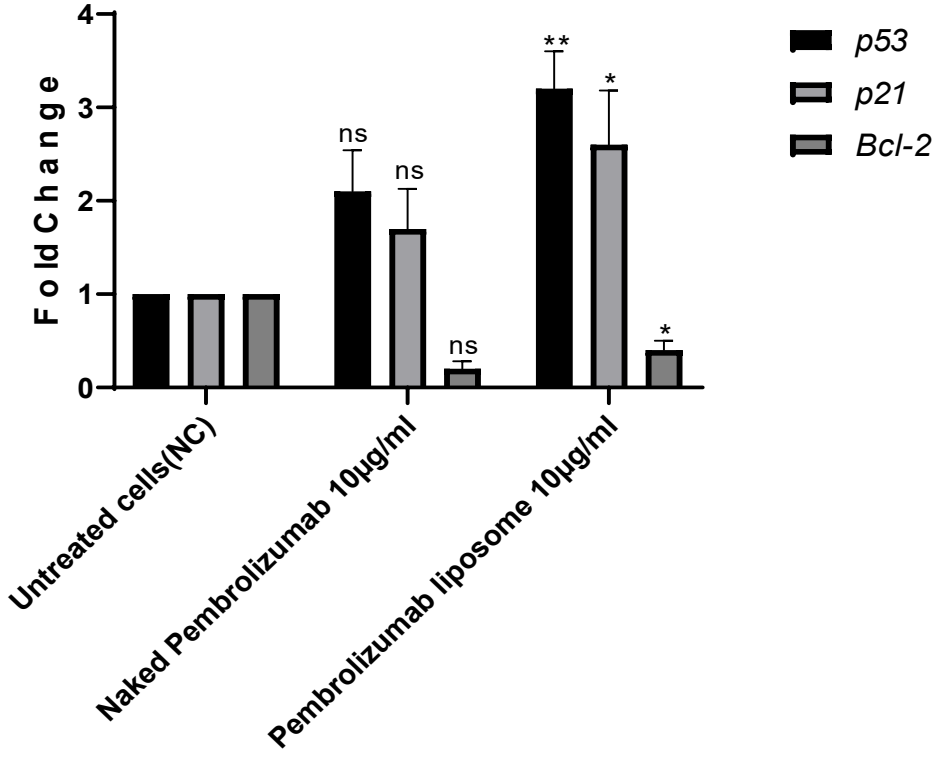


Figure 5-2 The effect of Pembrolizumab naked and liposome forms on the gene expression levels of *Bcl-2*, *P21* and *P53* in lymphocytes from HNC patients(n=3). The data were compared against the control group (C) and normalised against the house gene, GAPDH. Three groups included a control group, naked pembrolizumab 10 µg/ml and pembrolizumab liposome 10 µg/ml. *p53* and *p21* genes were significantly up-regulated; however, the *Bcl-2* gene was significantly down-regulated. (ns=not significant, *P<0.01, **P<0.001).

5.4 Western blotting Results

The effect of naked pembrolizumab 10 µg /ml and pembrolizumab liposome on p53, p21 and Bcl-2 protein expression levels in lymphocytes from healthy individual and HNC patient.

We used the WB test to investigate the level of protein expression for p53, p21, and Bcl-2 and compared it with the results that we got from RT-qPCR, and to investigate

the effect of naked and liposome pembrolizumab on protein expression levels of p53 tumour-suppressor and cell cycle regulator p21 and anti-apoptotic protein Bcl2, in lymphocytes from healthy individuals and HNC patients.

P53 is a tumour-suppressor protein that plays an important role in maintaining homeostasis in biological processes and is stimulated by oxidative stress to regulate DNA repair, cell death, and senescence (Lacroix et al. 2020).

Therefore, we investigated the p53 protein to verify its role in the naked and liposome pembrolizumab effect in lymphocytes from HNC patients. The results showed that naked and liposome pembrolizumab has a non-significant effect on expressing any of these proteins in healthy individuals' lymphocytes. Moreover, there are no differences between their regulation Figure 6.1 (A and B).

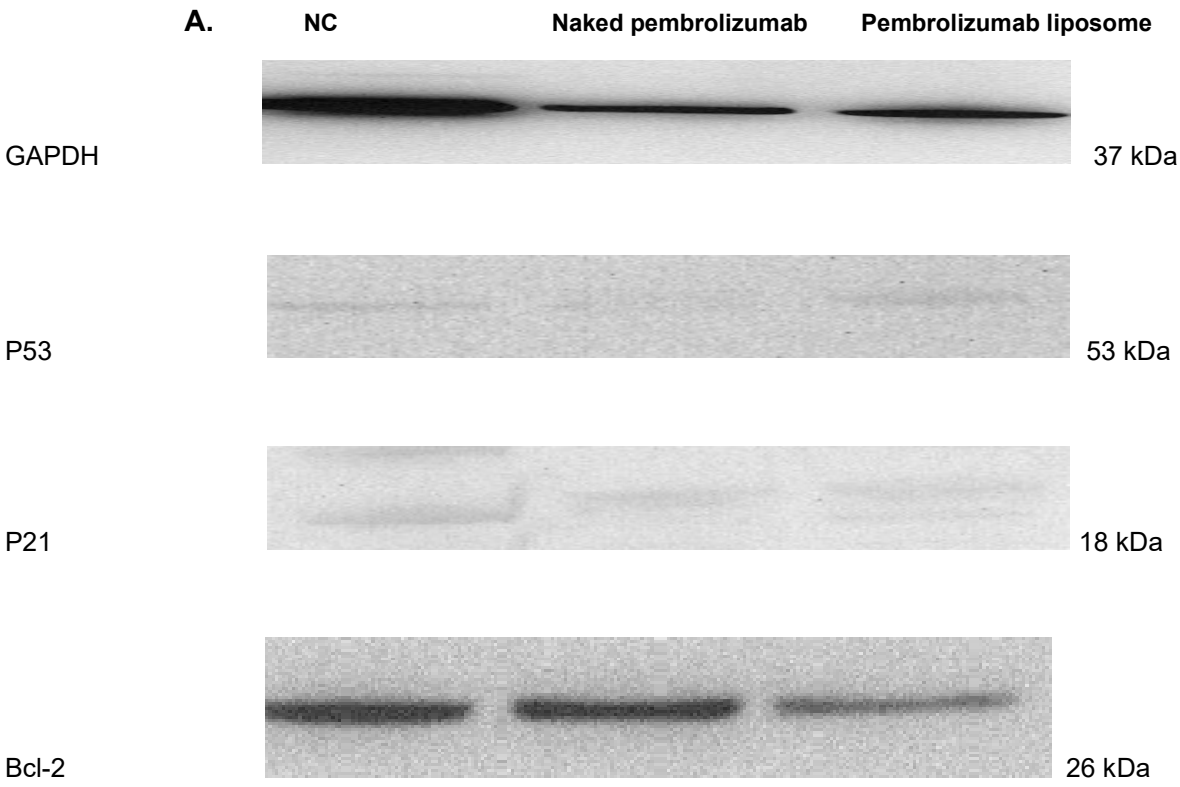
However, there was significant upregulation of p53 and p21 expression levels in lymphocytes from HNC patients Figure 6.2 (A and B).

