Using DNA damage in circulating blood cells as a biomarker for oesophageal adenocarcinoma risk.

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Summary (Abstract)

This thesis explored DNA damage in circulating blood cells as biomarkers for oesophageal adenocarcinoma (OAC) risk and treatment response using two assays: the cytokinesis-block micronucleus (CBMN) assay to measure lymphocyte micronucleus frequency (MN%) and the *PIG-A* mutation assay to assess *PIG-A* mutant frequency (MF) in erythrocytes.

Baseline MN% and *PIG-A* MF were significantly higher in OAC patients compared to those with gastroesophageal reflux disease (GORD), Barrett's oesophagus (BO), and healthy volunteers (HVs), aligning with previous studies linking increased MN% to cancer risk. The predictive value of *PIG-A* MF as a biomarker for overall cancer risk needs further investigation through large-scale studies.

The study examined DNA damage response to cancer therapy, finding an increase in MN% and *PIG-A* MF during treatment, though the prediction of therapy response was inconclusive.

Further investigation into the mechanisms behind the elevated MN% in OAC patients revealed complex interactions involving oxidative stress and inflammation. Pro-oxidant agents like hydrogen peroxide (H_2O_2) and deoxycholic acid (DCA) highlighted an adaptive response in patients, suggesting reduced sensitivity to subsequent *in vitro* DNA damage. Plasma from OAC patients also showed increased DNA damage induction in *in vitro* TK6 cells, indicating a potential genotoxic effect. Elevated levels of inflammatory markers such as IL-6 and IL-8 were associated with increased MN%, suggesting their potential role in biomonitoring OAC risk.

The study examined the cGAS-STING pathway *in vitro* and *ex vivo*, finding slightly elevated levels of 2'3'-cGAMP and IFNβ in OAC patient plasma. This suggested a link to increased MN formation and highlighted the pathway's role in genetic instability and inflammation. Inhibition of the STING complex in TK6 cells prior to ionising radiation resulted in a slight but statistically non-significant reduction in MN%.

Future research should focus on tissue-specific DNA damage and its correlation with blood-based biomarkers to assess their potential for predicting early cancer development.

Declarations and Statements

Declaration: This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed: 

Date: 09/08/2024

Statement 1: This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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Date: 09/08/2024

Statement 2: I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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Statement 3: The University's ethical procedures have been followed and, where appropriate, that ethical approval has been granted.

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List of Figures and Tables

Figures

Tables

Abbreviations

1. 2’3’-cGAMP – 2’3’ cyclic GAMP-AMP
2. ACTB – Actin Beta
3. ANCOVA - Analysis of covariance
4. ANOVA - Analysis of Variance
5. AP – Anchored protein
6. APC - Allophycocyanin
7. ATCC – American Type Culture Collection
8. AZA – Azathioprine
9. BD – Becton, Dickinsons and company
10. BER – Base excision repair
11. BMI – Body mass index
12. BO – Barrett’s oesophagus
13. BRCA – Breast cancer gene
14. BSA – Bovine serum albumin
15. CA – Carbohydrate antigen
16. CBMN – Cytokinesis-block micronucleus
17. CBPI – Cytokinesis block proliferation index
18. CEA - Carcinoembryonic antigen
19. cfDNA – Cell free DNA
20. cGAMP – Cyclic GMP-AMP
21. cGAS– Cyclic GMP–AMP synthase
22. CIN – Chromosome instability
23. CO_2 – Carbon dioxide
24. CRP – C-reactive protein
25. CT – Computed tomography
26. CTC – Circulating tumour cell
27. ctDNA – Circulating tumour DNA
28. Cyto-B – Cytochalasin-B
29. DAPI - 4,6-Diamidino-2-phenylindole
30. DCA – Deoxycholic acid
31. DCLK1 – Doublecortin Like Kinase 1

32. DDR – DNA damage repair
33. DH2O – Deionized water
34. DMSO - Dimethyl sulfoxide
35. DNA – Deoxyribonucleic acid
36. DQS – Dietary quality score
37. DSB – Double strand break
38. dsDNA – Double stranded DNA
39. EDTA - Ethylenediamine tetraacetic acid
40. ELISA - Enzyme-linked immunosorbent assay
41. EPIC - European Prospective Investigation into Cancer and Nutrition
42. ER – Endoplasmic reticulum
43. EUS – Endoscopic ultrasound
44. FAP – Familial adenomatous polyposis
45. FLOT - 5-FU, leucovorin, oxaliplatin, and docetaxel
46. GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase
47. GI – Gastrointestinal
48. GOJ – Gastroesophageal junction
49. GORD – Gastroesophageal reflux disease
50. GPI - Glycosyl phosphatidylinositol
51. GSH - Glutathione
52. H2AX - H2A histone family member X
53. H2O2 – Hydrogen peroxide
54. H2SO4 – Sulfuric acid
55. HGD - High grade dysplasia
56. HPI- Health promotion index
57. HPRT - Hypoxanthine-guanine phosphoribosyltransferase
58. HR – Homologous recombination
59. HS – Horse serum
60. HuMN – Human Micronucleus
61. HV – Healthy volunteer
62. IBD – Inflammatory bowel disease
63. ICS- Instrument calibration standard
64. IFN – Interferon
65. IL- Interleukin

66. IR – Ionising radiation
67. KCL – Potassium chloride
68. LGD - Low grade dysplasia
69. LINAC – Linear accelerator
70. MAP - MUTYH-associated polyposis
71. MN – Micronucleus/Micronuclei
72. MN% - Micronucleus frequency
73. MYC - MYC Proto-Oncogene, BHLH Transcription Factor
74. NAC – N-acetylcysteine
75. NBUDs – Nuclear buds
76. ND - Non dysplastic
77. NER – nucleotide excision repair
78. NHEJ – Non-homologous end joining
79. NHS – National health service
80. NNK - Nicotine-derived nitrosamine ketone
81. NPB – Nucleoplasmic bridges
82. OAC – Oesophageal adenocarcinoma
83. OECD - Organisation for Economic Co-operation and Development
84. OGD - Oesophagogastroduodenoscopy
85. OSCC – Oesophageal squamous cell carcinoma
86. PAH – Polyaromatic hydrocarbon
87. PBL – Peripheral blood lymphocyte
88. PBS - Phosphate buffered saline
89. PCR – Polymerase chain reaction
90. PE – Phycoerythrin
91. PET - Positron emission tomography
92. PHA – Phytohemagglutinin
93. *PIG-A* – Phosphatidylinositol Glycan Class A
94. PMBC - Peripheral blood mononuclear cell
95. PPI – Proton pump inhibitor
96. PSA - Prostate-specific antigen
97. PTEN - Phosphatase and Tensin Homolog
98. qPCR – Quantitative polymerase chain reaction
99. RBC – Red blood cell

100. REC – Research ethics committee
101. RET – Reticulocytes
102. ROS – Reactive oxygen species
103. RONS - Reactive oxygen and nitrogen species
104. RPD – Relative population doubling
105. RPMI - Roswell Park Memorial Institute
106. RT – Reverse transcriptase
107. SBUHB – Swansea Bay university health board
108. SOD – Superoxide dismutase
109. STING – Stimulator of interferon genes
110. SUMS – Swansea university medical school
111. TK - Thymidine kinase
112. TNF – Tumour necrosis factor
113. TP53 - Tumour Protein P53

Chapter 1 - General introduction

1.1 Cancer

The hallmarks of cancer

Cancer is a disease of uncontrolled proliferation by transformed cells, characterised by their unchecked growth and division. This leads to the formation of abnormal cell clusters known as tumours[1]. Cancer's defining traits are unique biological abilities that allow cancer cells to survive, multiply, and spread throughout the body. Douglas Hanahan and Robert Weinberg's previous work identified these traits to help explain the complex processes behind cancer development and progression[2, 3]. There are eight defined hallmarks of cancer determined by Hanahan *et al.,* (2022), two enabling characteristics, and four additionally proposed emerging hallmarks and enabling characteristics. As shown in **Figure 1.1**, the proposed hallmarks included the following: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming energy metabolism, and evading immune destruction. The enabling characteristics are genome instability and tumour-promoting inflammation. The emerging hallmarks involve unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells[3].

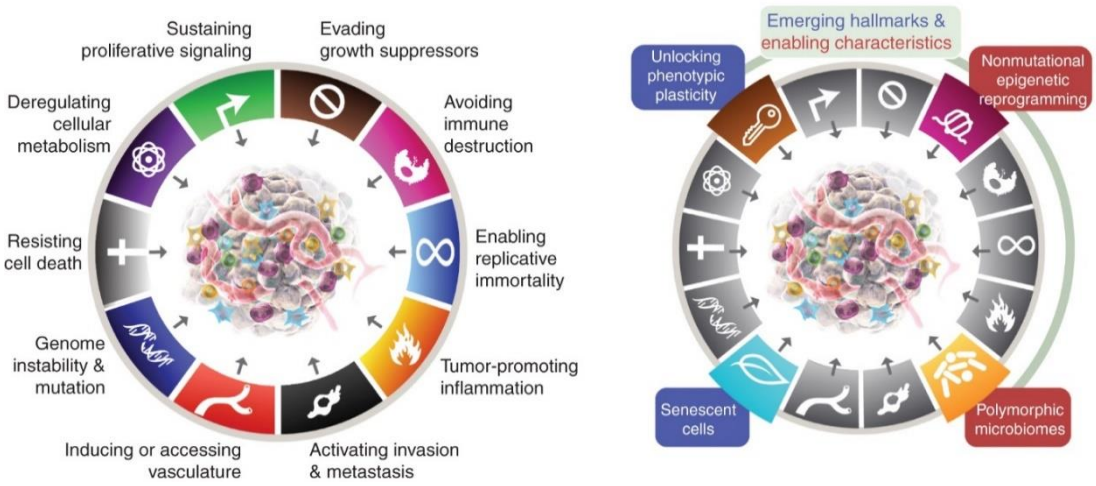

Figure 1.1 *Hallmarks of Cancer, circa 2022. Left: The Hallmarks of Cancer include eight hallmark capabilities and two enabling characteristics. The initial six hallmarks proposed in 2000 were expanded in 2011 to include reprogramming cellular metabolism and avoiding immune destruction, now part of the core set. The 2011 update added tumour-promoting inflammation alongside genome instability as key enabling characteristics. Right: The proposed emerging hallmarks and enabling traits in Hananah et al., (2022). Including unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells. Image taken from Hanahan and Weinberg (2022).*

DNA damage and cancer

Unrepaired damage to deoxyribonucleic acid (DNA) can cause mutations or chromosomal aberrations that activate oncogenes or deactivate tumour suppressor genes, leading to the malignant transformation of cells and the development of cancer[4]. DNA damage can happen spontaneously, or through the exposure to a mutagen. DNA damage arises from exposure to environmental hazards like toxic chemicals, or ionising radiation (IR), as well as endogenous factors such as reactive oxygen species (ROS) and replication errors[5]. Types of DNA damage include single-strand breaks, double-strand breaks, base modifications, DNA crosslinks, and clustered lesions, occurring directly or via reactive intermediates[6]. Persistent DNA damage that disrupts replication or transcription can trigger cellular senescence or apoptosis, both suppressing tumour development[4], and in regards to senescence – contributing to

tumorigenesis. However, improper DNA repair can lead to genomic instability and cancer initiation[4]. Deficiencies in DNA repair and impaired DNA damage response (DDR) pathways are frequently observed in cancer cells. Defects in DNA repair mechanisms, including nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ), increase cancer risk by allowing DNA damage to accumulate[4]. The accumulation of these changes to the DNA over a prolonged time results in the formation of cancer[7], hence why the most important risk factor to cancer is old age. Cancer cells often exhibit higher rates of genetic alterations, including chromosomal rearrangements, gene mutations, and epigenetic changes[8]. These alterations can disrupt essential cellular pathways, leading to uncontrolled cell growth and other cancer-related traits[9]. For example, mutations in the tumour suppressor gene *TP53*, (which encodes the p53 transcription factor), occur in over 50% of cancers[10]. The p53 tumour suppressor is often inactivated during tumourigenesis through mutations that produce a stable mutant protein[11]. This mutant p53 loses its tumour-suppressing function (as demonstrated in **Figure 1.2)** and gains oncogenic properties, aiding cell growth and survival[11].

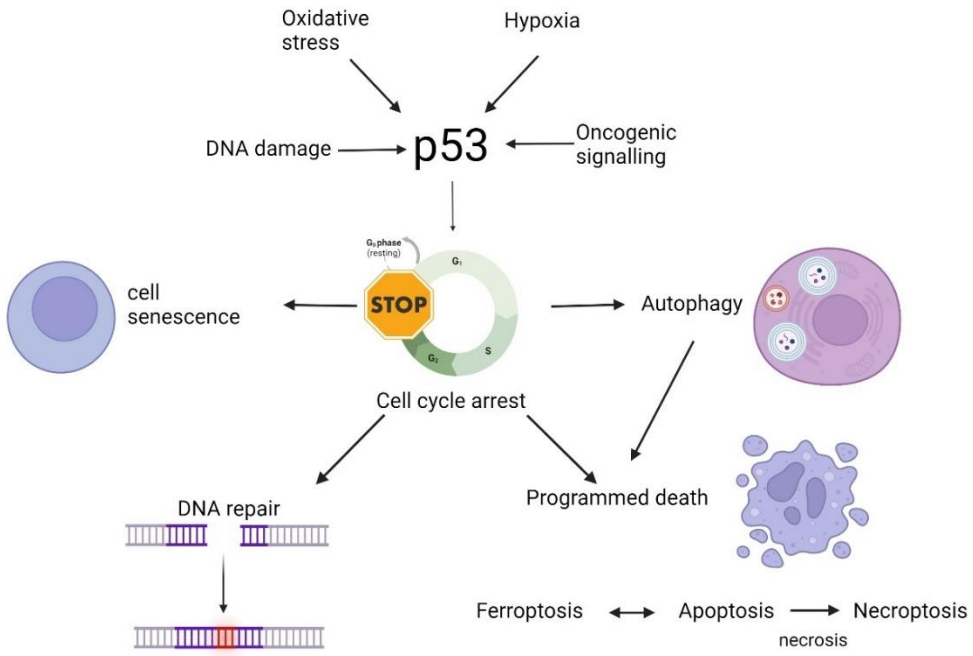

Figure 1.2. *Protective function of the transcription factor p53. p53 responds to various cellular stress signals, such as hypoxia, oncogene activation, DNA damage, and oxidative stress caused by reactive oxygen species (ROS). In response, p53's activity is enhanced. The typical response involves halting the cell cycle, facilitating DNA repair, and inducing cellular senescence or apoptosis through transcriptional and translational mechanisms. Adapted from Moulder et al., (2018)*[12]*. Created with Biorender.com.*

1.2 Oesophageal cancer

There are two main types of oesophageal cancer, oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma (OAC). OSCC occurs in the upper oesophagus, and shares traits with squamous cell carcinoma of the head and neck, whilst OAC occurs in the lower oesophagus and genetically resembles chromosomally unstable gastric cancer [13]. Whilst rates of OSCC are declining globally, OAC incidence has risen in recent decades. It is the 14th most common cancer in the UK, and the 7th most common cause of cancer death. This cancer has one of the poorest 5-year survival rates, with only 17% of patients in the UK surviving beyond five

years.[14]. The rapid increase in cases is hypothesised to be due to an increase in patients with gastro oesophageal reflux disease (GORD), whereby chronic exposure to refluxate constituents in patients leads to the condition known as Barrett's Oesophagus (BO)[15]. BO is the only known precursor lesion for OAC. Most BO and OAC cases are symptomless until advanced, leading to poor prognosis. Symptoms like dysphagia and weight loss prompt late medical attention, resulting in low 5-year survival rates[16]. Cancer Research UK statistics show that if OAC is diagnosed at an early stage 84% of people will survive for 1 year or more, with only 21% of people surviving the same amount of time with a late diagnosis[14]. This thesis will primarily focus on OAC, and the GORD to BO to OAC model.

Chronic reflux to cancer

GORD

GORD is a chronic condition whereby stomach contents repeatedly flow back into the oesophagus[17]. GORD occurs due to the lower oesophageal sphincter muscle becoming weakened or relaxing inappropriately. The resultant backwash, consisting of acid, bile, pepsin and pancreatic enzymes irritates the oesophageal lining, causing symptoms such as heartburn, regurgitation, and dysphagia[18]. Various risk factors contribute to GORD, including obesity, hiatal hernia, pregnancy, smoking, certain foods and beverages, as well as medications that relax the lower oesophageal sphincter (such as benzodiazepines)[19]. As well as lifestyle modifications (such as weight loss, and avoiding trigger foods) over-the-counter antacids and H2 blockers are prescribed to help with mild symptoms, while proton pump inhibitors (PPIs) reduce stomach acid production for more severe cases[20]. Surgery, such as fundoplication (whereby the top of the stomach is wrapped around the lower oesophagus to strengthen the valve), is considered for those who do not respond to medications[21]. Chronic, untreated GORD can lead to complications such as oesophagitis, oesophageal strictures, BO, and a slightly increased risk of OAC[22].

Barrett's oesophagus

BO is the only known pre-malignant lesion to OAC and is characterised by the replacement of normal squamous mucosa of the lower oesophagus with columnar-type mucosa[23] (**Figure 1.3**). There are multiple risk factors contributing to BO. Obesity (particularly central adiposity) is a significant factor, potentially due to mechanical

effects on reflux and the release of adipokines, with waist-to-hip ratio potentially indicating risk of BO more so than BMI[24]. Men are more likely to develop BO than women, with a male-to-female ratio of approximately 2-3:1[25]. Additionally, white individuals have a higher incidence of BO compared to other racial groups[26]. The risk of BO increases with age, with most cases occurring in individuals over 50 years old[27]. Alcohol is not a strong risk factor for BO, however, can worsen chronic reflux[26, 28]. Cigarette smoking is also not deemed a moderate risk factor, but it significantly increases the risk of progression to HGD and cancer in smokers with BO[24]. About 7% of BO cases show familial clustering, indicating a slight genetic predisposition. Genome-wide association studies have identified 16 risk loci for BO/OAC, with the strongest association within the CFTR gene (which codes for the cystic fibrosis transmembrane conductance regulator protein)[29]. Due to the nature of the protein as a chloride channel, the mucus membrane lining the oesophagus is altered, and 81% of cystic fibrosis patients have GORD, as well as a 3-fold increased risk of developing BO[30]. Interestingly*, whilst Helicobacter pylori* (*H.pylori*) infection increases an individual's risk for gastric cancer, it may reduce BO risk by lowering stomach acid production[24]. Hence, *H. pylori* eradication may increase risk of BO occurring.

Barrett's surveillance

As almost all cases of OAC are thought to arise from BO (the condition increases risk by 30-40 times[31]), it is important that patients with BO undergo endoscopic surveillance[32]. In the UK, high risk patients are recommended to have two yearly endoscopies with biopsies[27]. Biopsies are essential for histopathological confirmation, as they provide critical information about the presence of any dysplasia and/or cancer. This information includes the tumour's sublocation, and the presence and extent of BO. This enables the identification of high-risk factors for BO progression, such as segment length and mucosal abnormalities like oesophagitis grade[24]. BO segments are measured from the columnar mucosa extending proximally from the upper limit of the gastric folds at the gastro-oesophageal junction (GOJ)[24]. Longer segments indicate increased risk of progression[33]. Histological risk factors identified through analysis of biopsy tissue include intestinal metaplasia and dysplasia – e.g. No, low- or high-grade dysplasia (ND, LGD and HGD), as well as molecular events e.g. p53 overexpression are measured[34]. Chung *et al.,* (2007) studied p53 alterations in OAC, and concluded that these events are crucial for BO progression. The authors found mutations in 75%

of OAC cases, with 58% showing p53 protein overexpression[35]. Despite varied reported frequencies, p53's utility in monitoring BO is limited due to late-stage detection and inconsistent mutation detection by sequencing versus immunohistochemistry[35].

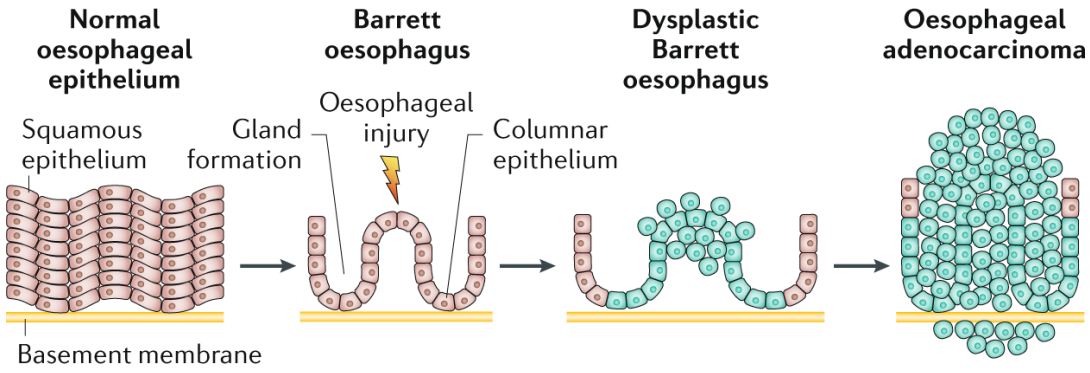

Figure 1.3. *The malignant transformation of tissue, from normal squamous epithelium to the formation of pre-malignant condition Barrett's oesophagus, to oesophageal adenocarcinoma[36]. Diagram taken from Peters et al., (2019)*

Diagnosis of OAC

The current diagnostic tests for OAC include several key procedures. Upper endoscopy (oesophagogastroduodenoscopy or OGD) is the primary diagnostic test, involving the insertion of a flexible endoscope through the mouth to visualise the oesophagus, stomach, and duodenum, allowing for direct observation of abnormal growths or lesions and enabling biopsies for histological confirmation of OAC[37]. A barium swallow is sometimes utilised as an initial alternative to endoscopy. This procedure involves swallowing a barium solution to visualise abnormalities or obstructions on X-ray imaging. This method is useful for detecting advanced OAC but less sensitive for early lesions[38].

If OAC is suspected, endoscopic ultrasound (EUS) is often performed alongside OGD to assess the depth of tumour invasion and involvement of adjacent structures, aiding in cancer staging and determining the appropriate treatment approach[39]. Computed tomography (CT) scans provide detailed images of the oesophagus and surrounding areas to evaluate tumour spread and lymph node involvement. Positron emission

tomography (PET) scans, often combined with CT (PET-CT), detect metabolically active cancer cells and identify distant metastases, assisting in accurate staging[39].

Endoscopic surveillance for BO is crucial for early cancer detection, yet the cost-effectiveness is debated due to the low rate of progression to cancer in those who have BO (0.33% annual risk)[40]. Furthermore, it is estimated that less than only 5% of those diagnosed with OAC had a prior BO diagnosis[41] with most BO patients being asymptomatic. This method of surveillance is incredibly invasive, and not always well tolerated by patients. It also comes with the potential for serious complications such as perforation of the oesophagus or infection. On top of this, expert gastro-intestinal input is required, and as diagnosis is based solely on dysplasia grading, there is clear inter-observer variation[42] Due to COVID-19 limiting non-essential procedures, the lists of patients requiring endoscopy are backlogged. Improved management and diagnostic tools are needed to ensure high-risk individuals receive appropriate monitoring, while minimising unnecessary procedures. For example, the development of non-endoscopic tests, tests run through primary care and better biomarkers for future improvements in BO diagnosis and management[43].

Molecular Characterisation of Oesophageal Adenocarcinoma

Recent genomic profiling efforts have greatly enhanced our understanding of the molecular alterations that underlie OAC. Large-scale studies, including work by The Cancer Genome Atlas (TCGA), have identified frequent mutations in TP53, CDKN2A, SMAD4, ARID1A, and amplifications in genes such as ERBB2 (HER2), MET, and FGFR2 — some of which represent actionable targets with therapeutic implications[44] For example, HER2 amplification has led to the use of trastuzumab in selected OAC patients. These findings are significant not only for understanding tumour biology, but also for guiding the development of predictive biomarkers that could improve early detection and individualised treatment strategies. Furthermore, molecular subtyping has revealed potential subgroups of OAC, including classifications based on chromosomal instability, DNA hypermutation, and specific mutational signatures[44]. Recognising and incorporating such subtypes into research and clinical workflows may help explain the variability in biomarker performance or patient response observed in studies and ultimately contribute to more tailored patient management approaches.

Despite these advances, the overall prognosis for OAC remains poor, and challenges in early detection, treatment resistance, and biomarker reliability persist. This underscores the need for continued research into novel diagnostic and therapeutic approaches that can improve patient outcomes.

The Cytosponge™

The Cytosponge™ is an example of a non-endoscopic diagnostic tool that is currently being evaluated for use in monitoring those with BO. This test involves swallowing a small capsule attached to a string; the capsule dissolves in the stomach, releasing a sponge that collects cells from the oesophagus lining when withdrawn. Combined with biomarker tests, it can help stratify patients' risk of developing dysplasia or cancer. For example, the cells can be analysed for Trefoil Factor 3 (TFF3), a protein indicating BO[45]. Clinical trials have shown that the Cytosponge-TFF3 procedure is safe, effective, and well tolerated. A large study found it has a 92.4% accuracy for diagnosing BO and a 79.9% sensitivity, which increases to 87.2% for patients with a higher cancer risk[46]. While Cytosponge™ is an effective screening tool, currently endoscopy remains the gold standard for diagnosis.

1.3 Liquid biopsy

Liquid biopsies as a non-invasive tool for early cancer detection, is an idea that has received great attention over the last decade due to the ease of sample acquisition. Potential cancer biomarkers can be found through the analysis of biological materials found in peripheral blood (and other body fluids) including; circulating tumour cells, metabolites, and cell-free DNA and RNA[47]. Compared to tissue biopsy, which is used to determine tumour characteristics, the analysis of biomarkers used in liquid biopsy are, as described by Payne *et al.,*2018 an "objective and quantitative test of disease progression"[48]. However, these biomarkers can also allow for early detection of cancer, to assess tumour dynamics and to monitor response in those receiving therapy or post-therapy[49].

Conventional and well established methods of population-wide cancer screening such as by mammogram (for detecting breast cancer) or the pap test (for detecting cervical cancer) are incredibly efficient, however they are specific to their cancer type[47]. The same can be said for liquid biopsy methods currently in place for early cancer detection, such as high levels of prostate-specific antigen (PSA) and carbohydrate

antigen 19-9 (CA19-9). PSA has been used to monitor progression of prostate disease since the 1980's, as high levels were found to be indicative of prostate cancer[50]. Similarly, high levels of circulating CA19-9 are used as an indication of pancreatic cancer[50]. Not only are these tests limited by specificity, they can also be elevated in a variety of benign conditions such as patients with gallstones presenting elevated CA19-9[51]. Using isolated individual biomarkers for cancer diagnosis can be inefficient due to the high number of false positives causing patients unnecessary anxiety and stress[51], however they are incredibly useful in prioritisation of patients for further investigation.

There are many advantages to using liquid biopsy as a method of screening BO for OAC risk, prior to conventional endoscopy and biopsy. It is minimally invasive and would prevent healthy individuals from undergoing a major procedure with multiple risks, allowing prioritisation of these healthcare resources for patients. Liquid biopsy approaches also have many applications –they can be used to monitor progression or spread of cancer, and genetic evaluation could provide clinicians with vital information for deciding on the best treatment. Whilst a tissue biopsy represents the molecular composition of the tumour from the time at which it was taken, liquid biopsy allows access to real time information on the continuously changing genetics of malignant neoplasms[47].

A paper by Cohen *et al.,* (2018), described a blood test named CancerSEEK, that can detect 8 common types of cancer including ovarian, liver, pancreatic, oesophageal, colorectal, lung, breast, and stomach[52]. This test requires the assessment of circulating protein levels, as well as mutations in cell free circulating DNA (cfDNA). The cfDNA assay is PCR based and can simultaneously assesses multiple regions of driver genes commonly mutated in many cancer types[52]. It was found to be more sensitive at detecting these mutations than conventional genome wide sequencing[52].The sensitivity of cancerSEEK ranges from 69-98% for detection of cancers that currently have no population-wide screening tests for individuals not deemed to be high risk of developing the disease such as ovarian, lung stomach and oesophageal cancer[52]. Despite this, the test was overall poor in detecting early-stage cancers, particularly oesophageal, therefore highlighting limitations to this assay.

DNA damage biomarkers detected through liquid biopsies have shown promise for early cancer detection, monitoring treatment response, and predicting outcomes. For example, ctDNA containing specific mutations or alterations in DNA repair genes like *BRCA1/2, ATM*, and *PARP* can be detected in blood samples[53]. Levels of DNA repair enzymes such as OGG1, MPG, and APE1 in blood can be combined into a "DNA Repair score," serving as a potential biomarker for cancer risk assessment[54]. Additionally, increased levels of tumour mutational burden and microsatellite instability, indicators of DNABL damage, can be detected in ctDNA and may predict response to immunotherapies[55]. Direct measurement of DNA / chromosomal damage in cells can be measured by several well developed techniques. The Comet Assay detects DNA single and double-strand breaks at the individual cell level, visualising damage as a comet-like tail after electrophoresis[56]. γH2AX detection identifies DNA double-strand breaks by detecting phosphorylated histone H2AX, enabling spatial localisation of damage within cells[57]. Quantitative PCR (qPCR) quantifies damage on specific genomic regions, crucial for studying repair mechanisms[58]. Additionally, the micronucleus assay measures micronucleus formation, indicating chromosomal instability and genotoxicity. These methods collectively enhance our understanding of DNA integrity and repair dynamics in cellular contexts. Indirect measurements of DNA damage include mutation tests such as the Ames test, *PIG-A* assay, and the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene mutation assay.

1.4 The Micronucleus assay

One test for DNA damage in human somatic cells is the micronucleus (MN) assay. According to Luzhna *et al.,* (2013), micronuclei (MN) are "extranuclear bodies that contain damaged chromosome fragments and/or whole chromosomes, not incorporated into the nucleus after cell division". MN were initially discovered in red blood cell precursors by Howell and Jolly in the late 19th century, where they were noted as residual cell nuclei present under pathological conditions. Their importance expanded in the mid-20th century when they were observed in bone marrow cells during disease, highlighting links to nutritional deficiencies such as in folic acid and vitamin B[59]. MN can also be measured in lymphocytes as markers for detecting chromosomal damage caused by genotoxic substances (**Figure 1.4**). Due to the low cost and high reliability of the assay, evaluating the expression of MN in *in vitro* cell lines for genotoxicity testing, as well as in peripheral blood lymphocytes (PBLs) for

biomonitoring has become a common and widespread practice. The cytokinesis-block micronucleus (CBMN) assay was developed in the 1980's, the basis of which allows MN frequency to be scored in binucleated cells that have recently completed cell division[59, 60] As part of the CBMN protocol, cytochalasin B is added to the cells to prevent cytokinesis[61], this allows for the elimination of potentially confusing results caused by the effects of cell division kinetics[60]. In circulating lymphocytes, it is especially important that MN are measured in binucleate cells to ensure cell division has occurred following mitotic stimulation with phytohemagglutinin (PHA). The CBMN assay also allows the measurement of biomarkers of chromosomal instability such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). NPBs can arise from dicentric chromosomes due to improperly repaired DNA double strand breaks or telomere end fusions. NBUDs form from the nuclear removal of unresolved DNA repair complexes and excess amplified DNA and can also result from the breakage of NPBs (**Figure 1.4**).

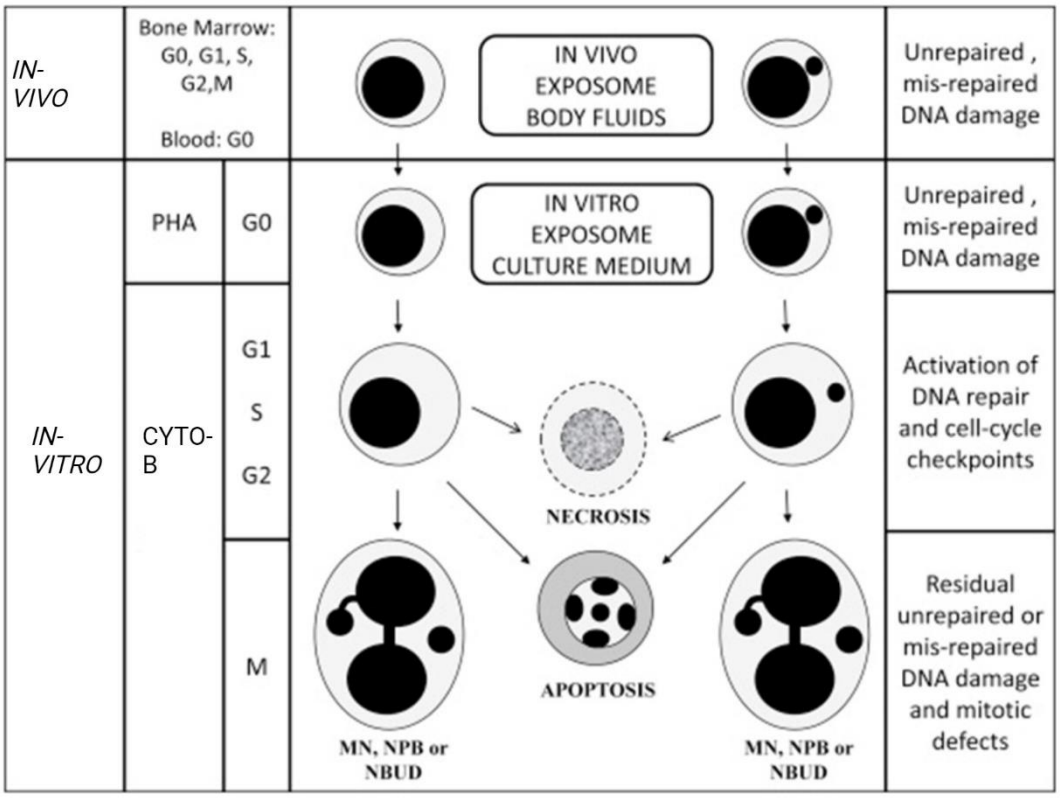

Figure 1.4. *Stages of the lymphocyte CBMN assay for measuring MN and other nuclear anomalies in cells exposed to genotoxicants in vivo. Cells are stimulated with phytohemagglutinin (PHA) in vitro for 44 hours prior to the addition of cytochalasin B (Cyto-B) to prevent cytokinesis. Measurements in mononucleated cells before mitosis and in binucleated cells after mitosis distinguish between MN expressed in vivo and ex vivo. Image taken directly from Kirsch-Volders et al., (2014)*

The HuMN project

Since the MN assay was first described, there have been many protocol variations used in laboratories internationally. The subjective nature of MN scoring results in significant inter- and intra-laboratory variability in the scoring of MN frequency at baseline levels[60]. This variation was highlighted by the Human Micronucleus (HuMN) project, an international database comparison of MN results. As described by Bonassi *et al*. (2001), this international collaboration was established in 1997 and included 25 laboratories and data from 7,000 subjects from various countries and populations[60]. The aim was to address the effect of different protocols and assess how these variations affected baseline MN frequency levels. They accumulated data from each laboratory to create an international database, intending to verify the importance of variable

protocols, as well as age and gender, to provide a reference range of normal values for lymphocyte MN frequency. Access to such information from a large study group was incredibly beneficial to researchers using this assay and contributed to biomarker validation. The potential issue with this data collection was that variation in quality and potential lack of subject information could result in problematic statistical analysis[60].

Origin of micronuclei

MN formation arises from either lagging chromosomes or acentric chromosome fragments that fail to integrate into daughter nuclei, instead becoming enclosed in separate nuclear envelopes[62]. Chromosome lagging can result from malfunctioning centromeres and kinetochores, improper kinetochore-microtubule attachments, and defects in mitotic spindle assembly[62]. For example, human and mice cells lacking functional kinesin motor gene *KIF18A* fail to align chromosomes correctly, leading to MN formation[62]. Other origins of MN containing whole chromosomes include hypomethylation, deficiencies in kinetochore proteins or their assembly, dysfunctional spindle apparatus, and defective anaphase checkpoint genes, all of which occur within the cell's natural processes[59].

Acentric chromosome fragments are the result of unrepaired DNA double-strand breaks (DSBs), which can be caused by exposure to ionising radiation (such as X-rays or gamma rays)[63] or certain chemicals such as polycyclic aromatic hydrocarbons (PAHs) found in coal tar and soot[64]. Endogenous processes, such as errors in DNA replication and responses to oxidative stress, also contribute to the occurrence of DSBs. DSBs can also form from closely aligned single strand breaks leading to fragmentation. Within eukaryotic cells, DSBs are a common occurrence and are typically corrected through two primary repair pathways: HR and NHEJ[63]. The diversity of factors causing DSBs results in a spectrum of DNA end configurations that require specific repair mechanisms. Deficiencies in these repair pathways, allowing the level of DSBs to exceed the repair capacity of dividing cells can leave DNA fragments unattached, potentially forming MN[63]. The impact of endogenous DNA damaging agents leading ultimately to lymphocyte MN formation *ex vivo* is uncertain regarding whether it primarily occurs in the body (*in vivo*) or during cell culture (*ex vivo*) due to persistent DNA damage[65]. Research suggests that MN found in

lymphocytes not yet divided after culture likely originate from *in vivo* exposure, while during *ex vivo* culture and forced lymphocyte division (using PHA), MN occur due to improperly repaired DNA damage[65]. For accurate interpretation in genetic toxicology and biomonitoring, understanding MN origins requires investigation into DNA repair dynamics and mutagen effects on chromosome integrity using tests like the L-CBMN assay.

MN and cancer

Chromosome instability (CIN) and aneuploidy are common characteristics of human tumours. Whilst aneuploidy is a specific manifestation of chromosome number abnormalities, CIN refers to the broader concept of increased genomic instability leading to various types of chromosomal alterations. The cascade of events initiated by chromosome instability in cancer cells includes errors in chromosome segregation, micronucleus formation, DNA damage, and chromothripsis. These abnormalities not only contribute to the genetic heterogeneity of tumours but also activate inflammatory pathways that may influence tumour progression and response to therapy[66]. For example, when DNA fragments are not properly repaired, they can trigger the cGAS-STING pathway, an innate immune response pathway that detects cytosolic DNA. Activation of cGAS leads to the production of cyclic GMP-AMP (cGAMP), which binds to the STING receptor, triggering the release of pro-inflammatory cytokines. This process plays a role in DNA damage-induced inflammation may promote a tumour-supportive inflammatory microenvironment[67].As well as being present in cancer cells, many studies have observed a relationship between PBL MN frequency (MN%) and cancer[68]. Research conducted by Bonassi *et al*., (2007) highlighted lymphocytes' MN% as a potential biomarker for cancer risk assessment. The study, spanning from 1980 to 2002 and involving 6718 subjects across multiple countries, observed a significant correlation between increased baseline MN levels in lymphocytes and higher incidences of various cancers decades later[68]. Particularly noteworthy were the associations found with urogenital and gastrointestinal cancers. Dhillon *et al.,* (2020) reviewed 19 studies to examine lymphocyte MN% in various cancers and found that MN levels were a biomarker for DNA damage and chromosomal instability in haematological and colorectal cancers, but not significantly elevated in skin, prostate, or oesophageal cancers[69].

Furthermore, cancer-prone patients with genetic disorders like Bloom syndrome and ataxia telangiectasia also exhibit high frequencies of MN in lymphocytes, reflecting their chromosomal instability[70, 71]. Many studies have also shown a correlation between exposure to MN-inducing agents, and risk of carcinogenicity[68]. Rothfuss *et al.,* (2000) investigated mutagen sensitivity in women with BRCA1 mutations, revealing heightened MN formation post gamma-irradiation and hydrogen peroxide exposure. During this study, while the comet assay found no difference in DNA repair rates, MN frequencies highlighted potential biomarker utility for screening BRCA1 mutation carriers in breast cancer families, underscoring its relevance in identifying genetic susceptibility to cancer[72]. Bonassi *et al.,* (2011) emphasised the need for further exploration into mechanisms, interindividual variability, and targeted preventive strategies to support the potential utility of lymphocyte MN frequency as a biomarker for various cancers[73].

1.5 The *PIG-A* assay

Phosphatidylinositol glycan class A (*PIG-A*) is a gene that encodes a catalytic subunit of the N-acetylglucosamine transferase complex[23]. The functionality of this complex is vital for the production of glycosyl phosphatidylinositol (GPI) molecules, which anchor key proteins e.g. CD55 and CD59 (involved in immune responses) to extracellular surfaces[23]. In those with paroxysmal nocturnal haemoglobinuria (PNH), a rare acquired genetic disease, patients present with a large fraction of *PIG-A* mutated erythrocytes in bone marrow and peripheral blood, phenotypically deficient in GPI-anchored proteins (GPI-APs), such as CD55[74]. PNH is diagnosed using flow cytometry; peripheral blood cells are labelled with fluorochrome conjugated antibodies against cell-type specific GPI-APs such as CD55 an CD59 which are usually expressed on the extracellular surfaces of red blood cells (RBCs) at high levels[75]. Mutant cells will not exhibit any fluorescence and can be enumerated to produce a diagnosis (**Figure 1.5**).

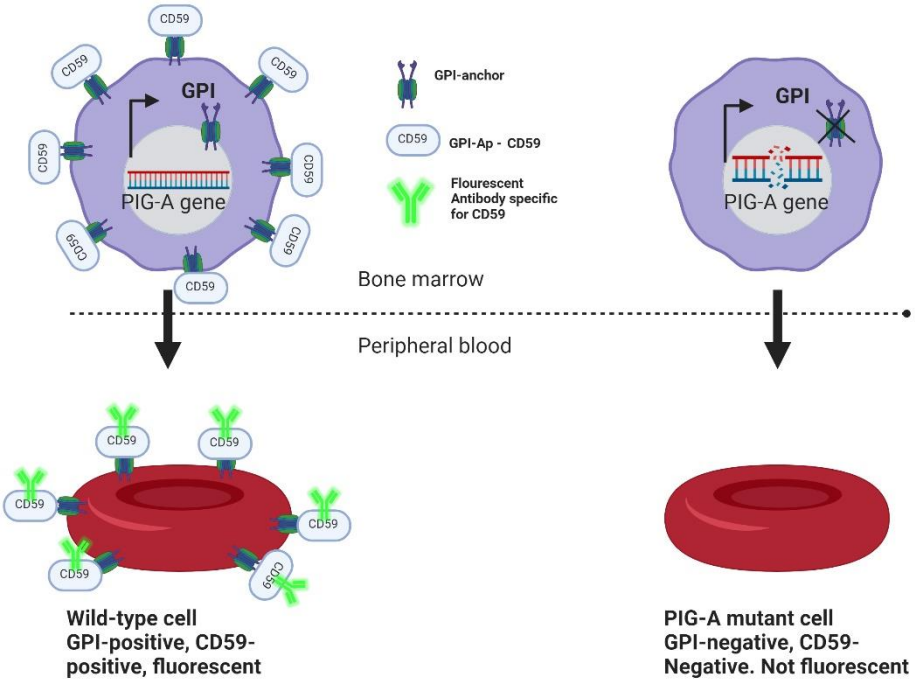

Figure 1.5 *Basis of the phosphatidylinositol glycan class A (PIG-A) mutant assay in erythrocytes. Functional PIG-A gene results in the production of GPI-anchors. Top left shows wild-type erythroid precursor cell with functioning PIG-A gene, resulting in the production of GPI molecules, and anchoring of the GPI-AP CD59. The resultant RBC (bottom left), when stained with fluorochrome-conjugated antibodies specific to CD59 will exhibit fluorescence when analysed with flow cytometry. Top right shows an erythroid precursor cell where the PIG-A gene has a loss of function mutation. This results in no GPI production and hence no CD59 anchored to the cell surface. The resultant RBC will show no fluorescence when analysed with flow cytometry. Diagram inspired by Gollapudi et al[76]. Created in BioRender*

The *PIG-A* assay is a recently developed mutation test, that is particularly prevalent in drug safety assessment due to how economic, rapid and accurate it is[23]. It was initially developed for use in rodents, and over 100 carcinogenic agents including chemicals, nano materials and ionising radiation, have been tested using this rodent *PIG-A* assay. The results from these tests have generally indicated that the assay has a remarkable sensitivity to mutagens[77]. Due to the conservation of the GPI synthesis pathway in mammals, Dobrovolsky *et al., (*2011) was able to develop the *PIG-A* assay for use on human RBCs, proving that it can also be used to measure mutation in human somatic cells[75]. This assay was further developed and improved by Dertinger *et al.,* 2015 who increased the reliability and reproducibility to measure human *PIG-A* gene mutant frequency (MF) by using both reticulocytes (RETs) and RBCs[78]. This enabled the capture of both recent and accumulated events. On top of this, they optimised the flow cytometry protocol—enhancing sensitivity and specificity through improved gating strategies and fluorescent labelling. They also implemented standardised procedures and automated data analysis tools, which reduced variability and made the assay suitable for large-scale and routine biomonitoring applications[78].

PIG-A and cancer

Cancer is a disease of mutations and the accumulation of gene mutations in somatic cells is the key to carcinogenesis and cancer development. Therefore, the measurement of mutation frequency and rate in said somatic cells can provide vital information about the risk of cancer development and progression, as well as exposure to carcinogenic agents in humans[79]. Peruzzi *et al.,* 2010 stated that for a gene to be used an as indicator of disease or to be an ideal "sentinel" gene for somatic mutation, it must meet three important requirements as closely as possible. These requirements are as follows; it must detect monoallelic mutations, have viable mutant cells, and can result in growth-neutral mutations. *PIG-A* meets all three of these requirements. It is a housekeeping gene situated at Xp22 with a single gene in men, and a single functional gene in women. Therefore, a single inactivating mutation may disrupt GPI synthesis and result in mutant cells lacking GPI-APs, indirectly measured through a lack of fluorescence when analysed using flow cytometry[75, 79].

There is a clear potential to use *PIG-A* assay to study the link between human somatic mutation and cancer risk, however, only a few reports of this nature have been

conducted and further work is still required to show if it can be effectively used for monitoring cancer risk in humans. A study by Haboubi *et al.,*(2019) evaluated the potential of *PIG-A* mutations of erythrocytes to be used as a potential surrogate marker of OAC, due to its ability to act as a biomarker for genomic instability[23]. Their results show that cancer patients with OAC showed significantly higher mutant frequency (MF) than those with non-dysplastic BO[23]. Whilst *PIG-A* mutations themselves are not a cause of OAC development, it is hypothesised to be a measure of underlying susceptibility to mutation. In genomically instable and heterogenic segments of BO, there is an increased risk of disease progression to OAC. Due to this, it is not surprising that there is an increased *PIG-A* mutant frequency as BO progresses to OAC[23]. They also showed that there was an increased mutation level in patients undergoing chemotherapy for OAC, likely due to the genotoxic effects of the chemotherapy to the bone marrow. They did however suggest that this work could be further investigated to measure *PIG-A* mutation pre- and post-treatment to identify chemotherapy-induced mutation[23]. Furthermore, an increase in erythrocyte *PIG-A* levels in patients with pancreatic cancer was shown in a study by Nichols *et al.,* (2024). As well as a biomarker of cancer risk the *PIG-A* assay in humans has many applications, including the study of occupational exposures and drug treatments.

1.6 Thesis Aim and objectives

The overall aim of this project was to use multiple markers of DNA damage to assess their potential at predicting risk of disease progression and response to therapy. In order to do this, understanding the mechanisms behind the formation of these biomarkers was deemed pivotal.

Objective 1. Assess Baseline Levels and Risk Correlation:

To evaluate the baseline levels of lymphocyte MN% and erythrocyte *PIG-A* MF and their association with the risk of developing OAC in patients with BO.

Objective 2. Measure Therapy-Induced Changes and Tumour Response:

To quantify the induction of lymphocyte MN% and erythrocyte *PIG-A* MF in cancer patients undergoing therapy (various chemo and radiotherapy) and determine if these changes reflect overall tumour response.

Objective 3. Investigate Mechanisms of Increased MN%:

To explore the mechanisms behind the increased lymphocyte MN% and sensitivity to DNA damage using a challenge assay and patient derived lymphocytes.

Objective 4. Examine relationship between inflammatory markers and patient lymphocyte MN%:

To investigate the relationship between lymphocyte MN% and inflammatory markers in blood plasma, with a specific focus on the cGAS-STING pathway.

Chapter 2 - General Methods and Materials

This chapter elaborates on the materials and techniques employed throughout this thesis to minimise repetition. Each subsequent chapter presents distinctive materials and methodologies specific to the corresponding data section.

2.1 Study design and patient recruitment

Ethical approval was in place for the recruitment of all individuals into this thesis. The details of each individual study are documented below. All samples were collected with informed and written consent. Participants involved were anonymous through the assignment of a unique code. All blood samples were collected in Ethylenediamine tetra acetic acid (EDTA) coated tubes, kept at an ambient room temperature, and processed within 24 hours of acquisition. It's important to note that the studies discussed throughout this thesis utilising clinical samples are pilot investigations. These preliminary studies are designed to assess the feasibility of research methods and procedures on a smaller scale before full-scale implementation. Due to their limited sample sizes, they are not powered to detect definitive statistical differences

2.1.1 Swansea university healthy volunteer study

Healthy volunteers were recruited under the research study given a favourable ethical opinion by Swansea University Medical School (SUMS) Research Ethics committee (REC), project reference 2022-0029. Patient information sheet, questionnaire and consent form are in **Appendix I-III**. Recruitment, consent, and sample collection was carried out by a trained phlebotomist in the health and well-being centre on Swansea University Singleton campus. Volunteers were sent the patient information sheet and questionnaire over 24 hours in advance prior to consent.

2.1.2 Singleton Hospital Endoscopy department

Patient recruitment from Singleton hospital endoscopy department was carried out between 1/09/2020-1/11/2022. Ethical approval was granted by Southwest Wales local research ethics committee (11/WA/0367) and sponsored in full by SBUHB research and development department. The study was designed by Dr Hasan Haboubi and Professor Gareth Jenkins and entitled: Studies in the molecular and lifestyle differences of patients with Gastro Oesophageal Reflux Disease & Barrett's Oesophagus. Patients were identified a week prior to their endoscopy, and an information pack was sent to them in the post. This pack contained the patient information sheet, a detailed questionnaire, and consent form (**Appendix IV-VI**).

Fully informed consent was taken by myself, Dr Rachel Lawrence, or Dr Hamsa Naser in person. Up to 20 ml blood was collected by a Swansea Bay University Health Board (SBUHB) staff member using EDTA coated blood vacuette tubes. Blood samples were transported using a Versapak Blood-in-Transit Bag. Patient questionnaires were used to deduce information about the individual's health and lifestyle before being filed along with consent forms in a locked cabinet.

2.1.3 Oncology department – Patient recruitment

Patients were identified and recruited to this study from Singleton Hospital Oncology department from 1/11/2020-1/01/2024. The study was designed by Professor Gareth Jenkins and Dr Sarah Gwynne and entitled: A novel blood-based approach to identify chemotherapeutic response in upper gastrointestinal (GI) tract cancer patients. Ethical approval was granted by the Research Ethics Committees Northern Ireland (BSO), HSC Research Ethics Committee's A (17/NI/0055). Screening occurred during the upper GI tract multidisciplinary team meeting. To meet the criteria, patients must have been diagnosed with oesophageal cancer, with treatment (chemo/radiotherapy) planned at Singleton Hospital Oncology. It was deemed essential that they had had no prior therapy within the last 10 years, and a suitable health status. Recruitment decisions rested with Dr. Sarah Gwynne, the clinical supervisor. Patients were introduced to the study during oncology clinic appointments, and consent was obtained in a subsequent appointment. Relevant documents were added to patient notes and the study file. Sample collection, initiated upon consent, followed the protocol of up to 20ml blood collected in an EDTA-coated vacuette tube. Patients verbally consented to ongoing involvement, with withdrawal not impacting treatment choices. The treatment regimens varied and are detailed in **Chapter 4**. Patients were coded and anonymised for confidentiality. Please see **Appendix VII-IX** for the relevant consent form, patient information sheet and questionnaire received by the oncology patients.

2.2 Equipment and materials

The reagents used throughout the studies are listed in **Table 2.1** below.

Table 2.1 *Common reagents used throughout this study, and the corresponding supplier.*

Reagents	Supplier
RPMI 1640 media	GIBCO™, Life Technologies, USA
L-Glutamine	GIBCO™, Life Technologies, USA
Horse serum	GIBCO™, Life Technologies, USA
Foetal bovine serum	GIBCO™, Life Technologies, USA
Sodium Pyruvate (100mM)	GIBCO™, Life Technologies, USA
DMSO (CAS:67-68-5)	Fisher Scientific, USA
Phosphate Buffered Saline (PBS)	GIBCO™, Life Technologies, USA
Giemsa's stain solution, Gurr™ for microscopical staining	VWR Chemicals, USA
Gurr Buffer Tablets (pH 6.8), tablets dissolved in D_2H_2O	GIBCO™, Life Technologies, USA
D_2H_2O	Milli-Q®, Merck Millipore, USA
Methanol (CAS:67-56-1)	VWR Chemicals, USA
DAPI (28718-90-3)	Sigma Aldrich (Merck), USA
Ethanol (CAS:64-17-5)	VWR Chemicals, USA
Trypan blue solution 0.4%	Thermo Fischer scientific, USA
Cytochalasin-B	Thermo Fischer scientific, USA
Acetic acid	VWR Chemicals, USA
Bovine serum albumin (BSA)	Sigma-Aldrich. USA
Flow-Check™ Pro Fluorospheres	Beckman Coulter life sciences, USA
Flow-Set™ Pro Fluorospheres	Beckman Coulter life sciences, USA

The equipment used throughout are listed in **Table 2.2**.

Table 2.2. *Equipment used throughout this thesis, and the corresponding supplier.*

Equipment	Supplier
Axiovert 40C Microscope	Zeiss, Germany
Biological safety cabinet class 2- Mars	Labogene (Scanlaf), Denmark
Centrifuge 5810R	Eppendorf Ltd, Germany
Luna-II™ automated cell counter	Logos biosystems, South Korea
CO_2 Air jacketed Incubator	NuAire, USA
ErgoOne Fast pipette controller	Starlab, Belgium
Grant sub aqua 18water bath	Eppendorf Ltd, Germany
Gilson pipette set (ACEA biosciences, Inc.)	Thermo Fischer Scientific, USA
Heraeus™ Pico™ 17 Microcentrifuge	Thermo Fisher Scientific, USA
Metafer	Metasytems, Germany
Nanodrop spectrophotometer ND1000	Labtech, UK
NovoCyte® flow cytometer	ACEA biosciences, Inc., USA
NovoCyte flow cytometer	Agilent technologies, USA
Pasteur pipette	Fischer Scientific, UK
T100 thermal cycler	Bio-Rad Laboratories, USA
Whirlmixer Vortexer	Thermo Fisher Scientific, USA
Cell Freezing chambers	Mr Frosty, Thermo Fisher Scientific, UK
2ml Cryovials	ELKAY laboratory products, UK
15ml centrifuge tubes	Corning Incorporated, USA
50ml unskirted falcon tubes	Corning Incorporated, USA
1000 µl tips (Sterile, filter tips)	TipOne, Starlab, Belgium
200 µl tips (Sterile, filter tips)	TipOne, Starlab, Belgium
20 µl tips (Sterile, filter tips)	TipOne, Starlab, Belgium
10 µl tips (Sterile, filter tips)	TipOne, Starlab, Belgium
FLUOstar Omega multimode microplate reader	BMG LABTECH Ltd, UK
T75 flasks	Cell Star®, Greiner Bio-One, Austria
T25 flasks	Cell Star®, Greiner Bio-One, Austria
6 well plates	Cell Star®, Greiner Bio-One, Austria

2.3 Cell culture

All cell culture occurred within a class II Mars biological safety cabinet (ScanLaf, UK). The human lymphoblastoid cell line - thymidine kinase (TK) 6 was obtained from American Type Culture Collection (ATCC), (Middlesex, UK).

2.3.1 *In vitro* Cell Culture - TK6 cell line

TK6 cells (passages 5-8) were stored in liquid nitrogen, suspended in 90% Horse Serum (HS, Gibco Invitrogen, Paisley, UK) with 10% dimethyl sulfoxide (DMSO, Fischer scientific, Loughborough, UK) at the concentration of $1x10^6$ cells/ml. TK6 cells were sustained in the following media composition; Roswell Park Memorial Institute-1640, (RPMI-1640) (Gibco Invitrogen, Paisley, UK) media with the addition of 1% L-glutamine (GIBCO Invitrogen, Paisley, UK) and 10% HS. The supplemented

RPMI-1640 (referred to throughout as growth medium), was stored at 4°C in the dark and warmed to 37°C in a water bath (Eppendorf, Stevenage, UK) prior to use.

Once taken from liquid nitrogen, TK6 cells were quickly defrosted in the water bath at 37°C, before being transferred into pre-warmed growth medium. Cells were immediately centrifuged at 107 *x g*for 5 minutes, before the supernatant was discarded and the cells transferred to 10ml growth medium in a T25 flask. Once confluent, cells were transferred to a T75 flask and maintained in a semi-confluent state to avoid entering cytostasis (<1x10^6 cells/ml). To passage (referring to the subculture of cells), cells were first counted using Trypan blue solution (0.4%, Thermofisher Scientific, US) – and the automated cell counter LUNA-II™ (Logos Biosystems). They were then seeded with the goal concentration of 1x10^5 for the new passage. Cells above passage 14 were deemed no longer fit for experimental use and disposed of.

2.3.2 Processing of blood samples

Samples were always processed within 24 hours of acquisition and remained at ambient temperatures. Whole blood was layered directly onto Histopaque®-1077 (Sigma-Aldrich Co. Ltd, UK) in a 1:1 ratio, using 15 mL falcon tubes. Centrifugation of blood at 400 *x g* for 30 minutes without sudden acceleration and breaking, resulted in the formation of four distinct layers: plasma, buffy coat, Histopaque®-1077, and red blood cells (**Figure 2.1**). The plasma accounts for 60% of the blood and primarily consists of water, while also encompassing a variety of crucial elements, including proteins (clotting factors, antibodies, enzymes, and hormones), sugars (such as glucose), and lipid particles[80]. The buffy coat contains peripheral blood mononuclear cells (PBMCs) including lymphocytes and monocytes, as well as platelets. The bottom layer consists of mainly red blood cells and granulocytes[80]. Whilst most of the blood was processed, around 100 µL was kept aside for the *PIG-A* assay

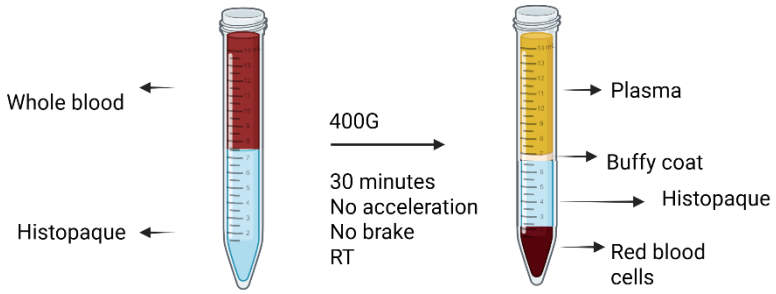

Figure 2.1. *Density dependent centrifugation of whole blood, using Histopaque®-1077. Blood is layered onto Histopaque®-1077 in a 1:1 ratio. The samples are centrifuged at 400 x g for 30 minutes at room temperature, with no acceleration or break. This results in the formation of four different layers - plasma, buffy coat containing peripheral blood mononuclear cells (PBMCs), Histopaque®-1077, and red blood cells. Created with Biorender.com.*

The plasma was removed with a Pasteur pipette and stored at -80^{O}C following a second centrifugation of 200 *x g* for 10 minutes. The buffy coat was also collected using a Pasteur pipette (Fischer Scientific, UK), and washed twice with 30 ml sterile 1X phosphate buffered saline 7.4 pH (PBS) (GIBCO, 10010023) (200 *x g*, 10 minutes). If red blood cells contaminated the PBMC pellet, Red Blood Cell Lysis Buffer (Merck, UK) was added prior to a third wash in PBS. The cells were then resuspended in lymphocyte growth medium (referred to throughout as L-Growth medium) consisting of RPMI-1640 with 1% Glutamine, 1% Sodium pyruvate (100mM, GIBCO®, Life Technologies) and 10% foetal bovine serum (FBS) (GIBCO®, Life Technologies), prior to enumeration.

<u>Culture of lymphocytes using phytohemagglutinin</u>

Live cells suspended in L-growth medium were diluted 1:1 in 0.4% Trypan blue solution and counted twice using the LUNA-II™ automated cell counter. The average count (live cells) was used to seed cells at a concentration of $1x10^6$ cells per 1ml in either a T25 flask, 6-or 12-well plate. The volume at which cells were seeded ranged from 1-10 ml dependent on the live cell count. Human circulating lymphocytes derived from peripheral blood are in a state of quiescence (G0), and hence require stimulation *in vitro*[81]. PHA - a lectin derived from red kidney beans -was first discovered by Peter C. Nowell in 1960. It was found that the lectin was able to stimulate metabolic activity and cellular division of lymphocytes, specifically T-cells[82]. PHA-M (Sigma-Aldrich, UK) was added to the L-growth medium at a concentration of 1% by volume, to stimulate proliferation of the lymphocytes.

2.4 The Cytokinesis-block Micronucleus assay

MN are small additional nuclei formed from chromosome fragments or whole chromosomes that are not incorporated into the main nucleus during cell division[61] (**Figure 2.2**). The MN assay is a widely employed genotoxicity testing method that assesses potential DNA damage caused by various agents, such as chemicals or radiation, by quantifying the frequency of MN. This thesis utilised the CBMN assay, however the mononucleate micronucleus assay was used exclusively for work involving the agonism and inhibition of the stimulator of interferon genes (STING) in **Chapter 6**. The CBMN assay was carried out as described by Fenech *et al.,* 2007[83] and following the Organisation for Economic Co-operation and Development (OECD) guidelines for the testing of chemicals[84]. This assay involved the addition of Cytochalasin-B (Cyto-B, Merck, UK) to cells at a concentration of 4.5µg/ml for 24 hours. After this time period, cells were harvested for MN scoring. Harvest and scoring protocols for MN in TK6 and Lymphocytes were identical and described in **section 2.4.3.** Cytotoxicity was also measured using either the cytokinesis-block proliferation index (CBPI), or relative population doubling (RPD) if satellite flasks could be seeded.

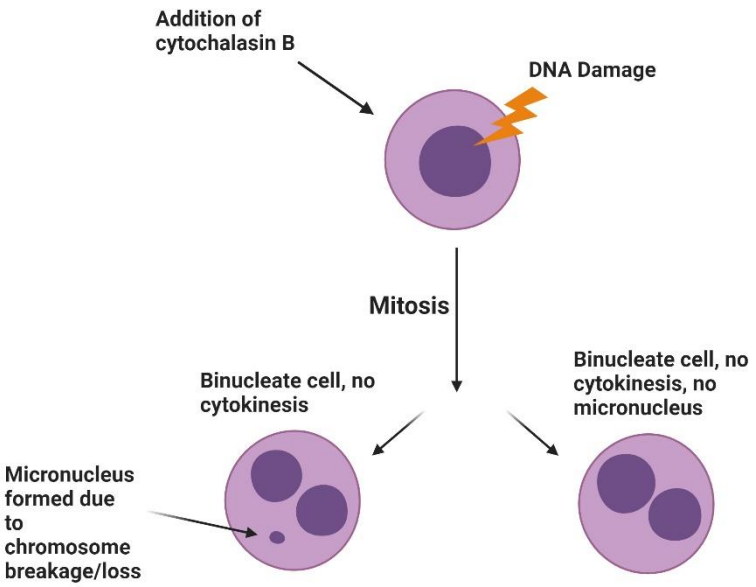

***Figure 2.2**. Basis of the cytokinesis-block micronucleus assay. Cytochalasin B is introduced into a peripheral blood lymphocyte to inhibit cytokinesis. The lymphocyte has a nucleus containing damaged DNA. The resultant cells are binucleated; the control (right hand side) exhibiting no micronuclei due to no damage to the DNA, whilst damaged binucleate cell (left hand side) shows a micronucleus. MN frequency is scored in binucleated cells only. Created with BioRender.com.*

2.4.1 CBMN assay in TK6 cells

TK6 cells were seeded at a concentration of $1x10^5$ and incubated at 37^OC at 5% CO_2. Before the exposure to a DNA-damaging treatment, cells were counted to ensure the population had grown overnight. Cells were then treated through addition of a genotoxic chemical, or any other test chemical, or exposure to IR. A negative control was always utilised, whereby the sample did not undertake this damaging exposure, however, was otherwise treated in an identical manner. Following treatment of varying times (starting from 4 hours, with a maximum exposure of 24 hours), cells were washed in PBS (107 *x g*, 5 minutes), before being transferred to fresh media in the presence of Cyto-B.

2.4.2 Lymphocyte cytokinesis-block micronucleus (L-CBMN) assay

By stimulating lymphocytes *in vitro*, the identification of damage accumulated *in vivo* is possible. To allow the scoring of MN in human lymphocytes, the CBMN assay must always be used. As well as avoiding confusion caused by cell kinetics, every individual varied in their response to PHA-M. Therefore the presence of binucleates confirmed division *in vitro*[83]. Following isolation and stimulation of lymphocytes with PHA-M, cells were incubated at 37^{O}C at 5% CO_2. Cyto-B was exactly 44 hours following PHA-M stimulation.

Hypoxic vs Hyperoxic culture of lymphocytes

In the standard cell culture incubator, O_2 concentration is typically maintained at 20% to 21%, and CO_2 at approximately 5% to 7%, mirroring atmospheric conditions[85]. Notably, lymphocyte culture *in vitro* commonly occurs in these hyperoxic conditions, diverging significantly from the environment of blood, which typically contains around 10-12% oxygen[86]. This dissonance in oxygen levels between hyperoxic cell culture and hypoxic blood could impact various cellular processes, such as cell proliferation, DNA repair, and oxidative stress, potentially posing challenges in drawing direct comparisons to physiologically relevant conditions[86, 87].

To address this concern, a preliminary experiment was conducted using lymphocytes from healthy donors. Following PHA stimulation, cells were transferred to a PHO2xBox (Labtech, UK) for cultivation at 37°C, with 10% oxygen and 4% CO_2. Cells were only removed briefly from the PHO2xBox for addition of Cyto-B 24 hours before harvest. As per **Figure 2.3**, there was no significant change in the baseline MN% of lymphocytes grown at 10% Oxygen compared to 20% Oxygen, and hence all subsequent lymphocyte culture occurred at 20% due to ease of culture and limited space in the PHO2xBox.

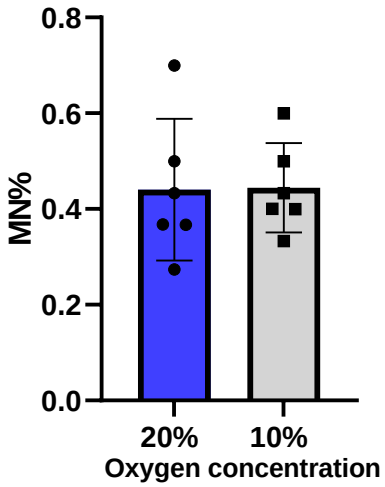

***Figure 2.3**. Lymphocytes cultured in different concentrations of oxygen, with 4% CO_2. There was no significant difference in the micronucleus frequency (MN%) of lymphocytes cultured from the same individual and grown at either atmospheric levels of oxygen (~20% n=6) or levels lymphocytes would be exposed to in the blood (~10% n=6). Average MN% was calculated following 3 replicates. (Error bars show SD).*

2.4.3 Harvest and scoring of MN in TK6 cells and patient lymphocytes

Harvest for manual scoring

Around 24 hours following addition of Cyto-B, cells were transferred into a 15ml falcon tube and washed once in PBS. A variable volume of PBS was added to the cells, depending on cell concentration, before 100µl of cell suspension was transferred onto Glass slides (frosted at one end) (Thermo Fischer scientific, UK) using the Cytospin 4 cytocentrifuge (Thermo Shandon, UK). Cell-density was determined using an initial slide, before 5 more slides were made per person or treatment. The cells were fixed to the slides through submersion in ice cold 90% methanol for 10 minutes. They were left to dry and stored at -20^{o}C before staining.

Giemsa-staining of cyto-dot

A 20% Giemsa solution was prepared through dilution of concentrated Giemsa using Phosphate Buffer M/15 (pH 6.8) reagent (Thermo Fischer Scientific, UK). Cells were stained for 10 minutes, before being washed 3 times with 6.8 pH phosphate buffer. Giemsa-stained cells were fixed once more using Xylene (Fisher scientific), before

DPX mounting medium (D/5330/05), Fisher Scientific) was used to fix coverslips to the slides. Slides were stored at 4°C in the dark prior to analysis.

Manual scoring of MN

Carl Zeiss™ Immersol™ Immersion Oil (Sigma- Aldrich, UK) was added to the slides, and cells were viewed under the highest magnification (100x) (**Figure 2.4**). For the manual scoring of MN, it was necessary to work systematically through the slide, making sure not to score the same cells twice. Using a differential cell counter, 1000 binucleated cells were counted in triplicate (using three slides) for each sample, as well as the ratio of mono- to bi- to multi-nucleated cells (500 cells counted per sample). The characteristics of a micronucleus used for scoring were as followed: round or oval, with a clear membrane, between a third and a sixteenth the size of the original nucleus, separated from the main nucleus but with similar staining intensity to that of the main nuclei. For the cases where satellite flasks could not be set up (lymphocyte treatment), manual scoring was always used to calculate the CBPI through the ratio of mono, bi and multi-nucleated cells.

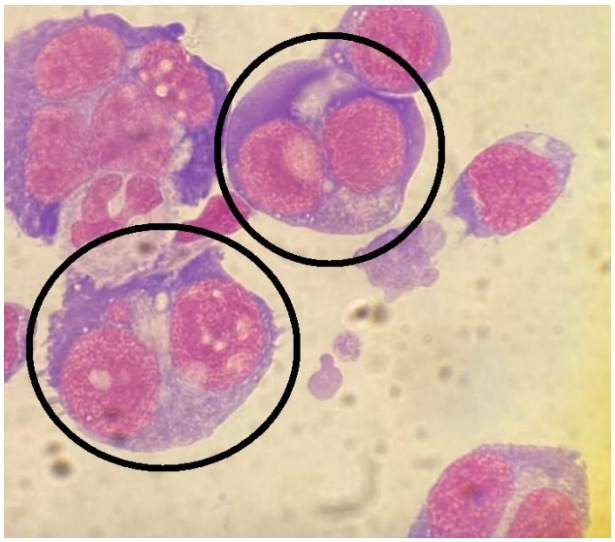

Figure 2.4. *Two binucleated lymphocytes, stained with Giemsa and viewed under a light microscope (1000x magnification). The upper circled cell shows a binucleate, whilst the lower left shows a micronucleate binucleated cell.*

The Semi-automated scoring of MN

This section describes the use of the Zeiss Metafer system in scoring MN. Whilst this is an automated microscopy platform, scoring of MN was only semi-automated, and still required subjective confirmation of MN in binucleate and/or mononucleate cells.

Fixing cells for visualising using fluorescent microscope

All centrifuge steps described in this section were as follows; 123 *x g*, 10 minutes, at 4^{O}C. Immediately following the preparation of cytodot, as described above, the remaining cells in PBS were pelleted and washed in pre-warmed 0.56% Potassium Chloride (KCL) solution. Cells were then resuspended in FIX 1 solution (20% acetic acid in methanol) for a 10-minute incubation at 4^{O}C. Cells were centrifuged, then resuspended in FIX 2 (5-parts methanol, 6-parts 0.9% Sodium chloride, and 1-part acetic acid). Cells were incubated at 4^{O}C for 10 minutes prior to centrifugation. Cells were washed twice in FIX2, before being stored in FIX2 at 4^{O}C until slide preparation.

Semi-automated scoring of MN using Metafer software.

Slides were kept in FIX2 for a minimum of 4 hours prior to slide preparation. Following rinsing of the slide, 100µl of cell suspension pipetted in a manner to ensure the cells were evenly distributed for scoring (**Figure 2.5**). Once dry, slides were stained with ~20 µL Vectashield® Mounting Medium with 4,6-Diamidino-2-phenylindole (DAPI) for 15 minutes whilst protected from light, then a 25 x 60 mm coverslip was added before they were imaged using a fluorescent microscope and Metafer system (Metasystems Metafer 4, Zeiss).

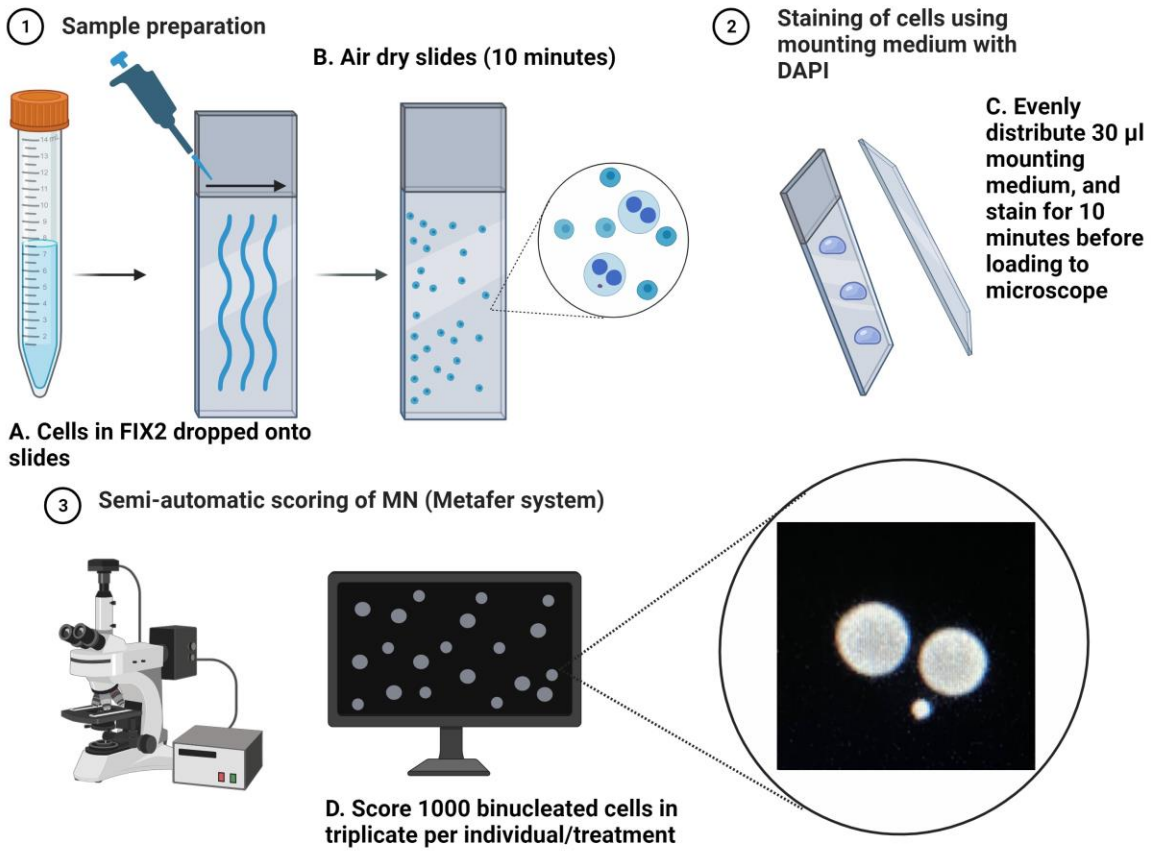

Figure 2.5 *Schematic demonstrating sample semi-automated scoring of micronuclei (MN) in binucleate cells using the Metafer and fluorescent microscope. Fixed cells, and microscope slides are stored in FIX2 prior to preparation. Cells are resuspended in variable volumes of FIX2, before being dropped across the top of the microscope and allowed to spread equally as the fixative dries. Following this, 30µl of mounting media with 4',6-diamidino-2-phenylindole (DAPI). DAPI is added to the slide, and a coverslip fixed in place on top. Given around 10 minutes, to ensure the cells are stained, the slides are loaded on the microscope and the system processes images taken of binucleate cells according to the classifier. A binucleated lymphocyte with a micronucleus is shown, taken directly from the Metafer.*

Classifiers developed by MetaSystems were modified to detect binucleated cells. The search definition classifiers were used to define the size and shape of nuclei and MN. The classifier specifications described in Table 2.3 were used to identify binucleate cells with/without a MN, both in TK6 cell, and in human lymphocytes.

Table 2.3 *Classifier settings on Metafer-System to define binucleated TK6-cells, and human lymphocytes as well as MN*

Binucleate cell in lymphocyte and TK6 cells	Nuclei	MN
Object threshold (%)	20	5
Minimum area (µm²)	100	1
Maximum area (µm²)	400	21
Maximum relative concavity of depth	0.15	1
Maximum aspect ratio	1.4	1.72
Maximum distance between (µm)	18	30
Maximum area asymmetry (%)	90	-

Cytotoxicity measurement

Cytotoxicity measurement ensures that MN are not formed as a biproduct of the toxicity of the DNA damaging agent. According to the OECD guidelines for the testing of chemicals[84] when choosing a concentration/ dose for the treatment of cells, the highest concentration/dose should aim to achieve no more than 60% cytotoxicity. RPD, and CBPI, were used to measure cytotoxicity. The calculations for which are shown in **Figure 2.6** and **Figure 2.7** respectively.

$$\mathbf{RPD} = \frac{\text{(No. of Population doublings in treated cultures)}}{\text{(No. of Population doublings in control cultures)}} \times 100$$

Figure 2.6*. The calculation for measurement of relative population doubling (RPD) as shown in the OECD guidelines for the testing of chemicals*[84]*.*

$$\% \text{ Cytostasis} = 100\text{-}100\{(CBPI_T - 1) \div (CBPI_C - 1)\}$$

And:

T = test chemical treatment culture
C = control culture

Where:

$$\textbf{CBPI} = \frac{((\text{No. mononucleate cells}) + (2 \times \text{No. binucleate cells}) + (3 \times \text{No. multinucleate cells}))}{(\text{Total number of cells})}$$

Thus, a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis.

Figure 2.7*. The calculation for measurement of percentage of cells in cytostasis, calculated using the cytokinesis-block proliferation index (CBPI) as shown in the OECD guidelines for the testing of chemicals*[84].

2.5 Enzyme-linked immunosorbent assays

All standard enzyme-linked immunosorbent assays (ELISA) kits were purchased from R&D diagnostics (and carried out according to the manufacturer's instructions. The glo-substrate reagent pack (R&D diagnostics) was purchased separately, and the 1M sulfuric acid (H_2SO_4) required to stop the reaction was purchased from Merck, UK. The absorbance was measured at 450 nm using the FLUOstar Omega multimode microplate reader, and the final protein concentration was calculated using the standard curve, generated from the known analyte concentrations. A standard curve was created for every plate analysed.

2.6 Cell cycle analysis

Fixing and staining cells

The cell cycle stage of a cell can be determined by staining DNA in fixed cells and quantifying DNA levels using a flow cytometer. The standard procedure for lymphocytes and TK6 cells was as follows: Around 1 million cells were harvested at variable time points and washed twice in PBS (centrifuged at 500 *x g* for 5 minutes). Following the second wash, cells were fixed by the gradual addition of ice cold 66% ethanol in PBS and stored at 4^0C prior to staining. On the day of analysis, the cells were washed in PBS before resuspension in 100 µl of FxCycle™ PI/RNase Staining Solution (Thermo Fisher Scientific,UK) and allowed to incubate in the dark at an ambient temperature for 30 minutes.

Cells were immediately analysed on the NovoCyte® flow cytometer (ACEA biosciences, Inc.) with a stop condition of 10,000 single cells of the desired population. Further descriptions of gating and analysis of results are described in data Chapters 5 and 6 for lymphocytes and TK6 cells respectively.

2.7 The *PIG-A* assay.

The X-linked *PIG-A* gene plays a role in the biosynthesis of glycosylphosphatidylinositol (GPI) anchors. A solitary mutation in this gene, results in the absence of GPI-anchors bound to the extracellular membrane (**Figure 2.8**), and therefore *PIG-A* mutant erythrocytes can be quantified using flow cytometry. The optimisation of this erythrocyte *PIG-A* mutation assay was previously conducted by Dr. Hasan Haboubi and Dr. Rachel Lawrence, both affiliated with Swansea University.

Cell staining

For this assay, 10$_{\mu}$L whole blood was stained directly with fluorescently conjugated antibodies Allophycocyanin (APC) conjugated CD235a and CD55/59 conjugated Phycoerythrin (PE) (details listed in Table 2.4), in triplicate. An unstained instrument calibration standard (ICS) was used to determine fluorescence thresholds from which to determine cell *PIG-A* status.

Table 2.4 *Antibodies used for PIG-A flowcytometric analysis.*

Antibody	Fluorochrome	Supplier	Clone	Isotype
Anti-Human CD235a	APC	BD Biosciences (BD Pharmingen™)	GA-R2 (HIR2)	Mouse IgG2b, κ
Anti-Human CD55	PE	BD Biosciences (BD Pharmingen™)	IA10	Mouse IgG2a, κ
Anti-Human CD59	PE	BD Biosciences (BD Pharmingen™)	Clone p282 (H19)	Mouse IgG2a, κ

APC and PE were selected based on their distinct excitation and emission spectra, with emissions at 660nm and 570nm, respectively (as shown in **Figure 2.8**), as well as their fluorescent intensity. The process of optimising the utilisation of APC and PE in the *PIG-A* assay was conducted by Dr. Hasan Haboubi, as detailed in his doctoral thesis.

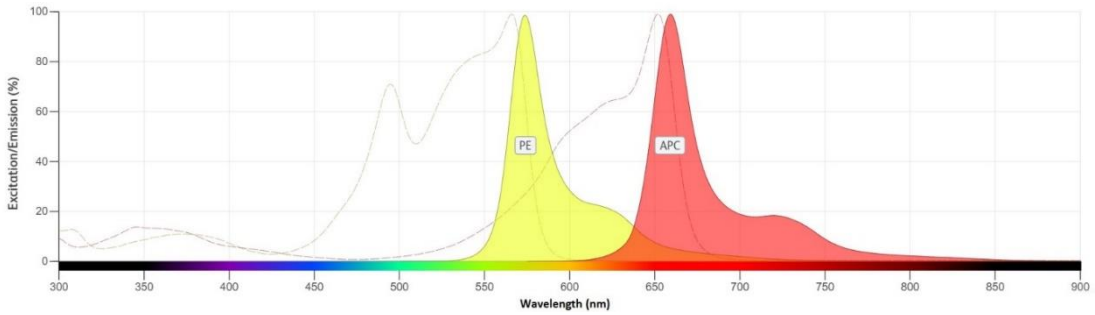

Figure 2.8 *The overlap of emission spectra for fluorophores phycoerythrin (PE), and allophycocyanin (APC). Fluorophore show peak emission at 570nm and 660nm respectively. Image was taken directly from bdbiosciences.com: accessed 17/01/2024 at https://www.bdbiosciences.com/en-gb/resources/bd-spectrum-viewer*

The samples were incubated with the antibodies for 30 minutes in the dark at room temperature, before centrifugation at 500 *x g* for 5 minutes. The excess, unbound antibodies were then removed. The cells were washed twice in 2ml antibody wash buffer (0.2% BSA in PBS) at 500 *x g* for 5 minutes. Cells were resuspended in 1ml of antibody wash buffer and immediately analysed using the flow cytometer (Navios, Beckman Coulter).

Quality control of NAVIOS flow cytometer

Prior to sample acquisition, quality control beads were run through the flow cytometer to ensure accurate and reliable flow cytometry data acquisition. The quality control tools for the Navios flow cytometer including the following: 1. Flow-Check™ Pro Fluorospheres for daily verification of the Navios™ optical alignment and fluidics system. 2. Flow-Set™ (Beckman Coulter) as an aid in standardizing the Navios™.

Gating strategy for enumeration of *PIG-A* mutant erythrocytes

CD235a (also known as Glycophorin A), is a transmembrane glycoprotein expressed by both erythroid precursors and mature erythrocytes. Through the addition of this antibody, we can differentiate the red blood cells from the other components of whole blood without the need for blood pre-processing.

CD55 and CD59 are glycoproteins anchored to the erythrocyte cell membrane through GPI linkage. Both glycoproteins work as complement regulatory proteins, with a role in regulating complement-mediated cell lysis [88, 89]. The absence of both CD55 and CD59 in this assay (both conjugated to PE) indicates a *PIG-A* mutant and lack of GPI

anchorage of these proteins to the RBC surface. The initial plotting and gating of a histogram with APC on the x-axis ensures the erythrocytes were being analysed. Through plotting APC (X-axis) against PE (Y-axis) the identification of *PIG-A* mutants (bottom right quadrant), and wild type cells (top right quadrant) was possible (gating strategy shown in **Figure 2.9**). One million cells were analysed in triplicate, and an average calculated to account for such rare events. Therefore, the unit used throughout for *PIG-A* mutants is 10^{-6}. If 6 in 1 million cells were mutants, the *PIG-A* MF is 6 x 10^{-6}.

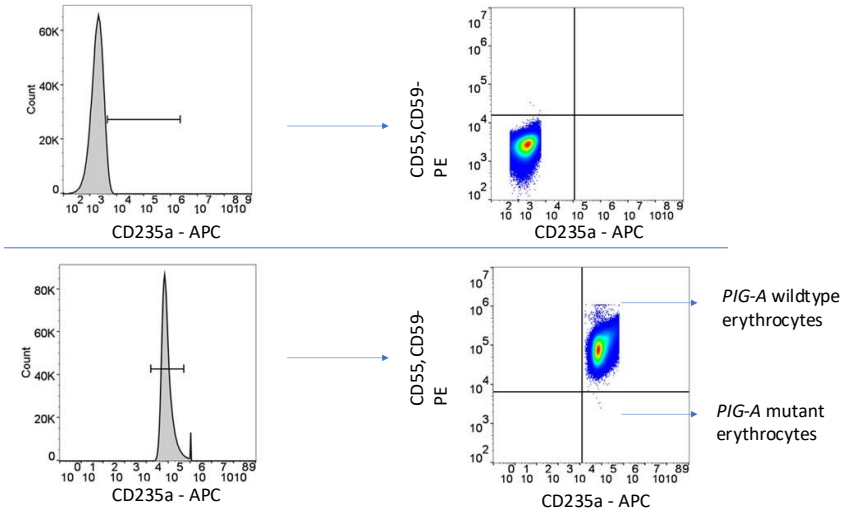

Figure 2.9. *Flow-cytometric gating strategy for enumeration of PIG-A mutant erythrocytes. The top panel shows an unstained Instrument Calibration Standard (ICS), where the cells do not emit light in the PE or APC channels. The bottom panel shows a whole blood sample stained with APC- and PE-conjugated antibodies against CD235a, CD55, and CD59. Initially, a histogram of APC fluorescence is plotted to gate erythrocytes. From this gate, a PE (y-axis) vs. APC (x-axis) plot is generated. Wild-type cells, expressing CD235a, CD55, and CD59, are located in the top right quadrant, emitting fluorescence in both PE and APC channels. Mutant cells, positive for CD235a but lacking CD55 and CD59, do not emit light in the PE channel and are in the lower right quadrant, allowing for their identification and enumeration.*

Chapter 3 - Baseline levels of lymphocyte MN% and *PIG-A* erythrocyte MF: Utility in biomonitoring and assessment of OAC risk

3.1 Introduction

3.1.1 MN and *PIG-A* in human biomonitoring

Diseases, including cancers, neurodegenerative, and cardiovascular disease, can be caused by genome instability, damage, and mutation. Genomic instability, defined as an increased tendency of the genome to acquire mutations, occurs when the processes involved in maintaining and replicating the genome are dysfunctional or when there is increased exposure to carcinogens[90]. Consequently, genomic instability is characterised by an elevated frequency of mutations, such as nucleotide substitutions, deletions, insertions, amplifications, DNA breaks, chromosomal translocations, and aneuploidy. The mechanisms leading to genomic instability include defects in DNA repair, DNA replication, cell cycle control, or chromosome segregation. Misrepair or erroneous replication can lead to mutations. When these mutations occur in critical genes like oncogenes or tumour suppressor genes, they can result in altered gene expression and further genomic instability, thereby promoting carcinogenesis[90]. Spontaneous mutations can arise from endogenous processes, such as replication mistakes, hydrolytic reactions, and oxidative damage[91]. Whilst endogenous DNA damage occurs thousands of times daily in human cells there are multiple DNA repair pathways that typically prevent these lesions from persisting[92]. Spontaneous mutations and chromosome rearrangements drive evolution by altering genome structure[91]. Due to DNA's inherent chemical instability, frequent replication, and vulnerability to mutagens, these spontaneous mutations are unavoidable[91]. As well as endogenous processes, various physical and chemical factors in daily life, like UV radiation, pollution, and exposures to carcinogenic chemicals, can adversely affect the genome[77]. These can consist of occupation exposures, or the result of lifestyle choice: for instance, the heavy metal lead, a long-utilized industrial toxin, can accumulate in the body with repeated exposure, heightening the risk of individuals in such environments to harmful, genotoxic substances. Another example is cigarette smoking, which is a definite cause of many different cancers, due to the carcinogens such as benzo(a)pyrene causing DNA damage in smokers[93].

Furthermore, as well as environmental and occupational factors, the role of diet and lifestyle in genome instability and cancer development cannot be understated.

Population-based studies play a crucial role in monitoring the effects of these factors on health outcomes. The European Prospective Investigation into Cancer and Nutrition (EPIC) study has assessed the relationship between diet and mortality from colorectal, breast, lung, and prostate cancer[94]. They found that a higher consumption of fruit, vegetables, fish, and yogurt had a protective effect against developing cancer. Excessive consumption of processed foods, red meat, sugary beverages, and alcohol can elevate cancer risk[94]. Alcohol can also work synergistically with tobacco to increase cancer risk[95]. Diao *et al.,* 2023 established an inverse relationship between total physical activity and the risk of breast, colon, lung, gastric and liver cancers, stressing the importance of a healthy lifestyle in lowering the risk of developing many different cancer types[96]. In individuals leading more sedentary and unhealthy lifestyles, the risk of obesity increases. It is well known that obesity increases risk of various cardiovascular diseases, however it also attributes to 4-8% of all cancers[97].