The p53 has increased by 1.7-fold with naked pembrolizumab 10 µg/ml and pembrolizumab liposome 10 µg/ml in lymphocytes from HNC patients. Furthermore, the p21 has upregulated to 1.8-fold for naked pembrolizumab 10 µg/ml and 1.7 for liposomal form compared to the control group.

The results have shown that naked pembrolizumab 10 µg/ml and pembrolizumab liposome 10 µg/ml can fight the cancer cells by stimulating the p53 expression levels and p21. It will have a protective and antitumour effect by stimulating DNA damage response (DDR).

Thus, it will contribute to survival through the repair of DNA damage. Furthermore, results from WB were consistent with the results of gene expression from the RT-qPCR technique.

A. Protein expression in Healthy individual



B.

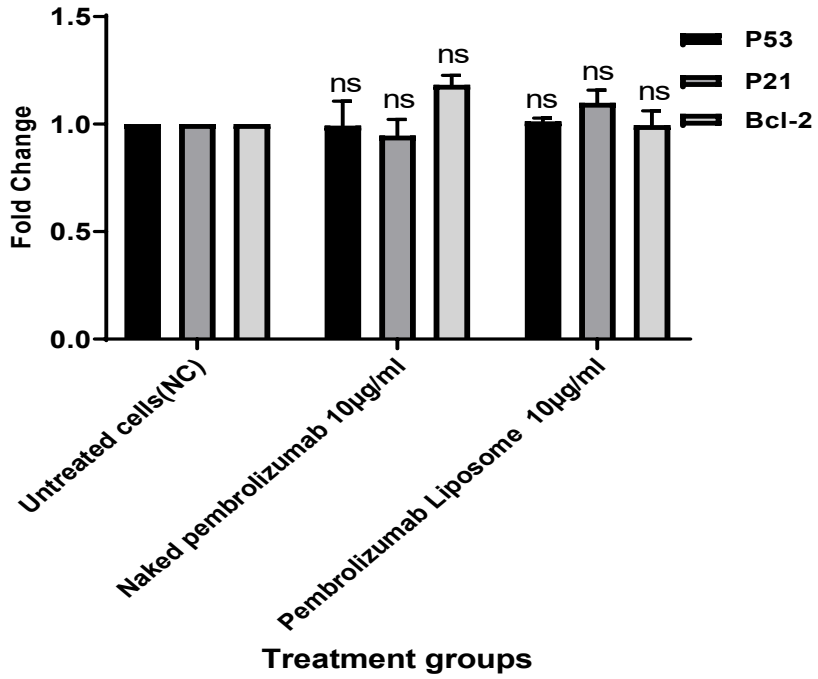
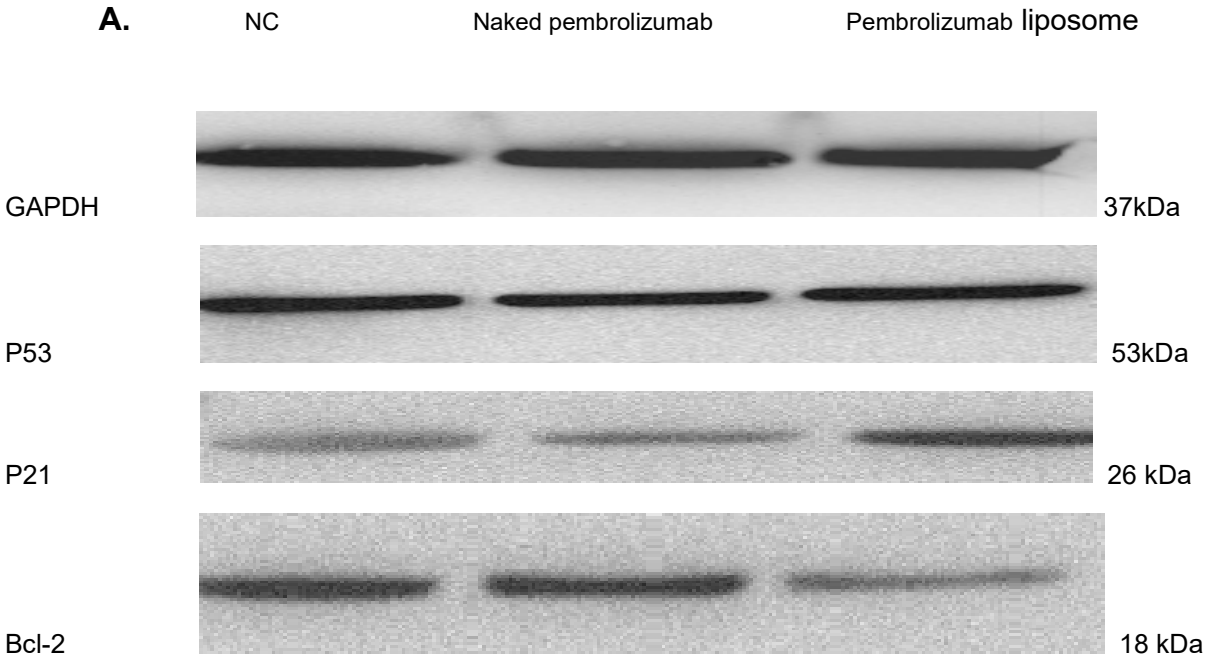


Figure 5-3 The effect of naked and liposome pembrolizumab on the protein expression levels of p53, p21 and Bcl-2 in lymphocytes from Healthy individuals(n=3). All data from the treatment groups were compared against the control group (C) and normalized against the internal home protein, GAPDH, untreated cells (NC), naked pembrolizumab 10 µg/ml and pembrolizumab liposome 10 µg/ml. Pembrolizumab 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 pembrolizumab 10 µg /ml and pembrolizumab liposome 10 µg/ml. (B) Bar graphs exhibiting fold changes in protein expression levels. Data are represented as the mean ± SEM of three experiments.

B. Protein expression in patient



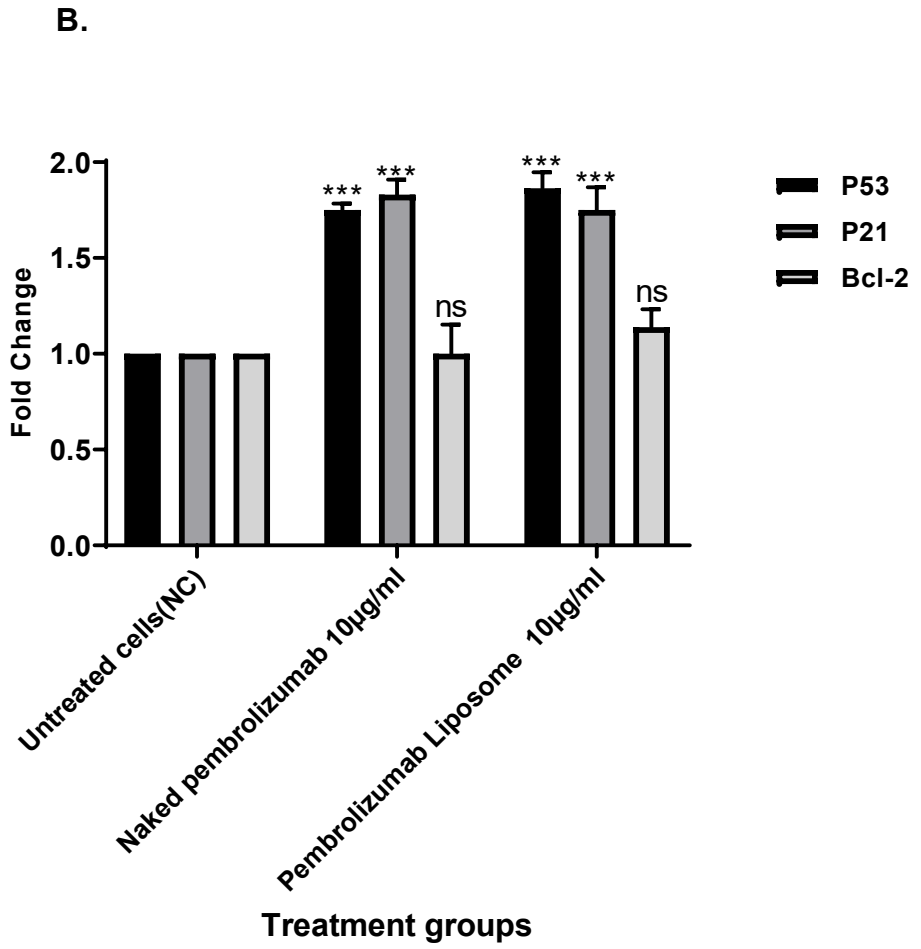


Figure 5-4 The effect of naked pembrolizumab 10 µg/ml and pembrolizumab liposome 10 µg/ml on the protein expression levels of p53, p21 and Bcl-2 in lymphocytes from HNC patients(n=3). All data from the treatment groups were compared against the control group (C) and normalised against the internal home protein, GAPDH, untreated (NC), naked pembrolizumab 10 µg/ml, and pembrolizumab liposome 10 µg/ml. Pembrolizumab in both forms significantly upregulated 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 HNC patients treated with naked pembrolizumab 10 µg/ml and pembrolizumab liposome 10 µg/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).

5.5 PCR & WB Discussion

The stability of genomic DNA is critical for all living organisms. However, DNA is prone to daily exposure to agents – both exogenous and endogenous – that impact the stability of the genome, and thus have a significant adverse effect on health (Williams and Schumacher 2016; Chatterjee and Walker 2017). The cellular mechanisms of repair work to mitigate DNA damage in healthy cells, allowing only sporadic errors to

occur (Barnes et al. 2018). Genetic or multifactorial disorders can thus be attributed to dysfunctionality among these repair mechanisms. The extent of this effect varies according to the type of cell, i.e. whether somatic or germline, thereby facilitating the genome alteration process; this also constitutes the main driver of evolution (Miles and Tadi 2020; Broustas and Lieberman 2014; Chatterjee and Walker 2017). Genomic instability is a hallmark of the majority of human cancers. It stems from acquired defects occurring in a DNA repair pathway, specifically BER, NER, HRR, DNA MMR, NHEJ, and DNA synthesis during translation (TLS) (Langie et al. 2013; Sadeghi et al. 2020). Genetic instability results from severely diminished MMR activity; this includes stronger anticancer-drug resistance (Hientz et al. 2017; Williams and Schumacher 2016).

A p53 mutation and its effects have been found to impact critical biological processes linked with cancer progression, proliferation, invasion, migration, immune system interactions, metabolic reprogramming, and pharmacological resistance (Alvarado-Ortiz et al. 2021; Hientz et al. 2017). The research has evidenced that p53 performs a variety of functions in mediated cell cycle arrest to facilitate genome repair and mitigate DNA damage propagation. Based on this, an increased level of p53 should allow the suppression of cancer cell proliferation via DNA repair, apoptosis, and cell cycle arrest (Boutelle and Attardi 2021).

In addition, in the study we examined the p53 at the gene and protein level, focusing on its capacity to encode for proteins that can interact with DNA and thus regulate gene expression, thereby precluding several genome mutations.

Our findings show that treating with naked pembrolizumab and liposome pembrolizumab for 24 hours increased the p53 level in the HNC patients' lymphocytes

at both the mRNA and protein levels. This indicates that pembrolizumab may trigger p53-mediated DNA repair and cell cycle arrest in HNC patients (***P<0.001, **P<0.01).

Prior research has shown that increased p53 expression correlates with p21 up-regulation (Shamloo and Usluer 2019), moreover, mutant p53 proteins often fail to transactivate p21, which is frequently linked to uncontrolled proliferation (Webber et al. 2019). This result is consistent with (Kuganesan et al,2021) who found that increased P53 level will stimulates the P21. Proteins level will be regulated by p53 a negative feedback cycle with MDM2 (García-Cano et al ,2020)

Key to the elimination of cancer cells, the mechanism of programmed cell death is controlled by the Bcl-2 family of proteins. This control is facilitated by the balance between the family's pro-apoptotic (e.g. Bad, Bax) and anti-apoptotic (e.g. Bcl-xl, Bcl-2) factors (Brunelle and Letai 2009).