Surveillance or "biomonitoring" is essential to evaluate the risk of developing diseases such as cancer and can possibly help to alleviate the burden[77]. Assessments of environmental, workplace conditions, healthy diets and lifestyles along with biological changes like early markers of carcinogenesis, can be conducted using *in vitro* tests analysing biomarkers of exposure and effect. Traditional cancer-related biomarkers, categorized into biomarkers of exposure (such as DNA adducts) and early biological changes (MN, *PIG-A*), are sensitive indicators mainly used for assessing bone marrow and peripheral blood exposure. The lymphocyte CBMN assay is a well-established cytogenetic *in vitro* method widely used for biomonitoring. Interest in utilising the human *PIG-A* erythrocyte assay in biomonitoring studies has risen over recent years[98]. Recent investigations into occupational exposures have combined the human *PIG-A* erythrocyte mutation assay with the lymphocyte CBMN assay for biomonitoring purposes. Yu *et al.,* (2019) demonstrated that workers with elevated lead levels in their blood exhibited increased MN frequency[99]. Conversely Cao *et al* 2020., employed the *PIG-A* assay alongside the CBMN for biomonitoring, utilising blood lead levels as a biomarker of internal exposure. They revealed a significant increase in *PIG-A* MF among those occupationally exposed to lead compared to the general population. They did not see any significant increase in MN frequency, or DNA damage as measured by the comet assay. They suggested *PIG-A* MF may be a more sensitive biomarker for lead exposure[77]. Cao *et al.,* 2021 conducted a study measuring *PIG-A* MF and

lymphocyte MN frequency in individuals occupationally exposed to PAHs. They saw PAH-exposure resulted in higher *PIG-A* MN and MN than controls, with *PIG-A* MFs significantly associated with urinary PAH metabolites[100].

These assays also hold validity in assessing a person's response to a drug or a therapy and testing drug safety during development. Cao *et al*., (2020) employed these assays for biomonitoring patients with inflammatory bowel disease (IBD) undergoing Azathioprine (AZA) treatment[101]. While AZA was deemed risk factor for inducing *PIG-A* mutation, the *PIG-A* results showed no correlation with treatment duration or exposure, unlike the CBMN assay, which exhibited a significant correlation between AZA treatment duration, exposure, and MN frequency[101]. There is also evidence of the benefit of measuring MN% and *PIG-A* mutations in blood cells in assessing the genotoxic events induced by diet and lifestyle habits. A review by Fenech *et al.,* (2011)[102] assessed the effects of diet and lifestyle factors on MN frequency in human PBLs. They reported that while increased physical exercise decreases MN%, smoking, alcohol and use of some recreational drugs can increase lymphocyte MN%. They reported that different nutritional factors required as part of a balanced diet can have various effects on MN%. This study revealed associations with intake of vitamin E, retinol, and calcium with decreased MN%, and higher MN% associated with increased riboflavin, pantothenic acid and biotin[102]. Research conducted on a large cohort by Lawrence *et al.,* (2020) suggested that increased consumption of vegetables, and engaging in physical activity exceeding one hour per week might lead to a reduction in levels of *PIG-A* mutant cells[103]. The presence of elevated MN% and *PIG-A* levels, influenced by the factors discussed here may indicate risk of future carcinogenesis.

3.1.2 Risk factors for oesophageal cancer.

Despite being one of the most common cancers in the UK, Cancer Research UK published that 59% of oesophageal cancer cases are preventable[14]. There are many risk factors associated with the elevated chance of developing oesophageal cancer. These include amongst others: age, male gender, obesity, and tobacco smoking (list of risk factors shown in **Figure 3.1**)

Risk factors	Magnitude of risk
ESTABLISHED RISK FACTORS	
Geographical region (Western countries)	++
Male gender	++
Caucasian race	++
Barrett's esophagus	++
Gastroesophageal reflux	++
Obesity	++
Tobacco smoking	+
Dietary fats	+
ESTABLISHED PROTECTIVE FACTORS	
Fruit and vegetables consumption	– –
Dietary antioxidants	– –
Non-steroidal anti-inflammatory drugs	– –
H. pylori infection	– –
NOT ASSOCIATED	
Total alcohol consumption	0
Carbonated soft drinks	0
EQUIVOCAL FACTORS	
Hot beverages (tea and coffee)	?

0, no association; +, low to moderate increase in risk; ++, moderate to high increase in risk; – –, moderate to high decrease in risk; ?, ambiguous studies.

Figure 3.1 *List of factors associated with oesophageal adenocarcinoma risk, and the magnitude of such risk. Published by Thrift et al., 2012[104].*

The association between ageing and increased susceptibility to cancer and other diseases is widely recognised[105]. It is thought to be attributed to the gradual accumulation of mutations, and impaired DNA repair within our tissues [106]. Moreover, as individuals age, there is a discernible increase in the prevalence of epigenetic modifications caused by exogenous exposures, particularly methylation-based gene repression[107]. As a result, older individuals are at a greater risk of neoplastic progression. This is underscored by the significant rise in cancer incidence during middle age. It is not surprising therefore that the majority of cases of both Barrett's oesophagus and oesophageal cancer occur in patients over 50 years old[108], with one study in Sweden identifying the highest 5-year risk of developing OAC occurring in those aged 70-74 years[109]. Rates of OAC are increasing in those younger than 50, however, and according to a study Codipilly *et al.,* 2021, this cohort of patients

experience a poorer prognosis[108]. As discussed in **Chapter 1,** most OAC cases arise from BO, a metaplastic transformation of the oesophageal lining associated with GORD. There are higher incidences of Barrett's oesophagus, and both subsets of oesophageal cancer in the Caucasian male population. The incidence of OAC in males is 6–10 times higher than females, and 2-3 higher for squamous cell carcinoma[110]. The exact causes of this are unknown, however several potential avenues have been explored. First, the role of hormones must be considered. It has been suggested that sex steroid hormones could potentially play a role in this phenomenon[22]. This idea is supported by research indicating the involvement of sex steroid hormones in inflammatory pathways as well as the expression of sex steroid hormone receptor proteins in oesophageal tissues[111].

Thrift *et al.,* 2014 found that elevated body mass index was associated with an increased risk of both BO and OAC. They suggest that this is partly due to the exacerbation of GORD symptoms, however the role of proinflammatory cytokines secreted by adipose tissue could play a role in promoting neoplasia independently of GORD[112]. Due to this association, diet and exercise also play a role in preventing OAC. Diet significantly influences OAC risks. Higher intake of fruits and vegetables reduces risk of OAC[104]. Conversely, increased consumption of pickled vegetables and red/processed meat elevates risks for both oesophageal cancers. Carbohydrates show a strong inverse association with OAC, while dietary fat intake mildly increases OAC risk. Poultry, fish, and dairy intake exhibit inconsistent associations. Micronutrient supplements' impact on OAC rates remains inconclusive, with potential protective effects observed in younger adults[104]. A study by Coleman *et al.,* 2012 found that BO patients who smoked tobacco had a 2-fold increased risk of developing OAC or HGD. Not only does smoking induce DNA damage on Barrett's mucosa but is also thought to worsen reflux due to its action in reducing lower oesophageal sphincter pressure[113].

3.1.3 Blood based biomarkers in OAC

Whilst surveillance of BO patients consists of a 2-yearly endoscopy and biopsy, a few blood-based biomarkers have been explored to assess an individual's risk of OAC progression. The presence of proteins or genetic materials detectable in blood, play pivotal roles in diagnosing, monitoring, and prognosticating various diseases, including cancers like OAC. The mutational of OAC is fairly well characterised, with a study by Abbas *et al*., (2023) highlighting several dominant mutational signatures

including those associated with P53 mutations, genomic instability, and increased proliferation. Notably, several DNA damage repair pathway defects—including HR, BER, and MMR—were shown to be prevalent in up to 50% of OAC cases[114]. Of these, BER deficiency was strongly associated with poor prognosis. Furthermore, certain mutations were identified that could have implications for targeted therapy development (e.g. KRAS). This work has suggested the existence of OAC subtypes, characterised by hypermutation, oxidative stress, and DDR impairment. These subtypes may underlie variability in disease behaviour and treatment response, underscoring the need for subtype-specific predictive biomarkers to inform and personalise patient management[114]. Recent research has also demonstrated the potential of cfDNA, (specifically ctDNA) as a non-invasive biomarker in OAC. Studies have shown that ctDNA can reflect tumour burden, predict recurrence, and monitor response to therapy, making it a promising tool to complement tissue-based mutational profiling[115]. Incorporating cfDNA analysis into clinical practice could enhance real-time tracking of tumour evolution and aid in the development of minimally invasive, biomarker-driven management strategies. However, the quantity of cfDNA obtained from plasma is often inadequate, particularly in early-stage cancers and precursor lesions, which can reduce detection sensitivity and may necessitate whole-genome amplification to augment sample volume[116]. In terms of proteins, Whorton *et al*. explored Doublecortin Like Kinase 1 (DCLK1) as a prospective serum biomarker for oesophageal cancer patients, aiming to evaluate its diagnostic or prognostic potential. Elevated DCLK1 expression was detected in the epithelium, stroma, and plasma of individuals with BO/OAC, suggesting it's use in minimally invasive biomonitoring[117]. PBLs reflect systemic changes, carcinogen sensitivity and cancer risk prediction, facilitating non-invasive biomonitoring in humans. One study found that telomere length in lymphocytes is indicative OAC risk independently of confounders, suggesting its potential as a predictive marker in cancer[118]. Analysis of lymphocyte micronucleus frequency and *PIG-A* erythrocyte mutation frequency also offer valuable insights into genomic instability and potential biomarkers for assessing the risk and progression of oesophageal adenocarcinoma. Only a few studies have assessed levels of MN in oesophageal cancer, and reports are differing. Chang-Claude *et al.,* in 1992 carried out an analysis of MN in oesophageal mucosa smears taken from individuals with normal or very mild to severe esophagitis[119]. They did not see any association between MN levels and disease severity. Whilst Karaman *et al.,* reported

increased lymphocyte MN frequencies in cultured lymphocytes in BO patients compared to controls[120], Mozdarani *et al.,(2005)* found no significant difference in lymphocyte MN frequency in those with oesophageal cancer compared to healthy controls[121]. *PIG-A* erythrocyte MF was assessed by Haboubi *et al.,* (2019) for their correlation to the progression of the gastro-oesophageal reflux disease GORD-BO-OAC model. This study involved 200 patients and 137 healthy volunteers, with results showing significantly higher mutant levels in OAC compared to non-cancer groups[23]. The combined analysis of lymphocyte MN frequency and *PIG-A* erythrocyte MF offers a promising approach for identifying biomarkers of genomic instability and mutation. Using both of these assays allows the evaluation of various types of genetic damage and exposure durations, offering a more complete understanding of genotoxic effects in human biomonitoring studies. They appear to offer the potential to assess the risk of progression to oesophageal adenocarcinoma. However, further research is warranted to reconcile differing reports and enhance the reliability of these findings.

3.1.4 Aim and objectives.

The primary aim of this research was to investigate the correlation between different risk factors associated with OAC and their potential genotoxic impact manifested in blood cells. Specifically, this study sought to evaluate the utility of biomarkers such as lymphocyte MN frequency and *PIG-A* erythrocyte MF in gauging the risk of disease progression, focusing particularly on OAC. By elucidating the relationship between these risk factors and their genotoxic effects, this research aimed to contribute to a deeper understanding of the pathogenesis of OAC and potentially inform more effective strategies for its management and prognosis assessment.

Objective 1: To evaluate the levels of *PIG-A* MF and lymphocyte MN% correlated to disease histology. This analysis also included the investigation of how demographics influence the relationship between these biomarkers and histological variations.

Objective 2: To investigate the impact of health, diet, and lifestyle exposures on the levels of *PIG-A* mutants and lymphocyte MN%.

Objective 3: To employ a longitudinal study to assess *PIG-A* MF and MN% in predicting risk of disease progression.

3.2 Materials and methods

3.2.1. Follow up of endoscopy patients

An anonymised list including patients recruited from 2016 onwards was compiled with hospital number as the only patient identifiable information. This list included the stage of oesophageal disease (GORD, BO, cancer) at the time of recruitment, as well as any co-morbidities. Staff members in Swansea University Health Board at Singleton hospital followed up patients and documented any further endoscopy, the status of oesophageal disease at the time of the most recent endoscopy and cancer co-morbidities. Patients were categorised into “progressors” or “non-progressors” based on the following changes in diseases state: GORD to BO, no dysplasia (ND) to low grade dysplasia (LDG) to high grade dysplasia (HGD), change in length of BO segment. The average follow- up time-period was 2.8 years.

3.2.2 Calculation of Health practice index and Dietary Quality Score

Participants recruited to the study through the endoscopy department answered a detailed questionnaire about diet and lifestyle. For the assessment of lifestyle practices the Health Practice Index (HPI), derived from Kusaka *et al.,* (1992), was employed. This method evaluates individual health behaviours by scoring eight specific health practices (see **Table 3.1**) with responses classified as either "good" or "poor" health practices. Each participant received a total score between 0 and 8, reflecting the number of "good" practices they follow. Based on these scores, individuals were categorised into three HPI groups: "poor" (0-3), "moderate" (4-5), and "good" (6 or higher). A dietary quality score (DQS) was also calculated for each endoscopy patient. The DQS score used here was adapted from Toft *et al.,* (2007). They developed an 8-item screener, to assess overall dietary quality and its association with cardiovascular risk factors in the Inter99 study. The DQS demonstrated reasonable validity compared to a longer 198-item food frequency questionnaire (FFQ) and was inversely associated with cardiovascular risk factors such as total cholesterol, triglycerides, and LDL cholesterol, indicating its effectiveness in ranking individuals by dietary quality and linking higher scores to a more favourable cardiovascular risk profile. **Table 3.2** shows the scoring method for DQS, signifying the higher the score, the better the quality of diet.

Table 3.1 *The Heath Practice Index score adapted from Kusaka et al., 1992*[122]

	Good health practice (1)	Poor health practice (0)
Physical exercise	≥Once per week	< Once a week
Alcohol Drinking	Sometimes/Never	Almost daily
Cigarette smoking	Non-smoker	Smoker
Nutritional balance	Balanced	Not balanced
Eating breakfast	Daily	Sometimes or no
Subjective stress	Slight/Moderate	Excess
Working hours	≤9 hours/ day	≥10 hours

Table 3.2. *The DQS score, adapted from Toft et al., 2006*[123].

Food	Frequency	Score
Vegetables (cooked or raw) and/or vegetarian dishes	≥5-7 servings/week	3 points
	The answers in between	2 points
	≤2 servings/week	1 point
Fruit	≥3 pieces a day	3
	≥3 pieces a week and ≤2 pieces/day	2
	≤2 pieces/day	1
Fish	≥ 200g/week	3
	The answers in between	2
	No intake	1
Fat (Two sections, then see summary)		
Use in bread	None (3 points)	3
	Margarine, vegetable margarine	2
	Butter, lard	1
Use in cooking	None, olive oil	2
	Vegetable margarine/oil	2
	Margarine, butter, lard (	1
FAT SUMMARISED	6 POINTS, summarised	3 points
	2-5 POINTS summarised	2 points
	2 POINTS summarised	1 point

3.2.3 Data analysis

To allow for adjustment due to the any confounding factors, SPSS® version 29 was used to carry out statistical analysis when comparing baseline biomarker levels between histological groups, with assumptions of normality and homogeneity assessed. This study utilised a General Linear Model to explore the relationship between demographic and clinical factors with MN% and *PIG-A* MF in patients. MN% data was log-transformed to address skewness, and effect sizes were gauged using Eta

Square. Age, gender, BMI, smoking status, and diagnosis category were included as independent variables and confounders to mitigate bias in the analysis. This multivariable approach allowed the estimation of the unique contribution of each factor while controlling for others. Planned Contrasts were used to explore differences between histology categories in the presence of confounders. Statistical significance was reported for all variables in the final model. In each data set analysed, age was the only statistically significant confounding factor. All other data analysis was performed using GraphPad Prism version 9. Normality was assessed, and subsequent non-parametric tests or one-way ANOVAs were performed with post-hoc Kruskal-Wallis or Dunn's test, respectively, for multiple comparisons. Parametric data were analysed using t-tests (independent or paired) when comparing two groups, and Tukey's multiple comparison test was used following ANOVA for group comparisons involving more than two groups. An R-value (correlation coefficient) was calculated for all correlation analyses to indicate the strength of correlation. Interpretation of correlation strength followed the thresholds described by Akoglu (2018)[124]. Error bars show either standard error of the mean (SEM) or standard deviation (SD). Figure legends contain relevant information.

3.3 Results

This section will explore the relationship between lymphocyte MN%, *PIG-A* MF and patient demographics including age and sex, as well as histology (healthy volunteers, GORD, Barrett's and OAC).

3.3.1 Patient demographics by histology

The demographics and characteristics of patients included in the lymphocyte MN and *PIG-A* studies are shown in **Table 3.3.** and **Table 3.4** respectively. The patient numbers in each study differ slightly as in some cases both assays could not be carried out with the sample from the same individual. Average values along with 95% confidence intervals are included for age (years) and body mass index (BMI, kg/m^2).

Table 3.3. *Demographics and characteristics of patients included in the MN study: healthy volunteers, patients with gastroesophageal reflux disease, Barrett's Oesophagus, and oesophageal adenocarcinoma.*

Characteristics	Healthy volunteers (n=28)	Gastroesophageal reflux disease (n=39)	Barrett's Oesophagus (n=22)	Oesophageal adenocarcinoma (n=20)
Median Age (years, 95% CI)	26 (25-32)	62 (56-66)	67 (59-75)	70 (64-76)
Male gender (%)	10/28 (36%)	17/39 (46%)	15/21 (71%)	18/20 (90%)
Median BMI (kg/m^2, 95% CI)	25.9 (24.6-27.3)	27.1 (25.8-28.5)	28.2 (26.4-30.5)	24.6 (22.9-26.7)
Smoking (% use)	3/28 (11%)	8/39 (21%)	2/21 (9.5%)	4/20 (20%)

Table 3.4 *Demographics and characteristics of patients included in the PIG-A study: healthy volunteers, patients with gastroesophageal reflux disease, Barrett's Oesophagus, and oesophageal adenocarcinoma.*

Characteristics	Healthy volunteers (n=27)	Gastroesophageal reflux disease (n=30)	Barrett's Oesophagus (n=23)	Oesophageal adenocarcinoma (n=19)
Median Age (years, 95% CI)	26 (25-32)	63 (54-66)	69 (59-75)	69 (64-73)
Male gender (%)	10/27 (37%)	14/33 (42%)	15/22 (68%)	16/18 (89%)
Median BMI (kg/m^2, 95% CI)	26.0 (22.2-28.8)	28.3 (25.0-21.6)	29.1 (24.7-33.6)	25.6 (22.5-28.6)
Smoking (% use)	2/27 (7%)	3/33 (9%)	2/22 (9%)	4/18 (22%)

3.3.2 The effect of patient demographics, habits and lifestyles, and medication on DNA damage in blood cells.

To ensure the precision of the analysis regarding the impact of diverse factors—ranging from patient demographics, health and lifestyle to medication—on overall DNA damage levels, the exclusion of cancer patients from the study was deemed necessary. This deliberate choice was driven by the potential confounding effects their presence could introduce to the dataset.

<u>Demographics</u>

It is well documented that DNA stability decreases with age[125], and therefore it was of interest to compare DNA damage levels with age of the patients. This relationship was assessed through correlation analysis (**Figure 3.2**). Whilst there is a moderate association between age and MN% in lymphocytes as shown in Figure 3.2A, there was no statistically significant relationship between *PIG-A* MF and age, as demonstrated in Figure 3.2B.

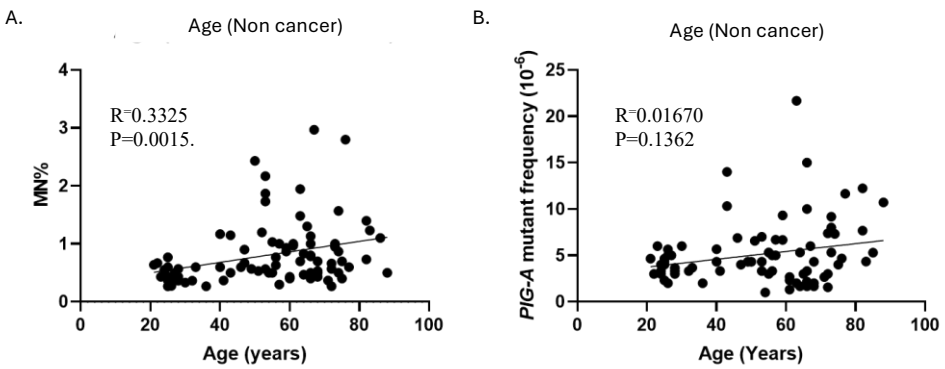

***Figure 3.2**. The correlation between DNA damage in blood cells and age. (A) There is a moderate positive correlation between the MN% of non-cancer individuals and age R=0.3325 and *p=0.0015. (B)Shows a comparison between the age of non-cancer group with their corresponding PIG-A MF. There is no significant correlation with R=0.01670 and P=0.1362*

Given that OAC manifests predominantly in white males[104], it was deemed appropriate to categorise individuals based on their biological sex. As previously mentioned, it has been observed in many studies that women have higher lymphocyte MN% than men[60]. Variation in DNA damage between sexes was analysed here as shown in **Figure 3.3**. In line with the literature, females demonstrated slightly higher MN% than males, (averaging at 1% and 0.79% respectively) (Figure 3.3A). However, the influence of sex on lymphocyte MN% was found to be statistically non-significant (P=0.55). When focusing on *PIG-A* MF, this sex disparity was lost, with both groups averaging identical *PIG-A* MFs of $5.1x10^{-6}$.

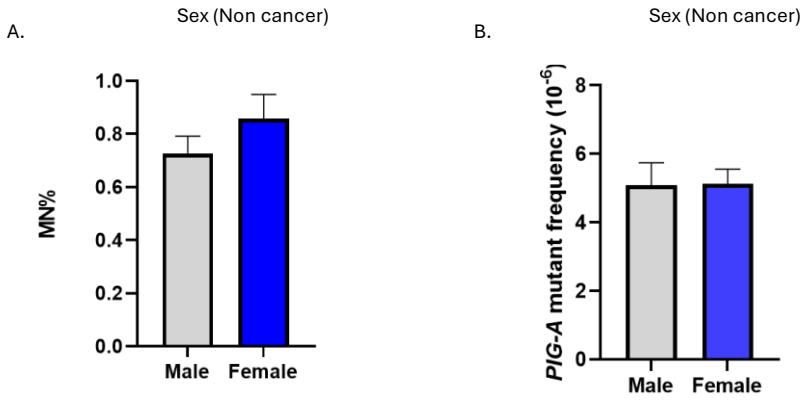

Figure 3.3 *Comparison of blood-based DNA damage between males and females. (A) This graph illustrates the average micronucleus frequency (MN%) between sexes. Females show marginally higher MN% compared to males. P=0.55 Males n=47, Females n=47. (B) PIG-A MF between sexes. Males and females have an almost identical PIG-A MF of 5.1. P= Males n=38, females n=43P=0.34. (Error bars show SEM).*

Health, diet, and lifestyle

The study gathered data from participants attending endoscopy through a comprehensive questionnaire, derived from the EPIC questionnaire[126]. This encompassed inquiries regarding dietary habits, lifestyle choices, and medical background, including co-morbidities and medications. Additionally, height and weight measurements were obtained from self-reported metrics provided by the participants. Cancer patients were excluded from the data analysis presented in this section to mitigate potential distortions in the results caused by their notably elevated average MN% and *PIG-A* MF. For each participant, an array of metrics was calculated and compared with corresponding MN% and *PIG-A* MF, including BMI (kg/m²), a HPI score, and a DQS, as illustrated in **Figure 3.4.** and **Figure 3.5** for MN and *PIG-A* respectively. Patients were stratified into three categories—"Poor," "Moderate," and "Good"—based on their DQS and HPI scores. Findings revealed a notable association between elevated MN% levels and obesity, characterised by a BMI of ≥ 30 kg/m² (Figure 3.4A). While HPI did not exhibit significant discrepancies among groups, a

pattern emerged where MN% decreased with increasing DQS (Figure 3.4B and C, respectively) (p=0.075).

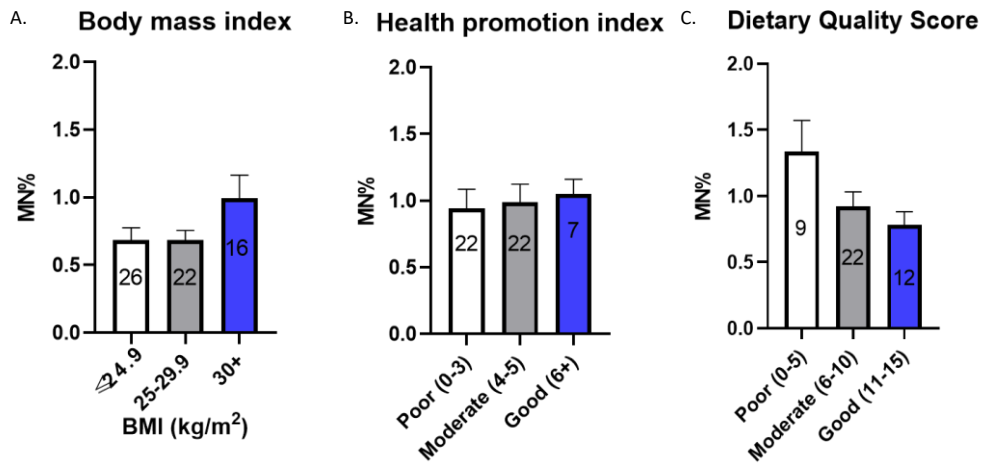

Figure 3.4. *The effects of body mass index (BMI), health promotion index (HPI) and dietary quality score (DQS) on lymphocyte micronuclei frequency (MN%). (A) Illustrates the impact of BMI on lymphocyte MN%. Obese patients have an increased MN% compared to healthy and overweight individuals. p=0.11. (B) Relationship between health promotion index and MN%. HPI scores were grouped into 3 categories ("Poor," "Moderate," and "Good") and compared with MN% plotted. P=0.30. (C.) Demonstrates relationship between dietary quality score and MN%. DQS were grouped into 3 categories ("Poor," "Moderate," and "Good") and MN% plotted. Those with a 'good' DQS' had lower MN% than those with poor, or moderate scores. This is however, not significant. P=0.075. (Error bars show SEM).*

Findings revealed no significant association between elevated *PIG-A* MFs and BMI (Figure 3.5A). While HPI did not exhibit significant discrepancies among groups, those classified as having a "moderate" health practice index had the highest average MF (Figure 3.5B) (p=0.30. The opposite was shown for DQS, whereby the same group had the lowest *PIG-A* MF (Figure 3.5C) (p=0.81).

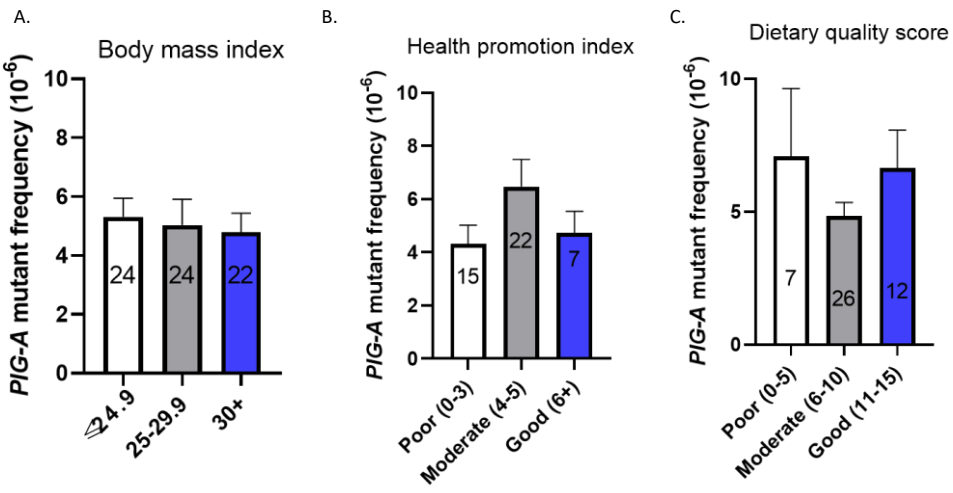

Figure 3.5. *The effects of body mass index, health practice index, and dietary quality score on PIG-A mutation frequency (MF) (A) Illustrates the impact of Body Mass Index on PIG-A MF P=0.81 (B) Relationship between health practice index (HPI) and PIG-A MF. HPI scores were calculated, and patients grouped into 3 categories ("Poor," "Moderate," and "Good") P=0.30(C.) Demonstrates relationship between dietary quality score (DQS) and PIG-A MF. DQS were calculated and patients grouped into 3 categories ("Poor," "Moderate," and "Good"). P=0.81. (Error bars show SEM).*

As both cigarette smoking and alcohol can cause DNA damage in cells, as well as increase individual risk of cancer, they were deemed important exposures to consider. The effect of cigarette smoking and weekly alcohol consumption on lymphocyte MN% and *PIG-A MF* was analysed (**Figure 3.6** and **Figure 3.7** respectively). Self-reported current smokers had higher lymphocyte MN% than current non-smokers (1% compared to 0.81% respectively, p=0.14, Figure 3.6A). There was no difference between those who drink alcohol and those who do not (Figure 3.6B), and no correlation of lymphocyte MN% with weekly units of alcohol consumed (Pearsons's correlation coefficient R=-0.1215, P-value=0.5, Figure 3.6C). Self-reported smokers show similar *PIG-A* MF to non-smokers (5.3 and 5.1 mutants per million respectively) as shown in Figure 3.7. In those who reported drinking no alcohol (0 units weekly) the *PIG-A* MF was slightly higher than those who reported weekly alcohol consumption *(*6.4 compared to 5 mutants per million in drinkers) (Figure 3.7B). There was a poor

negative between weekly alcohol units consumed and *PIG-A* MF (Figure 3.7C) (R=-0.1473 P=0.3125).

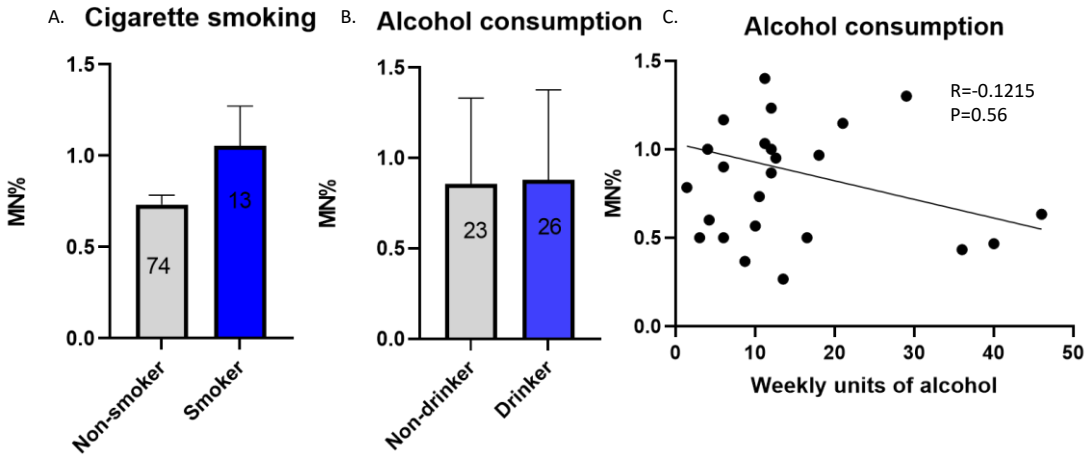

Figure 3.6. *Micronucleus Frequency (MN%) in different exposure groups. (A) Comparison of MN% between smokers and non-smokers p=0.14. (B) Lymphocyte MN% between those who do, and do not consume alcohol. P>0.05 (C) Scatter plot depicting the relationship between MN% in lymphocytes and weekly alcohol consumption in alcohol drinkers R=-0.1215, P=0.56. (Error bars show SEM).*

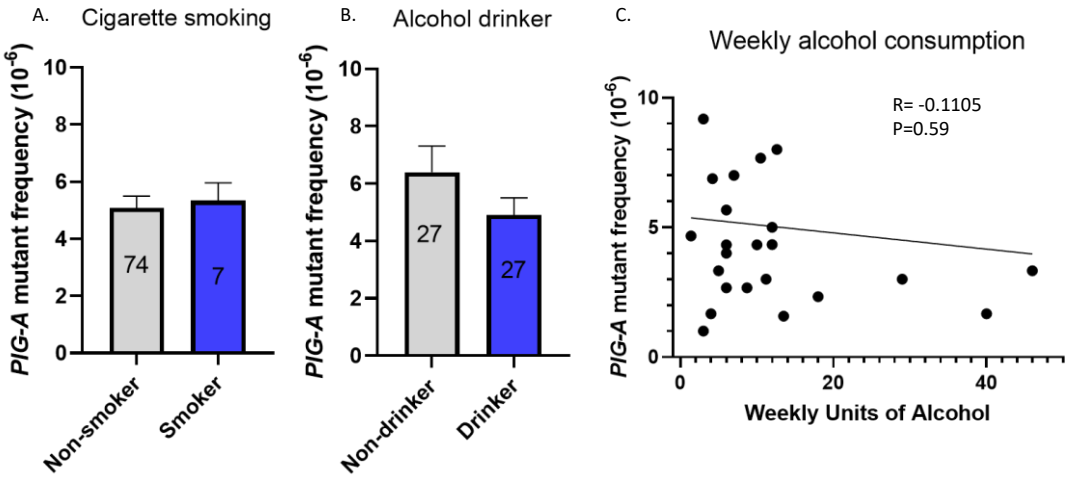

Figure 3.7. *PIG-A mutant frequency (MF) of erythrocytes in different Exposure Groups. (A) Comparison of PIG-A MF between smokers (n=74) and non-smokers (n=7). Self-reported smokers show similar PIG-A MF to non-smokers (5.3 x10-6 and 5.1 x10-6 respectively) p=0.2. (B) PIG-A MF in alcohol drinkers and non-alcohol drinkers reveals no significant disparity, despite MF showing an increase in the 'non-drinker' cohort (6.4x10-6 compared to 5 x10-6 in drinkers) p=0.28. (C) Scatter plot depicting the relationship between PIG-A MF and weekly alcohol consumption in drinkers. There was not a significant correlation between MN% and alcohol units consumed weekly (R= -0.1105, P=0.59). (Error bars show SEM).*

Medications: Aspirin and Proton Pump Inhibitors

Both aspirin and proton pump inhibitors are commonly prescribed to those with GORD/BO. While aspirin, as an anti-inflammatory agent has been associated with DNA damage protection, medications like omeprazole and PPIs have demonstrated contradicting effects[42, 43].In this study, the impact of aspirin and PPI usage, both individually and in combination, on MN% and *PIG-A* MF was investigated (Figure 3.8). This data set only includes the non-cancer patients attending endoscopy, not healthy volunteers, as this information was not always available in this cohort. No significant effects on MN frequency were observed with either aspirin or PPI usage (Figure 3.8A). No significant effects on *PIG-A* MF were observed with either aspirin or PPI usage (Figure 3.8B). For both endpoints, limited patient numbers in specific

categories, notably aspirin only and aspirin with PPI, prevent definitive conclusions from this dataset.

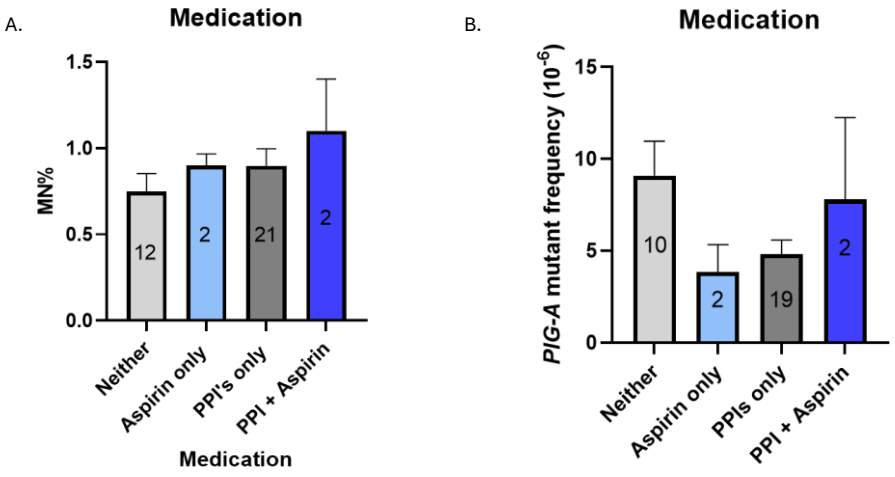

Figure 3.8. *The effects of aspirin and proton pump inhibitor (PPI) use on DNA damage in blood cells. (A) Micronucleus frequency (MN%) among patients attending endoscopy. Patients categorised by medication usage: those who take neither aspirin or proton pump inhibitors (PPIs), those who take either aspirin or PPIs, or both. p=0.50. (B) PIG-A MF among patients attending endoscopy. Patients categorised by medication usage: those who take neither aspirin or proton pump inhibitors (PPIs), those who take either aspirin or PPIs, or both. p=0.13. (Error bars show SEM).*

3.3.3 Patient Histology and DNA damage

Histology

DNA damage endpoints were measured in different histology groups. When carrying out the statistical analysis, adjustments were made for the confounding effect of age.. This was to control their influence on the relationship between disease histology, thus avoiding biased conclusions. The baseline MN% of PBLs was compared between the following groups: HV, GORD, different gradings of BO and OAC (**Figure 3.9**). The gradings of BO include ND, LGD and HGD. Those with OAC had significantly higher lymphocyte MN% than each non-cancer

histology. (Figure 3.9A). As shown in Figure 3.9B, when BO patients were categorised into 3 groups (ND, LGD and HGD) no significance was observed any of the groups. Some Barrett's patients had co-existing cancer in other tissues other than the oesophagus (e.g. prostate). Despite those with co-cancer's having higher MN% than those without (Figure 3.9C), this was also not significant.

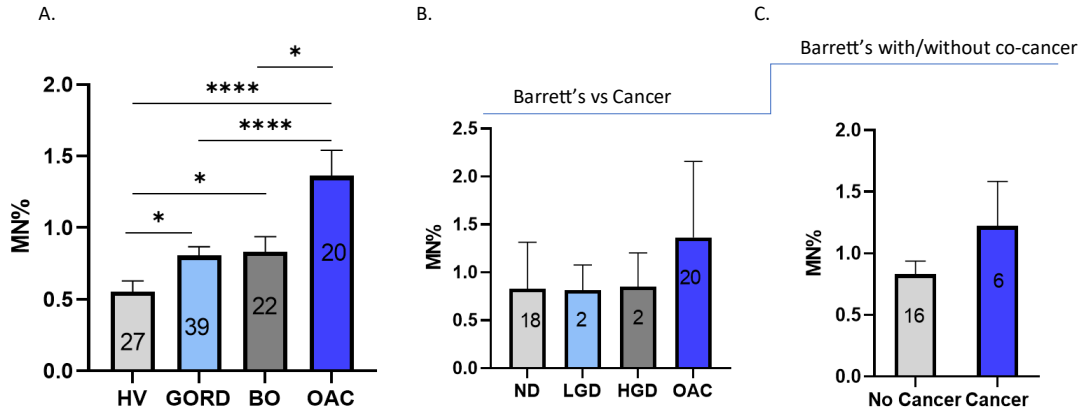

Figure 3.9. *Lymphocyte micronucleus frequency (MN%) between different histology groups. (A) Compares MN% of oesophageal cancer patients (OAC)of those with Barrett's oesophagus (BO) , gastroesophageal reflux disease (GORD) and healthy volunteers (HV). *p ≤ 0.05. **<0.01 ****<0.001(B) Compares lymphocyte MN% in different gradings of Barrett's oesophagus; no dysplasia (ND), low grade dysplasia (LGD), high grade dysplasia (HGD) and oesophageal adenocarcinoma (OAC). *p ≤ 0.05. (C) MN% between two groups of BO patient. Those with and without cancer diagnoses (excluding OAC). P=0.33. Patient numbers are displayed on the graphs. (Error bars show SEM).*

Those with OAC had more *PIG-A* mutant erythrocytes than each non-cancer histology, with significantly higher levels only shown when compared to those with GORD as shown in **Figure 3.10**. Figure 3.10B demonstrates that the *PIG-A* MF of those with ND BO is significantly lower than those with OAC (p=0.01). Like the MN analysis, due to the limited sample sizes in the LGD and HGD groups, it is challenging to accurately interpret the impact of varying dysplasia levels on MN% or *PIG-A* MF. Those with LGD had lower levels than those with HGD and OAC, despite no

significance (P=0.091 and P=0.086 respectively). Some BO patients had co-existing cancer, not including that of the oesophagus (e.g. prostate). The *PIG-A* MF of those without co-cancer in this cohort closely resembled that of BO patients with co-cancer, with only a slight difference that lacked significance. Graphs showing the endoscopy patients with co-cancer diagnoses removed from data sets are included in **Appendix X**, as this eradication had no impact on the overall results (6 BO patients involved in the *PIG-A* and MN study).

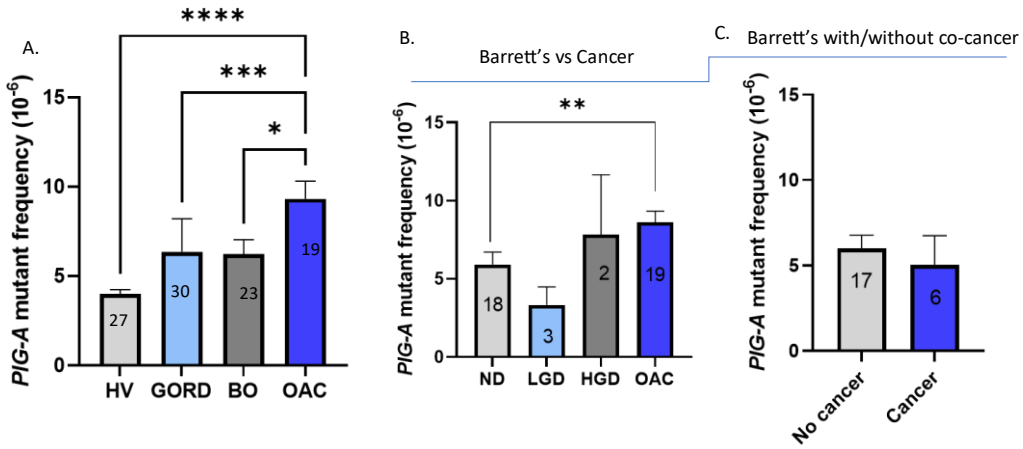

Figure 3.10. *PIG-A erythrocyte mutant frequency (MF) of different histology groups. (A) Cancer patients have the highest PIG-A MF with an average of 9.3 (n=19), significantly higher than those with Gastroesophageal reflux disease (GORD n=30, average MF: 6.3), Barrett's oesophagus (BO n=23 average MF= 6.2) and healthy volunteers (HV n=27, = 4). *p<0.05 ***p<0.005 ****p<0.001 (B) PIG-A MF between different gradings of BO as well as OAC. Non dysplastic (ND), low grade dysplasia (LGD) and high-grade dysplasia (HGD) included in analysis. **p<0.01. (C.) Shows the PIG-A MF of two groups of BO patients with and without cancer diagnoses (excluding OAC). P=0.41. (Error bars show SEM).*

<u>Endoscopy patients follow up.</u>

Patients who attended endoscopy at Singleton Hospital between March 2016 and December 2021, were followed up by clinical staff. Information on each patient's current oesophageal disease status, co-morbidities, reasons for and dates of death, and any further follow-up requirements were collected and compiled into a spreadsheet. This data was then refined, with the primary goal to assess baseline mutational

endpoint levels in relation to future (oesophageal) disease progression. Out of 260 patients followed up, only 77 had a repeat appointment in this time frame. Those with co-existing cancer outside of the oesophagus (e.g. breast, prostate, n=7) were excluded from the comparison of BO progression. Sixty patients showed no progression in their oesophageal disease (non-progressors), while 17 experienced disease progression (either died from OAC or had changes in their BO grading/length). Only 2 GORD patients developed BO in this time, however no MN% or *PIG-A* data was available for either. When excluding any oesophageal cancer patients who died from their condition, MN or *PIG-A* data were available for only 20 of the patients with follow-up details of the study, including 5 progressors and 15 non-progressors, presented in **Tables 3.5 and 3.6**. Although the MN% of progressors was comparable to that of non-progressors (0.95% versus 0.81%, respectively, p=0.51), average *PIG-A MF* was slightly but insignificantly higher in progressors compared to non-progressors ($9.3x10^{-6}$ versus $4.86\ x10^{-6}$, p=0.18). The small sample sizes in each group prevented a robust statistical analysis.