In this study, treatment with naked and liposome pembrolizumab did not significantly affect the Bcl-2 of the lymphocytes of HNC patients or healthy individuals. Due to the regulation of naked and liposome pembrolizumab of genes and proteins expression in the cells, Zeng et al (2020) mention that oxidative stress promotes the Bcl-2 expression in cells of pulmonary patients (DNMT).

Despite this, there was barely any p53 and p21 expression in healthy individual lymphocytes at the mRNA and protein levels after being treated with naked pembrolizumab and liposome pembrolizumab. The result of the current study agrees with the previous study that in healthy cells, p53 is kept at a low level due to the MDM2 proto-oncogene's action (Rivlin et al. 2011), and oxidative stress stimulates the increase of p53 level (Shi et al ,2021)

Our findings indicated that, by functionally promoting apoptosis, p53, p21, along with Bcl-2 , When DNA damage happens in lymphocytes in of HNC occurs , the p53-dependent signals induce Bax and cyto-c to directly inhibition of Bcl-2 (Hemann and Lowe, 2006) .As result p53, p21 play a key role in the successful response to repair DNA damage and may be considered as predictive factors of a better prognosis in patients with head and neck cancers.

CHAPTER SIX: General discussion and future work

6 General discussion and future work

6.1 General discussion

The current work was designed to assess the genotoxic effects of pembrolizumab liposome in human peripheral lymphocytes from healthy individuals and head and neck cancer patients after treatment with hydrogen peroxide, since previous studies reported that pembrolizumab alone had a significant role in DNA repairing of different cancers. The current study was designed to evaluate the potential of naked pembrolizumab and pembrolizumab liposome in DNA repairing of head and neck carcinoma. Lymphocytes have been chosen in this study as surrogate cells because of their exposure to different environments in the bloodstream, which enables them to reflect any damage to DNA induced by endogenous and exogenous genotoxins from chemical and physical agents (Najafzadeh et al., 2012). On the other hand, liposomes have physical and chemical properties relating to their size. The small size of liposomes allows them to penetrate the membrane of cells by different mechanisms and then they to diffuse through the nuclear membrane. For this purpose, we performed Comet assay to explore naked pembrolizumab and pembrolizumab liposome in terms of their genotoxic and genoprotective effects as well as their activity against DNA damage induced by H_2O_2 on lymphocyte cells taken from both HNC patients and healthy individuals Furthermore, the manner in which of naked pembrolizumab and pembrolizumab liposome affect the mitotic phase was evaluated using cytokinesis-block micronucleus assays, and the frequencies with which the main biomarkers occurred in the assay, i.e., micronuclei (MN), nuclear buds (NBUDs) and nucleoplasmic bridges (NPBS), were measured. In addition, Western blotting and RT-qPCR were employed to investigate how naked pembrolizumab and pembrolizumab

liposome impact p53, p21 and Bcl-2 genes expression at the mRNA and protein levels, respectively, in lymphocyte cells extracted from HNC patients and healthy individuals.

Cancer is a significant and worldwide public health issue. With the increasing cancer rates worldwide, there is an urgent need to develop strategies to prevent the disease (Sung et al. 2021). Against the backdrop of the alarming prevalence and outcomes of cancer, the medical field still focuses on its main therapeutic routes of radiotherapy, surgery and chemotherapy. Head and neck cancer patients have various options when it comes to prognosis and multimodal therapies. Which one is most suitable depends on the important epidemiological factors, the stage of the cancer, and its location in the body (Chow 2020). Thus, each subtype of HNC has its own specific aetiology and epidemiological trends, leading to a tailored therapy for each subtype. Despite treatment, advanced HNC patients generally experience a disease control rate of around 40% at 5 years, with multiple challenges emerging in terms of toxicity both acute as well as in the long term. However, there has been substantial therapeutic development in recent years, although the survival rates for HNC patients based on large samples are still not reported (Guo et al. 2021).

Chemotherapy bears numerous side effects, and cancers are becoming increasingly resistant to this method of treatment. Hence, efforts must be made to identify novel drug treatments that can address these issues. Significant progress has been made with respect to HNC treatment, with current methods including radiotherapy, surgery, chemotherapy, immunotherapy, and targeted therapy; these can be administered individually, but are often given in combination (Wang et al. 2019). Immunotherapy in particular is introducing new strategies into the management of HNC, especially in the advanced stage. Hereby, immunotherapy based on anti-PD-1 or anti-PD-L1 antibodies has been shown to significantly increase the rate of disease-free survival

in addition to overall survival in second-line therapy (Ferris et al. 2016; Colevas et al. 2018).

Previous research has also demonstrated significant disparities among the responses to treatment (Cadoni et al. 2017). A wavering pattern of incidence as well as increasingly enhanced survival rates were seen in HNC patients in the long run (Johnson et al. 2020a). While a variety of drugs are known to induce cytotoxicity for various cancer cell lines, little is known about pembrolizumab's potentially protective effect on lymphocyte cells from HNC patients. As a key component in immunotherapy, pembrolizumab contributes to the elimination of cancer cells by the immune system through its anti-proliferative and anticancer properties, whereby it inhibits the growth of tumours and promotes apoptosis.

Studies have evidenced its use in clinical settings, including in the management of various forms of cancer such as melanoma and Hodgkin's lymphoma (Seiwert et al. 2015; Toor et al. 2018). Nonetheless, to the authors' best knowledge, there are no prior studies examining either the effects of pembrolizumab *in vitro* or how different concentrations of it affect the DNA in lymphocyte cells taken from HNC patients.

Liposomes' circulation half-life can be determined by measuring their vesicle size and composition. For example, in an aqueous environment, the properties and size of certain liposome systems dictate their potential applications as drug carriers. Meanwhile, the parameter of vesicle size influences their drug encapsulation degree.

Liposomal formulations designed to increase the pharmacokinetics of pembrolizumab and to investigate the effect of pembrolizumab on biological activities were prepared with a particle size of 116.1nm and the PDI 0.227. The encapsulation efficiency was (99.3 %)

Thus, this study prepared liposomal formulations that could potentially strengthen the pharmacokinetic properties of pembrolizumab; then, it examined the subsequent impact on their biological activity. Because the liposomes have many advantages to improve cancer immunotherapy and immune responses by improving the type of drug delivery and enhancing drug efficacy and protecting the drug from degradation in circulatory system and from the effect of enzymes in the body, it is very important to improve the quality of liposomes for specific purposes, such as by targeting the specific site in the TME, and to reduce the side effects of drugs (Gu et al,2020).

Crucially, studies on cancer prevention strategies must evaluate how the drugs under study influence the various processes of carcinogenesis. The Comet assay hereby represents a good method for measuring genotoxicity with a high level of reliability. In comparison with other assays, it is also more sensitive at estimating DNA damage and repair while being simpler and more cost-effective (Anderson et al. 2013).

Consequently, we used it to investigate the DNA protecting effect of naked pembrolizumab and pembrolizumab liposome in lymphocytes from HNC patients compared to those from healthy individuals.

Both the Comet and micronucleus assays were used to examine the DNA damage, including chromosomal damage. This commonly implemented method is able to show to what extent a genotoxic substance can generate aneugenic and clastogenic effects during cellular division as well as other cell cycle phases (Fenech and Crott 2002).

There are several forms of DNA damage due to either genotoxicity or mutagenic changes, including DNA base alterations, DNA adducts, single- and double-strand breaks, cross-links and physical changes to the DNA (Karlsson et al. 2008).

The MN signify the DNA damage, specifically lost or fragmented chromosomes that are produced during the phase of nuclear division (Magdolenova et al. 2012). As a biomarker with numerous advantages, MN are widely used in both *in vivo* and *in vitro* studies of genome stability and chromosomes damage (Fenech and Crott 2002).

Particularly in genotoxic studies, the use of MNs as biomarkers allows the damage to the chromosomes of peripheral lymphocytes due to genotoxic agents to be evaluated (Luzhna et al. 2013). Despite this, there is still little knowledge on their effect and the underlying mechanism, and more research needs to be done on the key molecular mechanisms, including the cellular pathways and lymphocyte cells' self-repair abilities.

Then, a micronucleus assay was used to determine the effects of pembrolizumab in lymphocytes from HNC patients and compare with our results from the Comet assay. Based on the findings in (Hanahan and Weinberg 2000), there could be DNA damage in most of cancer patients' lymphocytes as their DNA repair mechanisms are compromised on the genetic level. This highlights the importance of using lymphocytes as model cells, supporting the emphasis on them in the current study.

Cancer susceptibility is significantly reliant on genome sensitivity, which can best be examined using peripheral lymphocytes (Collins 2004). Lymphocytes are thus often employed as surrogates to examine DNA damage when somatic cell types are not accessible. Lymphocytes offer multiple benefits in this respect as they are found across the body's circulatory system and are prone to DNA damage (Najafzadeh et al. 2009).

To determine the cell survival and cytotoxicity of naked and liposome pembrolizumab, we used cell counting kit 8 (CCK8) assay, in lymphocytes from both healthy individuals and HNC patients.

The two treatment groups were naked pembrolizumab and pembrolizumab liposome, untreated cells were used as negative control (NC) , and H_2O_2 (75μM) was used as a positive control (PC). The CCK-8 assay results indicated that for all considered treatment groups, cell survival remained above 80% in the lymphocytes taken from both healthy individuals and the HNC patients, Figure 3.5.

In both the groups investigated, H_2O_2 exposure led to substantial cytotoxicity levels. Significant levels of cytotoxicity were also observed in HNC patients with naked pembrolizumab and pembrolizumab liposome, although all groups showed a viability of above 80%.

As a result, this study used only non-cytotoxic doses, i.e., those demonstrating a viability above 80% (Henderson et al. 1998); none of the artefact activities were included due to toxicity. A Comet assay was used to determine the drug genotoxicity.

According to the results of the analysis, only ideal non-genotoxic concentrations (10 μg/ml) of naked pembrolizumab and pembrolizumab liposome, as demonstrated in the Comet assay by both the %tail DNA and OTM, were non-cytotoxic doses. Hence, they did not cause any statistically significant DNA damage at the basal level among the lymphocyte cells taken from healthy individuals.

Our findings from the Comet assay showed that the concentration of naked pembrolizumab and pembrolizumab liposome (10 μg/ml) was non-genotoxic and there was no considerable DNA damage to lymphocytes from healthy individuals compared with the control group.

In addition, the pembrolizumab liposome showed more reducing capacity of DNA damage when compared with naked pembrolizumab (Figure 3.6 and Figure 3.7). The patient group presented high levels of DNA damage during the *in vitro* study. However,

DNA damage has been declined in patient group after treated by pembrolizumab, it might be that the activity of PD-1 and ability to linked with ligands PD-L1 or PD-L2 is responsible for lymphocytes activation and increase of immune responses in HNC patients' lymphocytes (Han and Li, 2020). Hydrogen peroxide was used to induce stress in lymphocyte cells taken from both healthy individuals and HNC patients. A significant increase was found in terms of DNA damage compared to the untreated lymphocytes taken from both groups.

Nonetheless, this increase was lessened significantly through the application of naked pembrolizumab and pembrolizumab liposome. Those groups that had received naked pembrolizumab and pembrolizumab liposome co-administrated with H_2O_2 showed significantly less DNA damage than was found for the group treated with H_2O_2.

This is possibly due to liposome-coated pembrolizumab's induced solubility in an aqueous medium stabilising different therapeutic agent such as nucleotides and proteins. Liposome`s size, surface charge and composition are favourable conditions for obtaining effective results. Many hydrophilic and hydrophobic biomolecules can be encapsulated in liposomes and protected from interactions with the external environment. Liposomes induce the drug's therapeutic index through the inhibition of the drug's metabolism before reaching the target cells and protect the drug's interaction with the healthy cells (Alhawamdeh et al., 2021). It has been well known that the liposome form of any therapeutic drug shows enhanced pharmacokinetic properties and biodistribution compared to its naked form. The morphology of liposomes is similar to that of the cellular membranes, making them an ideal drug carrier system (Barenholz and Peer, 2012) and this might be due to the drug's binding to the receptors on the surface of lymphocytes to reduce DNA damage as well. In endocytosis, the liposome will be surrounded by the plasma membrane of cells. The

plasma membrane buds off within the cells to produce a vesicle containing the absorbed substance. After the endocytosed liposomes may be recycled to the surface or degraded into carbohydrates, fats, proteins, and other molecules into simple compounds. These can then return to the cytoplasm for use as new materials that cells are composed of (Khan et al ,2021; Khan and Steeg, 2021).