Table 3.5. *Baseline MN% and PIG-A MF of BO patients deemed "progressors" over an average of 2.8 years.*

Progressor ID	Initial Diagnosis	Progression	Time frame (Years)	Lymphocyte micronucleus frequency (%)	*PIG-A* mutant frequency *(MF)*
Prog1	BO (LGD)	BO (HGD)	1	-	17.11
Prog2	BO (LGD)	BO (LGD – increase in length)	5	0.5	4.16
Prog3	BO (ND)	BO (HGD)	2	1.45	-
Prog4	BO (LGD)	BO (LGD – increase in length)	5	0.93	6.67
Prog5	BO (HGD)	OAC	3	0.77	-
Prog6	BO (HGD)	OAC	1	1.1	-
Average (Mean)	**-**	**-**	**2.8 years**	**0.95**	**9.31**

Table 3.6. *Baseline MN% and PIG-A MF of BO patients deemed "non-progressors" over an average of 4.4 years.*

Non-progressor ID	Time Frame (Years)	Lymphocyte micronucleus frequency (%)	*PIG-A* mutant frequency *(x10^{-6})*
N-P1	3	-	10
N-P2	3	-	2.5
N-P3	7	-	5.67
N-P4	7	-	1.12
N-P5	7	-	3.67
N-P6	7	-	4
N-P7	7	-	8
N-P8	5	0.86	-
N-P9	5	1.1	1
N-P10	1	1.25	12.54
N-P11	1	-	2.67
N-P12	3	0.63	3.33
N-P13	3	0.5	4.33
N-P14	4	0.53	-
N-P15	2	-	4.33
Average (Mean)	**4.4**	**0.81**	**4.86**

In GORD and BO, the effect of co-existing cancers on DNA damage was also assessed (**Figure 3.11**). Both progressor and non-progressors were included in this analysis. MN% was higher in those with co-cancers with mean values of 0.95% and 1.51% respectively. (Figure 3.11A, p=0.3015), however this was not significant. *PIG-A* levels remained similar at around 5x10^{-6} in each group **(**Figure 3.11B, p=0.60).

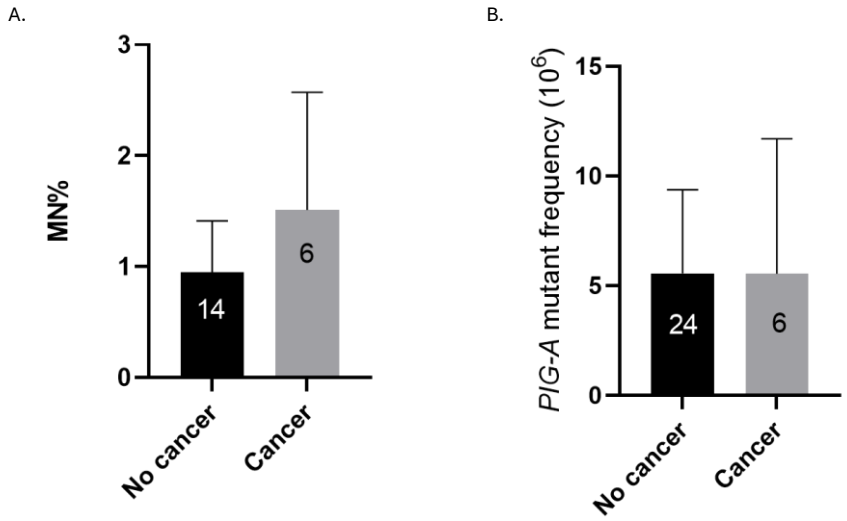

Figure 3.11. *Baseline levels of DNA damage in peripheral blood cells, in two groups. The two groups consist of those who developed cancer (excluding OAC) and those who did not, over a 7-year period. (A) Micronucleus frequency (MN%) in lymphocytes is higher in those with co-existing cancer (p=0.30). (B.) PIG-A mutant frequency is similar in those with and without co-existing cancer diagnoses (P=0.603). (Error bars show SD).*

3.3.4 Correlation between MN% and *PIG-A* MF.

The correlation between the two DNA damage tests described in this chapter was measured to assess if patients had both higher MN induction and *PIG-A* mutation concurrently. Data on *PIG-A* mutant frequency and MN% was available for 95 participants. As seen in **Figure 3.12**, there was a weak correlation between the two endpoints (R=0.253, P=0.013).

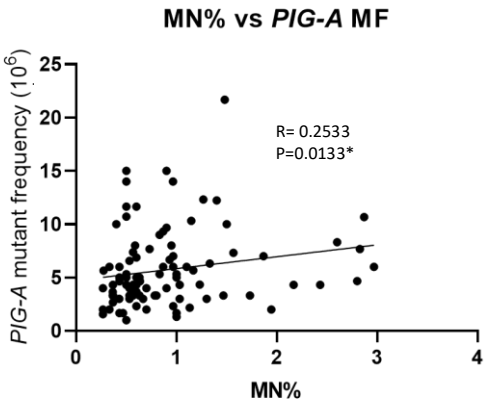

Figure 3.12. *Relationship between lymphocyte micronucleus frequency (MN%) and PIG-A mutant frequency ($10^{-6)}$ (MF). A weak correlation is shown, with R= 0.2544 and P=0.0133. n=95.*

3.4 Discussion

Patient demographics – age and sex

The patient cohort differs slightly in numbers between the micronucleus and *PIG-A* studies. This is due to a variety of reasons, including: volume of blood obtained not always allowing both tests to be performed, occasional lack of response of patient lymphocytes to PHA stimulation, contamination of lymphocytes during culture and issues with the flow cytometer measurement of *PIG-A* MF around the time of blood acquisition. Overall, individuals diagnosed with OAC tended to be older compared to those with non-cancerous histology's, and a significant majority were male (~90%). This pattern mirrors the demographic and epidemiological trends observed in OAC outside the scope of this study, wherein increasing age and male gender are recognised as established risk factors for developing OAC[127]. The average body mass index (BMI) was higher in those with BO than any other histology. Whilst obesity is considered a risk factor for developing oesophageal cancer due to the impact on GORD and BO[128], the cancer patient cohort had the lowest BMI. This is probably due to the fact that as the cancer progresses, OAC symptoms can include dysphagia and weight loss[129]. The histology with the highest proportion of cigarette smokers was OAC. As reported in a study by Coleman *et al.,* 2012 smoking can increase the risk of high-grade dysplasia and cancer amongst those with BO[113]. When comparing the complete cohort (including the OAC patients), sex was shown to have no impact on MN%. When the influence of the (majority) male cancer patient cohort was removed the MN% of females was marginally higher. According to the HUMN study, MN% in female lymphocytes are greater than those of males by a factor of 1.2-1.6[60]. Whilst this sex-based increase is not fully understood, it is hypothesised that it is due to the preferential incorporation of the inactive X chromosome in the MN[130]. As women age, their number of X chromosome-positive MN increases[130]. Whilst *PIG-A* MF appeared slightly increased in males when considering the entire patient cohort, sex had no obvious impact on *PIG-A* MF in the non-cancer group.

The relationship between age and DNA damage in blood cells was also assessed. Lymphocyte MN% was strongly associated with age in both the entire, and non-cancer cohort. This is unsurprising as it has been well reported that age is positively and significantly correlated with lymphocyte MN%[60, 131]. This positive relationship is due to an increase in genome instability due to an accumulation of DNA damage and

mutations that occur with age[131]. The positive and significant relationship between *PIG-A* MF is lost in the non-cancer cohort. It can therefore be assumed that the relationship shown in the entire patient cohort is caused by the increased age of the OAC patients.

Endoscopy study – health and lifestyle

When investigating the diverse array of factors affecting lymphocyte MN% and *PIG-A* MF, this thesis employed the non-cancer cohort. The rationale behind this choice lies in the significant DNA damage observed in the blood cells of cancer patients, which could potentially introduce biases to the data analysis. The study encompasses a total of 93 non-cancer participants, although complete information was not available for every individual, resulting in potential variations in the numbers presented across different sections.

For each participant, an array of metrics was calculated and compared with corresponding MN% levels and *PIG-A* MF, including BMI, a Health Practice Index (HPI) score, and a dietary quality score (DQS). Findings revealed a notable association between elevated MN% levels and obesity, characterised by a BMI of $\geq$ 30 kg/m^2. According to the World Health Organization criteria, a BMI of >30 is classified as obese. It is known obesity triggers inflammation primarily in adipose tissue, characterised by increased immune cell presence, particularly macrophages, in both visceral and subcutaneous regions[132]. Increased abdominal fat, or pressure from hiatus hernias also raises intragastric pressure, promoting reflux[133]. This therefore leads to higher levels of oxidative stress in individuals considered obese[134, 135] and could account for the mechanism behind this increased MN%. Whilst total lifestyle quality, as measured by HPI did not show any association with MN%, diet appeared to have an impact. The higher the dietary quality score, the lower the MN%. This finding could be explained through work carried out by Fenech *et al.,* 2005. Here, they discussed the protective effect of micronutrients like retinol, vitamin E, folate, pre-formed nicotinic acid, and calcium. These nutrients play pivotal roles in DNA methylation, chromosome segregation, and repair. Conversely, heightened genome instability was associated with increased riboflavin, pantothenic acid, and biotin concentrations[136]. These micronutrients are found in various foods as well as

supplements, highlighting the importance of a balanced diet in maintaining genetic stability.

PIG-A MF did not show clear correlations with BMI, HPI, or dietary factors. Limited research has explored how diet and lifestyle influence *PIG-A* MF. A study conducted by Lawrence *et al.,* 2020 utilised parameters such as diet, exercise, alcohol consumption, and stress levels to create a modified Health Practice Index (HPI) score. Their findings indicated a significant decrease in *PIG-A* MF among individuals with a high HPI score, suggesting a probable association with diet[103]. Specifically, the study noted lower *PIG-A* MFs in participants with higher weekly vegetable intake. The lack of association in the data presented here could be due to limitations such as sample size, or the integrity of patient-reported data on diet.

In the assessment of cigarette smoking and alcohol consumption's impact on MN% and *PIG-A* MF, Smoking status was linked to a slightly increased MN%, presumably due to exposure to the toxic substances in tobacco smoke, like polycyclic aromatic hydrocarbons, aromatic amines, tobacco related nitrosamines, and reactive oxygen species[60, 137]. These compounds induce DNA damage directly by forming adducts with DNA or indirectly by generating ROS, leading to oxidative stress and DNA strand breaks[137]. However, there was no relationship between *PIG-A* MF and smoking status, echoing some reports in the literature, (e.g., as documented in Lawrence *et al*., (2020)). There have been some studies that have established a relationship between elevated *PIG-A* MF in smokers (Haboubi *et al* 2019t(Cao *et al.,* 2016). The lack of relationship in the data presented here could be due to limited numbers of smokers in our cohort, or limited information regarding cigarette-pack years.

The effects of medicines commonly prescribed to those with GORD and BO was also assessed. PPIs such as omeprazole are frequently prescribed to those with chronic reflux, and BO. They block gastric acid secretion through inhibition of the hydrogen-potassium ATPase pump that resides on the luminal surface of the parietal cell membrane[138]. This reduces the concentration of acid backwash, therefore limiting oesophageal tissue damage and allowing the tissue time to heal[138]. Aspirin is another commonly prescribed chemoprotectant medicine for those with BO, due to its anti-inflammatory properties[139]. The AspECT trial reported that both medications in combination significantly improved outcomes in those with Barrett's oesophagus[140],

despite the reduction in absorption of aspirin due to its favourable absorption in an acidic environment[141]. MN% and *PIG-A* MF appeared to be slightly reduced in the group taking aspirin despite the lack of statistical significance, impacted by low numbers of aspirin-takers in the cohort. The protective nature of aspirin has been well documented. An *in vitro* study utilising human lymphocytes by Dandah *et al.,* 2018 showed a decrease in MN% and comet assay DNA damage following treatment with aspirin[142]. A study by Jiang *et al.,* 2023 demonstrated that outside of aspirin's anti-inflammatory properties, it also promotes DNA repair due to its acetylating potential[143]. Through the enhancement of chromatin de-condensation following the acetylation of histone 4 lysine 16, the recruitment of homologous recombination repair factors to damage sites is improved[143]. The use of PPIs reduced *PIG-A* MF, while slightly increasing average MN%. While PPIs can prevent neoplastic transformation in those with Barrett's esophagus through the reduction in acid reflux into the oesophagus, long-term PPI use is linked to systemic risks such as osteoporosis, infection, and nutrient malabsorption, as well as raising concerns for increased gastric cancer risk[144]. Limiting factors to this study include variations in the dose and duration of treatment among those taking PPIs, which may influence the observed effects on *PIG-A* and MN. Only a minority of patients in this study were using both PPIs and aspirin upon enrollment. The MN% in this subgroup closely resembled that of the PPI group, while surpassing that of each medication group for *PIG-A* MF. This inconsistency is probably attributable to the insufficient number of patients, preventing a thorough comparison between groups.

As biomarkers, the observed associations between lymphocyte MN% and *PIG-A* MF in relation to various health and lifestyle factors present promising avenues for predicting future cancer risk. MN% levels, influenced by factors such as obesity and smoking, offer insights into the extent of DNA damage and the potential for cancer development. Meanwhile, despite the lack of clear associations with *PIG-A* MF in this cohort, further exploration of its relationship with lifestyle factors and cancer risk could enhance its predictive utility. These findings underscore the importance of comprehensive biomarker profiling in understanding individual susceptibility to cancer and devising targeted prevention strategies

Blood cell DNA damage and OAC risk

All the above health and lifestyle factors can influence both MN% in lymphocytes, and erythrocyte *PIG-A* MF. When carrying out statistical analysis between patient groups, the influence of confounding factors had to be determined using regression-based approaches. Age was identified as the only statistically significant confounding factor for both the MN% and *PIG-A* analysis. Therefore, the statistical analysis corrected for the confounding effects of age. As well as comparing MN% and *PIG-A EMF* of prospective patients recruited during this study as a cross-sectional approach, patients enrolled in the endoscopy study dating back to 2016 underwent follow-up evaluations by clinicians. This involved reviewing current endoscopy and biopsy results to identify any progression from GORD to BO, as well as changes in Barrett's segment length or stage (e.g., transition from absence of dysplasia to low-grade dysplasia). Furthermore, occurrences of BO advancing to cancer was recorded. BO patients were then divided into two main categories: 'progressors' or 'non-progressors' based on changes in their disease state from the time of enrolment until April 2023.

MN%

Through statistical analysis taking age into consideration as a confounding factor, it was determined that cancer patients have significantly higher MN% than each of the non-cancer histology's. The increase of lymphocyte MN% is a well-observed phenomenon in many cancer types. For example, El Zein *et al*., (2006) indicated that increased MN% in lymphocytes is predictive of lung cancer risk[145]. A study by Bonassi *et al.,* 2011 presented an overview of the baseline lymphocyte MN% found in a variety of cancers, including (but not limited to) breast, lung, and gastroenteric. It was concluded in this study that MN% in lymphocytes is predictive of future cancer risk, however the author stressed that knowledge about the role of individual susceptibility limits the applicability of MN% as a biomarker for cancer screening[73]. Increased MN% in lymphocytes could be a result of an increase in genetic instability or reduced DNA repair capacity[120]. In the context of oesophageal cancer, there are few studies assessing the relationship with lymphocyte MN. Whilst a study by Karaman *et al.,* 2010 found higher levels of MN in 30 BO patients compared to 30 healthy volunteers[120], Mozdarani *et al.,* 2005 saw no significant difference in lymphocyte MN% in oesophageal cancer patients compared to healthy controls[121]. Analysis in a larger cohort (including the patients involved here) on those recruited as far back as 2016 was carried out by myself and Dr Rachel Lawrence. The MN% was significantly

higher in those with OAC compared to each patient group, even when correcting for confounding factors such as age. Further work (including mechanistic studies) is required to fully understand the mechanisms behind the elevation of MN in the lymphocytes of oesophageal cancer patients.

PIG-A

Similarly to MN%, age was deemed a confounding factor when comparing histological groups. Following the multivariate analysis, it was determined that OAC have significantly higher *PIG-A* MF compared to each of the non-cancer groups. When considering the variations in the grading of BO, there was no statistical differences between gradings of dysplasia, however OAC patients had significantly higher *PIG-A* MFs than ND BO patients. There are few other studies that measure *PIG-A* MF as a predictive marker for cancer risk. Haboubi *et al*., (2020) employed larger cohort than used here and identified significantly higher levels in those with OAC compared to HV, GORD, and those with non-dysplastic Barrett's oesophagus[23]. Nichols *et al*., (2023) observed higher levels of *PIG-A* mutant erythrocytes in treatment-naïve pancreatic cancer patients compared to a non-cancer group , suggesting a potential link between individual susceptibility to this type of mutation and cancer risk[146]. According to Dobrovolsky *et al*., (2011), the pre-treatment *PIG-A* MF in cancer patients was similar to that of healthy controls[75]. Horibata *et al*., (2016) reported that healthy volunteers had a *PIG-A* MF range of 0.00–5.00 mutants/million, while cancer patients (with a range of cancer types) displayed a wider range of 0.00–49.67 mutants/million[147].

Longitudinal analysis of endoscopy patients

Previous studies by Murgia *et al*., (2008) and Bonassi *et al*., (2011) observed increased baseline MN% in initially disease-free individuals, correlating with future cancer incidence and reduced cancer-free survival. However, the mechanistic link between solid tumour development and the induction of circulating *PIG-A* mutation levels remains unclear. Patients recruited in 2016 were longitudinally followed to investigate the association between DNA damage levels and the potential prediction of disease progression. After refining the data, and accounting for the confounding effect of co-existing cancers in patients, comparisons of MN and/or *PIG-A* levels was possible in 21 patients. There was no discernible difference in MN% between BO patients who

progressed and those who did not; however, among 260 patients with GORD, only 2 developed BO, and this data was unavailable for analysis. While *PIG-A* mutant levels were elevated in progressors, there was a limited numbers of progressors (n=3) compared to non-progressors (n=14). To comprehensively evaluate the predictive potential of *PIG-A* or MN, longer-term follow-up is necessary due to the low annual risk of cancer development in non-dysplastic BO patients (0.12%[148]). Additionally, data availability posed challenges, with some patients having only one endpoint or none, due to issues such as contamination, lost data, or biopsy-only samples. The eradication of patients with co-existing cancers also decreased this sample size.

Correlation between *PIG-A* and MN%

A weak correlation between MN% and *PIG-A* levels was observed when comparing 95 individuals here (including HV, GORD, BO and OAC). Previous work generated within the *in vitro* Toxicology group at Swansea University, and published by Nichols *et al.,* 2023 did not observe any significance in their correlation between the two markers of DNA damage in a pancreatic cohort, however this included substantially less patients (n=20). This suggests that in some patients, higher MN levels may signify heightened chromosomal damage and genomic instability, reflected in the corresponding high *PIG-A* MF potentially due to inefficient DNA repair mechanisms. However, in other patients who display high MN%, the *PIG-A* MF is low and vice versa. This lack of strong correlation may be due to the fact that these mutational endpoints (MN and *PIG-A*) are regarded as complementary elements of genotoxicity covering chromosome level damage (MN) and point mutations (*PIG-A*) respectively. Understanding this correlation (or in some cases lack of correlation) can provide insights into the underlying mechanisms of carcinogenesis and may aid in the development of novel biomarkers for cancer risk assessment and early detection. Moreover, it highlights the importance of monitoring both MN and *PIG-A* levels as complementary indicators of genomic integrity and DNA repair efficiency, which can ultimately inform personalised treatment strategies and improve patient outcomes.

Conclusion

The investigation into the potential link between MN% and *PIG-A* MF and oesophageal disease status offers the promising prospect of these events being developed as suitable biomarkers of disease. They also offer insights into disease

progression, lifestyle factors, and exogenous exposure. Analysis of patient demographics reveals notable age and sex disparities, with older individuals, particularly males, exhibiting a known higher predisposition to OAC. Lifestyle factors that can increase risk of OAC such as obesity were considered, with patients with a BMI of >30 displaying increased MN%. Similarly, preliminary associations between *PIG-A* levels and cancer were observed, although further research is warranted to elucidate its full relevance. Longitudinal follow-up studies to assess the potential utility of MN% and *PIG-A* as predictive biomarkers for disease progression was difficult to assess due to the small number of patients followed up who showed progression of their oesophageal disease. The association between elevated lymphocyte MN% and cancer, particularly when considering patients with diagnoses outside of OAC, suggests lymphocyte MN% is a biomarker for cancer risk and progression. By uncovering the molecular processes contributing to elevated MN% levels in cancer patients, its utility as a specific biomarker for cancer risk assessment and progression monitoring can be refined. The observed correlation between MN% and *PIG-A* MF levels suggests an interplay between genomic instability and DNA repair mechanisms, or even exogenous exposures in some patients. Understanding this correlation could enhance cancer risk assessment and inform personalised treatment strategies tailored to individual patients.

Chapter 4 – MN% and *PIG-A* MF in response to cancer treatment

4.1 Introduction

Chemo and radiotherapy are two of the primary treatments for cancer and have been used for the past few decades[149]. They employ different mechanisms, however, are commonly used together in many treatment plans. Chemotherapy is a systemic mode of treatment, using cytotoxic drugs to kill rapidly dividing cancer cells[150]. These chemotherapeutic drugs interfere with DNA, RNA, or protein synthesis[150]. Cisplatin, for example, inhibits DNA synthesis through binding to DNA to form intra-strand DNA adducts[151]. Radiotherapy uses directed X-rays or protons to damage DNA and kill cancer cells, it can be delivered to a patient either externally or internally[152]. Both methods of treatment exploit the higher proliferation rate and defective DNA damage responses of cancer cells compared to normal cells in order to destroy tumours[153].

4.1.1 Monitoring response to treatment

Endoscopy is routinely used to detect pre-cancerous dysplastic lesions in both OAC and OSCC, enabling early intervention. Upon identification, patients with early-stage disease may undergo endoscopic resection and, if appropriate, local ablative therapy—a strategy recommended by the National Institute for Health and Care Excellence (NICE) for high-grade dysplasia and superficial tumours[154]. In more locally advanced cases, oesophageal cancers have a high risk of recurrence if managed with surgery alone; therefore, multimodal treatment is standard, as outlined in **Figure 4.1**. According to NICE, perioperative chemotherapy or chemoradiotherapy is advised to improve long-term outcomes[155]. The FLOT regimen—consisting of 5-fluorouracil, leucovorin, oxaliplatin, and docetaxel—is currently favoured for fit patients with resectable OAC, as it has been shown to significantly improve overall survival. Each agent in FLOT acts synergistically: 5-FU inhibits thymidylate synthase, leucovorin enhances its activity, oxaliplatin forms DNA crosslinks, and docetaxel disrupts mitotic spindle function. Alternative regimens such as FOLFOX, carboplatin with paclitaxel, or capecitabine-based combinations are used based on individual tolerability the mechanisms of action of which are shown In **Table 4.1**. In unresectable or metastatic disease, systemic palliative chemotherapy is offered, and in select cases, targeted therapies such as trastuzumab (for HER2-positive tumours) or immune checkpoint inhibitors like nivolumab may be added[156]. Radiotherapy also plays a critical role, especially in non-surgical candidates, by inducing DNA damage and apoptosis in

tumour cells. This comprehensive, stage-based approach underpins the current NHS standard of care for managing oesophageal cancer.

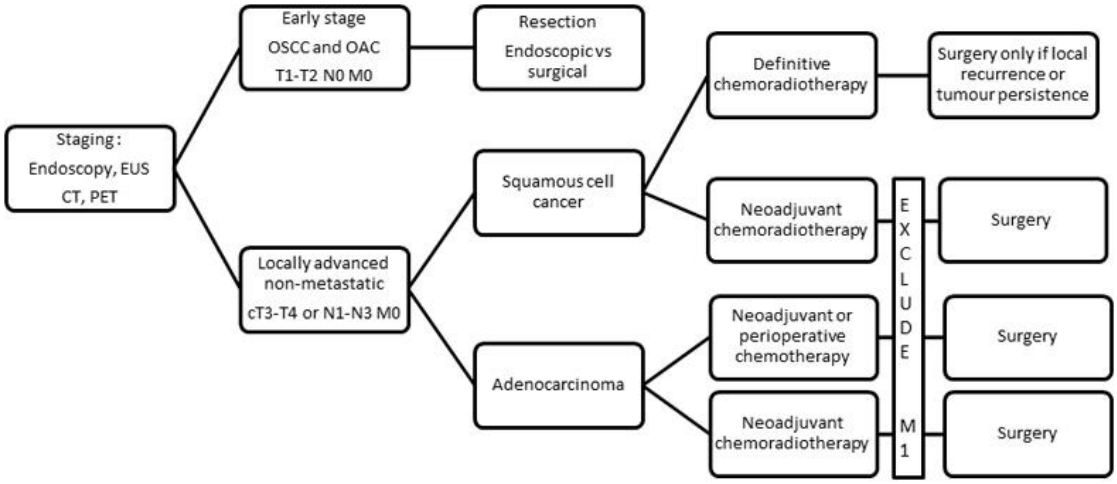

Figure 4.1. *Flow chart demonstrating the management of oesophageal cancer. Diagram was taken directly from Smyth et al., 2017: Oesophageal cancer*[13]

Table 4.1*. Mechanism of actions of chemotherapies and radiotherapy used in treatment of OAC*

Type of treatment	Mechanism of action
FLOT (5-FU, Leucovorin, Oxaliplatin, Docetaxel)	- 5-FU: Pyrimidine analogue; inhibits thymidylate synthase → blocks DNA synthesis - Leucovorin: Enhances binding of 5-FU to thymidylate synthase (potentiator) - Oxaliplatin: Platinum compound; causes DNA crosslinking → apoptosis - Docetaxel: Stabilizes microtubules → inhibits mitosis
FOLFOX (5-FU, Leucovorin, Oxaliplatin)	Same as above (subset of FLOT without Docetaxel)
Carboplatin	Platinum analogue; forms DNA crosslinks → inhibits DNA replication and transcription
Paclitaxel	Binds β-tubulin; stabilizes microtubules → prevents mitotic spindle breakdown → mitotic arrest
Oxaliplatin	Binds DNA to form intra- and inter-strand crosslinks → disrupts DNA replication and repair
Raltitrexed	Thymidylate synthase inhibitor (direct) → disrupts DNA synthesis (similar to 5-FU but more targeted)
Radiotherapy	Ionizing radiation causes double-strand DNA breaks, produces free radicals, and disrupts cellular repair → leads to apoptosis of rapidly dividing tumour cells

Less than one third of patients with OAC respond to neoadjuvant therapy meaning there is an urgent need for biomarkers to predict response to therapy[157]. Surveillance following chemoradiotherapy typically involves a series of medical procedures

including regular OGD with biopsies, EUS, CT scans, and Fluorine 18 fluorodeoxyglucose (FDG) positron emission tomography (PET-CT)[158]. PET-CT has demonstrated feasibility and accuracy in assessing the response to neoadjuvant therapy in oesophageal cancer. Nevertheless, its effectiveness in detecting non-response remains uncertain[158]. Investigations into alternative biomonitoring techniques for evaluating treatment response in oesophageal cancer patients have extended beyond traditional approaches. For instance, a study by Lynam-lennon *et al*., (2016) delved into the methylation patterns of microRNAs as potential indicators. They found specific microRNA methylation patterns in OAC patients pre-treatment associated with treatment outcomes[159]. Previously, research has expressed interest in innovative blood markers like ctDNA and circulating tumour cells (CTCs). CTCs can provide insights into tumour driver mutations, which could guide the selection of targeted therapies, similar to their use in cancers like breast and lung. This approach could enable more personalised treatment strategies in OAC by identifying mutations that influence drug response. Challenges with using these biomarkers in a clinical setting include small datasets and low ctDNA detection rates. While some studies suggest correlations with neoadjuvant therapy response, limitations hinder robust conclusions. CTCs' predictive potential, primarily studied in other cancers, faces barriers like the need for improved detection devices and protocols. More traditional biomarkers like carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 for predictive purposes in OAC have also been explored. A study by van der Kaaij *et al*., 2019 demonstrated that elevated CEA and CA19-9 predict early treatment failure and reduced survival in OAC patients[160]. The ongoing pursuit of more effective biomarkers remains imperative for refining treatment strategies in oesophageal cancer management.

4.1.2 DNA damage and cancer therapy

Chemotherapy and radiotherapy, aim to eradicate cancer cells by inducing various types of DNA damage, including breaks, crosslinks, and base changes[161]. There are various mechanisms aimed at restoring DNA integrity, collectively referred to as the DNA damage response (DDR)[162]. The DDR orchestrates repair mechanisms to restore DNA integrity, crucial for preventing genomic instability and mutations associated with cancer development. Despite their efficacy, cancer cells often develop resistance to therapy by dysregulating the DDR. Defective DDR pathways contribute to tumour

heterogeneity, therapeutic resistance, and cancer progression[162]. For instance, cisplatin induces DNA crosslinks repaired by NER and HR[163]. Resistance to cisplatin is associated with elevated DNA repair enzyme expression and NER activity[163]. Understanding the intricate relationship between chemotherapy-induced DNA damage and its associated toxicities in healthy cells is paramount for mitigating long-term side effects and enhancing the quality of life for cancer survivors[161]. By gaining insights into the impact of DNA damage on both cancerous and healthy cells, clinicians can refine therapeutic approaches to minimise harm to patients while maximising treatment efficacy.

4.1.3 Effect of cancer therapy on lymphocyte MN induction and *PIG-A* MF

Due to the genotoxic nature of cancer therapy, both tumour cells and normal tissues undergo significant cellular and DNA damage, leading to adverse effects on healthy tissues. Lymphocyte MN% and *PIG-A* erythrocyte MF represent promising biomarkers due to their direct reflection of DNA damage and repair capacity following chemotherapy or radiotherapy. These markers could offer insights into treatment efficacy, toxicity, and overall patient response. The effect of chemo/radiotherapy on both lymphocyte micronucleus induction and *PIG-A* mutant frequency in circulating blood cells has been assessed in a small number of studies, however the question of whether the alteration in levels of DNA damage is representative of patients' response to treatment remains unsolved. Radiotherapy increases MN in lymphocytes, as confirmed by several studies ([164],[165]), with persistent elevation in some patients indicating lasting genetic damage post radiotherapy[166]. Dröge *et al.,* (2021) assessed the use of the MN% of lymphocytes in the context of rectal cancer treatment. The study aimed to assess the prognostic value of cytogenetic damage markers, particularly lymphocyte MN%, in predicting patient outcomes. Despite observing an increase in cytogenetic damage during therapy, the study did not find a correlation between lymphocyte MN% and patient outcomes in locally advanced rectal cancer[167].

It is widely known that chemotherapeutic agents have genotoxic effects on cells. A 2008 study by Minicucci *et al.,* evaluated DNA damage in lymphocytes of children undergoing a chemotherapy with a range of drugs and cancers, and saw a significant increase in MN% (a fold change of around 2.7)[168]. Cytogenetic damage in lymphocytes was also reported in those undergoing 131-iodine treatment for thyroid cancer, and those treated with chemotherapy (cisplatin and bleomycin) for testicular cancer [169,170].

Osanto *et al.,* (1991) saw elevated MN levels for up to 9 years following treatment, indicating the implication of DNA damage to healthy cells in the increased risk of secondary tumours[170]. In the context of oesophageal cancer, a study by Emamgholizadeh Minaei *et al.,* (2016) investigated the combined effects of chemoradiotherapy on MN in PBLs, comparing RT alone to combined chemoradiotherapy. They found that MN frequency increased significantly after 12 RT fractions in both groups, and at the end of treatment MN% was higher in chemoradiotherapy group compared to RT alone[171].

The exploration of *PIG-A* mutant levels in peripheral blood cells has more recently begun. Studies by Bonetto *et al.,* (2021), Horibata *et al.,* (2016), and Dobrovolsky *et al.,* (2024) investigated the impact of cancer treatments on mutation levels. Bonetto *et al.,* 2021 found no significant change in granulocyte *PIG-A* mutant levels in breast cancer patients undergoing limited radiotherapy[172]. Horibata *et al.,* (2016) lacked pre-treatment samples, hindering evaluation of treatment effects on erythrocyte mutant cell levels in a diverse cancer cohort[147]. Dobrovolsky *et al.,* (2024) found that cisplatin chemotherapy led to a moderate increase in the frequency of *PIG-A* mutant erythrocytes, particularly immature erythrocytes (reticulocytes), suggesting an induction of mutagenesis in these patients' hematopoietic tissue[173]. Maximising study durations and utilising appropriate assay timing could enhance treatment sensitivity assessment, with a test battery potentially providing comprehensive insights into genotoxic exposure effects and treatment response. Analysing *PIG-A* mutations and MN% in PBLs helps understand DNA repair mechanisms and cellular sensitivity to cancer treatments, offering insights into treatment efficacy and potential side effects. Whilst it is clear these endpoints can be induced during therapy, the extent to which it reflects clinical outcome has not been properly explored. Given the association between these DNA damage endpoints and the development of oesophageal cancer, there arose an intriguing opportunity to investigate the susceptibility to damage induction through cancer therapy and its potential correlation with clinical response and survival outcomes.

4.1.4 Aim and objectives.

The primary aim of this pilot study is to assess whether measurements of DNA damage in the blood cells (lymphocyte MN% and erythrocyte *PIG-A* MF) of patients with oesophageal cancer is reflective of advance disease, and response to treatment.

Objective 1: To analyse pre-treatment DNA damage levels in terms of cancer characteristics, and treatment outcome.

Objective 2: To assess whether chemo/radiotherapy induces higher levels of DNA damage in the blood cells of oesophageal cancer patients.

Objective 3: To assess whether fold change in levels of DNA damage during and following treatment is indicative of outcome and response to treatment.

4.2 Methods

4.2.1 Categorising patients by clinical stage

Staging oesophageal cancer is a complex process influenced by the type of cancer (either OSCC or OAC), the cellular grade (indicating how abnormal the cells appear), and whether the staging is based on clinical evaluations or post-surgical pathological assessments. The TNM (Tumour, Node, Metastasis) system, central to this process, classifies the cancer into stages 0 through 4. This system evaluates the tumour's size and extent (T), lymph node involvement (N), and the presence of distant metastasis (M).Based on the TNM score available for each patient, a stage number was deduced using the cancer research UK website[174], with a summation shown in **Table 4.2.** Patients were generalised as stage 2, 3, or 4 instead of being broken down into subcategories like 2A, due to the limited number of patients in each subgroup.

Table 4.2.*The Tumour, Node, Metastasis (TNM) scoring system, clinical stage, and description of OAC as described by Cancer Research UK.*

Stage	Tumour (T)	Nodes (N)	Metastasis (M)	Description
0	Tis	N0	M0	High-grade dysplasia
1	T1	N0	M0	Tumour limited to the innermost layers of the oesophagus, no lymph node involvement, no metastasis
2A	T2 or T3	N0	M0	Tumour invades muscularis propria or adventitia, no lymph node involvement, no metastasis
2B	T1 or T2	N1	M0	Tumour invades lamina propria, muscularis mucosae, or submucosa (T1) or muscularis propria (T2), with minimal lymph node involvement, no metastasis
3A	T4a or T3 or T1	N0 or N1 or N2	M0	Tumour invades pleura, pericardium, or diaphragm (T4a), or adventitia (T3), or submucosa (T1), with 0-2 lymph nodes involved, no metastasis
3B	T2 or T3	N2	M0	Tumour invades muscularis propria (T2) or adventitia (T3), with 3-6 lymph nodes involved, no metastasis
3C	T4a or T4b or any T	N1 or N2 or N3	M0	Tumour invades pleura, pericardium, or diaphragm (T4a), or adjacent structures (T4b), with 1-3 lymph nodes involved, no metastasis
4A	T1-T4a or T4b or any T	N2 or N0-2 or N3	M0	Tumour of any size or extent, with extensive lymph node involvement, no metastasis
4B	any T	any N	M1	Tumour of any size or extent, any lymph node involvement, with distant metastasis

4.2.1 Medical treatment and follow up schedule of oncology patients.

Recruitment of patients from oncology is described in general materials and methods. Following a pre-treatment blood sample, further blood collections were co-ordinated dependent on the patient treatment schedule. Not all patients had multiple blood samples taken due to several reasons including suspension of the study for several months, illness on the patients' behalf or withdrawal from the study. **Table 4.3** details the individual treatment plans and time points of blood acquisition. Chemoradiotherapy for oesophageal cancer often involves starting chemotherapy before radiotherapy and continuing it concurrently. Treatment typically occurs in cycles: for example, chemotherapy on Days 1, 8, and 15, followed by a week's break. A cycle lasts about 3 weeks, with three preoperative and three postoperative cycles[175].

Table 4.3. *Cancer, treatment plan and cycle time point of blood acquisition of oncology patients included in this study, including those with oesophageal adenocarcinoma (OAC), squamous cell carcinoma (SCC) and tumours at the gastro oesophageal junction (GOJ).*

Patient ID	Cancer type and stage	Treatment plan	Time of blood acquisition
CX100	OAC, stage 4	Chemo only (FLOT)	Pre, Cycle 2,5
CX101	OAC, stage 3	Chemo only (FLOT 8 cycles)	Pre, Cycle 1,4, Post
CX103	OSCC, stage 4	ChemoRadio (Carboplatin/Paclitaxel, 50 GY in 25 fractions)	Pre, Cycle 1,5, Post
CX104	OAC, stage 2	ChemoRadio (Carboplatin/Paclitaxel, 50 GY in 25 fractions)	Pre, Cycle 3,6, Post
CX105	OAC, stage 3	Chemo only (FLOT – 8 cycles)	Pre, Cycle 2,6, Post
CX107	OAC, stage 3	Radiotherapy (50 GY in 25 fractions)	Pre, Cycle 1,4, Post
CX108	OAC-GOJ, stage 3	FLOT (only received 1/5 intended cycles)	Pre, Cycle 1
CX109	OAC-GOJ, stage 3	FLOT (1 cycle – adverse reaction) Raltitrexed and oxaliplatin	Pre, Cycle 1,5, Post
CX110	OAC, stage 4	Chemo only - FLOT (cycle 1) Folfox (5 cycles)	Pre, Cycle 2,6, Post
CX111	OAC-GOJ, stage 4	Chemo only (FLOT – 8 cycles)	Pre, Cycle 3,6, Post
CX112	OAC, stage 3	ChemoRadio (Carboplatin/Paclitaxel, 50 GY in 25 fractions)	Pre, Cycle 1,4, Post
CX113	OAC, stage 2	Patient discharged back to surgical team	Pre-only

4.3 Results

4.3.1 Oncology study: pre, during and post treatment levels of DNA damage

Blood samples were taken from patients before, during and after chemo/radiotherapy. For each patient, a maximum of 4 blood samples was acquired. For ease of analysis, samples taken during treatment were categorised into two groups. Cycles 1-3, whereby the blood was acquired after cycles 1, 2 or 3 of treatment, however prior to cycle 4. Cycle 4-6 uses information from blood taken after cycles 4, 5, and 6. The post treatment blood sample was taken >1 month following final treatment cycle for each patient. A total of 16 patients were consented to this study, 12 patients gave a pre-treatment sample, however due to complications or withdrawal, a total of 10 patients completed the study, meaning they donated the required pre, during and post samples. Details of specific treatment plan and time point of sample acquisition are listed in **Table 4.3** above.

The first half of this section will compare pre-treatment samples, of which there were 12 samples. Amongst the oncology patients, 1 patient had SSC whilst the rest had OAC/ adenocarcinoma of GOJ. Due to the small patient numbers, no comparisons could be drawn. Due to this, as well the similar clinical handling of the two, both subgroups were included in the subsequent analysis.

4.3.2 Pre-treatment sample demographics – *PIG-A* erythrocyte mutant frequencies and lymphocyte micronucleus frequencies.

Patients were categorised into four different age groups (40-50, 51-60, 61-70, and 71-80), and pre-treatment DNA damage levels in circulating blood cells were compared across these groups. No clear difference could be observed between MN% or *PIG-A* MF and age in cancer patients, due to limited numbers in each group. Regarding sex, only one female patient was included in the pre-treatment analysis, which limited direct comparisons between sexes.

4.3.3 Comparing DNA damage in circulating blood cells with tumour characteristics.

Different tumour characteristics were compared against pre-treatment *PIG-A* MF and lymphocyte MN% for each of the 12 individuals. Patients were grouped according to

cancer stage (stage 2, 3, or 4) prior to treatment. There was no significant difference drawn between groups. DNA damage levels were also assessed in patients with non-metastatic versus metastatic cancer. No significant differences were observed in either *PIG-A* MF or lymphocyte MN% between these two groups.

4.3.4 *PIG-A* MF and MN% before, during, and after treatment

Pre-treatment vs outcome

Pre-treatment *PIG-A* MF and MN% were compared to post-treatment outcome, as recorded in April 2024 (**Figure 4.2**). The patients were categorised into three groups; those who passed away (n=4), patients with a poor prognosis (n=2), and therefore under palliative care. The third category includes the patient responding to treatment, therefore remained under curative care (n=5). These patients are still under surveillance by the oncology team. When comparing pre-treatment *PIG-A* MF and MN% there was no significant differences between groups (Figure 4.2A and B respectively).

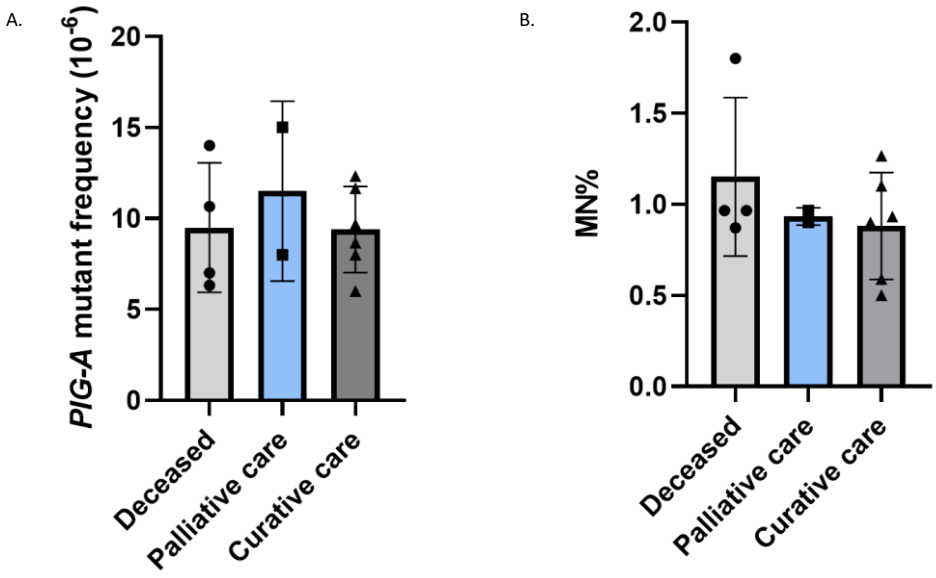

Figure 4.2. *Pre-treatment DNA damage levels compared to overall patient outcome. The outcomes include dead, under palliative care, and remaining under curative care. (A) Average erythrocyte PIG-A mutation frequency. Deceased, n=4 Palliative care n=2, Curative care n=6*, p=0.71 *(B) Average lymphocyte micronucleus frequency* (MN%) *Dead n=4, Palliative care n=2, Curative care n=6.*, p=0.72. *(Error bars show SEM).*

Change in DNA damage during and after treatment.

Raw *PIG-A* and MN% values were compared before, during, and after treatment (**Figure 4.3**). Analysis of *PIG-A* MF (Figure 4.3A) revealed a subtle increase during and after treatment, with a mean MF of 9.7 pre-treatment, compared to 11.8 at cycle 4-6 and 11.1 post treatment. However, the raw MN% significantly increased during treatment, returning to similar pre-treatment levels thereafter (see Figure 4.3B).

It is important to note that baseline mutant frequencies (both MN% and *PIG-A*) varied between patients, which could obscure true treatment-related changes. To address this, we calculated fold changes in mutant frequency relative to each patient's own pre-treatment baseline (see **Figure 4.4**). This within-subject normalization approach allowed each patient to serve as their own control, thereby reducing the confounding effect of inter-individual variability in background frequencies.

While raw values did not demonstrate statistically significant changes, fold change analysis revealed a significant increase in *PIG-A* MF during treatment (**Figure 4.4A**). Notably, post-treatment levels remained significantly elevated compared to pre-treatment baselines. This supports the utility of fold change as a sensitive metric for detecting treatment-induced biological effects in patient populations.

Similarly, MN% fold change increased significantly during treatment but reverted to near baseline post-treatment (Figure 4.4B). Lastly, mid-cycle fold-change levels of DNA damage were compared with the current patient status (deceased, receiving palliative care, or undergoing curative treatment). Mid cycle fold changes were used here as these showed the maximum increase in DNA damage observed.