The stability and integrity of genomic DNA are crucial for all living organisms as there are fundamental to their proper functionality and survival. As a biomolecule, DNA is, however, susceptible to a variety of genotoxic agents, both within the organism itself and in its environment, potentially leading to genome instability (Williams and Schumacher 2016; Chatterjee and Walker 2017). Multiple genotoxic insults are constantly affecting DNA, ranging from toxic substances in the environment to radiation, leading to stresses that can cause errors in DNA replication and produce endogenous metabolites. As a result, it is extremely difficult to ensure that the genome is maintained at all times. Various DNA repair mechanisms have therefore evolved in living organisms that are able to identify and repair damaged DNA, thereby removing genetically flawed cells or blocking their progression. Despite these mechanisms, not all DNA damage is suppressed as some evade detection or enter error-prone pathways. This damage further causes mutagenesis, which, aside from driving evolution, is the primary instigator of ageing, inheritable disease, and cancer (Volkova et al. 2020).

Two complex and intertwined regulatory pathways, namely the DNA repair pathway and the quality control system for proteins, control the integrity and stability of the genome and proteome. A disruption in either the genome or the proteome will inevitably have an impact on the other. Therefore, they offer great therapeutic potential for the treatment of diseases due to genomic instability (Biau et al. 2019). Thus, the

repair of DNA is a crucial biological pathway that contributes to the maintenance of the homeostasis of the cell (Demirbağ-Sarikaya et al. 2021). However, as not all damage is detected or repaired, mutagenesis can occur and also cause cancer (Volkova et al. 2020).

The regulation of the cell cycle, which is crucial in cell repair, growth and proliferation, relies on the complex interactions between enzymes, proteins, and cytokines. Any disruptions to it are associated with the emergence and growth of tumours as well as tumour metastasis. Hence, there is significant synergy to be found in regulating the cell cycle together with the application of chemotherapy, both through promotion and inhibition. Specifically, tumour cells' sensitivity to chemotherapy can be enhanced by integrating strategies for cell cycle regulation into the treatment course (Sun et al. 2021).

Some research has associated treatment with pembrolizumab as first-line therapy because PD-1 offers great potential as a repair mechanism for numerous cancer types as it has been shown to stimulate the G2-phase processes of DNA repair. (Kakoti et al. 2020). Specifically, PD-L1 upregulation entails both the cGAS/STING cascade and micronuclei in the event that cells are released from the G2/M checkpoint during cell cycle arrest after DNA damage; this is an obvious biomarker of DNA damage and genome instability (Kakoti et al. 2020). The effects of naked pembrolizumab and pembrolizumab liposome on lymphocyte cells taken from healthy individuals and head and neck patients were examined via the CBMN assay. This assay is useful in that it can efficiently investigate genotoxicity at the chromosomal level among both genoprotective and genotoxic compounds by measuring various parameters. These parameters are the micronuclei, which indicate broken, rearranged or lost DNA, nucleoplasm bridges and DNA apoptosis or necrosis, and the nuclear buds, which are

biomarkers of amplified genes. The CBMN assay uses these as biomarkers of genomic instability, including in the long-term cultures of lymphocyte cells (Fenech and Crott 2002; Fenech 2007).

This study revealed that MNi frequency was especially detected amongst the various cell types assessed, i.e. mono-nucleated cells, bi-nucleated cells and multi-nucleated cells, in the treatment groups. Moreover, they counted MNi in both mononucleated and binucleated cells to respectively estimate the pre-existing damage and evaluate the damage to chromosomes following the treatment. The compound mitomycin C (MMC) is genotoxic and clastogenic (Fenech 2008).

The MNi count (per 1000 counted cells) in bi-nucleated cells taken from both (HNC) patients and healthy volunteers was significantly less after treatment with naked pembrolizumab and pembrolizumab liposome in comparison to the respective control groups. Hereby, pembrolizumab was revealed to be more effective. The MNi frequency was very low in mono-nucleated cells taken from healthy volunteers. However, it was higher in the patient group, which suggests that the disease had caused pre-existing basal damage (Olusanya et al. 2018).

The MNi frequency in the mononucleated cells was significantly diminished following treatment with naked pembrolizumab and pembrolizumab liposome for the HNC patients in comparison to the healthy individuals, highlighting their potential as a strategy to reduce the basal damage in lymphocyte cells among HNC patients. Meanwhile, lymphocytes taken from healthy volunteers that remained untreated presented lower levels of DNA damage compared to the untreated lymphocyte cells taken from cancer patients. This observation from the *in vitro* study is in line with numerous prior studies (Miloševic-Djordjevic et al. 2010).

The MN assay was used to examine the effects of H_2O_2 on the stability of the genome for both healthy individuals and HNC patients. Treatment with H_2O_2 led to a significant rise in MNi formation compared to untreated cells. However, co-administration of H_2O_2 with either naked pembrolizumab or pembrolizumab liposome statistically significantly reduced the initiation of MNi when compared with the PC. Pembrolizumab liposome was hereby found to have a stronger effect than naked pembrolizumab.

Significant oxidative damage and stress were found in lymphocyte cells taken from healthy individuals and HNC patients following treatment with H_2O_2. Meanwhile, it was revealed that pembrolizumab can significantly mitigate the adverse impact of exposure to H_2O_2. In summary, pembrolizumab shows great potential as a therapeutic strategy as it offers protective effects against DNA damage caused by exposure to H_2O_2 in lymphocyte cells taken from healthy individuals and HNC patients. These findings agree with Kakoti et al (2020) who mention that DNA repair and signalling are implicated in the control of PD-L1 expression. where the ATM/ATR/Chk1 signalling pathways, will stimulation of the immune response after DNA damage, activate the immune system. Therapies targeting DDR alone or in conjunction with RT have immunostimulatory potential via micronuclei formation and Type-I IFN production. Consequently, DNA damage can be a potent stimulant for immunological activation. Therefore, the use of anti-PD-1/PD-L1 antibodies will be successful, especially when combined with cancer therapies associated with DNA damage, such as radiotherapy and DNA-damaging chemotherapy (McLaughlin et al., 2020).

Genome instability reflects an increased likelihood that the genome will acquire certain mutations, such as nucleotide sequence changes or chromosomal rearrangement, gain or loss (Langie et al. 2015). Meanwhile, the other form causes mortality in cells, with DSBs being among the most dangerous forms of DNA damage. In adequately

repair of such lesions can have a detrimental effect in terms of genome instability, reducing the rate of cellular survival (Podhorecka et al. 2010). With their highly deleterious nature, even a single DSB, unless repaired, is enough to cause cell death. Cancer cells evidence a greater DSB burden than healthy cells as a result of the replication stresses derived from oncogenes in addition to the deficiencies in the DNA damage response (DDR) mechanism (Trenner and Sartori 2019).

In the current study, our results from the Comet, and micronucleus assays showed that naked and liposome pembrolizumab forms (10 μg/ml) do not induce DNA or chromosomal genotoxicity of any kind in lymphocytes from healthy individuals and HNC patients assessed at basal levels.

Pembrolizumab has been demonstrated to increase the capacity for cell repair and promote genome integrity. The current research has demonstrated that pembrolizumab promotes a significant decrease in the DNA damage induced by exposure to H_2O_2. This may be due to the early stimulation of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) following cells' exposure to a pro-apoptotic agent. PARPs are crucial to a variety of cellular processes, including recombination, replication, DNA repair and chromatin remodelling. It should be noted that they also regulate certain inflammatory processes, and it has been suggested that DNA repair deficiencies in combination with chronic inflammation may make cells more prone to the development of cancer (Keung et al. 2019; Pazzaglia and Pioli 2019).

The transcription factor p53 is a tumour growth suppressor that regulates multiple genes possess a range of biological functions, most importantly those that involve regulating the cell cycle and transcription (Allen et al. 2014).

Numerous stress signals induce the activation of p53, including oncogene activity and DNA damage. It generally leads to apoptosis and the arrest of the cell cycle (Chen 2016).

Defects in the checkpoints in the G1/S phases in cancer cells tend to correlate with the inactivation of the p53 pathway, which is generally considered to relate to the suppression of the apoptosis and cell cycle arrest processes (Benedict et al. 2018).

Based on this, p53 both induces the G1 phase and cell cycle arrest and motivates the expression of the cyclin-dependent kinase inhibitor (CDKI) p21. This crucial and multifaceted protein, a founding member of the CDKI, contributes to the regulation of the cell cycle and genome stability maintenance (Kreis et al. 2019; Mamoor 2020).

Despite its role as a transcription factor, p53 also relates to crucial apoptosis-linked genes; for example, it downregulates Bcl-2 (an anti-apoptotic protein) expression (Li et al. 2015b) and activates the apoptotic pathway by triggering the pro-apoptotic protein Bax. Studies have shown that to effectively remove cancerous cells, it is preferable to downregulate Bcl-2 while concurrently overexpressing Bax (Naseri et al. 2015).

It is well-established that p53 tumour suppressor gene inactivation is a common characteristic found in tumour development. In general, the gene acquires a mutation, producing mutant p53 proteins with good stability. Beyond losing their tumour suppression function, these altered proteins frequently acquire oncogenic features that allow cancerous cells to experience increased survival and growth rates. Hence, the accumulation of these proteins is a well-established characteristic of cancer cells.

Notably, the *p53* gene is known to acquire mutations at any point in the various phases of malignant transformation, which is a multistep process. As a result, *p53* gene

mutation can have a differential effect on the initiation, growth, metastasis and aggressiveness of tumours (Rivlin et al. 2011).

P53 primarily regulates apoptosis induction by transcriptionally activating certain genes (Fridman and Lowe 2003). Moreover, genes regulated by p53 generate proteins, prevent damage to DNA, repair damaged DNA, and produce feedback loops to inhibit or strengthen the p53 activity. They also communicate with other signal transduction pathways via stress signals. These are sent via positive and negative regulators and can relate to hypoxia, DNA damage, and neighbouring cells being deprived of nucleotides (Sionov et al. 2001; Levine et al. 2006).

Stresses that can adversely affect cell division, especially DNA replication fidelity, can trigger the p53 pathway (Harris and Levine 2005). This is likely to prevent mutations by initiating certain repair pathways prior to the mitosis phase (Shaw 1996).

Studies have shown that after stimulation by p53, p21 sets the machinery of excision repair in motion (Al Bitar and Gali-Muhtasib 2019), via an interaction with the proliferating cell nuclear antigen (PCNA) complex (Moldovan et al. 2007; Vuong et al. 2012).

The p53 protein activates the Mdm2 gene, providing feedback-based control of the activity of p53. During early development, this is probably the primary aim of Mdm2 (Gupta et al. 2019).

The effects of naked and liposome pembrolizumab on P53 protein expression at post-translational and gene expression in lymphocytes from HNC patients and healthy people were studied, as well as their effects on some of the p53-regulated genes, such as p21 and Bcl-2, by estimating their regulation in lymphocytes from HNC patients and healthy individuals.

Protein and gene expression in lymphocytes from HNC patients and healthy individuals were investigated using WB and qRT-PCR. In cells from healthy people, a 24-hour treatment with naked and liposome pembrolizumab showed no effect on p53 and p21 expression at protein and mRNA levels. The manifestations of these genes were scarcely detectable. Our findings are in line with a prior study that found that the MDM2 proto-oncogene keeps p53 at a low level in normal cells (Reisman et al. 2012).

However, p53 protein levels in HNC patients' lymphocytes increased significantly after treatment with naked and liposome pembrolizumab (Figure 6.2), which is consistent with PCR results showing that the p53 gene was up-regulated in lymphocytes from HNC patients after treatment with naked and liposome pembrolizumab, implying that pembrolizumab causes cell cycle arrest by p53 that leads to DNA repair in HNC patients' lymphocytes. And this agreed with previous studies which reported that high levels of p53 cause cycle arrest (Kreis et al. 2019; Mamoor 2020). DNA damage in HNC patients might stimulate protein kinases such as ATM to phosphorylate p53 and increase p53 level. Because Mdm2 expression is stimulated by p53, the raise of p53 expression also increases Mdm2, but it has no effect although p53 is phosphorylated. After repairing of DNA damage, the ATM kinase will be not activated. p53 will be rapidly dephosphorylated and damaged by the Mdm2 accumulation. When DNA damage occurs the P53 stimulate P21 then P21 inhibits the enzyme Cdk2 that is required in S phase and M phase, and it is very important to induce growth arrest.

Furthermore, the p21 expression result was similar to p53 expression, with p21 significantly up-regulated in lymphocytes from HNC patients when treated with both naked and liposome pembrolizumab at both the mRNA and protein levels (Figure 6.2), in agreement with previous studies that show that increased p53 gene expression is often accompanied by up-regulation of the p21 gene (Ye et al. 2016; Shamloo and

Usluer 2019). Low levels of p53 protein are extremely susceptible to the activation of p21 expression (Vousden and Prives 2009).