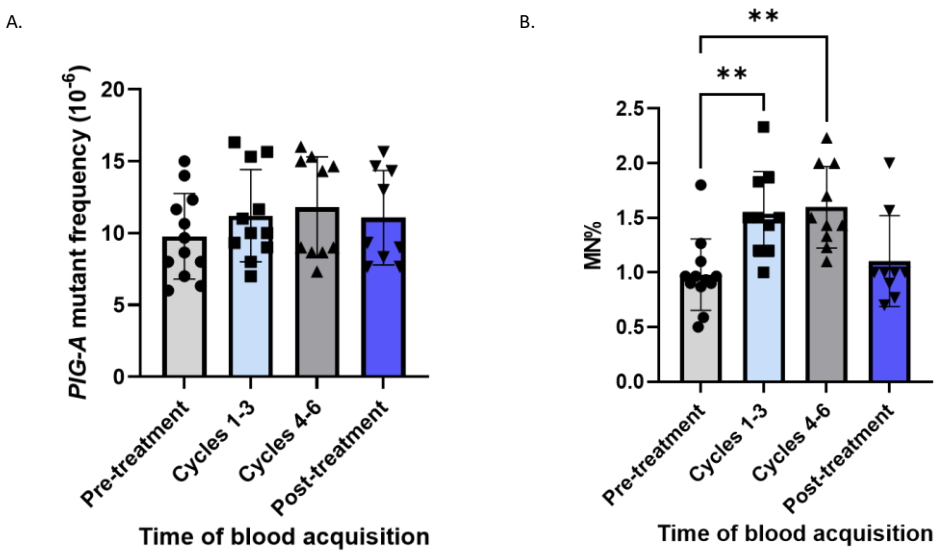

Figure 4.3. *Raw DNA damage levels before, during, and after chemo/radiotherapy treatment. (A) Average erythrocyte PIG-A mutation frequency. Pre-treatment n=12, Cycles 1-3 n=11, Cycles 4-6 n=10, Post treatment n=9. P>0.05. (B) Average lymphocyte micronucleus frequency. Pre-treatment n=12, Cycles 1-3 n=10, Cycles 4-6 n=10, Post treatment n=9. **P<0.01. (Error bars show SEM).*

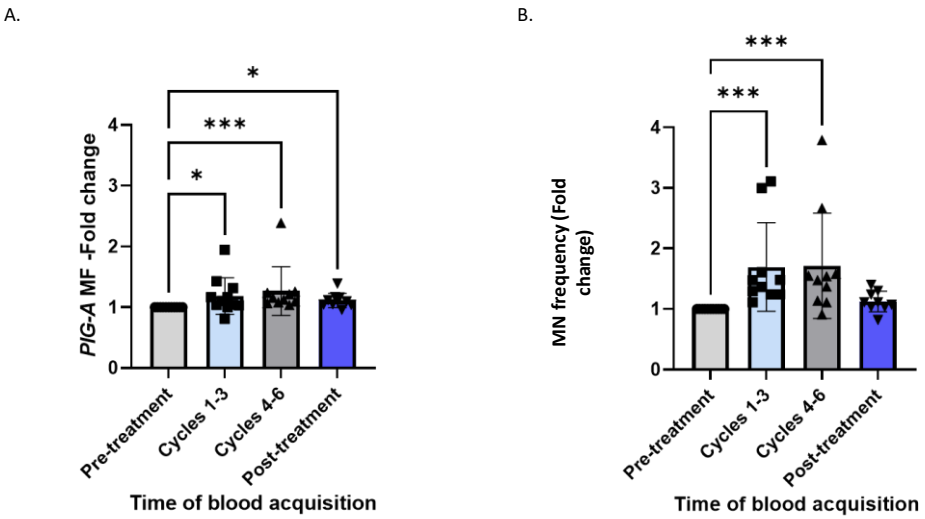

Figure 4.4. *Fold change of DNA damage levels before, during, and after chemo/radiotherapy treatment. Fold change calculated in comparison to pre-treatment value. (A) Erythrocyte PIG-A mutant frequency (MF) fold change. Pre-treatment n=12, Cycles 1-3 n=11, Cycles 4-6 n=10, Post treatment n=9. (B) Lymphocyte micronucleus frequency (MN%) fold change. Pre-treatment n=12, Cycles 1-3 n=10, Cycles 4-6 n=10, Post treatment n=9. *p<0.05, ***p<0.0005 ****P<0.0001. (Error bars show SEM).*

With help from clinical staff, patients were split into three groups based on the tumour response assessment. These groups consist of "Poor" response, "Partial" response and "Complete" response. The levels of DNA damage induction, as measured by fold change during/post treatment was compared to tumour response. Due to the limited number of patients in each group, no firm conclusions could be drawn at this time.

4.4 Discussion

Whilst over the course of the recruitment for the study, 16 patients were deemed suitable and consented. Pre-treatment blood samples were taken from 12 patients, with 10 patients contributing mid-treatment samples and 9 patients donating the total pre, mid and post treatment blood samples. Several hurdles prevented collection of the complete set of blood samples from each patient. These included but were not limited to, difficulty in blood acquisition or patient co-ordination, patient withdrawal due to their choice, illness, or death.

As a consequence of the limited availability of patient samples during chemotherapy for this portion of the research, the pre-treatment data was utilised for analysing tumour characteristics. DNA damage levels were measured in cancer patients across various demographics and tumour characteristics. As there was no obvious difference in DNA damage levels comparing OAC to SSC, both were included in the cancer therapy response analysis. Despite observing age-related increases in *PIG-A* mutation frequency and slight differences across tumour stages for both end points, numbers within each group were not large enough for statistical analysis to be performed. Tumour characteristics, such as stage and metastasis, did not show strong associations with DNA damage. In the literature observations have previously been made between metastasis and MN%. Joseph *et al.,* (2009), saw that among thyroid cancer patients, those who had metastatic cancer had higher levels of DNA damage (MN) compared to patients without metastasis[176]. This was not shown here as sample size constrained the ability to detect significant differences. The study examined the relationship between pre-treatment DNA damage levels and post-treatment outcomes in cancer patients. Patients were categorised into three groups: deceased, under palliative care, or receiving curative treatment as of April 2024. Palliative care focuses on symptom management in advanced cancer, while curative care aims at disease control. Patients under curative care no longer require active treatment but are still monitored by the oncology team.

Among the 12 patients followed up, 4 were deceased, 2 were under palliative care, and 6 remained under curative care. There was no discernible trend observed in the baseline *PIG-A* MF depending on the outcome. Surviving patients (both under palliative and curative care) had lower baseline MN% compared to deceased patients. However, this discrepancy is likely due to patient variability, particularly influenced

by one patient who skewed the results with a particularly high MN% of 1.8%. It's crucial to recognize that baseline *PIG-A* MF/ MN% may not solely predict response to treatment; rather they reflect underlying systemic levels of damage. Therefore, merely assessing pre-treatment levels might not accurately indicate how patients will respond to treatment. Understanding the response to treatment requires considering additional factors beyond baseline DNA damage levels. Pre-treatment levels of DNA damage were compared with mid- and post-treatment levels to evaluate if there was a significant induction in *PIG-A* and MN% and to assess sensitivity to treatment, tumour response, and patient outcome.

Upon examining the raw data, there was no significant change in *PIG-A* levels in patients undergoing treatment for cancer, whilst the MN% were significantly increased. This is likely due to variations in the baseline mutant levels of *PIG-A* in this cohort; therefore, fold change was considered a more appropriate method of assessing response to treatment, whereby each patient was normalised to their own baseline data. This approach reduces inter-individual variability and allows for more accurate detection of treatment-induced changes by using each patient as their own control. *PIG-A* MF and MN% fold change was significant compared to the pre-treatment sample for both time points (Cycles 1-3 and Cycles 4-6). Whilst MN% levels returned almost back to pre-treatment levels post treatment, post-treatment *PIG-A* MFs remained elevated. This is likely due to the life span of each cell type in circulation. Red blood cells have a life-span of around 120 days in circulation[177], whilst T-cells, the subset of lymphocytes stimulated by PHA, have a shorter lifespan[178]. On average CD8+ T cells live 32 days, whilst CD4+ T-cells live for 44 days[178].

Whilst multiple studies have demonstrated a significant increase in the DNA damage endpoints described here ([10-14,16,17,18,21]), there are not many that have related these results to the clinical outcome/response to treatment. In an attempt to do so, fold change during and post-treatment was compared with overall patient outcome and treatment response. For *PIG-A*, there was no difference in average mutant frequencies for patients within each different outcome or response category, despite one patient, with a "complete" response to therapy expressing a higher induction of DNA damage. This patient (CX105) was on a FLOT regimen, with samples taken after cycles 2 and 5. The lack of a clear relationship between these molecular changes and clinical outcomes suggests that additional factors, such as timing and duration of treatment,

may play crucial roles in determining the clinical impact of DNA damage on treatment responses, as well as lack of patient numbers. Lawrence *et al.,* (2023) highlighted in their literature review that the rise in *PIG-A* mutant frequency seen in cancer patients receiving therapy is attributed to the systemic genotoxic agents such as chemotherapy drugs inducing genetic mutations in bone marrow hematopoietic stem cells[179]. They emphasise the importance of timing between treatment and mutant appearance.

Few studies have observed a link between lymphocyte MN% and patient response to cancer treatment. Hahn *et al.,* (1996) found that in dogs getting chemotherapy for osteosarcoma, higher DNA damage levels (increased MN after cisplatin exposure) were linked to better treatment response[180]. Nadin *et al.,* (2006) found increased DNA damage in lymphocytes (as measured through the comet assay) following cisplatin-based chemotherapy associated with better response, however these results were not reflected with doxorubicin[181]. Nikolouzakis *et al., (2019)* showed conflicting results, with responders' MN levels decreasing over time, stable patients showing initial drops then rises, and non-responders experiencing significant increases[182]. These varying outcomes underscore the complexity of treatment response in cancer and the need to consider different factors, including specific cancer and chemotherapy types as well as DNA damage assessment methods. The MN assay's ability to predict radiotherapy response remains unclear. Encheva *et al*.,(2011) investigated if it could predict radiation morbidity in gynecological cancer patients[183]. Despite higher baseline levels, it couldn't reliably forecast radiosensitivity. In a study by Dröge *et al.,* (2021) on rectal cancer, they found significant variation in lymphocyte damage with no correlation to treatment outcomes, suggesting limited predictive value for radiochemotherapy response[167]. When examining MN induction in lymphocytes here, no statistical significance could be drawn due to lack of patient numbers.

Overall, baseline data analysis revealed no obvious differences related to tumour characteristics, and fold-change analysis during and post-treatment showed significant increases in *PIG-A* MF and MN%, despite the lack of significance in *PIG-A* raw data. *PIG-A* induction does not appear to correspond strongly with patient response to treatment, or overall patient outcome. Predicting treatment outcomes solely based on pre-treatment DNA damage levels may be insufficient, and monitoring changes during and after treatment could provide valuable insights for personalised therapy strategies, however further work is required. It is worth noting that individuals involved in this

study had different treatment plans, and therefore this could contribute as to why MN/*PIG-A* appears to be a more sensitive indicator of treatment response in some individuals than others. As a pilot study with low patient numbers in each tumour response group, it wasn't possible to draw conclusions about changes in DNA damage and tumour response. Overall, a larger study with a broader patient population would be instrumental in drawing firmer conclusions and generalising the findings. As a pilot study, despite its limitations, it sheds light on the potential utility of monitoring DNA damage levels throughout cancer therapy. Further research with increased sample sizes and diverse treatment cohorts could validate and expand upon these initial observations, ultimately enhancing the clinical management of cancer patients.The next two chapters will focus on the mechanisms behind micronucleus formation in those with oesophageal disease.

Chapter 5 - Challenging patient lymphocytes to assess individual susceptibility to DNA damage and repair.

5.1 Introduction

5.1.1 The role of GORD in oxidative stress

The exposure and subsequent damage to the oesophageal mucosa caused by reflux in those with GORD, has many implications in terms of oxidative stress, inflammation and disease progression to Barrett's oesophagus. The mechanism behind this damage is complex, as the origins include not only the reflux of gastric acid, but also of bile acids, pancreatic juices, and the pro-inflammatory immunological response to resultant irritation of the oesophagus[184].

In 1997, an analysis into reflux constituents by Kauer *et al.,* discovered a significantly elevated presence of bile acids in those with GORD, far surpassing those found in the general population[185]. Among these bile acids, the study particularly spotlighted taurine-conjugated and deconjugated bile acids, deoxycholic acid (DCA), and chenodeoxycholate as the most detrimental to the oesophagus.[185]

Further investigations have linked DCA to the progression of GORD, with a study by Jenkins *et al.,* in 2007 highlighting the genotoxic properties of this chemical at both neutral and acid pH levels[186]. It is hypothesised that DCA causes damage to mitochondria and subsequently releases reactive oxygen species (ROS) within the cytoplasm of exposed cells[186]. A further study into Barrett's tissue demonstrates mitochondrial swelling in response to DCA exposure, supporting this theory[187].

As well as being mechanistically induced through the action of bile acids, ROS are produced in the oesophageal mucosa by the action of pro-inflammatory immune cells recruited to the site by pro-inflammatory cytokines such as interleukin (IL)-8, IL-6 and monocyte chemoattractant-protein 1[184]. These recruited immune cells produce reactive oxygen and nitrogen species (RONS) in order to fight a suspected infection, but subsequently also damage oesophageal cells. This dramatic increase in ROS triggered by chronic exposure of the oesophageal tissue to reflux, causes high levels of oxidative stress in this environment. Oxidative stress is the state that occurs when there is an imbalance between the production and accumulation of ROS, and the ability of a biological system to remove these products[188] through enzymatic mechanisms (catalase, superoxide dismutase and glutathione peroxidase) as well as non-enzymatic mechanisms (glutathione, vitamins C and vitamins E)[189].

ROS include superoxide radicals (O_2•−), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$)[190]. These species can initiate lipid peroxidation, damaging cell membranes by breaking down unsaturated fatty acids and oxidising proteins, impairing their structure and functionality[191]. Reactive oxygen species can also cause oxidative damage to DNA, which is particularly concerning because it can lead to mutations and hence the initiation of carcinogenesis[189].

Oxidative DNA damage

Oxidative stress is a significant cause of DNA damage *in vivo*. ROS can cause DNA lesions in many ways. These include: base modifications, single-strand, and double-strand breaks, as well as inter-strand crosslinks[189]. If not properly repaired, this damage can lead to mutations, chromosome aberrations and chromosome loss, and subsequently the formation of MN in cells[189].

One of the most well- studied ROS-related modifications to DNA is the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) through interaction of the nucleobase with the hydroxyl radical[192]. This kind of adduct has detrimental consequences on genomic stability, as the change in configuration of 8-OHdG can mispair with adenine instead of cytosine and lead to transversions – a type of point mutation [193](**Figure 5.1**).

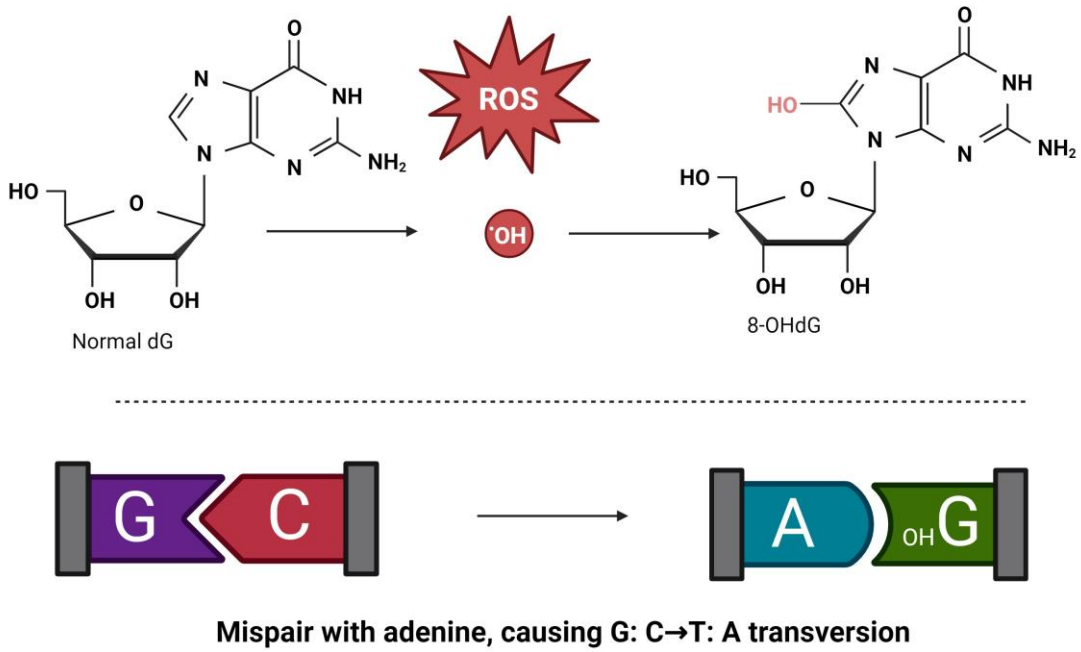

Figure 5.1. *Modification of nucleobase Guanine by hydroxyl radical, results in 8-hydroxy deoxyguanosine. Failure to repair this alteration, results in Guanine mispairing with adenine, instead of cytosine, leading to a point mutation. Created with Biorender.com*

DNA repair pathways have evolved to effectively manage various types of DNA damage caused by ROS. One key pathway is the BER pathway, which is responsible for repairing DNA base damage such as deamination, oxidation, and alkylation[194, 195]. However, if the damage exceeds the repair capacity or if there are errors in the repair process, it can negatively impact genomic stability and contribute to the development of diseases (including cancer). Additionally, heterozygous germline mutations in genes involved in DNA repair can increase the risk of cancer[196]. For example, *MUTYH* encodes the MutY homolog enzyme, vital in the BER pathway. Mutations in *MUTYH* have been shown to raise cancer risk. For instance, homozygous or heterozygous germline mutations can lead to *MUTYH*-associated polyposis (MAP), an autosomal recessive disorder[197]. This condition results in adenomatous colorectal polyps, increasing the risk of colorectal cancer risk[198].

5.1.2 Mechanisms behind MN induction in PBLs

Whilst the focus has been on reflux-induced DNA damage in oesophageal tissue, it's essential to consider its broader implications on systemic DNA integrity. In the context of human biomonitoring, evaluating the frequency of MN in lymphocytes is a crucial method to assess the extent of the chromosomal/DNA damage obtained *in vivo.* DNA damage can be caused by occupational exposure to genotoxic agents or susceptibility to mutation due to inherent genetic defects. Exposures to exogenous agents have the potential to cause damage to lymphocytes, as they circulate in the body and become activated or detoxified by different organs, as well as through damage to precursor cells in the bone marrow[199]. For example, exposure to benzene, commonly found in industrial settings such as chemical manufacturing or gasoline refining, is known to damage bone marrow precursor cells[200]. This damage can subsequently result in MN formation[200].

Various studies have demonstrated a clear link between increased MN% in PBLs and cancer incidence. A study by Bonassi *et al.,* (2007) suggested that increased MN formation in PBLs may predict future cancer diagnosis[68].

As discussed in **Chapter 3**, it is evident that the MN% in human PBLs increases in cases of OAC. Factors leading to the formation of MN could include oxidative stress, exposure to substances that break or rearrange chromosomes, disruption to the machinery responsible for chromosome segregation, genetic anomalies in cell cycle regulation, or inherited deficiencies in DNA repair genes[68].

Mechanistic evidence for MN induction using complementary end points is required to provide valuable information about the pattern of chromosome damage in cancer patients and the reliability of risk estimates for MN frequencies[73]. Centromere staining via *in-situ* hybridisation, for example, can distinguish between clastogenic or aneugenic events underlying the DNA damage which can be informative[199].

As exogenous and endogenous exposures can contribute to carcinogenesis, another important consideration is individual susceptibility to these carcinogens and DNA damage/repair. A study by El-Zein *et al.,* (2006) assessed the influence on nicotine-derived nitrosamine ketone (NNK) in cigarette smoke on individual cancer risk. They found heightened sensitivity to NNK's genotoxic effects in the lymphocytes of lung cancer patients, correlating with increased cancer risks[145]. In essence, this suggests that

individuals with lung cancer may exhibit greater vulnerability to the induction of MN by NNKs due to potential deficiencies in DNA repair mechanisms. However, it's essential to discern whether these effects solely stem from NNK or encompass other carcinogens present in cigarette smoke, as discussed in their study. Moreover, the heightened susceptibility to NNK's genotoxic effects raises concerns about increased cancer risks beyond lung cancer in these individuals.

These studies emphasise how understanding the complex mechanistic aspects of MN formation is crucial for validating them as a biomarker of individual cancer risk.

5.1.3 Challenging human lymphocytes

Enhancing our comprehension of the underlying mechanisms responsible for the heightened levels of DNA damage observed in lymphocytes of those with oesophageal malignancies provides us with valuable information. This information is necessary to assess the utility of MN% as a biomarker of disease progression risk in individuals with pre-malignant diseases such as GORD and BO. *In vivo*, most of the DNA damage that could manifest as MN in lymphocytes is typically repaired[65]. However, *in vitro* observations of MN are influenced by experimental conditions, such as treating cells with PHA, which induces cellular division before adequate repair mechanisms can take place[65]. Whilst lymphocyte MN% can serve as a measure of unrepaired DNA damage, this measurement does not necessarily reflect an individual's repair capacity, as elucidated by the fact that various potential exposures can influence systemic MN levels[201].

One method of assessing an individual repair capacity involves the exposure of an individual's cells to a harmful or damaging agent followed by the quantitative examination of cellular changes e.g., DNA damage, MN induction, and chromosomal aberrations. This technique is known as a "challenge assay" and employing live lymphocytes in these assays can provide a precise and individualised health risk assessment. Lymphocytes contain all the tools for DNA repair[202] (in those without inherited DNA repair deficiencies), meaning their response to damage induced *ex vivo* is finely tuned to the individual's distinct characteristics e.g. their inherited genetic profile[202]. As well as aligning with the natural repair capacity of the individual, lymphocytes can give an insight to individual sensitivity to specific DNA damaging agents, the exposure to which can potentially increase the repair workload and cause

variations in efficiency[203]. Some external factors may even temporarily or permanently impair lymphocyte DNA repair function, underscoring the significance of investigating these dynamics. For example, a study by Wang *et al.,* (2021) showed that workers occupationally exposed to benzene had reduced DNA repair capacity (increased MN) following treatment of blood with bleomycin[204]. Understanding individual sensitivity to DNA damaging agents *in vitro* can provide crucial insights into why patients might have an increased baseline MN, providing an insight into how their unique genetic and environmental interactions affect DNA repair capacity and cellular response to damage.

5.1.4 Aim and objectives

The overall aim of this chapter was to use a challenge assay to assess whether the lymphocyte response to DNA damage measured by the induction of MN, cell cycle progression, and DNA repair capacity differs in those with OAC compared to non-cancer individuals.

The objectives were as follows:

Objective 1: To choose appropriate aneugenic and clastogenic chemicals to incorporate into the challenge assay.

Objective 2: To assess MN induction and growth of cells following challenge with the chosen chemicals – hydrogen peroxide, deoxycholic acid, and vinblastine

Objective 3: To assess cell cycle progression following treatment with the above chemicals.

Objective 4: To develop an assay that measures the DNA repair capacity of lymphocytes by allowing an extended time for recovery before analysis.

Objective 5: To assess how induction of MN correlates with both disease histology and baseline MN frequency of lymphocytes (pre-challenge) and explore any relationships with clinical parameters.

5.2 Methods

5.2.1 Recruitment of patients and processing of patient samples and isolation of lymphocytes

Described in **Chapter 2: General Materials and Methods**. The samples from cancer patients included in this chapter overlap with **Chapter 3** and **Chapter 4** and were treatment naïve.

5.2.2 Determining the optimal concentrations of chemicals for the challenge assay using TK6 cells

The challenge assay involved the utilisation of three selected chemicals: H_2O_2 (30% (w/w) in H_2O, containing stabilizer, (Merck, UK), sodium deoxycholate (Sigma-Aldrich, UK), and vinblastine sulfate salt (Merck, UK). The execution of the CBMN using TK6 cells, as outlined **Chapter 2**, included the seeding of satellite flasks for RPD analysis. The concentrations chosen were 20µM for H_2O_2, 150µM for DCA, and 0.9nM for vinblastine.

5.2.3 Assessing lymphocyte susceptibility to DNA damage using the challenge assay.

Following the isolation of PBMCs from whole blood, the cells were seeded at a concentration of $1x10^6$ per ml and stimulated using PHA (10µl/ml -1% by volume) (Merck, UK) for 24 hours. As shown in **Figure 5.2**, the cells were exposed to 20µM H_2O_2, 150µM DCA, and 0.9nM vinblastine for 20 hours. As the cell number isolated from each patient sample differed, not every donor was exposed to all three chemicals. Following chemical treatment, the cells were moved to fresh media in the presence of Cyto-B for 24 hours. Cells were harvested for CBPI and MN scoring using the methods described in **Chapter 2**. MN%-fold change was calculated by the division of the MN% in the treated culture (B) by the MN% in the untreated control (A), as demonstrated below.

Untreated control MN% (A) = 0.5%

H_2O_2 treatment MN% (B) = 1%

Fold change = B/A = (1/0.5) = 2-fold.

There were two main controls in place regarding the potency and consistency of treating patient lymphocytes. The first control was to assess H_2O_2 stability. The second control was the parallel treatment of TK6 cells alongside patient lymphocytes. This was to provide information about the potency of the chemical as time progressed and ensure any abnormal response to the chemical in an individual was not due to human error. The data obtained was also used to normalise the lymphocyte data. The controls are further described below (**section 5.2.4**)

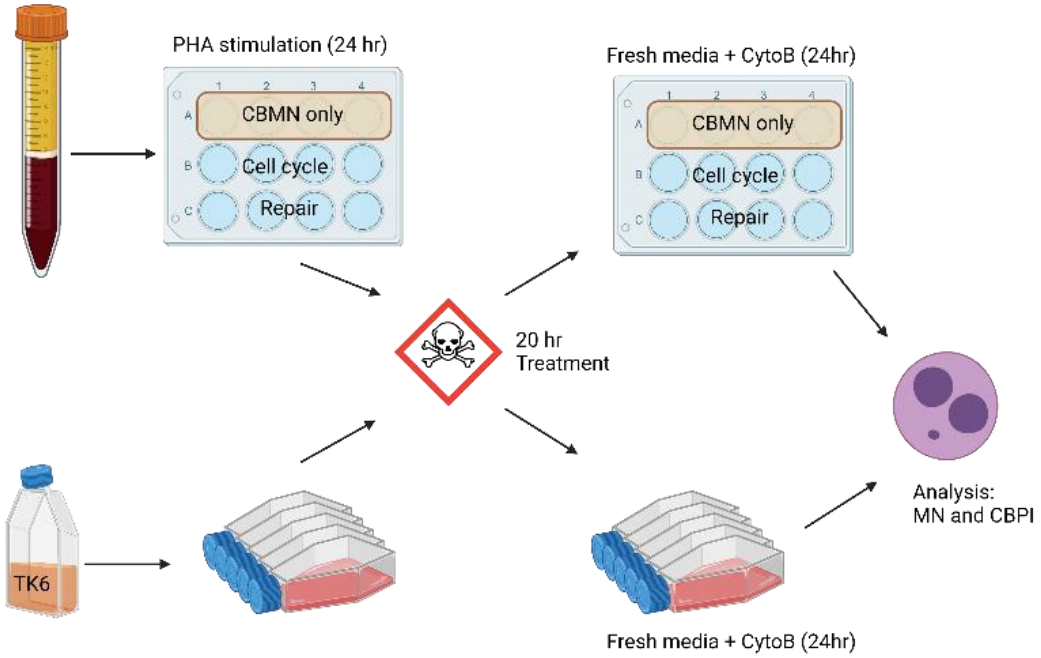

Figure 5.2. *Schematic demonstrating challenge assay experimental set up. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density dependent centrifugation. Lymphocytes were stimulated with phytohemagglutinin (PHA) for 24 hours before being exposed to a DNA damaging agent. Following a further 20 hours, cells were placed in fresh media in the presence of Cytochalasin B (Cyto-B). After 24 hours cells are harvested for micronuclei (MN) analysis and calculation of cytokinesis-block proliferation index (CBPI). Diagram created with Biorender.com.*

5.2.4 Challenge assay controls

<u>H_2O_2 stability</u>

To assess the potency and decide the cut off point for using the same bottle of stabilized H_2O_2, TK6-cells were treated with 20µM every five days for three weeks and MN% and RPD were measured. **Figure 5.3** shows the MN%-fold increase following treatment with 20µM H_2O_2, as well as the RPD. Whilst RPD does not change significantly, there is a significant drop in MN induction from 15 days onwards. A bottle of hydrogen peroxide was therefore used only until 10 days following the initial use.

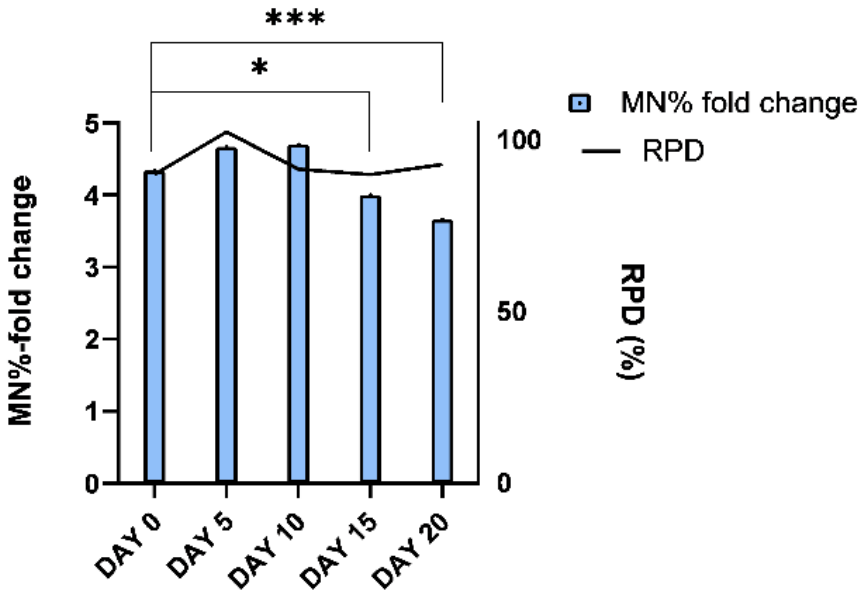

Figure 5.3. *Testing the potency of a bottle of stabilised hydrogen peroxide over a 20-day period, using relative proliferation index (RPD) and Micronucleus frequency (MN%). MN% was calculated in the treated and untreated TK6 samples, to calculate a MN%-fold change every five days following the initial use of a bottle of hydrogen peroxide. Induction of MN drops significantly at day 15. There was no significant change in RPD. Statistical analysis carried out using GraphPad Prism on MN% fold change only. GraphPad Prism, including normality and lognormality testing, and One-way ANOVA (parametric) with post-hoc analysis including Tukey's multiple comparisons test. P-value of ≤0.05=*, ≤0.005= *** (n=1)*

Using TK6 cells as a parallel control

TK6 cells were seeded simultaneously alongside patient lymphocytes during the establishment of the challenge assay. Except for the introduction of PHA, the TK6 cells received identical treatment to the lymphocyte cultures and were harvested concurrently for MN and CBPI assessment. Following the compilation of all TK6 MN data, the average MN% fold-change was computed for each chemical across all replicates. A normalisation factor was determined for each control sample based on its deviation from the average. This normalization factor was then applied to the L-MN% fold change to generate a normalized value. This allowed for any significant variations in chemical potency and/or potential human errors. The reproducibility of the TK6 cells' response to chemical treatment is shown in **Figure 5.4**, where the average MN-fold change is presented for H_2O_2, DCA, and Vinblastine. Reproducibility was assessed through the calculation of the coefficient of variation (CV%). The calculation for CV% was as follows: CV% = 100a/mean, where a = standard deviation. A CV% beyond a 30 percent threshold was considered a limit and may suggest experimental issues or non-reproducibility. The CV% for H_2O_2, DCA, and Vinblastine was 16.85%, 6%, and 14.55%, respectively, indicating good reproducibility of the results.

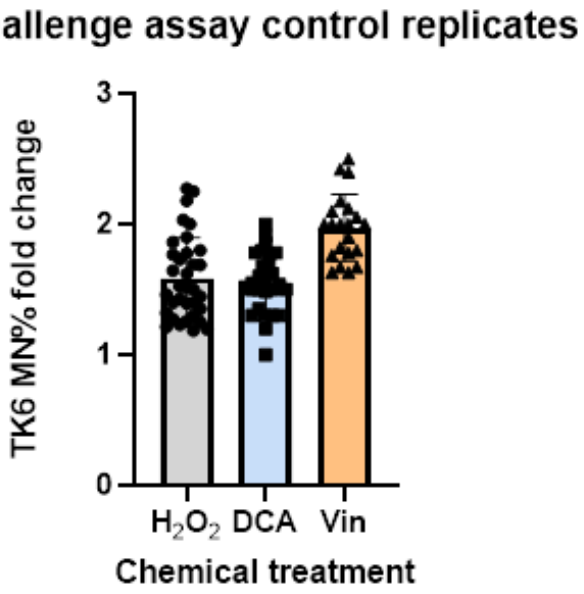

***Figure 5.4**. The micronucleus frequency (MN%) fold change of all TK6 control replicates following each chemical treatment; hydrogen peroxide (H_2O_2), Deoxycholic acid (DCA) and Vinblastine (Vin). (Error bars show SEM).*

5.2.5 Assessing DNA repair using the challenge assay

To assess individual's DNA repair capacity, additional wells of lymphocytes were seeded at the same cell density and treated as described above. The 'repair' wells were allowed a 24-hour recovery period prior to the addition of Cyto-B. This resulted in the harvest of the challenge assay wells 24 hours post treatment (before repair, BR), and the repair wells at 48 hours post treatment (after repair, AR) (**Figure 5.5**). A calculation for DNA repair capacity was derived from a study by Fernandez-Bertolez *et al,*. (2022) [205], and was as follows:

%Repair capacity = ((MN%$_{BR}$ – MN%$_{AR}$)/MN%$_{BR}$ *100).

For example, when MN%$_{BR}$ = 1, and MN%$_{AR}$ = 0.5, (1-0.5/1*100) = 50% repair capacity.

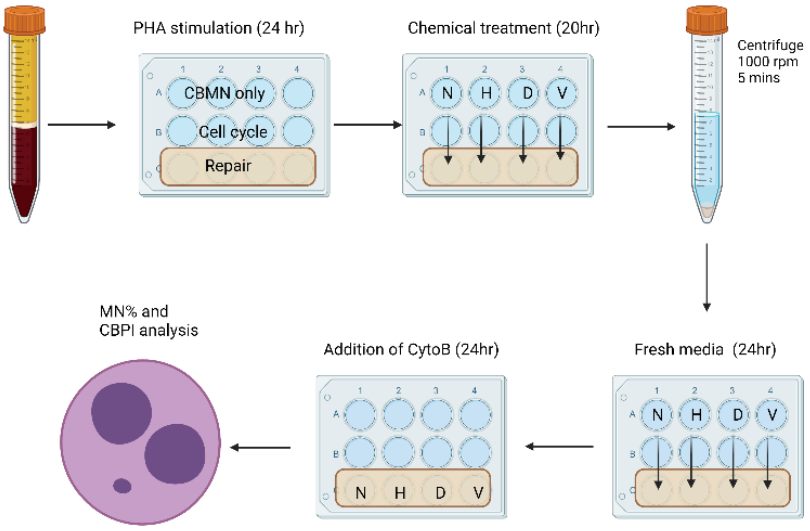

***Figure 5.5**. A schematic demonstrating the modification to the challenge assay, allowing cells an extra 24 hours to repair any DNA damage before adding cytochalasin-B (Cyto-B). This adjustment aimed to evaluate the efficacy of DNA repair mechanisms by comparing micronucleus (MN) formation before and after the repair period.*

5.2.6 Cell cycle analysis of cultured lymphocytes

Cells were cultured according to **5.2.3**, without the addition of Cyto-B at any point. Cells were fixed for cell cycle analysis 72 hours following PHA stimulation. The fixing

of cells prior to staining is described in **Chapter 2**. FxCycle™ PI/RNase Staining Solution (Thermofisher Scientific, UK) was used to stain the DNA of approximately $1x10^6$ cells for 30 minutes before the cells were acquired on NovoCyte® flow cytometer (ACEA biosciences, Inc.). A stop condition of 10,000 dividing cells was applied to the NovoCyte®. FlowJo version 10.3 software (Becton Dickson, USA) was used to analyse the data, and extract the percentages of cells within each stage of the cell cycle. The cell numbers were normalized to achieve a total count of 100%, ensuring proportional representation across all stages. The gating strategy for dividing lymphocytes was derived from Riberio *et al*., (2013) where it was stated that when comparing forward scatter (FSC) to side scatter (SSC) of PBMCs stimulated with PHA, there are two distinct groups of cells. PBMCs with reduced FSC and SSC light dispersion properties are non-dividing, whilst PBMCs with increased FSC and SSC are actively dividing[206]. The gating strategy for lymphocytes is shown in **Figure 5.6**. The increase in SSC/FCS of the dividing population of lymphocytes differed inter-individually.

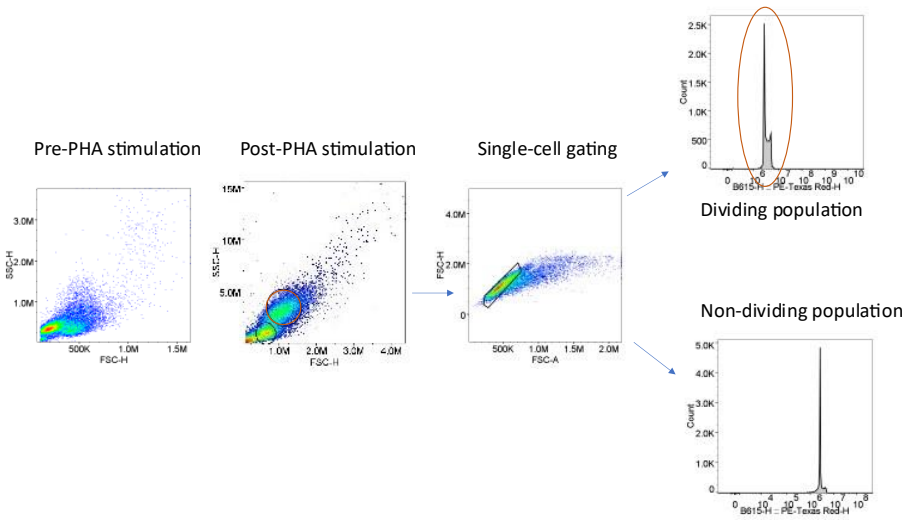

Figure 5.6. *Gating strategy for the cell cycle analysis of dividing lymphocytes. Forward (FSC) and side scatter (SSC) analysis is compared for pre-phytohemagglutinin (PHA) non-dividing lymphocytes compared to post-PHA treated lymphocytes. In the post PHA treated cells, there are two distinct populations, actively dividing (gated in orange), with increased SSC and FSC, and non-dividing (gated in black). Single cells are gated through plotting FSC-H against FSC-A. Finally, a histogram of PE-Texas-RED-H is plotted. The dividing cells show 3 distinct cell cycle stages (G1, S, and G2) depending on the concentration of DNA stained. The non dividing population has only one peak, representing cells in G0 or G1.*

5.2.7 Measuring glutathione concentration in patient plasma

Glutathione levels can provide information about levels of oxidative stress in patient plasma. Plasma samples from patients were stored at -80^{0}C prior to use in all plate assays. Glutathione was measured using the glutathione assay kit (Sigma-Aldrich, UK). The samples were first deproteinized through addition of 5% 5-Sulfosalicyclic Acid Solution followed by centrifugation to remove precipitated protein. The supernatant was added to the plate without dilution. Standard concentrations of reduced glutathione (GSH) are shown in **Table 5.1**, whilst the reaction set up is shown in **Table 5.2**. Every sample was added in duplicate.

***Table 5.1**. Preparation of glutathione standard solutions by serial dilutions of the stock 50µM glutathione solution.*

Well number	1	2	3	4	5
GSH Concentration (µM)	50	25	12.5	6.25	3.125
GSH solution (µl)	50	25 (from well 1)	25 (from well 2)	25 (from well 3)	25 (from well 4)
5% SSA (µl)	None	25	25	25	25
nmoles GSH in a 10µl sample	0.5	0.25	0.125	0.0625	0.0312

***Table 5.2**. Reaction scheme for glutathione plate assay*

	Mix and incubate 5 minutes			Start
Sample measured	Sample volume	5% SSA	Working mixture	NADPH (0.16mg/ml)
Reagent blank	-	10µl	150µl	50µl
Standard curve (various dilutions)	10µl	-	150µl	50µl
Unknown sample	Xµl	10µl -X	150µl	50µl

Absorbance was measured on the FLUOstar Omega multimode microplate reader (BMG LABTECH Ltd, UK) at wavelength of 412nm with kinetic reads at 1-minute intervals for 5 minutes. To calculate nmoles GSH per ml of sample, the glutathione standard solutions were first used to determine a standard curve and calculate the ΔA_{412}/min equivalent to 1nmole of reduced glutathione per well. To calculate the nmoles of GSH in the plasma the following equation was used:

GSH concentration (nmoles/ml) = ΔA_{412}/min(sample) x dilution factor or original sample ÷ ΔA_{412}/min (1 nmole) x volume of sample in reaction in ml

5.2.8- 8-OHdG measurement using competition ELISA

8-OHdG measurement can provide an indication of levels of oxidative stress, as this base modification is produced by the oxidative damage of DNA through reactive oxygen and nitrogen species. Plasma samples used for 8-hydroxy 2 deoxyguanosine measurement were stored at -80^{0}C prior to analysis.

Levels of both free and DNA incorporated 8-OHdG were measured in plasma (defrosted on ice) using the 8-hydroxy 2 deoxyguanosine competition ELISA Kit (abcam, 201734), and was carried out according to the manufacturer's instructions, a schematic of which is shown in **Figure 5.7.**

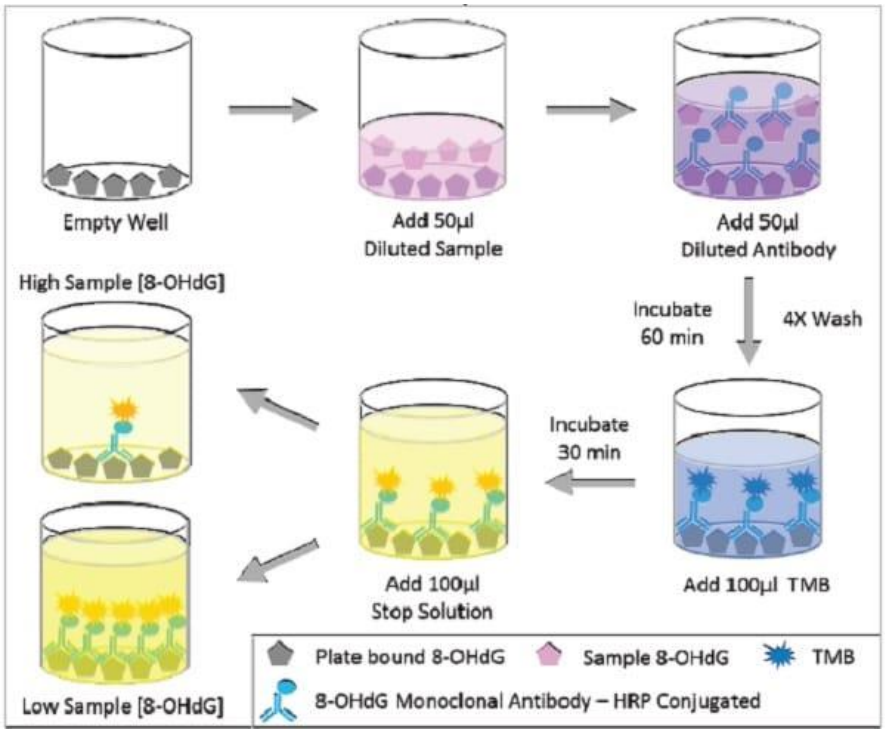

Figure 5.7. *Abcam's 8-hydroxy 2 deoxyguanosine (8-OHdG) ELISA kit (ab201734 schematic). This competitive ELISA assay measures the concentration of 8-OHdG in plasma by detecting signal interference. The sample antigen competes with a reference antigen for binding to a pre-coated multi-well plate. The sample, pre-incubated with labelled antibody, is added to the wells, where free antibodies are available depending on the antigen concentration. Higher antigen levels result in weaker detection of the reference antigen and a weaker signal. Diagram taken from abcam.com*

5.2.9 Statistical analysis.

In this study's relatively small cohort, age did not emerge as a statistically significant confounding variable in the analysis of baseline biomarker levels between histological groups. While age was initially included in the General Linear Model alongside gender, BMI, smoking status, and diagnosis category, its effect did not reach statistical significance. This may be due to limited sample size reducing statistical. All statistical analysis was conducted using GraphPad (Prism, version 9). For datasets with sample sizes smaller than 50 ($n < 50$), the Shapiro-Wilk test was chosen due to its increased power to detect deviations from normality. When comparing patient groups, non-parametric Kruskal-Wallis tests were applied to datasets that failed the normality test, followed by Dunn's multiple comparisons test for post-hoc analysis. For datasets that passed the normality test, one-way ANOVA was performed, followed by Tukey's multiple comparisons test. Statistical significance was defined as $p \leq 0.05$ and denoted

by asterisks: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$). Correlation analysis utilised Pearson correlation coefficients for normally distributed data and Spearman correlation for non-normally distributed data, as determined by normality testing. An R-value (correlation coefficient) was calculated for all correlation analyses to indicate the strength of correlation. Interpretation of correlation strength followed the thresholds described by Akoglu (2018)[124].