In lymphocytes from healthy individuals, naked and liposome pembrolizumab regulated p53 and p21 gene expression differently than in lymphocytes from HNC patients. This is because pembrolizumab has a greater effect on lymphocytes from HNC patients than on lymphocytes from healthy individuals, and because lymphocytes from patient groups are more susceptible to pembrolizumab than lymphocytes from healthy individuals owing to genomic instability and malignancy. These results agree with Fang et al (2019) who found that p53 activation could increase antitumour immunity caused by anti-PD-1 therapy in the tumours of mice treated with a combination of APG-115 (a MDM2 antagonist) with pembrolizumab.

The naked and liposomal versions of pembrolizumab, on the other hand, had no statistically significant influence on Bcl-2 expression in lymphocytes from HNC patients and healthy individuals (Figure 6.1 and Figure 6.2).This is because pembrolizumab did not influence the apoptotic pathway and instead aided a p53-mediated repair mechanism in HNC patients' lymphocytes to limit proliferation. This result agrees with Naseri et al, 2015 who found that the increases of p53 level causes decline of the Bcl-2 level.

To summarize our findings, both naked and liposome versions of pembrolizumab showed genoprotective antioxidant activity in lymphocytes from HNC patients and healthy individuals. In both groups, they proved that they are powerful antioxidants by safeguarding the cell against H_2O_2 and DNA damage. Pembrolizumab, both naked and liposomal, has been found to have genoprotective effects in lymphocytes from

HNC patients by activating the p53 and p21 genes, which drive cell cycle arrest and DNA repair, respectively.

Liposomes are an excellent choice for an optimum drug-carrier system since their shape is similar to that of biological membranes and they may include a range of chemicals.

Their value as optimal methods for the administration of biologically active compounds in vitro and in vivo derives largely from their technological and biological advantages. Furthermore, formulations based on liposomes have lower toxicity than single-drug formulations, as well as better pharmacological characteristics as a drug delivery mechanism.

This might be related to pembrolizumab liposome's increased solubility in aqueous media, which helps to stabilise diverse therapeutic agents including proteins and nucleotides. Liposomes can encapsulate a wide range of biomolecules, both hydrophobic and hydrophilic, and protect them from the environment. Furthermore, they obstruct the drug's metabolic effects until it reaches the target cell, preventing it from interacting with healthy cells. This improves the drug's therapeutic index even further.

Therapeutic medicines in the liposomal form have been shown to have enhanced biodistribution and pharmacokinetic features when compared to their free counterparts, underlining the advantages of this drug delivery strategy.

The present study's overall findings suggest that liposomes can possibly enhance the interaction of pembrolizumab with cancer cells resulting in better therapeutic effects and ultimately proposing a better drug delivery system.

Finally, we would like to mention to some limitations in this study such as the size of sample was small and there were big different of ages between healthy and H&N cancer patients. These limitations must be considered in the future studies to give clear picture about the relationship between the ages and Head and neck cancer.

6.2 Future work

The findings of this study revealed that pembrolizumab liposomes decreased DNA damage in human lymphocytes from HNC patients *in vitro*. It was also shown that pembrolizumab protected cells from DNA damage induced by oxidative stress produced by H_2O_2. The findings further imply that in the presence of H_2O_2, pembrolizumab liposome reduced oxidative stress in lymphocytes from HNC patients. Micronucleus FISH (Fluorescence *in situ* hybridisation) is one of the methods that may be used to validate these findings and determine the type of chromosomal damage lesions. According to results of p53, p21, and Bcl-2, the pembrolizumab liposome exerts a regulatory impact. Still, additional study with other genes and proteins is required to validate potential in animal and human level. Future studies might also be conducted to find other possible mechanisms of action rather than the PD-1 binding mechanism.

Furthermore, it was shown that pembrolizumab liposomes decreased H_2O_2 related oxidative stress which could be proven effective in treating HNC patients where during the treatment, oxidative stress caused by various factors can be controlled to a minimum level promoting the effectiveness of treatment. This research might also be replicated using lymphocytes from patients with other forms of cancer and HNC tissue to see whether the outcomes are similar or different when compared to HNC results. Cancer-enabling traits such as genomic and chromosomal instability are a driving factor behind tumour heterogeneity and evolution, resulting in medication resistance

and worse clinical response rates. Our findings showed pembrolizumab improved cellular repair capability and increased genomic stability point to the possibility of testing pembrolizumab's efficacy in delaying the establishment of drug resistance in various cancer cells. Furthermore, the major implication of the current study is that pembrolizumab can potentially be used in combination with other chemotherapeutic agents which are known toxic to lymphocytes ultimately could help to protect cancer patients against the toxic effects of chemotherapy. Finally, more research into the liposomal formulation's long-term stability, loading levels, and release kinetics is needed.

Limitations of Thesis

1- Due to the Covid 19 epidemic the University of Bradford and the laboratory facilities remained closed for more than 4 months, we could not work in the lab during this time, which caused a lot of stress and back log.
2- Also, due to the epidemic, the patients could not come the hospital as before so that it was difficult to collect the blood sample from the patients and we had to work out with whatever was available during that time. We were not able to age-match the investigative groups. That's why age between the healthy individual group and patient group different considerably.
At the time of study initiation, the best possible match samples available (especially from the patient group) varied in age significantly between the healthy subject group and the cancer subject group. Most healthy individuals were young compared with cancer patients who were all above 50. Because the healthy samples were obtained from student and our friends so that the most of doners were young, while the most of patient more 50 years.
3.I was ill. I did an operation. I had struggled a lot both physically and mentally, but I tried to give my best and tried to manage my time effectively.

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Chapter 8

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

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		RESULT	DATE
WEIGHT			OTHERS		
HEIGHT					
BMI					

8.3 Appendix 3

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

Professor Diana Anderson, Established Chair in Biomedical Sciences, BSc, MSc, PhD, DipEd, FIBiol, FATS, FRCPath, FIFST, FBTS, FHEA, University of Bradford, Richmond Road, Bradford, BD7 1DP and Honorary Research Consultant to Bradford NHS Trust.

E-mail: d.anderson1@bradford.ac.uk

Dr Mojgan Najafzadeh MD, PhD, Post-doctoral Research Fellow, Division of Medical Sciences, University of Bradford, Richmond Road, Bradford, BD7 1DP and Honorary Research Consultant to Bradford NHS Trust.