Error bars show either standard error of the mean (SEM) or standard deviation (SD). Figure legends contain relevant information

5.3 Results

5.3.1 Baseline MN% and CBPI of lymphocytes following treatment with PHA.

Prior to employing the challenge assay approach, it was essential to measure the baseline MN% and CBPI of untreated lymphocytes following PHA stimulation (**Figure 5.8**). This preliminary step provided crucial baseline data for comparison. Subsequently, the challenge assay evaluated various parameters, including MN induction (via MN% fold change), CBPI, alterations in the cell cycle, and the assessment of repair capacity. **Table 5.3** shows the demographics of the patients whose samples were used in this challenge assay. Amongst the 44 individuals included in the challenge assay, the OAC and BO patients had significantly higher MN% compared to the healthy volunteers (Figure 5.8A). However, there was no significant difference between OAC patients and BO patients (p=0.58) or between either and the GORD patient group (p=0.82 to BO and 0.17 to OAC). The CBPI data showed no change amongst patient groups, meaning they all responded similarly to the PHA stimulation (Figure 5.8B). **Table 5.3** shows the demographics of the patients whose samples were used in this challenge assay.

Table 5.3. *Demographics of the individuals included in the challenge assay.*

Characteristics	Healthy (n=18)	GORD (n=9)	Barrett's Oesophagus(n=9)	OAC (n=11)
Median Age (years, 95% CI)	26.6 (22–31)	60.89 (51-71)	67.11 (60–74)	67.91 (62–74)
Male gender (%)	44.44% (8/18)	22.2% (2/9)	88.89 (8/9)	81.82% (9/11)
Median BMI (kg/m^2, 95% CI)	25.41 (23.88–26.94)	33(29.93–36.07)	26.83(24.88–28.78)	25.08 (22.53–27.63)
Smoking (% use)	5.56% (1/18)	22.2% (2/9)	11.11% (1/9)	54.44% (6/11)

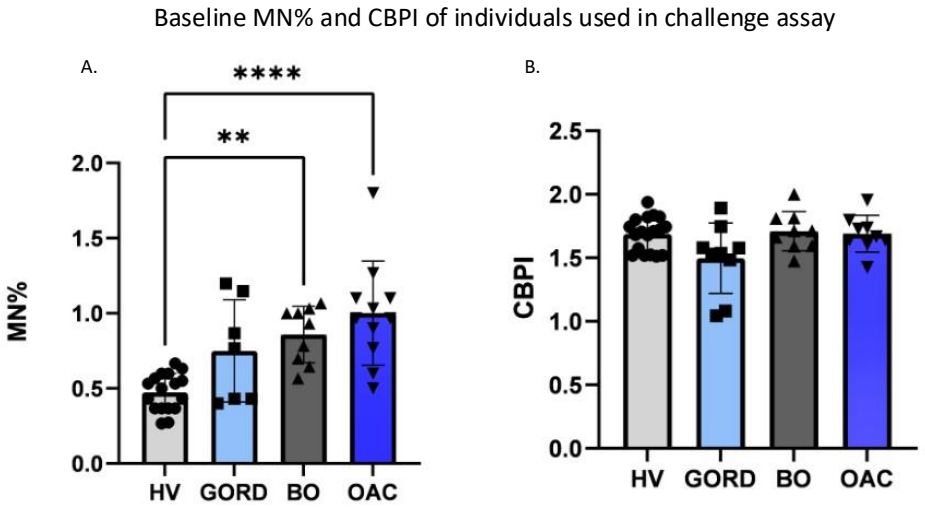

Figure 5.8. *The baseline Lymphocyte Micronucleus Frequency (MN%) and Cytokinesis-Block Proliferation Index (CBPI) of untreated lymphocytes following Phytohemagglutinin (PHA) stimulation ex vivo. (A) Comparison of the MN% of untreated lymphocytes between histology groups, revealing significantly higher MN% in Oesophageal Adenocarcinoma (OAC) and Barrett's Oesophagus (BO) groups compared to Healthy Volunteers (HV). p<0.005**, p <0.0001****. (B) The CBPI of lymphocytes of different histology groups, following treatment with PHA. P=0.09 In both graphs: HV (n=17), GORD (n=7), BO (n=9), and OAC (n=11). (Error bars show SEM).*

5.3.2 Finding an optimal concentration of H_2O_2, sodium deoxycholate and vinblastine.

Using TK6 cells, various concentrations of H_2O_2, sodium deoxycholate (DCA), and vinblastine (5µM-30µM, 100-200µM, and 0.5-1.2nM, respectively) were tested prior to the treatment of patient lymphocytes. MN% and RPD were measured. The criteria for lymphocyte treatment necessitated a significant induction of MN, coupled with RPD of ≥40 (therefore cytotoxicity levels remaining below 60%) as per OECD guidelines (OECD 2010b)[84]. Consequently, concentrations of 20µM H_2O_2, 150µM DCA, and 0.9nM vinblastine were deemed the appropriate concentrations for challenging lymphocytes, using the data presented in **Figure 5.9.** At these concentrations, the MN% fold change and RPD for each chemical is displayed in **Table 5.4.**

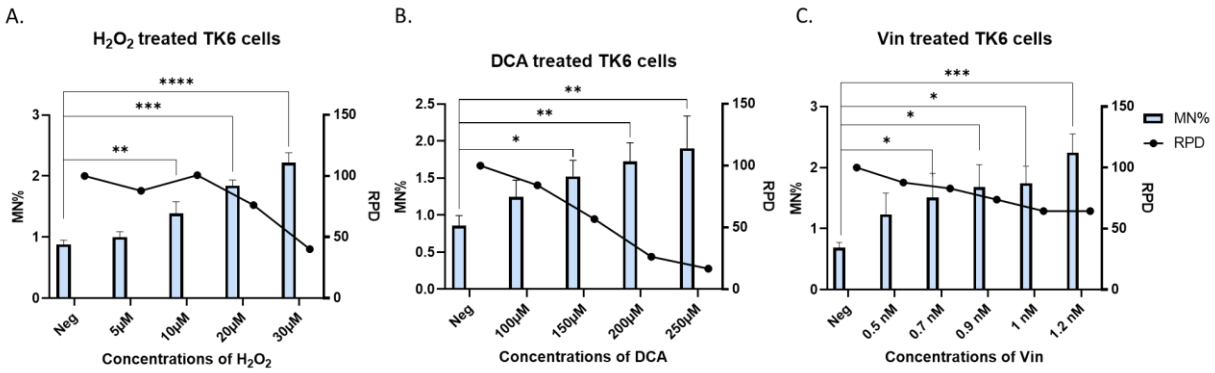

Figure 5.9. *Determining the optimal concentration of chemicals prior to treatment of lymphocytes. A. Concentrations 5-30μM of hydrogen peroxide were tested, with significant MN% occurring at 10μM. 20μM is the highest concentration used before dose becomes too cytotoxic. B. Concentrations of sodium deoxycholate tested on TK6 cells. 150μM induced significant increase in MN%, without reaching cut off levels of cytotoxicity measured using RPD. C. Vinblastine concentrations. No strong cytotoxicity seen with TK6, 0.9nM chosen as it significantly increases MN% without much cytotoxicity. P*<0.05**<0.01***<0.005****<0.001. (Error bars show SD).*

Table 5.4. *TK6 MN% fold change and RPD following chemical treatment.*

Chemical	Concentration	MN% fold change	RPD (%)
Hydrogen peroxide	20μM	2	76.2%
Sodium deoxycholate	150μM	1.78	57%
Vinblastine	0.9nM	2.4	74%

5.3.3 Challenging patient lymphocytes using pro-oxidant chemicals (H_2O_2 and DCA) and vinblastine.

Treatment of lymphocytes with 20μM hydrogen peroxide

MN% fold change and CBPI were calculated compared to the baseline levels, following treatment of cells with 20μM H_2O_2 (**Figure 5.10**). The MN% fold change showed no significant differences between different histology groups ranging from 0.9

to 3.4-fold change, p=0.61 (Figure 5.10A). When comparing the baseline MN% in untreated lymphocytes to the MN% fold change in challenged lymphocytes, a statistically significant negative correlation was identified (R=-0.64, p=<0.0001, n=40) (Figure 5.10B). This suggests that in some of these patients, the higher their baseline MN% the lower their susceptibility to DNA damage following treatment with H_2O_2 *in vitro*. This observation suggests an adaptive response. The CBPI of GORD patients was lower than the other patient groups, with significance occurring between the GORD and OAC physiologies (p=0.015) (Figure 5.10C). The observed decrease in CBPI in GORD compared to OAC could indicate a slower growth rate of cells in GORD, when treated with H_2O_2 than the lymphocytes from other histology groups.

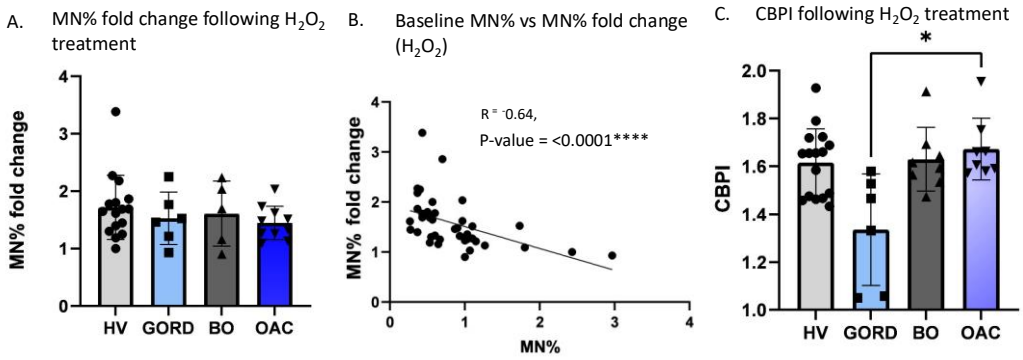

Figure 5.10. *Treatment of patient lymphocytes with 20µ*M hydrogen *peroxide (H_2O_2). (A). The micronucleus frequency (MN%) fold change following treatment with 20µM H_2O_2 between different histology groups. No significant changes observed* (p=0.61) *(B). The baseline MN% of the untreated sample* vs *MN% fold change following treatment with H_2O_2. R=-0.64 suggesting strong negative correlation, p= <0.0001****, n=40 (C). The cytokinesis-block proliferation index (CBPI) of lymphocytes from each histology group. Significance shown between GORD and OAC (p-value =0.0148*). (Error bars show SEM).*

Treatment of lymphocytes with 150µM DCA

MN% fold change and CBPI was calculated in DCA treated lymphocytes in comparison to the untreated control. (**Figure 5.11**). Whilst the MN% fold change showed no significant differences between histology groups, ranging from 0.77-2.5-fold change, p=0.91 (Figure 5.11A), when comparing the baseline lymphocyte MN% to the MN% fold change after challenge with DCA, a statistically significant strong negative correlation is identified (R=-0.50, P-value=0.008, n=27) (Figure 5.11 B). This suggests that in some of these patients, the higher their baseline MN% the lower their susceptibility to DNA damage following treatment with DCA *in vitro*. Similarly to H_2O_2, this observation also fits with the concept of an adaptive response. Like with H_2O_2 treatment, the CBPI of GORD patients is lower than the other patient groups, with significance occurring between the GORD and OAC histology groups (Figure 5.11C) (p=0.0112). The observed decrease in CBPI in GORD compared to OAC could indicate a slower growth rate of cells from patients with GORD, when treated with DCA compared to the other histology groups.

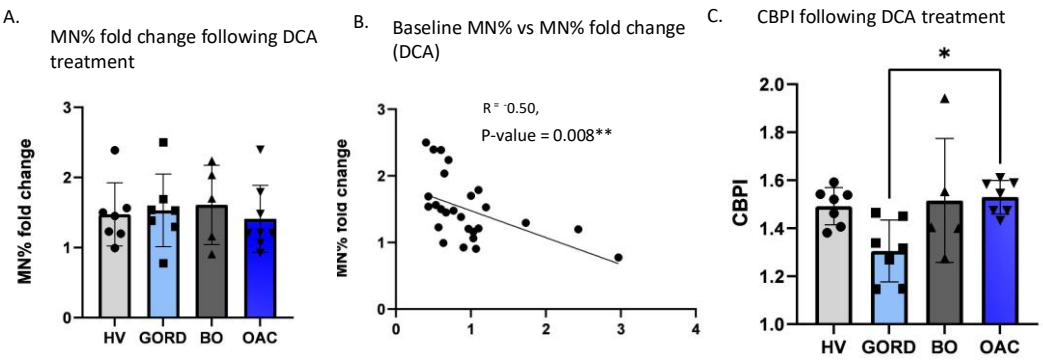

Figure 5.11*. Treatment of patient lymphocytes with 150µM* sodium deoxycholate *(DCA)* (*A*). *The micronucleus frequency (MN%) fold change following treatment with sodium deoxycholate between different histology groups. No significant changes observed*, *p=0.91 (*B). *The baseline MN% of the untreated sample, versus the MN% fold change following treatment with 150uM sodium deoxycholate, showing a strong and significant negative correlation R=-0.5, p=0.008**, n=27.* (*C*). *The cytokinesis-block proliferation index* (CBPI) *of lymphocytes from each physiological group. Significance shown between GORD and OAC *=p-value=0.01. (Error bars show SEM).*

Treatment of lymphocytes with 0.9nM Vinblastine

Due to the later addition of Vinblastine into challenge assay chemical cohort, there are fewer patient lymphocytes challenged with this chemical (n=24) (**Figure 5.12**). Lymphocytes were treated with 0.9 nM Vinblastine for 20 hours, and subsequent MN% fold change and CBPI was calculated. OAC patients appeared to be slightly more susceptible to DNA damage following treatment with vinblastine compared to the HV and GORD however there was no significance observed (p=0.08) (Figure 5.12A). There was a wide variation in patient response in those with BO. When comparing fold change to baseline MN%, there was a weak correlation (R=0.26, P=0.3, n=24) (Figure 5.12B). There were no differences observed in the CBPI between histology groups following treatment of lymphocytes with vinblastine (P=0.56) (Figure 5.12C).

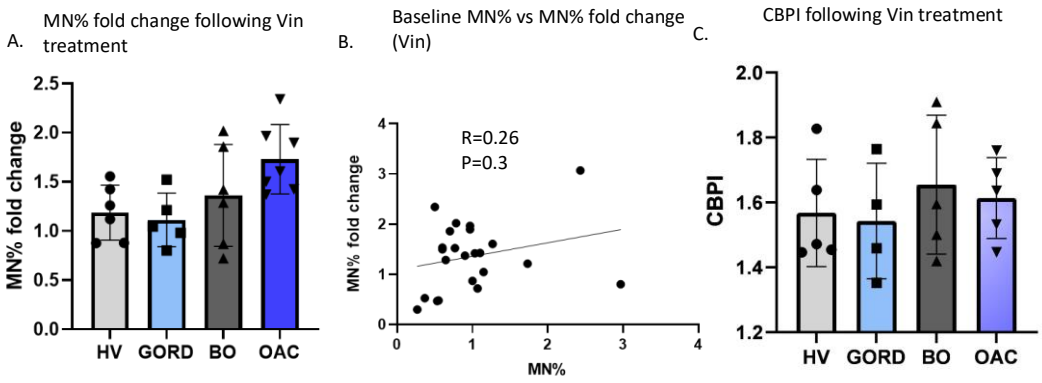

Figure 5.12. *Treatment of patient lymphocytes with 0.9nM Vinblastine (Vin) (A). The MN% fold change following treatment with 0.9nM vinblastine between different* histological groups. *Overall, no significant changes were observed (P=0.08), however OAC patients appear to be slightly more sensitive to vinblastine than HV or GORD groups. (B). The baseline MN% of the untreated sample, versus the MN% fold change following treatment with vinblastine, with no significant correlation observed. R=0.23, P=0.29, n=24 (C). The cytokinesis-block proliferation index (CBPI) of lymphocytes from each histological group in the presence of 0.9nM vinblastine. No significant changes were observed between the different groups (p=0.56). (Error bars show SEM).*

5.3.4 Effect of chemical treatments on cell cycle kinetics of PHA-stimulated lymphocytes in cancer versus non-cancer patients.

Propidium iodide staining was used to determine the percentage of cells in each stage of the cell-cycle (including G1, S, and G2/M) in a small cohort of individuals (cancer patients n=4, non-cancer patients n=4). The cells were harvested for staining 68 hours following stimulation with PHA and 44 hours after chemical treatment. The percentage of cells in each stage measured (G1, S and G2/M) was measured in each treatment group and the untreated sample (**Table 5.4**). PBLs are in G0 stage prior to PHA activation[81]. For the untreated cells, there was a higher proportion of cancer derived lymphocytes in 'S' phase compared to non-cancer patients, however this difference showed no significance (p=0.43). H_2O_2, DCA and vinblastine increased the percentage of cells in G1 for both groups, therefore reducing the percentage of cells in S and

G2/M. There were no significant differences between the response of lymphocytes derived from cancer or non-cancer patients in this small cohort of patients.

***Table 5.5**. Mean percentage of lymphocytes (from cancer patients and non-cancer patients- GORD patients) in each phase of the cell cycle following chemical treatment in vitro.*

Chemical treatment	Cell cycle phase	Average percentage of lymphocytes (cancer)	Average percentage of lymphocytes (non-cancer)
Negative control	G1	63.94	70.21
	S	20.13	14.45
	G2	16.18	15.35
Hydrogen peroxide (20μM)	G1	67.37	73.34
	S	17.91	12.57
	G2	14.47	14.84
Sodium deoxycholate (150μM)	G1	74.58	78.61
	S	13.43	11.46
	G2	12.5	11.12
Vinblastine (0.9nM)	G1	73.08	72.62
	S	16.8	17.83
	G2	10.86	10.55

5.3.5 Correlation between age and MN%-fold change following chemical treatment.

Age is known to have a negative impact of DNA repair capability[207] hence having the potential to confound the above results. Age in years was compared with MN induction (measured using MN% fold-change) following treatment with each of the three chemicals (**Figure 5.13**), and no significant correlation was found for H_2O_2 (Figure 5.13A), DCA (Figure 5.13B) or Vin (Figure 5.13C) ($p=0.49, 0.23$, and 0.42 respectively). This suggests that the age of patients does not confound the challenge assay data.

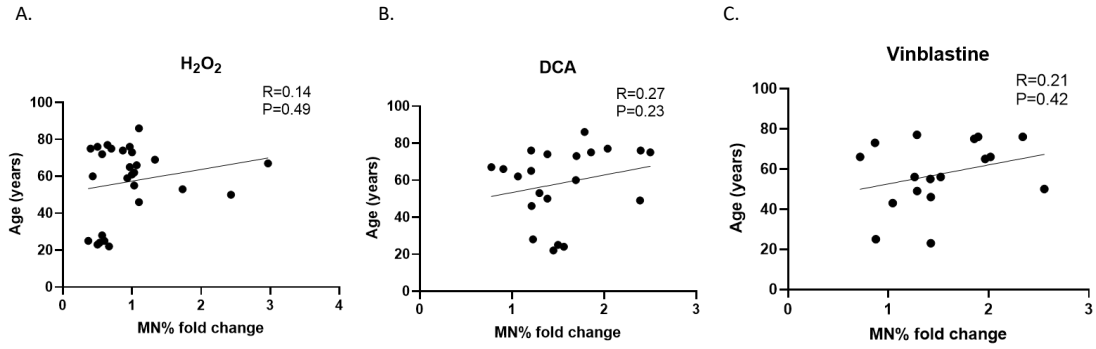

Figure 5.13*. The effect of age on* micronucleus (*MN*) *induction* measured by MN frequency (MN%) fold change, following *chemical treatment of lymphocytes.* (*A*). *Age versus MN% fold change following treatment with 20µM hydrogen peroxide*. R=0.14, P=0.49, n=27 *B. Age versus MN% fold change following treatment with 150 µM* sodium deoxycholate (DCA). R=0.27, p=0.23, n=21. (*C*). *Age versus MN% fold change following treatment with 0.9nM vinblastine.* R=0.21, p=0.42, n=17. *(Error bars show SEM).*

5.3.6 Inter-individual variation in lymphocyte DNA repair capacity

In an attempt to measure the inherent DNA repair capacity of lymphocytes, additional wells were added to the challenge assay to allow an additional recovery period of 24 hours post-exposure. The comparison of the MN% of cells "before repair" and "after repair" allowed a percentage repair capacity to be calculated.

Only five individuals were utilised in this experiment, meaning a comparison of histology groups was not possible. The patients included consisted of 3 healthy volunteers (HAB015, HAB533, and HAB091) and 2 cancer patients (treatment naïve) (CX107-A and CX109-A). **Figure 5.14** shows the difference in repair capacity following treatment with both H_2O_2 and Vinblastine in the five individuals. The repair capacity calculates the repair based on the drop in MN% at 48 hours compared to 24 hours. Hence a capacity of 30% illustrates a 30% reduction in MN% (and therefore repaired DNA damage). HAB015 showed no difference in repair capacity to

vinblastine or H_2O_2. HAB533 appeared to more efficiently repair DNA induced by H_2O_2, while the opposite was seen for CX107-A, HAB091 and CX109-A. There was a clear difference in repair capacity between individuals for both chemicals, however this was not statistically significant (p=0.07).

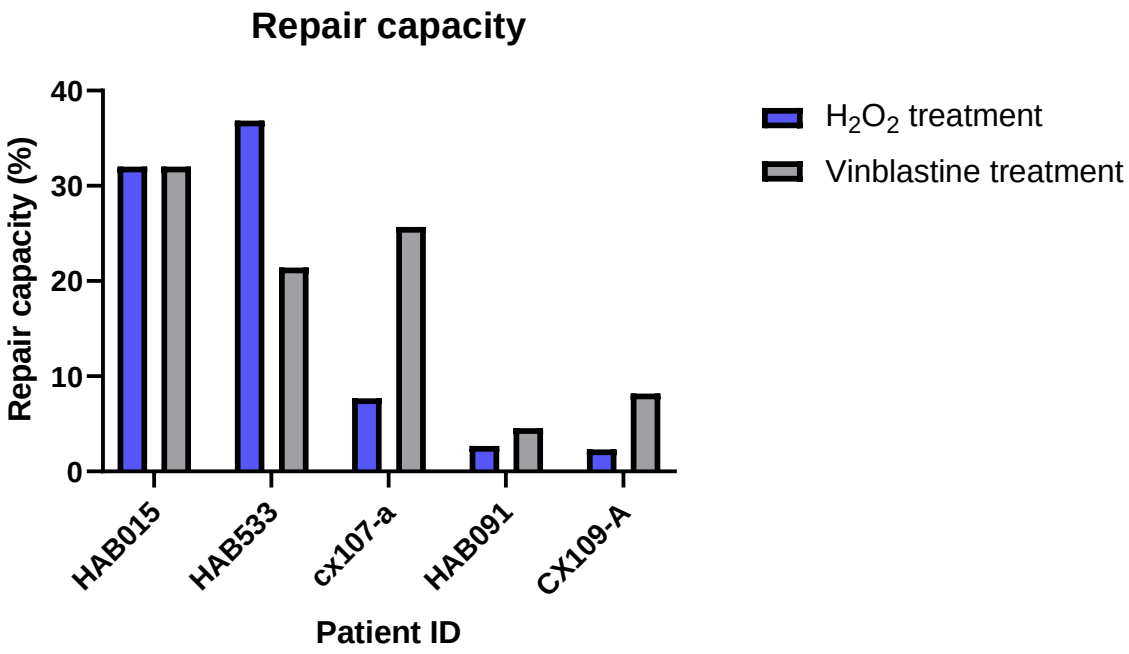

***Figure 5.14**. Repair capacity of five individuals following treatment with hydrogen peroxide (H_2O_2) and vinblastine. Each patient sample appears to differ in terms of their repair capacity for damage induced by H_2O_2, as well as the aneugen vinblastine, measured by the drop in micronucleus frequency (MN%) after an additional 24-hour culture period. Following a 2-way ANOVA, variation across individuals* and *across chemical treatments was not significant (*p=0.07 and *p=0.*72 respectively)

5.3.7 Investigating the adaptive response of lymphocytes to pro-oxidant chemicals

Glutathione levels in plasma

As an adaptive response was noted following challenge with H_2O_2 and DCA, this could suggest that the lymphocytes had adapted to a ROS-rich environment making them less sensitive to ROS mediate MN induction. To explore this further, the plasma levels

of ROS as well as antioxidant factors was assessed. Glutathione is an intracellular antioxidant and hydrogen peroxide scavenger[208]. The concentration of glutathione in plasma is generally much lower than within cells, as cells require higher levels for their internal functions[209]. The presence of glutathione in plasma highlights its importance in maintaining overall health, managing oxidative stress, and supporting detoxification processes in the body[209]. GSH was measured using a glutathione ELISA plate assay with patient plasma and compared to MN% (**Figure 5.15).** GSH represents the reduced form of glutathione and is often reduced in times of oxidative stress as it is oxidised by the ROS generated. There was a weak (R=0.21)) correlation between GSH levels and corresponding patient MN% (n=36) (Figure 5.15A). There are no statistically significant changes in glutathione levels between patient histology groups (p=0.272) (Figure 5.15B)

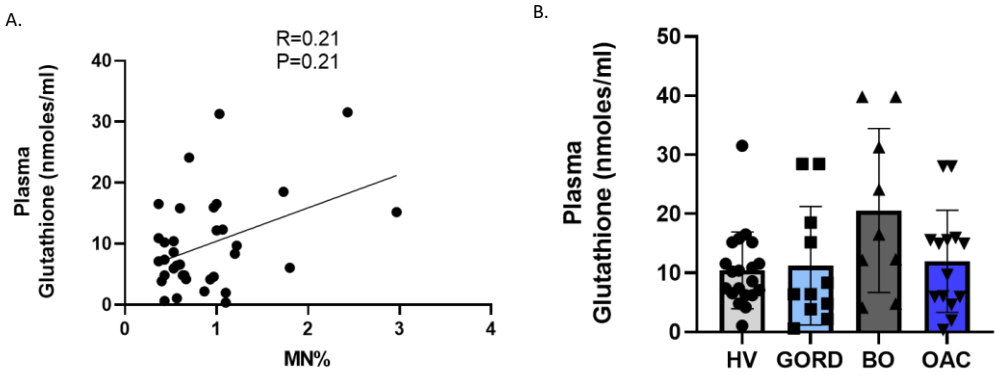

Figure 5.15. *Glutathione levels in patient plasma.* (*A*). *The MN% of the untreated patient samples was compared to the nmole/ml of glutathione in the same patients*. *A weak, insignificant trend was observed, with R=0.21 and p=0.21*, n=36 (*B*). *The levels of reduced glutathione in nmoles/ml in plasma of each histology group. No significant differences were identified (p=0.27). (Error bars show SEM).*

5.3.8 Investigating oxidative DNA damage through measurement of 8-Hydroxydeoxyguanosine.

To further investigate the link between ROS, oxidative DNA damage and increased MN% in the lymphocytes of these patients, 8-OHdG was measured in patient plasma.

The concentration was then compared between histological groups as well as versus MN% in lymphocytes (**Figure 5.16**). This assay measured both free and DNA incorporated 8-OHdG, as complex samples such as plasma contain a mixture of both[210]. When comparing plasma levels of 8-OHdG between histology groups, OAC patients had the highest levels although this was not statistically different from the other groups (p=0.55) (Figure 5.16A). The HV, GORD and BO patients have similar levels of 8-OHdG in plasma (average of 21, 25 and 23ng/ml respectively) (p=0.99). On average, OAC cancer patient's plasma exhibited elevated levels of 8-OHdG in comparison to the non-cancer groups (41ng/ml). However, it is important to note that there is a substantial variation in 8-OHdG concentrations among the OAC plasma samples, with the highest recorded level being 104ng/ml and the lowest recorded level being 15.686 ng/ml. When comparing the MN% of patient lymphocytes to the corresponding plasma levels of 8-OHdG (Figure 5.16B), there is a moderate statistically significant correlation, with an R of 0.437. (P=0.037, N=23)

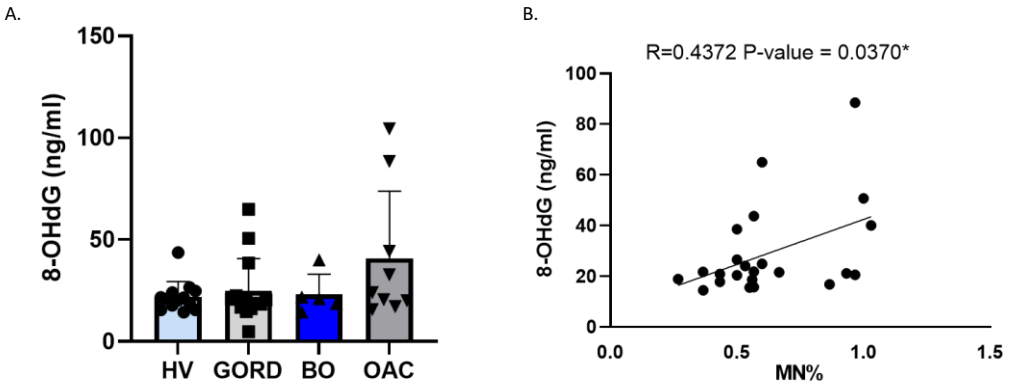

Figure 5.16. *Levels of 8-hydroxy-2' -deoxyguanosine (8-OHdG) in patient plasma. (A). Levels of 8-OHdG between patient groups. There are no significant differences, however on average oesophageal adenocarcinoma (OAC, n=9) patients have higher levels of 8-OHdG in their plasma than healthy volunteers (HV, n=13), and patients with gastroesophageal reflux disease (GORD, n=14) and Barrett's oesophagus (BO, n=5) (41ng/ml compared to 21, 25 and 23ng/ml respectively). P=0.55 (B) Levels of 8-OHdG compared to the corresponding frequency of MN% in patient lymphocytes. There is a moderate statistically significant correlation between the two, with an R=0.437 (*p=0.037). (Error bars show SEM).*

5.4 Discussion

The results in **Chapter 3** indicated that MN and *PIG-A* levels were higher in the lymphocytes of OAC patients compared to non-cancer individuals. Therefore, a challenge assay was optimised to investigate the mechanistic basis for the increase in MN% observed with the progression of oesophageal disease. Since *PIG-A* and MN assess different types of genetic damage—chromosome breakage/loss for MN and mutations for *PIG-A*—it was determined that MN would be the more appropriate marker for experimental use. This decision was based on the fact that the time required for the expression of a mutated gene in erythrocytes is significantly longer, making MN a more practical and timelier indicator of genetic damage. The aim of this assay was to assess the differences in MN induction after exposure to DNA damaging agent's *in vitro*, both in individual cases and between histology groups (HV, GORD, BO and OAC).

Using TK6 as a control

The number of lymphocytes derived from patient samples differed greatly depending on the individual and the volume of blood acquired. Some patients provided 20ml, whilst others only 4ml. This made technical replicates difficult even when seeding at low concentrations (1 ml minimum), and therefore TK6 cells were employed to establish optimised approaches for our study. Initially, these cells served as a valuable resource for determining the optimal concentration of the chemicals to be tested. TK6 cells are lymphoblastoid in origin, and express functioning p53 protein, making them a gold standard in genotoxicity studies[211], as well as a physiologically relevant comparison to PBLs. This optimisation process aimed to ensure that the selected chemical concentrations could induce micronuclei without causing excessive cytotoxicity, (as recommended in OECD guidelines for the testing of chemicals[84]) thereby preventing the unnecessary depletion of precious patient samples.

During the implementation of the challenge assay, lymphocytes were induced to undergo division through the addition of phytohemagglutinin. The volume used for culture of the cells was adjusted according to the quantity of cells obtained from whole blood, ranging from 1ml to 5ml, while maintaining a consistent concentration of $1x10^{-6}$/ml. Concurrently, preparations were made for the experimental use of TK6 cells. Both lymphocytes and TK6 cells were treated identically. The parallel configuration

involving TK6 cells played a crucial role in acting as a control when treating patient lymphocytes. This setup ensured that any significant elevation in MN induction could be confidently attributed to the unique repair capacity of the cells, rather than being influenced by variations in chemical concentration or potency. The MN fold change was analysed for both cell types. For TK6 cells the MN fold change was calculated, and a normalization factor was determined based on the deviation from the overall average in fold-change across all the control samples. This factor was then applied to the lymphocyte MN fold change data for the matched sample, to obtain normalised results. This normalisation process ensured accurate comparisons between TK6 cells and lymphocytes in terms of MN induction, accounting for experimental variability. The graphs produced using the normalised data are included in **Appendix XI-XIII**, as pattern observed, and the statistical significance of the data remained unchanged.

Baseline MN% and CBPI of cohort

Before the response to chemical treatment is discussed it is first important to address the baseline MN% and CBPI of the lymphocytes. The results showed only a significant increase in the MN% from healthy volunteers to both BO and OAC patients. As a significantly smaller cohort of individuals were involved in the challenge assay (44 in comparison to 108) discrepancies between the larger and smaller cohorts could stem from a combination of factors, including sample size and underlying biological variability. Sample size plays a crucial role in the statistical power of the analysis. The significance shown in chapter two came from a larger number of participants, increasing the power of the study and its ability to detect smaller effects. This could lead to the detection of significant differences that might not be apparent in a smaller cohort. The differences in significance could also be reflective of the underlying biological variability within the groups. In the larger cohort, the differences in micronuclei frequency between the groups are more pronounced and consistent, leading to significant results. Additionally, it is important to consider that biological variation may be influenced by everyday exposure to different mutagens, diverse lifestyles, and varying diets, contributing to the observed variability. The CBPI represents the ratio of multi- to bi- to mono-nucleated cells following PHA stimulation and addition of cyto-B. The lack of difference between the groups suggests that lymphocytes from the different groups respond similarly to PHA-stimulation *in vitro*. The CBPI represents the ratio of multi- to bi- to mono-nucleated cells following PHA

stimulation and addition of cyto-B. The lack of difference between the groups, suggesting that lymphocytes from the different groups respond similarly to PHA-stimulation *in vitro*.

Lymphocyte response to pro-oxidant species *in vitro*

All lymphocytes were slightly more sensitive to treatment with DCA than hydrogen peroxide, with a reduction in division measured through CBPI. Following treatment with both pro-oxidant chemicals, GORD patients had a lower CBPI than the other groups, with statistical significance compared to cancer patients.

Patients with GORD exhibited significantly lower CBPI compared to other conditions, especially cancer. Chronic inflammation, stemming from stomach acid and bile reflux into the oesophagus, likely contributes to this inhibition of *in vitro* cell growth in GORD patients. DCA is a bile acid commonly refluxed into the oesophagus in those with GORD. In the context of GORD, where lymphocytes are already compromised by chronic inflammation, the presence of DCA could further inhibit their ability to divide. It has been shown that patients with Barrett's oesophagus have higher levels of anti-inflammatory compounds like interleukin-10 (IL-10) and interleukin-4 (IL-4)[184]. In contrast, GORD patient lymphocytes may be exhausted by chronic inflammation[212], lacking sufficient time to enhance antioxidant effects, and struggle to divide in the presence of DCA *in vitro*.

There was no obvious change in the *in vitro* induction of MN when comparing diagnostic groups, however when the MN% fold-change is compared to the untreated lymphocyte MN%, a negative correlation is apparent for pro-oxidant chemical treatments (H_2O_2 and DCA). In these patient lymphocytes an increase in oxidative stress could be result in the increase in MN%. If the cells are exposed to high levels of oxidative stress while *in vivo,* they could adapt to avoid any further damage. Adaption to high levels of oxidative stress is a concept that was discussed in a study published by Kumar *et al*, (2016) which explored the relationship between occupational exposure to IR and blood plasma levels of antioxidants such as GSH, and Superoxide Dismutase (SOD)[213]. This study reported increased levels of total antioxidant capacity, glutathione, and SOD levels in blood plasma in individuals that had been occupationally exposed to IR. They also suggest, that in these individuals the susceptibility of the circulating lymphocytes to DNA damage (measured by MN in

lymphocytes) is associated with plasma antioxidant levels. This effect is reflected within our study, as those with higher MN%, appear less susceptible to DNA damage *in vitro*. There was a weak correlation with plasma GSH and increased lymphocyte MN%.

Furthermore, Kumar *et al.,* (2016) suggests that this increase in antioxidants within blood plasma is an adaptive mechanism to protect cells from further damage caused by oxidative stress. Similarly, Sebastià *et al., (*2020)[214] carried out a study analysing blood levels of antioxidants in those occupationally exposed to IR. Conversely, they saw that those exposed to higher levels of IR had higher markers of oxidative stress (e.g. nitrites and nitrates, and lipid peroxidation) and reduced antioxidant capacity, despite an increase in dietary antioxidants in the IR exposed group[214]. They did not however measure DNA damage in lymphocytes.

To further explore the relationship between MN% and oxidative stress, levels of 8-hydroxy deoxyguanosine were measured in the patient plasma. 8-OHdG is formed when guanosine is oxidised and then excreted into the plasma and urine following its excision during DNA repair[215]. The presence of 8-OHdG excreted in urine can be considered a biomarker for high levels of oxidative stress[216]. In this study, a weak correlation was shown between MN% and plasma levels of 8-OHdG, with cancer patients exhibiting the highest levels. Previous studies have shown that there are higher levels of 8-OHdG in the urine of those with SSC[217], with a study by He *et al.,* (2014) demonstrating that levels of 8-OHdG in oesophageal tissue, are not only a risk factor for the carcinogenesis of oesophageal cancer but also serve as a good marker for predicting the outcomes of postoperative patients with oesophageal cancer. In addition, the average age is higher in the cancer patient group (72 years) compared to the non-cancer group (average of 53 years). Lodovici *et al.,(*2000*)* observed a correlation between 8-OHdG levels in human leukocytes and age, which could play a role in these results[218]. The age of the patients, however, showed no significant correlation with the fold-change following treatment with H_2O_2 or DCA.

Lymphocyte response to vinblastine

Whilst the action of the pro-oxidant species employed in this study act via clastogenic mechanisms[186, 219], leading to MN containing chromosomal fragments[219], MN can also contain whole chromosomes not incorporated into the nucleus during cell division due

to error in chromosomal segregation (aneugenic mode of action)[59]. These errors can stem from issues such as mitotic spindle failure, kinetochore damage, centromeric DNA hypomethylation, or abnormalities in cell cycle regulation[59].

Aneuploidy represents a significant characteristic of cancer and is observed in approximately 90% of human tumours[220]. In the research conducted by Hadjinicolaou *et al*., (2020) their findings underscore the pivotal role of aneuploidy in oesophageal tissue as the sole predictive biomarker for the neoplastic progression of non-dysplastic Barrett's oesophagus[221]. Given the significance of aneuploidy in the development of OAC, it was deemed important to incorporate an aneugenic chemical into the challenge assay. This approach aimed to assess whether individuals with BO or OAC exhibit heightened susceptibility to DNA damage via this mechanism. Whilst a study carried out by Baciuchka-Palmaro *et al.,* (2002) revealed significantly higher levels of acentromeric MN in the lymphocytes of cancer patients (10 patients of multiple diagnoses vs 10 healthy controls)[222], work carried out by Dr Rachel Lawrence suggested a slightly increased frequency of centromere positive MN in those with OAC, suggesting defects in chromosomal segregation in this cohort of cancer patients (**Appendix XIV**).

From the resulting fold-change, it does appear that the cancer patients included in this study are slightly more susceptible to vinblastine than the healthy volunteers, and GORD patients. As the average age of cancer patients is higher than the non-cancer patients (see table 5.3), this could have a confounding effect on the results, however, as shown above there does not appear to be a relationship between age and fold change following treatment with vinblastine.

A study conducted by Leopardi *et al.,(2002)* investigated whether susceptibility of PBLs to vinblastine was determined by age, however the authors deduced that while age may affect spontaneous errors, it has limited influence on the induced chromosome errors caused by vinblastine in lymphocytes[223]. This study states that chromosomes display varying susceptibilities to malsegregation, both spontaneously and when exposed to aneugenic agents. The study observed a gender-related effect on vinblastine susceptibility, highlighting that chromosome X exhibited a higher vulnerability to induced chromosome errors in female PBLs compared to their male counterparts, indicating a potential gender-specific response to vinblastine exposure[223]. As the

substantial majority (80%) of the patient cohort studied here were males, it is reasonable to suggest that the increased vulnerability of chromosome X in female PBLs to vinblastine, as described in the study by Leopardi *et al.,* may not substantially contribute to the heightened susceptibility observed in this particular demographic.

Assessing DNA repair

To further investigate individual susceptibility of lymphocytes to chemical treatment, the challenge assay was adapted to include an additional one-day interval, allowing the cells to undergo a period of rest and repair before being harvested for analysis. By comparing the levels of DNA damage in the group harvested at 24 hours to those allowed more time for repair, we can assess how well the cells manage DNA damage over a longer duration. This approach offers a more comprehensive understanding of the cell's DNA repair capability, considering both immediate responses and the ability to address damage over an extended period. It helps us gain insights into how cells cope with and recover from DNA damage caused by a genotoxic agent, potentially revealing nuances in the dynamics of repair mechanisms.

This experiment had multiple limitations, first imposed by the number of available lymphocytes necessary for expanding the number of cultures. Additionally, the study involving recruitment of patients from the local endoscopy department had to be discontinued and therefore recruitment was halted from October 2022. This meant we could not recruit further patients with GORD or BO, and a reduced number of OAC patient samples were available. A total of 5 individuals (3 healthy volunteers, and 2 cancer patients) were utilised for the repair assay, and while each individual showed a unique repair capacity to both oxidative stress, and mitotic disruption, the sample size was too small for any significant statistical analysis.

Cell cycle progression

Another endpoint considered was cell cycle kinetic dysregulation following chemical treatment. If DNA damage is not properly repaired during the ‘S’phase of DNA replication, this is picked up by the G2 /M checkpoint, to prevent division of the cell and hence the incorporation of damaged DNA into the daughter cell. If the cell evades this checkpoint, and enters mitosis with damaged DNA, the cell becomes more susceptible to forming MN[224].

Similarly to the above, the halt in recruitment as well the number of lymphocytes was a limiting factor for this experiment. To analyse the cell cycle kinetics, no Cyto-B can be present and hence satellite cultures were required. The cohort included 4 non-cancer patients and 4 cancer patients. No change was seen regarding disturbances in cell cycle kinetics in PHA stimulated only, DCA, H_2O_2, or Vinblastine treated samples between cancer and non-cancer patient samples.

5.4.1 Conclusion

The study employed an optimised challenge assay to investigate the difference in MN% between healthy controls and patients with GORD, BO, and OAC, reflecting various stages of oesophageal disease. TK6 cells served as controls for chemical concentration optimisation. The CBPI indicated uniform lymphocyte responses to PHA stimulation across physiologies. GORD patients demonstrated lower CBPI, potentially linked to chronic inflammation. Pro-oxidant species treatment revealed a potential relationship between oxidative stress and higher lymphocyte MN%. Analysis of 8-OHdG in patient plasma showed a moderate correlation with MN%, with a stronger effect seen in cancer patients. Vinblastine treatment suggested slightly higher susceptibility in cancer patients, with age showing no correlation with treatment response, suggesting an age-independent reduction in repair capacity. In a very small sample of patients, a technique has been optimised to measure both DNA repair capacity and cell cycle progression, with no firm conclusions drawn due to a limited sample size and lack of age-matched controls. Further work should include increasing study patient numbers. Due to the apparent relationship between oxidative stress and levels of DNA damage, the role of inflammation in plasma will be further explored in the next chapter.

Chapter 6 - The influence of plasma, inflammation, and the cGAS-sting pathway on MN% in lymphocytes and TK6 cells

6.1 Introduction

6.1.1 Plasma and the role of inflammation in biomonitoring

Blood plasma, comprising 55% of total blood volume, acts as a fluid medium transporting essential nutrients, hormones, and waste products, alongside blood cells. It consists primarily of water, incorporating proteins, glucose, clotting factors, electrolytes, and hormones[225]. Key plasma proteins include albumin, globulins (e.g., immunoglobulins), and fibrinogen, crucial in clotting[225]. It can serve as a useful matrix for biomonitoring purposes through various markers of inflammation. Certain pro-inflammatory cytokines that can be measured in blood plasma include IL-6, IL-8 and IL-1β, all of which play an important role in acute and chronic inflammation[226]. The presence of C-reactive protein (CRP) in plasma, in response to inflammation, is frequently used clinically to monitor inflammation in a range of diseases[227]. CRP is synthesized predominantly by hepatocytes in the liver in response to inflammatory stimuli like IL-6, tumour necrosis factor-alpha (TNF-α), and IL-1-beta[227]. In Crohn's disease, elevated CRP levels correlate with disease severity and predict outcomes such as the need for colectomy[227]. Metabolic syndrome, characterised by chronic inflammation also manifests with increased circulating CRP levels[228]. In Parkinson's disease CRP has also shown promise as a primary biomarker for monitoring inflammation and disease progression[229]. A study by Picod *et al*., (2022) however, suggested that concentration of plasma IL-6 is a better biomarker for predicting activity of diseases such as cancer, compared to CRP[230]. Higher plasma levels of both CRP and IL-6 have been associated with an increased risk of progression to OAC in a prospective study by Hardikar *et al* (2014)[231]. These studies emphasise how blood plasma, particularly its proteins like CRP and cytokines, serve as crucial biomarkers for monitoring various diseases. Blood plasma components, including antioxidants, micronutrients, as well as direct exposure to genotoxic compounds, impact lymphocyte MN levels. Fenech *et al*. (1994) demonstrated a correlation between higher plasma folate and vitamin B12 levels and reduced micronuclei rates, suggesting protection against chromosome damage[232]. Meanwhile, Wang *et al*. (1999) identified elevated plasma benzo(a)pyrene (B(a)P) in coke oven workers, which correlated with increased micronuclei rates, emphasising plasma's role in genetic instability[233]. These findings highlight the intricate relationship between plasma components and genetic integrity, particularly significant as plasma flows through tissues, delivering nutrients

and removing waste. This underscores the importance of considering plasma factors in understanding DNA damage mechanisms. Such insights are crucial for assessing health risks associated with environmental exposures, health and lifestyle.

6.1.2 The cGAS-STING pathway

Cyclic GMP-AMP synthase (cGAS) is an innate immune receptor, involved in the detection of cytosolic double-stranded DNA (dsDNA)[234]. This DNA can either be derived from invading microbes or pathogens, as well as self-DNA released from damaged cell nucleus or mitochondria[234]. Following the binding of DNA, cGAS undergoes a conformational change and catalyses the synthesis of the second messenger 2'3'-cyclic GMP-AMP (2'3'cGAMP) from adenosine triphosphate (ATP) and guanosine triphosphate (GTP)[235]. Following its production, cGAMP binds to the endoplasmic reticulum (ER) protein known as the stimulator of interferon genes (STING). This results in another conformational change, allowing STING to traffic to the Golgi complex[236]. Activated STING recruits and activates the kinases TBK1 and IKK, which in turn phosphorylates the transcription factors IRF3 and NFkB respectively. These phosphorylated transcription factors then translocate to the nucleus, where they induce expression of type 1 interferons including interferon-beta (IFN-β) and interferon-alpha (IFN-α) and other pro-inflammatory cytokines (**Figure 6.1**)[234]. The products of this pathway, through recruiting immune cells and promoting antigen presentation create an antiviral and antitumour immune response[234]. Recent studies have established that extracellular cGAMP has paracrine activity as an "immunotransmitter" that can be imported into cells, with ABCC1 identified as an ATP-dependent exporter of cGAMP[237]. This paracrine transfer of cGAMP facilitates the propagation of the cGAS-STING pathway from one cell to neighbouring cells, holding significant implications for both anti-tumour immunity and anti-viral responses[238]. Due to the inflammatory nature of this pathway, its regulation is tightly controlled to prevent excessive signalling. Dysregulation of cGAS-STING pathway is implicated in various autoimmune, inflammatory and cancer pathologies[234].

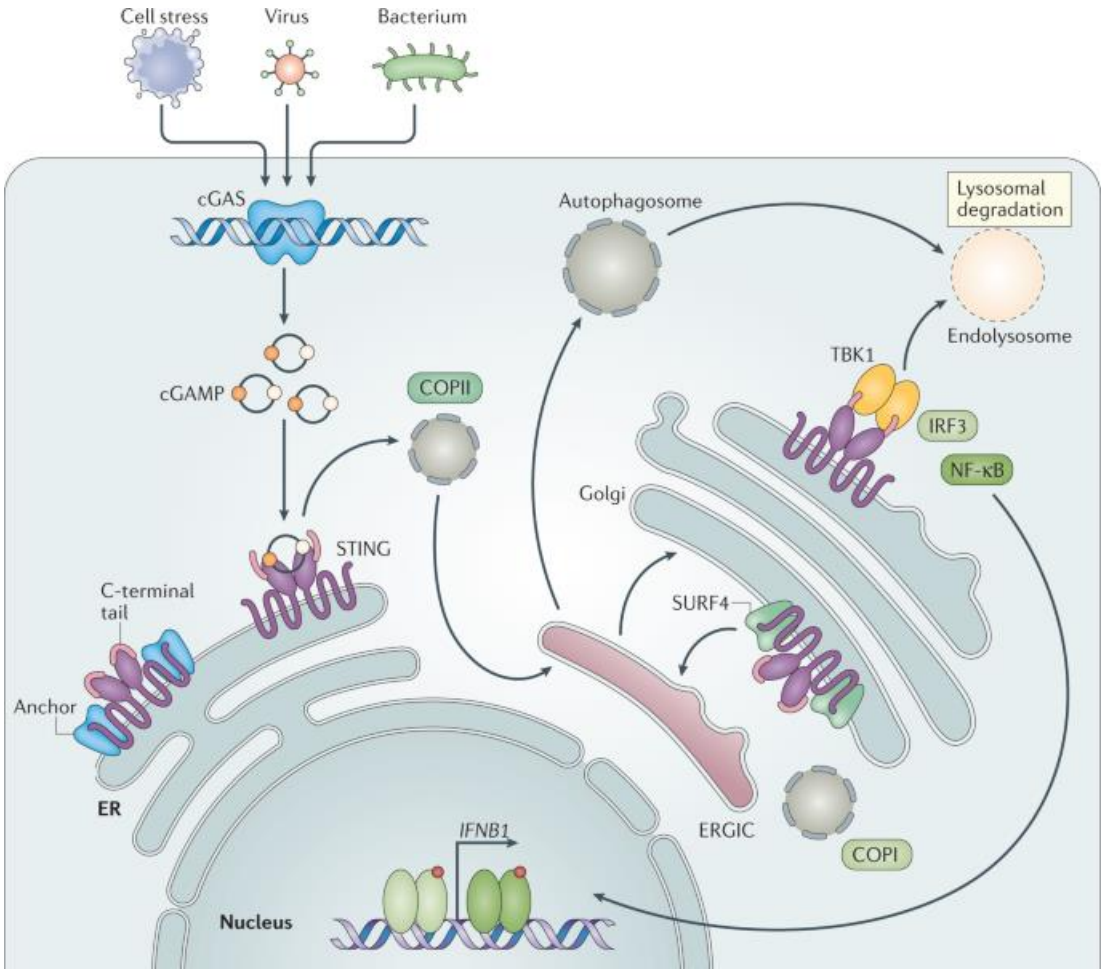

Figure 6.1. *Overview of the* Cyclic GMP-AMP synthase – Stimulator of interferon genes (*cGAS-STING) pathway. Double-stranded DNA (dsDNA) triggers cytosolic cGAS activation, stimulated by pathogen infection or cellular stress. Upon binding dsDNA, cGAS dimers assemble, activating cGAS to synthesize 2'3' cyclic GMP–AMP (cGAMP). cGAMP binds STING dimers on the endoplasmic reticulum (ER) membrane, inducing conformational changes triggering STING oligomerization. This initiates a cascade, recruiting TBK1, phosphorylating IRF3, leading to gene expression of type I interferons, ISGs, and inflammatory mediators. Figure taken directly from Decout et al., (2021).*

6.1.3 cGAS-STING, MN, and cancer

Micronuclei are prone to nuclear envelope rupture, allowing the leakage of genomic DNA into the cytosol[67]. The cytosolic DNA from ruptured micronuclei can be sensed by the DNA sensor cGAS, leading to its activation[67]. Dysregulation of the cGAS-

STING pathway in response to micronuclei has been implicated in autoimmune disorders and cancer, where genome instability and micronuclei formation are common[239].

The cGAS-STING pathway plays a complex and multifaceted role in cancer, exerting both pro-tumour and anti-tumour effects depending on the context. Playing a vital role in immune surveillance, this pathway enhances anti-tumour immunity by recognising cytosolic DNA from tumours[240]. It initiates production of type I interferons and inflammatory cytokines, facilitating T-cell activation and immune cell infiltration into the tumour microenvironment[240]. Additionally, activation of the cGAS-STING pathway prompts cellular senescence and autophagy, mechanisms that suppress tumour growth[241]. Due to this, STING agonists are emerging as promising cancer immunotherapeutic tools to aid anti-tumour immunity[242]. Conversely, persistent stimulation of cGAS-STING due to chromosomal instability in cancer cells may exacerbate inflammation, potentially fuelling tumour advancement and metastasis[243]. Not only this, but studies have shown that elevated STING levels in certain cancers show association with unfavourable prognosis and diminished infiltration of immune cells[242]. This underscores the necessity for a comprehensive grasp of this pathway and its activation across diverse cancer types.

6.1.4 Aim and objectives.

Aim: This study aimed to delve into the mechanisms underlying MN formation in the context of oesophageal disorders, shedding light on potential biomarkers and pathways involved in disease progression. The primary objective of this research was to explore the intricate relationship between blood plasma/plasma-based inflammation and lymphocyte MN% in individuals diagnosed with OAC, with a particular emphasis on investigating the involvement of the cGAS-STING pathway.

Objective 1: To assess the ability of plasma derived from individuals with GORD, BO, and OAC to induce MN in TK6 cells, thereby elucidating potential differences in genotoxicity among various disease subtypes.

Objective 2: To investigate whether levels of different plasma markers associated with the cGAS-STING pathway and inflammation, vary across distinct histological groups of oesophageal diseases. Additionally, to examine the correlation between these

plasma marker levels and lymphocyte MN%, providing insights into the molecular mechanisms underlying MN formation.

Objective 3: To test the relationship between activation of the cGAS-STING pathway and MN% through evaluating the impact of STING inhibition using H-151 on TK6 MN% after IR exposure.

6.2 Methods and materials

6.2.1 Plasma treated TK6 cells.

Prior to experimental use, plasma previously stored at -80^{0}C was defrosted fully on ice. TK6 cells were seeded at concentration of 1x10^5 in 3ml of media. After 24 hours, the cells were resuspended in modified media whereby the HS was replaced by 10% patient plasma (**Figure 6.2**.). Following 4 hours of exposure to human plasma, the cells were then transferred to fresh complete media containing 4.5 µg/ml Cyto-B for 24 hours prior to harvest. The MN% and CBPI of TK6 cells was then assessed, and a fold-change calculated based on the negative control. To correlate fold change of TK6 versus corresponding lymphocyte MN%, the lymphocyte CBMN- assay was utilised as described in **Chapter 2**. This work was carried out by myself, MSci student Rhiannon Wright under my supervision and Dr Hamsa Naser.

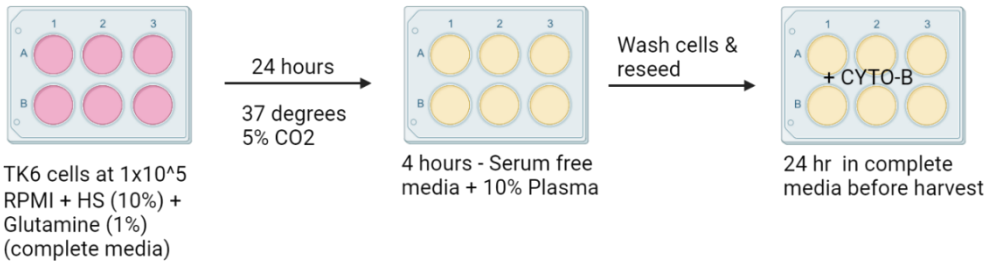

Figure 6.2. *Schematic demonstrating the exposure of TK6-cells to patient plasma. TK6 cells were cultured for 24 hours in complete media - RPMI with 10% horse serum (HS) and 1% glutamine. Following this, cells were cultured for 4 hours in a modified media, whereby HS was replaced with 10% patient plasma. Following a further 24 hours in complete media in the presence of cytochalasin-B (Cyto-b), cells were harvested for analysis. Created with Biorender.com*

6.2.2 Stimulation of STING in TK6 cells

TK6 cells were cultured as described in **Chapter 2**. The stimulator of interferon genes (STING) was stimulated through the addition of 25nM cGAMP disodium salt (ab144865) (Abcam, UK) for 24 hours.

6.2.3 qPCR array

To ensure the STING complex could be effectively activated in TK6 cells, a qPCR array was carried out. A qPCR array is a high-throughput method commonly used to

measure the expression levels of multiple genes simultaneously[244]. The qPCR array, as described in **Figure 6.3**, was designed to include genes involved in the cGAS-STING pathway. This included the following genes: *IFNA1, IFNB1, IL-8, MYC, PTEN, TNF, TP53.* Housekeeping genes included *ACTB* and *GAPDH.*

RNA extraction and quantification

RNA extraction from TK6 cells was carried out using the RNeasy micro kit (74004, Qiagen), according to manufacturer's instructions. The RNA was then tested for yield and quality on the NanoPhotometer® (IMPLEN, Germany) and stored at -80°C until the complementary DNA (cDNA) synthesis step. The NanoPhotometer required 1µl of RNA eluant to quantify the material (ng/µl). Purity readings were also given through two ratios, 260/280[245] (contamination of protein) and 260/230 (purity of sample regarding salts and other contaminants) – the numbers corresponding to the absorbance at the wavelengths at 230, 260, and 280nm.

Synthesis of cDNA

From this stage onwards, all work was carried out in a laminar flow PCR hood, with all equipment and the hood itself cleaned thoroughly with 70% ethanol and RNase Zap (BioRad) before use. The process was carried out according to the manufacturer's instructions for the iScript gDNA Clear cDNA synthesis kit (172-5034, Bio-rad). All components of the kit, and the RNA samples were defrosted on ice prior to experimental use. A DNase master mix was prepared as described in **Table 6.1**.

Table 6.1. *DNA master mix per reaction*

Component of master mix	Volume per reaction (µl)
iScript DNase	0.5
iScript DNase buffer	1.5
Total	2.0

An equal amount of RNA was used in each cDNA synthesis reaction (1µg).

For each RNA sample, an RNA/DNase reaction mix was prepared according to the volumes laid out in **Table 6.2** A reverse transcription control was prepared using 1 µl of the control RNA template, included with the PCR array plates (Biorad,

UK). Following centrifugation of the samples in microcentrifuge (Starlab, UK) (to ensure the removal of bubbles), the samples were transferred to the T100 Thermal cycler (Bio-Rad). The DNase reaction protocol that followed is described in **Table 6.3**.

***Table 6.2**. RNA/DNase reaction mix*

Component of RNA/DNase reaction mix	Volume per reaction (µl)
DNase master mix	2
RNA template (1 µg)	Variable
Nuclease free water	Variable
Total	16

***Table 6.3**. DNase Reaction protocol*

Step of DNase reaction	Temperature (°C)	Time (minutes)
DNase Digestion	25	5
DNase inactivation	75	5
Storage	4 (or in ice)	Until RT PCR step

The DNase treated RNA template was combined with the iScript reverse transcription (RT) supermix (or the no RT control) to produce the reverse transcription master mix (Described in **Table 6.4**). For each RNA sample prepared, a no-RT control was also carried out.

***Table 6.4.** Reverse transcription (RT) master mix set up*

Component of master mix	Volume per reaction (µl)
iScript RT supermix (or no RT control)	4
DNase treated RNA	16
Total	20

The samples were mixed thoroughly by pipetting and centrifugation to remove bubbles. The reaction tubes were incubated in the T100 thermal cycler as described in **Table 6.5**.

***Table 6.5.** cDNA synthesis thermal cycler conditions*

Thermal cycler stage	Thermal cycler conditions
Priming	5 minutes at 25°C
Reverse Transcription	20 minutes at 46°C
RT inactivation	1 minute at 95°C
Hold	4°C

The cDNA was then stored at -80°C prior to the next step.

qPCR using the customised PCR array.

Real time PCR is conducted as per manufacturer's instructions using SYBR green super mix (1725121, Biorad) and PCR array (10025137, Biorad) as shown in **Figure 6.3.**

***Figure 6.3**. Plate layout of polymerase chain reaction (PCR) array, including genes of interest: Interferon-Alpha 1 (IFNA1), Interferon-Beta 1 (IFNB1), MYC Proto-Oncogene (MYC), Phosphatase and Tensin Homolog (PTEN), Tumour Protein P53 (TP53), housekeeping controls: Actin Beta (ACTB) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). (PrimePCR™ Assays). Controls included: Genomic DNA (gDNA) to detect contamination, Reverse Transcription Efficiency Controls (RQ1 and RQ2) to assess consistency and efficiency of reverse transcription, PCR control to confirm successful amplification, and RT control to ensure amplification is from cDNA and not contaminating genomic DNA. Created with Biorender.com.*

The PCR array plates were brought to room temperature, and all reagents and cDNA samples were thawed at 4^0C before use. Each cDNA sample was diluted to give a total of 100 µl using nuclease free water. A qPCR reaction master mix was made up, with each reaction requiring the volumes as described in **Table 6.6** below.

Table 6.6. *qPCR reaction master mix*

Component of master mix	Volume per reaction (µl)	Final Concentration
SYBR green supermix	10	1x
PCR primer	Lyophilised on plate	-
cDNA sample/cDNA controls (RT or no RT)	1	10 ng
Nuclease free water	Variable	-
Total volume	20	-

A total of 20 µl qPCR reaction mix containing the samples of interest was pipetted into each well of the PCR array plate. The gDNA (no RT control) and RT controls were made as per **Table 6.7**, using the cDNA synthesised in section 5.3 and 20µl pipetted into the corresponding wells as show in figure 5.

A PCR control well was also required, whereby 1 µl of the PCR control tube was pipetted into the PCR control well with the 20 µl of master mix (total volume of 21 µl). The plate was then sealed with a plate sealer and centrifuged for 2 minutes at 2600 x g at room temperature. The plate was run using the Bio-Rad CFX qPCR machine as per the conditions in **Table 6.5.**

Table 6.7. *PCR cycling protocol*

qPCR protocol	Temperature (°C)	Time (s)	Number of cycles
Activation	95	120	1
Denaturation	95	5	40
Annealing/Extension	60	30	
Melt curve	65-95 (0.5°C increments)	5 (s/step)	1

The values produced for the PCR array quality controls were then checked to ensure the data could be used. A total of 4 repeats were used, in duplicate on each plate.

6.2.4 Finding the optimum concentration of STING inhibitor H-151

STING inhibitor H-151 (chemical name N-(4-Ethylphenyl)-N'-1H-indol-3-yl-urea) was purchased from TOCRIS, UK. H-151 binds to STING at Cys91, preventing the assembly of STING clusters by blocking palmitoylation[246] as shown in **Figure 6.4**. This optimisation experiment was carried out by BSc student Sonja Collaku under my supervision. RPD was used as a measure of cytotoxicity following treatment with a range of concentrations. The optimum was determined as the highest inhibitor concentration without any cytotoxicity. As a study by Haag *et al*., used 0.5µM on THP1cells[246], therefore a broad range of concentrations from 0.5-15µM was first assessed in TK6 cells here, before a narrowed down range of 1-6µM was decided to be more appropriate. TK6 cells were seeded at a concentration of $1x10^5$ cells per ml in 10ml using T25 flasks and left to incubate for 24 hours at 37^0C and 5% CO_2. They were counted using trypan blue staining, and LUNA-II™, before being treated with a range of concentrations of H-151, or equal volume of DMSO (vehicle control). After 24 hours, the cells were moved to fresh media following a wash in PBS, and subsequently counted a further 24 hours later. The prior and final cell count were used to calculate the RPD and assess cytotoxicity.

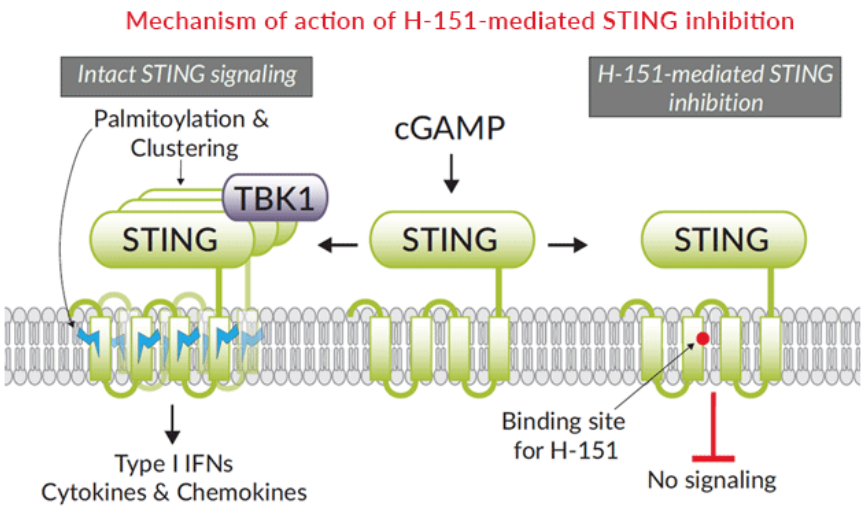

Figure 6.4. *The mechanism of action of STING antagonist H-151. H-151 covalently binds to Cys91 residue of STING, preventing palmitoylation and clustering, thereby inhibiting upregulation of type 1 IFNs, cytokines and chemokines. Image taken directly from https://www.invivogen.com/h151. Accessed November 2023.*

6.2.5 Assessing the influence of STING inhibition on MN formation following DNA damage.

TK6 cells were treated with a STING inhibitor for 2, and 6 hours prior to undergoing IR. Cells were harvested for: micronuclei, RPD, and cell cycle analysis 48 hours later,

Inducing MN using IR

TK6 cells were seeded into T25 flasks at a concentration of $1x10^5$ cells/ml in 10ml and incubated for 24 hours at 37^0C and 5% CO_2. Cells were subsequently counted to ensure growth, then incubated for 2, and 6 hours with 1μM of H-151. All cells were then transported to the radiology department in Singleton hospital in a polystyrene box with hot water bottles.

Radiation of the cells was carried out by Dr Richard Hugtenburg and Sigrid Mathiesen of the radiology department. The layout of cells for irradiating, and the calculation of radiation dosage (as calculated by Sigrid Mathiesen) is shown in **Figure 6.5**. Flasks were arranged 3x2 in the coronal plane, with the flasks resting in the isocentre, the source to surface distance was 100cm with a 30x30 cm field size and 10cm of solid water phantom (Bart's Health NHS Trust, UK) posterior and 5cm build-up anteriorly to the flasks (**Figure 6.5**).

A dose of 1.73 ± 0.0016Gy of radiation (calculation shown as table in **Figure 6.5**) was delivered to the cells using the Elekta versa HD linear accelerator (LINAC, Elekta, Sweden). A medical LINAC generates high-energy X-rays or electrons which can be precisely targeted at tumours for therapeutic radiation. This dose was chosen through trial and error, using the *in vitro* dose of 2gy used by Widel *et al.,* (1999) as a reference[247]. The chosen dosage provided a statistically increased MN%, without hitting the cytotoxicity limit of 60%.

A.

B.

Specifics	LINAC
Source to cell distance	5
Field number	1
Depth (cm)	5
SSD	100
Source-cell distance	105
Energy (MV)	6
Total Mus	200
Number of fraction	1
Field size	30x30
Equivalent square	30
Output Factor	1.074
M.	0.911
%DD	0.883
Total cGY =	173
Total GY =	1.73

Figure 6.5. *Radiation of TK6 cells in Singleton hospital (A). The layout of flasks for radiation with 1.73GY using the Elektra versa HD LINAC in Singleton hospital radiology department. Cells were arranged in a 2x3 layout with solid water phantoms. (B). Table of calculations used to work out the dosage of radiation in GYs used to treat the cells by Sigrid Mathiesen. SSD = Source-Skin Distance, MUs = Motor units, M. = monitor unit, %DD= Percentage Depth Dose, Total cGY = Total cumulative dose in centigray, Total GY= total dose in grays.*

During radiation, all control cells were removed from the room. Following radiation, cells were immediately returned to the incubator for 48 hours at 37^0C at 5% CO_2 before being counted for RPD and being harvested for cell cycle and MN analysis.

Mononucleate MN assay

Cell cycle progression is required for STING activation[248], and therefore Cyto-B could not be used to allow the scoring of MN in binucleated cells. This meant that MN were scored in mononucleate cells. Four thousand cells per treatment were scored for presence of MN, and the experiment was carried out in triplicate. For cytotoxicity measurement, RPD was used. Harvest of cells was carried out as described in **Chapter 2**, in regard to washes in fixatives, preparation of slides, and staining with DAPI. The scoring of cells was blinded - carried out by myself, and Sonja Collaku.

Cell cycle in TK6 cells

The harvest and fixing of cells for cell cycle analysis is described in chapter 2. On the day of analysis, cells were washed once in 1ml PBS, and around $1x10^6$ cells were resuspended in 100µl of FxCycle™ PI/RNase Staining Solution (Thermofisher Scientific, UK) for 30 minutes before the cells were acquired on the NovoCyte® flow cytometer (ACEA biosciences, Inc.) with a stop condition of 10,000 live cells. The gating strategy for TK6 cells is shown in Figure 6.7. FlowJo version 10.3 software (Becton Dickson, USA) was used to analyse the data, and extract the percentages of cells within each stage of the cell cycle. The cell numbers were normalized to achieve a total count of 100%, ensuring proportional representation across all stages.

6.2.6 ELISA for measurement of type 1 interferons and proinflammatory cytokines

All ELISA's utilised for this chapter, except the measurement of 2'3'cGAMP, were purchased from R&D and carried out according to manufacturer's protocol, generally described in **Chapter 2**. Some plasma samples were derived from the same patients and volunteers in previous chapters, however some were previously collected and stored in -80^{o}C prior to the start of this study.

6.2.7 Measurement of 2'3'cGAMP in patient plasma

Prior to use in ELISA, patient plasma was stored at -80^{0}C, and thawed on ice. The 2'3'cGAMP ELISA kit was acquired from Cayman Chemicals, UK and carried out according to the manufacturer's guidance. **Table 6.8** shows list of reagents included in the kit, and the preparation required before use, whilst **Figure 6.6** shows a schematic of the assay's principal.

Table 6.8. *Reagents supplied and used in 2'3'cGAMP ELISA.*

Reagent provided	Preparation required	Reagent used in assay
Immunoassay buffer C Concentrate (10X)	Dilute with 90ml ultrapure water	Immunoassay buffer C (1X)
Wash buffer concentrate (400X)	Dilute to a total volume of 2L with ultrapure water and add 1ml Polysorbate 20	Wash buffer (1X)
2'3'-cGAMP-HRP Tracer (1x)	None	2'3'-cGAMP-HRP Tracer (1x)
2'3'cGAMP ELISA Polyclonal Antiserum (1X)	None	2'3'cGAMP ELISA Polyclonal Antiserum (1X)
2'3'cGAMP standard	Serially dilute in Immunoassay Buffer C (1X), to get a range of concentrations (6.1pg/ml → 100,000pg/ml)	Series of standards, diluted in Immunoassay Buffer C (1X).Final concentration 6.1pg/ml → 100,000pg/ml

Figure 6.6*. Schematic of 2'3'cGAMP ELISA. Taken from product booklet https://www.caymanchem.com/product/501700/2'3'-cgamp-elisa-kit,accessed 08.11.2023.*

A 96 well plate, precoated with mouse anti-rabbit IgG and blocked with a proprietary formulation of proteins (Figure below) was first washed 5 times with 1X wash buffer, before the addition of 2'3'cGAMP standards, plasma samples (diluted 1:2 in triplicate), and Immunoassay buffer to NSB (100µl) and B_0 (50µl) wells, As well as a blank in duplicate, this assay required multiple controls including non-specific binding (NSB), total activity (TA), and maximum binding (B_0) wells as displayed in **Figure 6.7**.

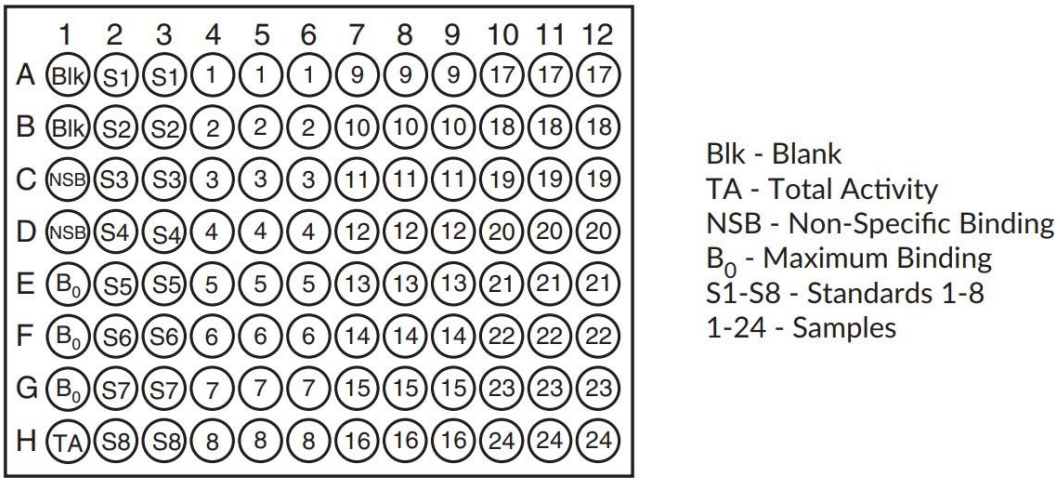

Figure 6.7. *Sample plate format. Taken from product booklet, from https://www.caymanchem.com/product/501700/2'3'-cgamp-elisa-kit. accessed 08.11.2023*

A 2'3'cGamp-HRP Tracer was added to all wells (except blank and TA wells), followed by the addition of 2'3' cGAMP ELISA polyclonal Antiserum within 15 minutes of addition of the tracer (all wells except TA, NSB and blanks).

The plate was then covered and incubated overnight at 4^0C. The next morning, the wells were aspirated and washed 5 times, before TMB substrate solution was added to each well, and the 2'3'-cGAMP-HRP Tracer (1x) added to the TA wells. The plate was covered and allowed to incubate on an orbital shaker at room temperature for 30 minutes. Following the incubation, HRP stop solution was added to the plates, and the absorbance was measured using plate reader at 450 nm.

2'3'cGAMP-data analysis

To determine the concentration of cGAMP in an assay, the following steps were followed: First, the average absorbance readings from the NSB wells and the B0 wells

were calculated separately. Next, the NSB average was subtracted from the B0 average, which yielded the corrected B0 or corrected maximum binding value. Then, for the remaining wells (S1-S8 and all sample wells), the B/B0 (Sample or Standard Bound/Maximum Bound) was calculated by subtracting the average NSB absorbance from the well's absorbance and dividing by the corrected B0 obtained in the previous step. If a logistic four-parameter fit was to be used, these values were multiplied by 100 to obtain %B/B0. It was important to note that TA values were not used in standard curve calculations but served as a diagnostic tool. Low or no absorbance in a TA well might indicate issues in the enzyme-substrate system. A standard curve was plotted by graphing %B/B0 for standards S1-S8 against 2'3'-cGAMP concentration using linear (y) and log (x) axes and a four-parameter logistic fit was applied. Finally, the sample concentration was determined by calculating the B/B0 (or %B/B0) value for each sample and using the equation derived from the standard curve plot. It was ensured that any dilution of the sample concentration was accounted for before adding it to the well.

6.2.8 Statistical analysis

All statistical analysis described in this chapter was carried out using the analyse function on GraphPad Prism version 9, without adjustment for age when comparing between histology groups. Normality and lognormality tests were carried out initially to assess the spread of data. Following this, one-way ANOVA or nonparametric tests were carried out, with post-hoc or Kruskal-Wallis tests. An R-value (correlation coefficient) was calculated for all correlation analyses to indicate the strength of correlation. Interpretation of correlation strength followed the thresholds described by Akoglu (2018)[124]. Results were considered significant at a p-value of less than 0.05. Error bars show either SEM or SD. Figure legends contain relevant information.

6.3 Results

6.3.1 Induction of MN in TK6 cells through exposure to patient plasma.

As an increased L-MN% is an indication of genotoxic exposure in many biomonitoring studies[249], an experiment was developed to investigate whether a blood-borne genotoxic factor could be contributing to the increased L-MN% in cancer patients. TK6 cells were cultured in modified media, whereby the horse serum was replaced with 10% patient plasma, previously stored at -80^{0}C, and subsequently MN% was measured (**Figure 6.8**). Whilst the effect of plasma versus serum was considered, plasma was ultimately chosen as *in vivo* the circulating lymphocytes would only come into contact with serum at the time of clot formation[250]. Exposure to plasma derived from each patient group had the ability to alter MN% in comparison to the vehicle control. When comparing patient groups, the plasma from those with BO or OAC had a significantly higher MN induction compared to the effect of plasma derived from HVs (Figure 6.8A). When comparing the TK6 fold change, to the corresponding patient lymphocyte MN%, there was a statistically significant moderate correlation, suggesting a relationship between the lymphocyte MN% with the effect of corresponding plasma (Figure 6.8B). CBPI was measured to ensure cell division and any potential cytotoxicity induced by patient plasma (Figure 6.8C). There was no difference in the growth of cells cultured with plasma from any of the patient groups.

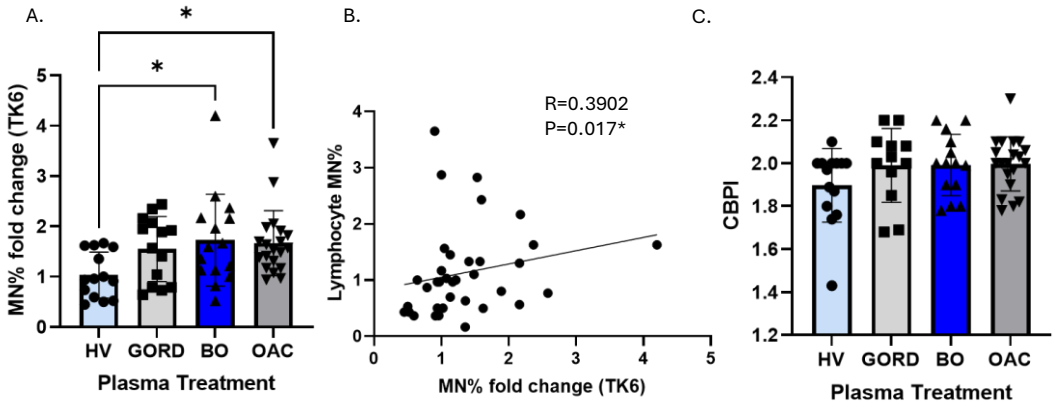

Figure 6.8. *The effect of patient plasma on in vitro cell line TK6.* (*A) Micronucleus* (MN) *induction following plasma treatment, as measured by fold change* (MN% fold change) *compared to untreated TK6 cells. TK6 cells treated with plasma from healthy volunteers (HV, n=14), patients with gastro-oesophageal reflux disease (GORD, n=14), Barrett's oesophagus (BO, n=15) and Oesophageal adenocarcinoma (OAC, n=19) *p<0.05.* (*B) Relationship between the induction on TK6 cells by patient plasma, against the corresponding patient baseline micronucleus frequency (MN%). Moderate correlation identified between 37 individuals, R=0.3902, p<0.05. C) Cytokinesis block proliferation index following plasma treatment. Healthy volunteers (HV, n=14), patients with gastro-oesophageal reflux disease (GORD, n=14), Barrett's oesophagus (BO, n=15) and Oesophageal adenocarcinoma (OAC, n=19). (Error bars show SEM).*

6.3.2 The correlation between some inflammatory markers in plasma and the corresponding lymphocyte MN%

As inflammation drives carcinogenesis, especially in the context of oesophageal adenocarcinoma, it was deemed appropriate to explore the relationship between inflammatory markers in the plasma, and the corresponding lymphocyte MN%. Moreover, recent literature indicates an association between micronuclei and the cGAS-STING pathway. This pathway triggers the upregulation of pro-inflammatory cytokines and type I interferons, which became a primary focus of this research.

ELISA was used to measure the quantity of several different markers; 2'3-cGAMP (**Figure 6.9**), IFN-β (**Figure 6.10**), IFN-α (**Figure 6.11**), IL-8 (**Figure 6.12**), IL-6 (**Figure 6.13)** and IL-1β (**Figure 6.14**).

cGAMP: Around 80 samples were analysed for the quantification of 2'3'-cGAMP, 41 of which had detectable levels. HVs had the lowest concentration of 2'3'-cGAMP (average concentration of 27 pg/ml), with concentrations gradually increasing by histology, with OAC patients exhibiting the highest levels (average of 48 pg/ml) (Figure 6.9A). This was almost reaching significance (p=0.06). There was moderate correlation between lymphocyte MN% and corresponding plasma levels of 2'3'-cGAMP (R=0.42, P=0.029, n=27), suggesting a relationship between cGAS activation and DNA damage levels (Figure 6.9B).

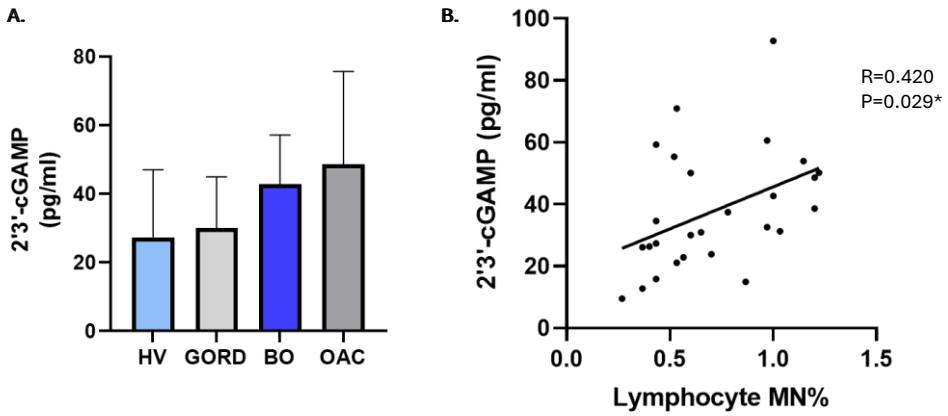

Figure 6.9. *Concentration of 2'3'-cyclic GMP-AMP (cGAMP) in patient plasma.* (*A*). *Plasma concentration of cGAMP in different histological groups. Healthy volunteers (HV) n=11, Gastroesophageal reflux disease (GORD) n=10, Barrett's oesophagus (BO) n=8, Oesophageal adenocarcinoma (OAC) n=12.* P=0.06 (*B*). *Correlation between lymphocyte micronucleus frequency (MN%) and cGAMP concentration in plasma n=27, R=0.4202, *p=0.029. (Error bars show SD)*

IFN-β: Whilst 120 samples were tested for IFN-β concentration, only 52 had measurable quantities. HVs had the lowest concentration of IFN-β (average

concentration of 32 pg/ml), with concentrations gradually increasing by histology, with OAC patients exhibiting the highest levels (average of 51 pg/ml) (Figure 6.10A). OAC patients had significantly higher levels than those with GORD and HVs, whilst BO exhibited significant levels in comparison to HVs. No relationship was seen however between lymphocyte MN% and concentration of IFN-β (R=0.05, P=0.7, N=25)(Figure 6.10B).

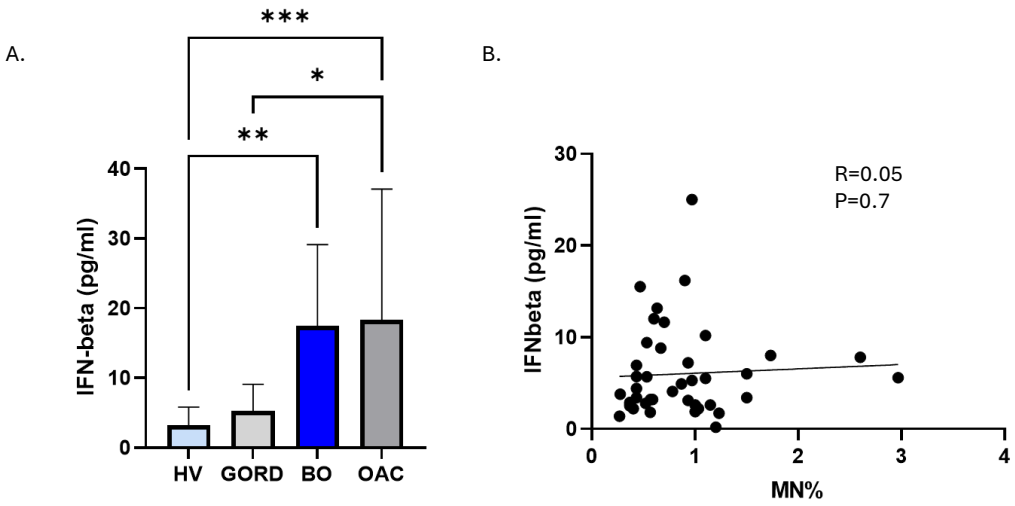

Figure 6.10*. Concentration of Interferon-β (IFN-β) in patient plasma. A) Plasma concentration of IFN-β in different histological groups. Healthy volunteers (HV) n=17, Gastroesophageal reflux disease (GORD) n=15, Barrett's oesophagus (BO) n=7,. Oesophageal adenocarcinoma (OAC) n=13 *P=<0.05, **=<0.01, ***=<0.005. B) Correlation between lymphocyte micronucleus frequency (MN%) and IFN-B concentration in plasma. R=0.05, p=0.7. (Error bars show SD).*

IFNα: A similar pattern was observed with IFNα, lacking statistical significance (p=0.25). The average concentration of healthy volunteers was 3 pg/ml, with concentrations gradually increasing by histology. OAC patients had an average of 6 pg/ml (Figure 6.11A). No relationship was seen however between lymphocyte MN% and concentration of IFN-α (R=0.03, P=0.86, N=34) (Figure 6.11B). Despite testing 120 samples, only 47 had measurable levels of IFN-α.

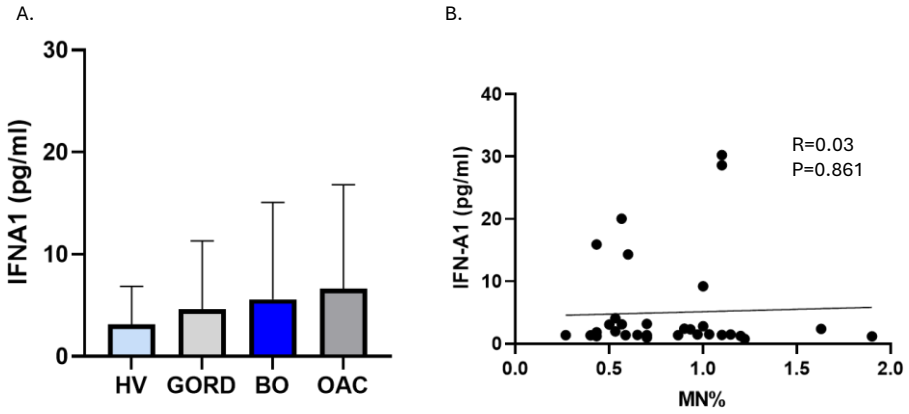

Figure 6.11. *Concentration of Interferon-α (IFN-A1) in patient plasma. A) Plasma concentration of IFN-A1in different histological groups. Healthy volunteers (HV) n=11, Gastroesophageal reflux disease (GORD) n=11, Barrett's oesophagus (BO) n=12, Oesophageal adenocarcinoma (OAC) n=13. p=0.25 (B) Correlation between lymphocyte micronucleus frequency (MN%) and IFN-A1 concentration in plasma. R=0.03 P=0.86. (Error bars show SD).*

IL8|: Overall, ELISA was carried out using 80 patient samples, 32 of which had measurable levels. HVs had a significantly lower concentration of plasma IL-8 compared with OAC patients (average concentrations of 26 pg/ml and 177 pg/ml respectively, P=0.038). Levels were elevated in those with GORD and BO (average of 140 pg/ml and 121 pg/ml respectively (Figure 6.12A). In 32 patients, there was a significant correlation between lymphocyte MN% and IL-8 concentration in plasma (R=0.44, P =0.0133, N=32) (Figure 6.12B).

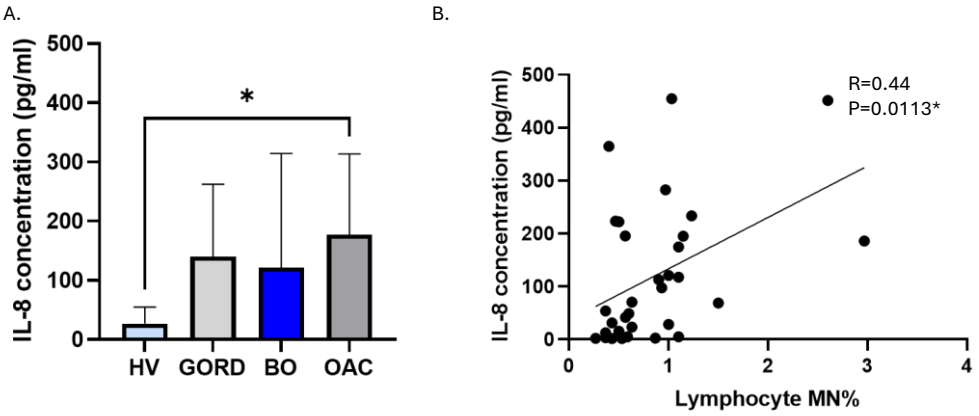

***Figure 6.12**. Concentration of Interleukin-8 (IL-8) in patient plasma. A) Plasma concentration of IL-8 in different histological groups. Healthy volunteers (HV) n=11, Gastroesophageal reflux disease (GORD) n=12, Barrett's oesophagus (BO) n=5, Oesophageal adenocarcinoma (OAC) n=9. *P=0.038 B) Correlation between lymphocyte micronucleus frequency (MN%) and IL-8 concentration in plasma. A moderate correlation shown, indicating those with higher MN% have higher levels of IL-8 in their plasma. R=0.44 P=0.013*. (Error bars show SD).*

IL6: A total of 80 patient samples were analysed for concentration of IL-6, of which 44 had measurable levels. HVs had a significantly lower concentration of plasma IL-6 compared with OAC patients (average concentrations of 6 pg/ml and 33 pg/ml respectively, p=0.047). Levels were similar in those with GORD and BO (average of 19 pg/ml and 20 pg/ml respectively (Figure 6.13A). In 27 patients, there was no correlation between lymphocyte MN% and IL-6 concentration in plasma (R=0.04, P =0.818) (Figure 6.13B).

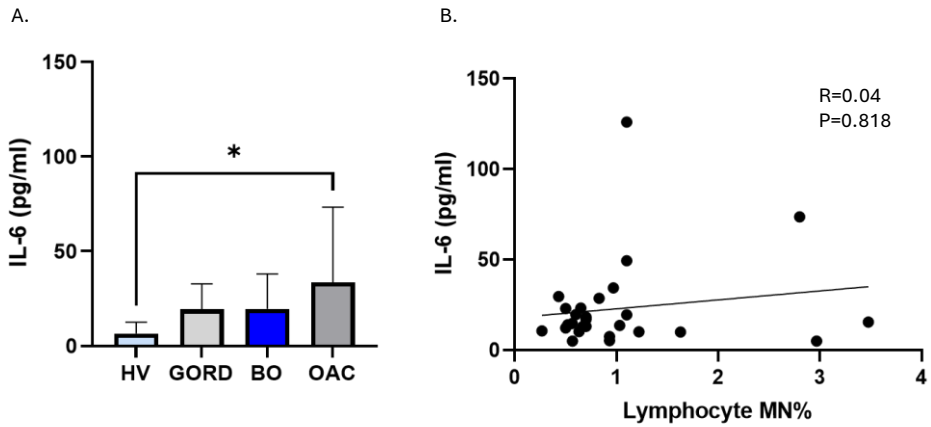

Figure 6.13. *Concentration of Interleukin-6 (IL-6) in patient plasma.* (*A) Plasma concentration of IL-6 in different histological groups. Healthy volunteers (HV) n=9, Gastroesophageal reflux disease (GORD) n=12, Barrett's oesophagus (BO) n=12, Oesophageal adenocarcinoma (OAC) n=11. *P=0.047* (*B) Correlation between lymphocyte micronucleus frequency (MN%) and IL-6 concentration in plasma. R=0.04, P =0.818. (Error bars show SD).*

IL1B: Finally, 80 samples were tested for IL-1B concentration, 34 of which gave a reading. Whilst a similar pattern emerged, there was no significance in the different average levels between histology groups (p=0.55) (Figure 6.14A). In 21 patients, there was no correlation between lymphocyte MN% and IL-1β concentration in plasma (R=0.08, P =0.712, N=21) (Figure 6.14B).

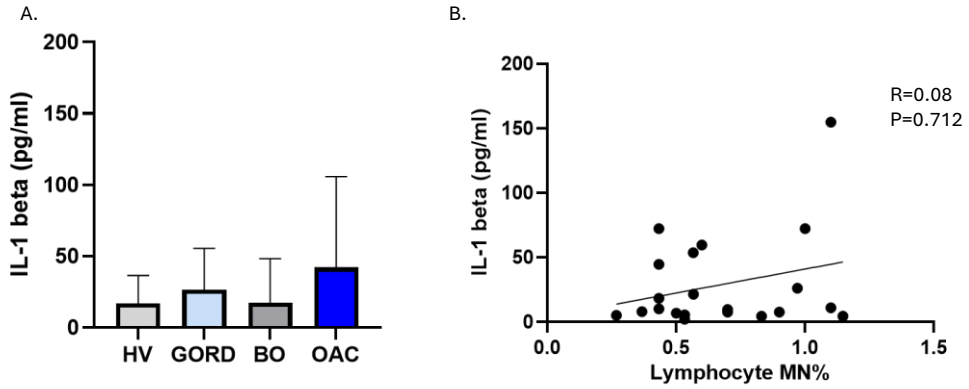

Figure 6.14. *Concentration of Interleukin-1β (IL-1B) in patient plasma. (A) Plasma concentration of IL-1B in different histological groups. Healthy volunteers (HV) n=13, Gastroesophageal reflux disease (GORD) n=11, Barrett's oesophagus (BO) n=5, Oesophageal adenocarcinoma (OAC) n=5. P=0.55 (B) Correlation between lymphocyte micronucleus frequency (MN%) and IL-1B concentration in plasma. R=0.08, P=0.7. (Error bars show SD).*

6.3.3 Assessing the effect of STING inhibition on DNA damage following ionizing radiation

Micronuclei can trigger the cGAS-STING pathway, leading to increased expression of interferons and inflammatory cytokines. Due to the correlation between cGAMP, and several markers of inflammation with MN in lymphocytes, a preliminary *in vitro* experiment was designed to further test the relationship between STING activation and MN induction in cells of lymphoblastoid origin. TK6 cells were first treated with an agoniser of STING (cGAMP disodium salt) and ionizing radiation (IR) (at 1.73gy). PCR array confirmed the significant upregulation of *IFNB1*, and *IFNA1* in these cells following treatment of both. Average upregulation of *IFNB1* and *IFNA1* WERE 30-fold and 10-fold respectively following treatment of TK6 cells with 25 nM cGAMP disodium salt, and 70-fold and 7-fold following ionizing radiation (**Figure 6.15**). Next, TK6 cells were cultured in the presence of a STING inhibitor (H-151) for varying time points prior to undergoing IR (2 and 6 hours, **Figure 6.16**). Cells were allowed a 24-hour recovery before harvest for analysis. This experiment was repeated 4 times. MN

induction was reduced when treated with H-151 for 6 hours prior to IR (p=0.0503), with no effect observed in the treatment with a 2-hour incubation. STING inhibition, however, did not appear to have a significant impact on RPD or cell cycle progression.

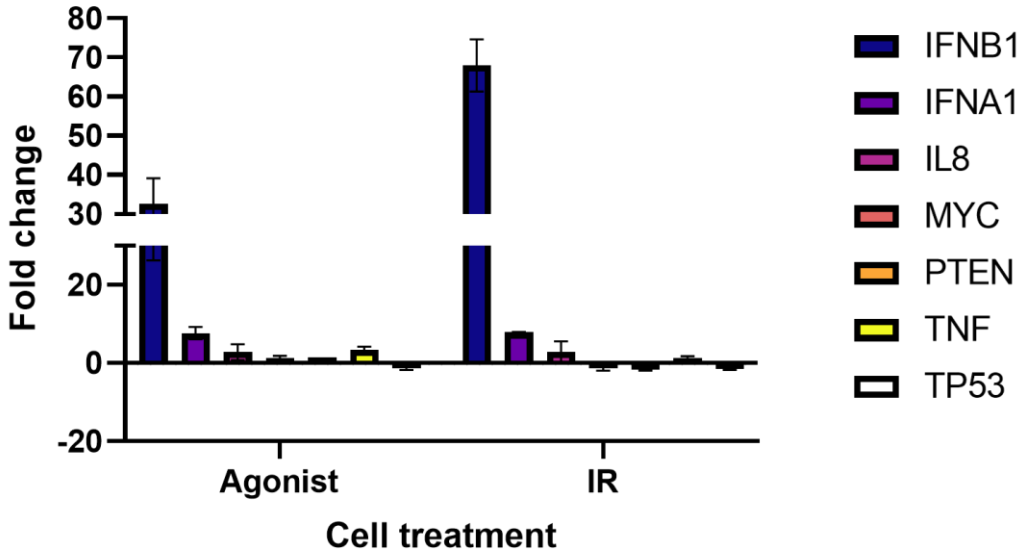

Figure 6.15. *Change in gene expression following treatment with STING agonist in TK6 cells, as measured by qPCR. Data includes 4 repeats, with average upregulation of IFNB1 and IFNA1 being 30-fold and 10-fold respectively following treatment of TK6 cells with 25 nM cGAMP disodium salt, and 70-fold and 7-fold following ionizing radiation (1.73gy). Genes included: Interferon-Alpha 1 (IFNA1), Interferon-Beta 1 (IFNB1), MYC Proto-oncogene (MYC), Phosphatase and Tensin Homolog (PTEN), Tumour Protein P53 (TP53), housekeeping controls: Actin Beta (ACTB) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). (n=3, Error bars show SD).*

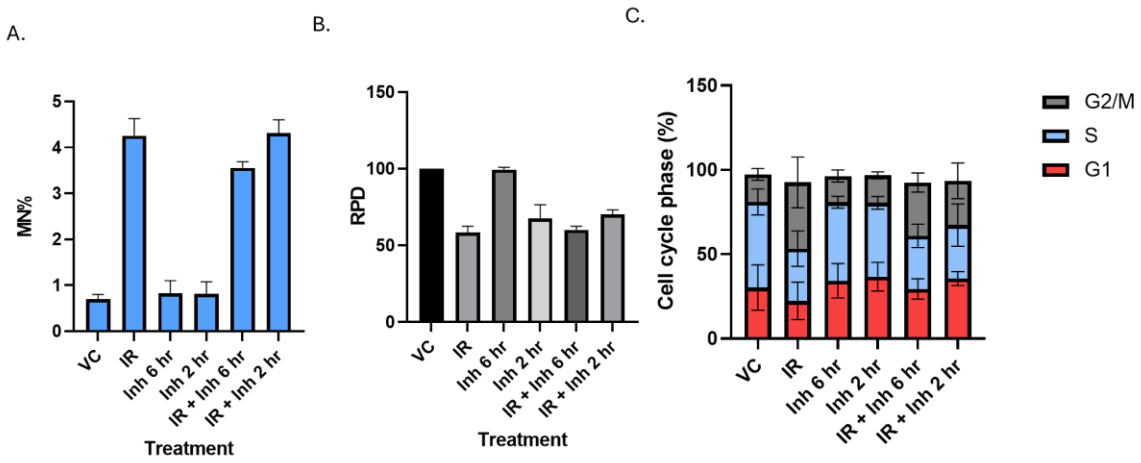

Figure 6.16. *Using H151 to inhibit the stimulator of interferon genes (STING) complex prior to ionizing radiation (IR). (A). Micronucleus frequency (MN%) of TK6 following ionizing radiation (IR) of 1.73gy. Treatment with H-151 (STING inhibitor) slightly reduced MN induction (p=0.0503) (B). Relative population doubling (RPD) IR of 1.73gy. Treatment with inhibitor has no significant effect on the cytotoxicity of IR (C). Cell cycle stage of TK6 cells, 48 hours following IR exposure. Treatment with IR increases the proportion of cells in G2/M stage, the effect of which is not significantly altered by the inhibition of STING. (Error bars show SD).*

6.4 Discussion

In this chapter, the role of plasma and inflammation (with specific focus on the cGAS-STING pathway) on MN% was explored. First of all, the hypothesis that there could be a blood-borne genotoxic element present in the plasma was investigated. An experiment was set up involving TK6 cells, whereby they were exposed to patient plasma. Subsequently, MN% was measured in the treated cells, and a fold change calculated using the untreated control. TK6 were utilised for this experiment, as they are lymphoblastoid in origin and therefore share physiological characteristics with PBLs. Additionally, they express the wild-type p53 tumour suppressor gene[251], and are commonly used in genotoxicity testing, therefore giving a true indication of the potential genotoxic effect of the plasma. MN induction was compared in the treated samples to the negative control, with a fold change calculated. Each individual sample had a varying effect on the overall MN-fold change of TK6 cells, with some potentially having a protective effect (fold change of less than 1), and others inducing DNA damage (fold change of more than 1). When comparing the fold change of each histology group, it was shown that the damage induced by BO and OAC plasma samples was significantly higher than that induced by the plasma of healthy volunteers. The observed correlation between MN induction in TK6 cells by plasma and the MN% in the lymphocytes within this study suggests that there could be a relationship between the plasma induced effects and MN induced in lymphocytes. The increase in inflammation, characteristic in those with GORD, BO, and cancer[184], as well as certain lifestyle factors and exposures could contribute to this DNA damaging effect through the exacerbation of oxidative stress. While reviewing the literature, it became apparent that there is a scarcity of studies that aim to directly employ plasma *in vitro* to observe its impact on MN induction. Maffei *et al*. (2011) assessed the activity of antioxidant enzymes and clastogenic factors in the plasma of CRC screening participants[252]. They utilised ultrafiltered plasma to treat primary lymphocytes from a healthy volunteer, and measured MN induction. Here they showed that plasma from CRC patients exhibited elevated chromosome-damaging substances, potentially contributing to cancer development. Even plasma from polyp patients (at increased risk of CRC) and those with positive faecal occult blood tests showed chromosome damage, indicating potential future cancer risk[252]. The clastogenic factors described here could include

TNF-α and lipid peroxidation products, both associated with oxidative stress[253], and indicate utility as a biomarker for increased risk of CRC.

As discussed previously, diet and lifestyle plays an important role in lymphocyte MN% and therefore metabolites present in the plasma could have an effect on the induction in TK6 cells. For example, whilst the presence of B[a]P correlates with increased MN in lymphocytes[233], dietary interventions incorporating antioxidants such as vitamin C, vitamin E, beta-carotene, and folic acid have demonstrated efficacy in diminishing MN levels in lymphocytes[254]. This could account for the reduction in MN observed in some plasma treated TK6 cells. This cocktail of genotoxic metabolites and chemoprotective factors in plasma will vary on a case-by-case nature.

The role of diet, lifestyle factors, medication, and smoking in this cohort could be further analysed to assess any relationship with MN induction. Screening plasma by liquid chromatography and mass spectrometry to identify potential genotoxic metabolites would also be informative. Additionally, incubating TK6 cells with and without antioxidants like N-acetylcysteine (NAC) in the presence of patient plasma can help determine if ROS are responsible for the observed increase in MN in certain patients.

Markers of inflammation

ELISA was used to quantify various inflammatory markers in patient plasma. Although the ideal scenario would have involved using the same plasma samples from patients as those used in the TK6 cell experiment, logistical constraints such as limited volume or concerns regarding freeze-thaw cycles sometimes rendered this impractical. Consequently, a decision was made to compare inflammatory markers via histology and correlate them with lymphocyte MN%, allowing for broader conclusions to be drawn. Unfortunately, this approach did not permit a direct comparison with the potential of inflammation in plasma to induce micronuclei *in vitro*. Whilst over 500 samples were included for ELISA analysis, only 250 yielded measurable readings. This reflects the significant impact of preanalytical and analytical factors such as sample collection, processing, and biological variability on cytokine quantification in plasma samples. For example, factors like prolonged storage at -80°C and repeated freeze-thaw cycles may have compromised sample integrity, affecting result reproducibility[255].

This study focused on the cGAS-STING pathway due to its recent association with MN formation and inflammation. Extracellular cGAMP exists in a free, soluble form that can be hydrolysed by the enzyme ENPP1 present on cancer cells or in the tumour microenvironment[256]. The high extracellular cGAMP levels produced by cancer cells could activate the STING pathway in immune cells like CD14+ PBMCs to induce an anti-tumour immune response, therefore also show relevance in biomonitoring of cancer patients[256].

The results reveal a gradual increase in 2'3'-cGAMP levels from HVs to OAC patients, which correlates positively with lymphocyte MN%. This suggests two possibilities. Firstly, heightened inflammation associated with DNA damage may elevate MN in lymphocytes, reflecting increased cGAMP levels. Alternatively, the correlation implies that lymphocyte DNA damage, reflected in MN%, mirrors genome instability within tumour cells, which in turn activate this pathway. This suggests that lymphocyte MN% could serve as a marker for tumour genome instability and potential cancer progression, highlighting a role for the cGAS-STING pathway in these patients. Measurement of certain aspects of this pathway in oesophageal tissue would provide valuable insights.

Conversely, while IFN-β and IFN-α levels increased with histology, no significant correlation with MN% was observed. Given their susceptibility to influences from various factors like viral infections[257], autoimmune disorders[258], medications[259], and chronic stress[260], IFN-β and -α levels in plasma may lack the consistency needed for reliable biomonitoring of cancer progression when assessing their association with MN%.

IL-6 and IL-1β also showed no significant correlations with MN%, despite variations in their plasma concentrations across histological groups.

The expression of various cytokines in the oesophageal mucosa is likely to trigger oxidative stress through the recruitment and activation of inflammatory cells and the upsurge in reactive oxygen species production[184]. This oxidative stress induction, driven by the reflux of gastric acid and duodenal contents (including bile acid and pancreatic juice) into the oesophagus, is implicated in initiating inflammation and carcinogenesis[184]. Involvement of pro-inflammatory elements, such as IL-6 and -8, along with leukocytes and oxidative stress, is well-documented in GORD development

and progression to BO and OAC[184]. Notably, a moderate correlation between IL-8 concentration in plasma with lymphocyte MN% was shown here, with the highest levels occurring in those with OAC. This could indicate its potential as a marker of inflammation linked to DNA damage during disease progression. Additionally, in OSCC, there are findings linking elevated levels of IL-8 with aggressive disease behaviour. Bhat *et al*. (2021) identified IL-8 as one of the interleukins that promote metastasis in oesophageal cancer, especially in cases involving lymph node infiltration[261]. Similarly, in breast cancer, elevated IL-8 levels correlate with advanced stage, metastasis, and poorer survival[262].

These findings underscore the intricate interplay between inflammatory mediators and genotoxicity in OAC, indicating that specific markers such as IL-8 could potentially serve as indicators of disease severity. Moreover, they may shed light on the mechanisms underlying the increase in lymphocyte MN% as the histology of the disease progresses. Understanding these mechanisms could help shed light on the link between inflammatory processes and genotoxic events such as MN formation, providing valuable insights for clinical practice.

STING inhibition and MN induction

Finally, an *in vitro* experiment was undertaken to elucidate the correlation between MN% and the cGAS-sting pathway. By inhibiting the STING complex, the goal was to reduce the inflammatory response following IR-induced DNA damage, with the induction of MN used to assess this effect. IR was chosen here as a potent inducer of DNA damage and MN. Before continuing with the inhibitor experiment, firstly TK6 cells were treated with cGAMP and exposed to IR. Subsequently, RNA extraction was performed, followed by a qPCR array encompassing various cGAS-STING related genes to validate pathway activation in this cellular context. This dataset both confirmed activation of this pathway in TK6 cells and informed the selection of cytokine ELISAs to investigate cytokine expression in plasma, particularly the upregulation of IFNβ and IFNα (as discussed above).

Given the limited literature on the STING inhibitor H-151 at the time of the experiment, two distinct timepoints were chosen for pre-radiation cell incubation with H-151: 6 and 2 hours. Prior to experimental use, an optimal concentration was also chosen. This work was designed by myself, however carried out by BSc student Sonja

Collaku. It was decided that the highest concentration before any cytotoxicity was observed would be chosen for experimental use.

Following IR, several endpoints were assessed: MN%, RPD, and cell cycle phase. Treatment with a STING inhibitor after irradiation showed no alteration in cell cycle progression or cell division relative to the vehicle control as measured by RPD. A decrease in MN frequency was noted in the cells treated with H-151 for 6 hours prior to IR. While this suggests that the cGAS-STING pathway activation may promote MN formation, potentially through driving inflammation, Basit *et al*. (2020) demonstrated the contrary. In HeLa and U2OS cancer cells, they saw that knockdown of STING, TBK1, or IRF3 actually elevated micronuclei formation[263]. This effect was thought to be due to the decreased p21 levels in knockdown cells which accelerated the G2/M transition and micronuclei formation. While not directly assessing the correlation between cGAS-STING activation and MN levels due to inflammation, Mackenzie *et al*. (2017) noted the inflammatory response triggered by cytoplasmic MN detection. They observed elevated cGAS-STING activation in diseases with high MN levels, such as RNase H2 deficiency or Aicardi-Goutières syndrome, suggesting a role in inflammation[67]. A recent study by Sato *et al*., (2024) reported that the accumulation of cGAS in MN during mitosis does not invariably translate to its activation or downstream STING signalling in the subsequent interphase[264] and that despite the potential of MN to activate the cGAS-STING pathway, this phenomenon is governed by intricate regulatory mechanisms[264]. These include chromatin compaction and nucleosome binding, which can hinder cGAS activation, mitotic phosphorylation of cGAS, preventing its activation during mitosis, and the rapid degradation of cytosolic DNA originating from ruptured MN, thereby restricting cGAS activation[264].

In future studies, it's essential to refine experimental procedures to gain a thorough understanding of how MN interact with the cGAS-STING pathway. As stated in Sato *et al*., (2024) employing a reporter system to investigate whether micronuclei effectively activate cGAS is pivotal[264]. This system should enable the real-time observation of MN formation, cGAS localization, and STING activation in living cells.

Exploring additional biomarkers post-treatment, such as upregulation of type 1 interferons and pro-inflammatory cytokines may provide deeper insights into the relationship between CGAS-STING activation and MN formation.

To conclude, this study further elucidated the relationship between plasma, inflammation, and MN% in lymphocytes, particularly in patients with GORD, BO, and OAC. The TK6 cells exposed to patient plasma revealed varied effects on MN induction, suggesting a link between plasma factors associated with inflammation and genotoxicity, as well as hinting at potentially protective effects in some patients whereby the fold change was reduced compared to the untreated control. Elevated 2'3'-cGAMP levels correlated positively with lymphocyte MN%, indicating a potential relationship between cGAS activation and individual genome instability displaying as lymphocyte MN%. Additionally, IL-8 showed a moderate correlation with MN%, suggesting its potential as an inflammation marker in disease severity. *In vitro* experiments indicated a possible role of cGAS-STING pathway activation in promoting MN formation however as an initial study, optimisation is required. These findings highlight the complexity of inflammation-driven genotoxic events in MN production, offering insights for further research and potential clinical applications.

Chapter 7 - General Discussion

7.1. DNA damage in circulating blood cells as biomarkers in OAC

During this PhD, two different tests were used to measure DNA damage. Lymphocyte MN% was used to measure chromosomal damage/loss, while the *PIG-A* mutation assay allowed the analysis of mutations expressed phenotypically in red blood cells. Lymphocyte MN% was used here in challenge assays and mechanistic work as they are nucleated cells with the ability to culture *in vitro*.

7.1.1 Baseline levels and cancer risk

MN%

In **Chapter 3**, the relationship between histological conditions and blood-based markers of DNA damage was examined, revealing a significant increase in lymphocyte MN% amongst patients with OAC compared to those with GORD, BO, and HVs, even when adjusting for age as a covariate. This same relationship was observed in a larger cohort of 155 individuals (**Figure 7.1**). The larger cohort consists of the patients included in **Chapter 3**, as well as patients previously recruited to the study (prior to September 2020). A similar trend was observed in the baseline MN% of the smaller cohort utilised for the challenge assay in **Chapter 5**, although the statistical significance between GORD patients and those with BO and OAC was not observed. This was likely due to the smaller cohort size reducing the statistical power of the analysis.

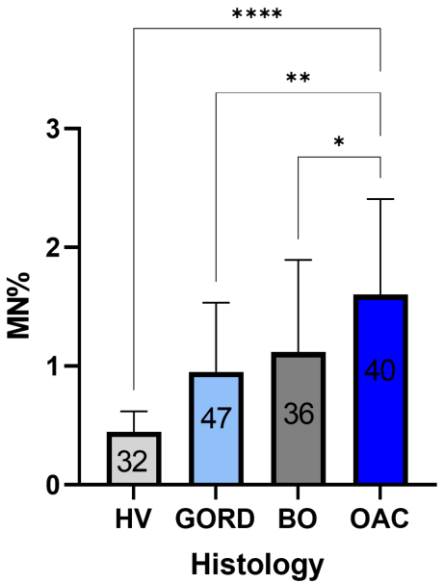

Figure 7.1. *Micronucleus frequency (MN%) of different histology groups attending endoscopy (Gastroesophageal reflux disease – GORD, Barrett's Oesophagus- BO, and oesophageal adenocarcinoma – OAC) and healthy volunteers (HV). This larger cohort includes individuals included in this study (n=108), as well as those recruited prior to September 2020 (n=47). Patient numbers displayed on the graph. P<0.05*<0.01**<0.001****. .(Error bars show SD).*

In **Chapters 3** and **4**, baseline MN% levels were correlated with progression of BO patients, and prognosis of OAC patients. Among cancer patients recruited from oncology clinics, the results revealed a slightly increased baseline MN% in patients deceased at the time of follow up, although this lacked statistical significance (p=0.72). The follow-up of BO patients, who were initially recruited from endoscopy as early as 2016, showed a slightly increased average MN% in those whose condition had worsened (e.g. increase in segment, change is dysplasia levels, or progression to cancer). However, this increase was not statistically significant, likely due to the limited number of patients who experienced disease progression and for whom data was available, making a meaningful comparison difficult. A higher average baseline MN% was also observed in BO patients who went on to develop different cancers, not including OAC, suggesting the broad use of MN% as a biomarker for health and biomonitoring. The results here align with published literature, where research has

extensively explored the correlation between lymphocyte MN frequency and the risk of cancer. Many studies indicate that an increased MN% in lymphocytes could be used as a biomarker of cancer due to the increased genomic instability and chromosomal damage associated with the disease ([59, 69, 73, 222, 265]). A significant study by Bonassi *et al.,* (2007) highlighted the utility of MN in lymphocytes as a marker of cancer incidence and risk. This extensive international study involved over 6700 individuals demonstrated that those with elevated baseline MN% in their lymphocytes had a notably heightened risk of developing cancer decades later and experienced poorer survival rates compared to those with lower MN%. This correlation held true across diverse geographical locations and various types of cancers, including those affecting the gastrointestinal system[68]. Increased lymphocyte MN% can be indicative of genetic damage resulting from various factors such as exposure to chemicals, radiation, genetic deficiencies in DNA repair mechanisms, or deficiencies in essential micronutrients required for DNA maintenance[69]. Initially uncommon (0-2 per 1000 cells at birth[69]), MN% in lymphocytes tends to increase with age, which is a very important risk factor for cancer[105]. While the strength of evidence supporting this association varies among different types of cancer, ongoing studies must investigate the potential of lymphocyte MN% as a predictive biomarker for assessing cancer risk through understanding the mechanisms underlying MN formation and their implications in inflammation and cancer.

As well as MN, NPB, and NBUDs are prevalent biomarkers associated with chromosome instability, and commonly observed in cancer cells. NPBs are particularly noteworthy for initiating breakage-fusion-bridge cycles, where chromosomes repeatedly break and fuse, exacerbating chromosomal instability and promoting cancer progression. Fenech *et al*. (2011) identified a gap in the literature regarding whether NPBs could offer greater predictive value for chromosomal instability and cancer risk compared to MN or NBUDs[266]. Research by Podrimaj-Bytyqi *et al.,* (2018) demonstrated elevated levels of MN, NPBs, and NBUDs in lymphocytes of patients with urothelial cell carcinoma compared to non-cancer patients[267]. Similarly, Dhillion *et al.,* (2023) observed increased NPBs in lymphocytes of individuals with prostate cancer[268]. Combining MN measurements with other markers like NPBs could enhance understanding of molecular mechanisms underlying DNA repair and mitotic processes, leading to genomic instability. As noted by Bonassi *et al.,* (2011),

combining these findings with mechanistic evidence supports the potential use of MN frequency in cancer screening programmes. However, to fully realise this potential, challenges such as interindividual variability and the implementation of preventive strategies in high-risk groups need to be further addressed[73].

PIG-A

Multiple studies across different cancer types and carcinogen exposures have demonstrated the potential of using the *PIG-A* mutant frequency as a biomarker for detecting cancer, monitoring treatment effects, and predicting carcinogen exposure risk ([23, 75, 146, 147, 173]). **Chapter 3** revealed that patients with OAC exhibited higher levels of *PIG-A* mutants compared to HV, GORD and BO patients. These findings reflected those reported by Haboubi *et al.,* 2020, who studied a larger cohort undergoing endoscopy (n=329) and found significantly higher *PIG-A* MFs in OAC patients compared to HVs and individuals with GORD and ND BO. Long-term monitoring of BO patients (outlined in **Chapter 3**) indicated that *PIG-A* MF might serve as a more sensitive marker for predicting the risk of progression of disease (specifically worsening BO or OAC developing) rather than predicting overall cancer risk. Notably, BO patients whose disease progressed, showed on average higher baseline *PIG-A* MF levels, although this trend did not reach statistical significance (p=0.25), likely due to the relatively small sample size included in the analysis. It is mentioned in Haboubi *et al.,* 2020 that the increase in MF could be due to an increased susceptibility to mutation in general, in those who develop cancer. However, a study by Meuser *et al.,* (2024) shed light on the potential role of *PIG-A* mutations and cancer, and the potential role it could play in progression from pre-malignant conditions in Gl cancers. This study focused on understanding the pathogenesis of duodenal tumours in familial adenomatous polyposis (FAP) and MUTYH-associated polyposis (MAP) syndromes. Researchers identified recurrent somatic mutations in the *PIG-A* gene in 27% of duodenal adenomas from FAP and MAP patients, alongside established driver mutations in APC and KRAS genes[269]. These findings suggest that *PIG-A* mutations and subsequent loss of GPI anchors could offer new insights into and potential interventions for duodenal tumorigenesis in patients with FAP and MAP syndromes. Understanding the increase in *PIG-A* mutants in patients with OAC could identify its potential pathogenic mechanisms which would aid in early detection and surveillance of BO patients. To comprehensively evaluate the potential of the *PIG-A* assay as a

biomarker for cancer risk assessment overall, large-scale population-based studies akin to those conducted for lymphocyte MN by Bonassi *et al., (*2007), are necessary. Such studies would be easier to carry out due to the small volume of blood required, and more rapid nature of this assay. They could help provide robust evidence on the utility of the *PIG-A* assay in biomonitoring and assessing cancer risk in clinical settings.

7.1.2 Predictors of treatment response

DNA damage following anti-cancer therapy is to be expected due to the cytotoxic and genotoxic nature of chemo and radiotherapy. The ability of the lymphocyte CBMN assay to predict radiotherapy response remains uncertain, with various studies reporting conflicting findings ([164, 166, 167, 170, 171, 180, 247]). **Chapter 4** assessed MN% induction (through fold change) in those undergoing chemoradiotherapy and attempted to assess any relationship between this induction and overall response to therapy. In this small cohort MN% fold change was significantly higher post treatment compared to the pre-treatment sample for both time points (Cycles 1-3 and Cycles 4-6). Differences in MN induction among lymphocytes are observed across treatment response groups, particularly in patients with partial or complete response, although not reaching statistical significance. **Chapter 4** also examined the efficacy of *PIG-A* MF as a marker for cancer therapy response. Although chemoradiotherapy increased *PIG-A* MF, overall, no clear correlation emerged between mutation induction and treatment response. The effect of chemotherapy on *PIG-A* MF has previously been considered. Dobrovolsky's studies, particularly his 2011 publication, showed minimal MF changes during and after therapy, except for one patient on Cisplatin and Etoposide, who experienced a threefold increase[75]. Another study in 2024 indicated increased mutations in blood cells during cisplatin treatment for head and neck cancer[173], potentially suggesting therapy-related induction rather than predicting inherent risk. A broader cohort encompassing diverse regimens and cancer types would enhance understanding of both MN% and *PIG-A* MF's clinical relevance in therapy response assessment. Overall, these findings collectively suggest that lymphocyte MN% could serve as a valuable biomarker for monitoring disease progression and treatment response in the context of OAC, however more data is needed to properly validate this.

7.1.3 Clinical utility of the assays

Combining DNA damage markers like *PIG-A* MF and lymphocyte MN% offers promising clinical utility due to their affordability, ease of use, and minimally invasive nature. The weak correlation of the two endpoints as demonstrated in **Chapter 3** implies that simultaneous evaluation of both markers could provide a more comprehensive understanding of DNA damage and repair dynamics in biological systems. These assays require just 10ml of peripheral whole blood (with less than 100µl utilised in the *PIG-A* assay), enabling repeat testing and wider acceptability. Increasing study numbers to understand mechanisms behind the increase of those at risk would provide a better understanding of test sensitivity and specificity. Although the CBMN assay for measuring MN is cost-effective, its labour-intensive nature may limit clinical feasibility, suggesting the need for alternative approaches like flow cytometry, automated imaging, and FISH, which offer quicker and less subjective manual MN scoring. Another technique commonly used to measure DNA damage is the buccal cell MN assay. This assay involves measuring MN in exfoliated cells from the inside of the cheek. MN and NBs can be detected in these cells which have large nucleus-to-cytoplasm ratios and distinct staining properties (**Figure 7.2**) [270].

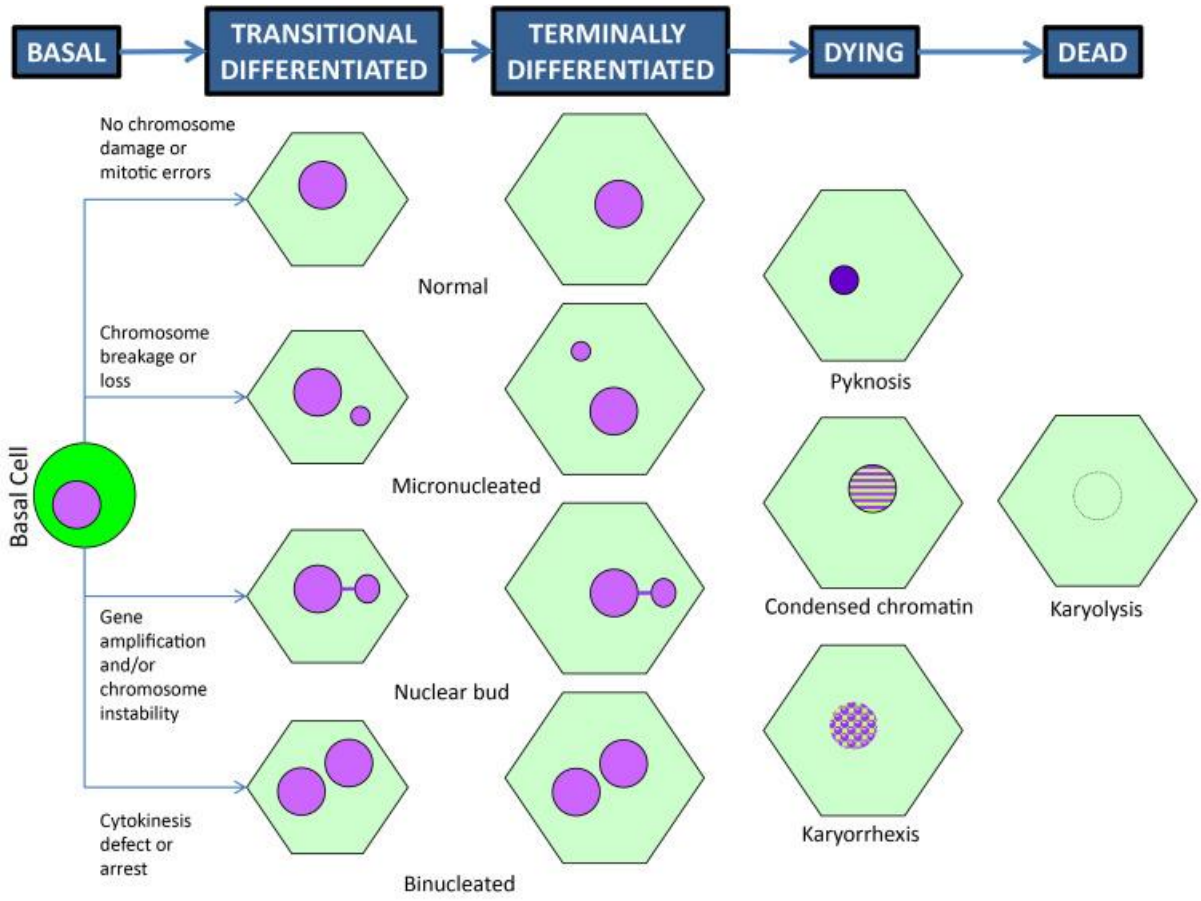

Figure 7.2. *Buccal micronucleus model. Schematic illustration of different buccal cell types and the processes leading to their formation. Diagram taken directly from Bolognesi et al., (2013)*[270].

The buccal cell MN assay could provide a useful non-invasive alternative for assessing genotoxic effects, which could be particularly advantageous in large-scale epidemiological studies or for monitoring populations at risk of disease[270]. There is some evidence of correlation between buccal cell and lymphocyte MN levels, however the relationship is not consistently strong across all studies. Ceppi *et al.,* (2010) found a strong positive correlation between MN in lymphocytes and buccal cells, with their results showing an increase in the frequency of MN in both peripheral lymphocytes and buccal cells among groups exposed to genotoxins or with various diseases[271]. A study by Burgaz *et al.,* (2011) saw significantly higher levels of MN in both buccal and lymphocytes of head and neck cancer patients compared to HVs. Whilst they saw a correlation between buccal cell and lymphocyte MN in HVs, this was not reflected in the cancer group[272]. Comparison studies would be required to assess the utility of

this assay for those at risk of OAC. Furthermore, when considering which assay to use, the nature of the exposure being investigated should be taken into account. For example, intravenous chemotherapy may increase lymphocyte MN% without necessarily affecting buccal cell MN%, whereas smoking and ingestion of genotoxins could have a different impact.

In terms of the *PIG-A* assay, whilst it is rapid and cost effective, it indirectly detects mutations by assessing the presence or absence of the GPI anchor, primarily capturing mutations causing phenotypic changes. However, it is unable to detect silent mutations and those affecting non-coding regions or altering bases but not amino acids. Gene sequencing for comprehensive mutation analysis could be beneficial in the context of increased risk of progression to OAC and early detection.

7.2. Adaptive Responses, Oxidative Stress and Lymphocyte MN

The connection between oxidative stress and DNA damage has been extensively documented, with heightened levels of oxidative DNA damage observed across various medical conditions including GORD, BO, and OAC[273]. DCA, strongly implicated in the disease progression, stimulates ROS production increasing DNA damage and activation of oncogenic pathways, such as the excessive activation of the mitogen-activated protein kinase (MAPK) pathway[274]. This can contributes to tumour development through various mechanisms, including the suppression of tumour suppressor genes and activation of anti-apoptotic proteins through genetic mutations[273]. Oxidative stress serves as a catalyst for widespread DNA damage, while interventions involving antioxidants such as vitamin E and N-acetylcysteine (NAC) have shown potential in mitigating such damage[273, 275].

Chapter 5 investigated the mechanisms behind the increase in lymphocyte MN%, focussing on the response of lymphocytes to two pro-oxidant DNA damage inducers (H_2O_2 and DCA) and the aneugen vinblastine. While lymphocyte treatment with vinblastine indicated heightened susceptibility to whole chromosome loss preferentially among cancer patients, there was no correlation between overall susceptibility and baseline MN%. However, an interesting trend emerged following treatment with pro-oxidant DNA damage inducers. A negative correlation was observed between the induction of MN% post-H_2O_2 and DCA treatment and higher baseline MN%. The measurement of 8-OHdG in plasma of the challenge assay cohort

also saw a moderate correlation with MN%, suggesting that in some patients, oxidative stress is strongly linked to the increase in DNA damage. These findings together suggested a potential adaptive response in these patients. GSH, an antioxidant and H_2O_2 scavenger was measured in the plasma of these patients, with a weak correlation observed with lymphocyte MN%, further underscoring the complex interplay between oxidative stress, DNA damage, and cellular responses. The concept of adaptive responses, wherein cells exposed to chronic low doses of genotoxic agents develop decreased sensitivity/increased resistance to subsequent or higher doses[276] has been considered in PBLs previously. Occupational exposure, for example to ionising radiation, X-rays, or gamma rays, has been shown to induce an adaptive response in lymphocytes[277],[278],[214]. Olivieri *et al*. (1984) first reported a reduced frequency of chromosomal aberrations after a high dose in cells pre-treated with very low (priming) doses of ionising radiation. Lymphocytes circulate throughout the body, encountering inflammatory tumour environments (in the case of cancer patients), circulating ROS released from tissues due to pro-oxidant exposures e.g. refluxed chemicals like DCA into the oesophagus, and tissue generated ROS such as H_2O_2. Exposure to low levels of DCA and ROS may increase MN in lymphocytes and subsequently induce an adaptive response, reducing the susceptibility of lymphocytes when challenged *in vitro*. The lack of statistical significance between fold change of challenged lymphocytes from histology groups suggests that this adaptation could be seen in not only cancer patients, but also in those with chronic inflammation, such as GORD and BO. This is further supported by the lack of statistical significance between baseline levels of MN% in this specific cohort. Whilst this effect is seen in lymphocytes, the opposite has been shown in oesophageal mucosa. A study by Jiménez *et al*. (2005) stated that reduced SOD antioxidant activity (resulting in higher mucosal levels of superoxide anion and peroxynitrite radicals), may contribute to the development of oesophageal damage and BO in patients with GORD[279]. It suggests that administering SODs may be a therapeutic option for treating patients with oesophagitis and BO. Exploring whether the antioxidant capacity of the mucosa, as reflected by lymphocytes and blood plasma, correlates with the development of oesophageal damage and Barrett's oesophagus warrants further investigation

7.3 The Importance of Plasma and Its Effect on Micronuclei

Inflammation and plasma

Chronic inflammation is a well-known contributor to the development and progression of cancer. Inflammation can cause DNA damage by generating RONS, which harm DNA. Conversely, DNA damage can worsen inflammation, forming a cycle regulated by DNA repair pathways and cellular signals. When these processes are disrupted, it can lead to uncontrolled cell growth and potentially cancer[280] (**Figure 7.3**).

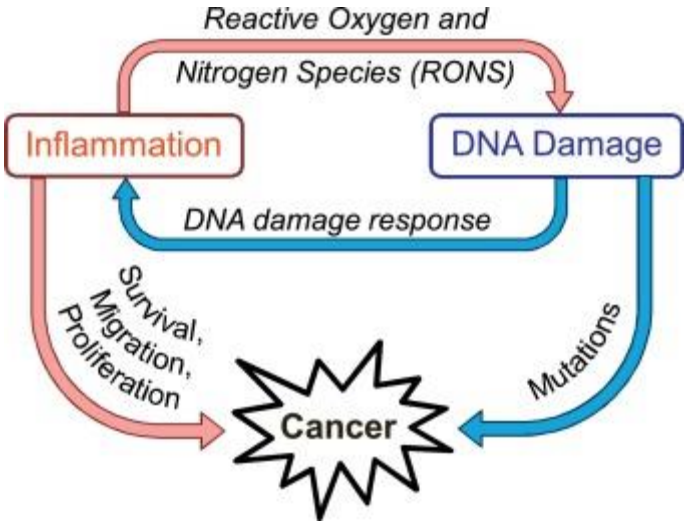

Figure 7.3. *The relationship between inflammation and DNA damage. Image taken from Kay et al., (2019)[280]*

Lymphocytes continuously circulate between the blood and lymphatic system[281],with plasma making up around 55% of blood[225]. Plasma contains a diverse array of proteins and other biomolecules that can serve as potential biomarkers for cancer detection and monitoring. For example, proteins secreted or shed by tumour cells into the bloodstream can act as cancer biomarkers, e.g. prostate specific antigen (PSA) for prostate cancer, or CA-125 for ovarian cancer. Markers of inflammation are also of interest in terms of predicting risk of cancer, with results varying vastly depending on the cancer type. For example, whilst Bao *et al.,* (2013) found no significant association between circulating CRP, IL-6, and TNF-αR2 levels and pancreatic cancer risk[282], several studies have observed a link between plasma inflammatory markers and colorectal cancer risk. Urbiola-Salvador *et al.,* (2023) identified changes in 202 plasma proteins (including IFN- γ and IL-32) that related to CRC pathogenesis and cancer-related inflammation[283]. These included levels. Jimi Kim *et al.,* (2020) highlighted elevated levels of sTNFR-2, IL-8, and IGF-1 as CRC risk factors, suggesting that they were modifiable by lifestyle factors[284]. Su *et al.,* (2023) measured higher plasma concentrations of multiple inflammatory biomarkers in CRC and adenoma patients

including TNF-α, indicating potential in early detection and risk assessment[285]. Plasma was used throughout this thesis primarily to understand the relationship it has with the increased MN% of lymphocytes in those with cancer, whilst also comparing levels in different patient groups. Whilst in **Chapter 5** plasma was used to test oxidative DNA damage, in **Chapter 6** several markers of inflammation were tested. These levels were then correlated with MN% of the corresponding patient lymphocyte. Increased levels of IL-8 and IL-6 were observed in individuals with OAC compared to healthy controls. Both of these cytokines are associated with poor prognosis in OSCC[286]; however, there is a lack of documentation in the literature regarding levels in OAC and how they may correspond with progression or prognosis. IL-6 and IL-8 are key cytokines involved in the body's inflammatory response and are linked to oxidative stress[191]. IL-6 regulates inflammation and immune responses through IL-6R-mediated signalling, promoting both pro-inflammatory effects via trans-signalling (with sIL-6R) and anti-inflammatory responses[287]. IL-8 (CXCL8) attracts neutrophils and supports inflammation, influencing monocyte-macrophage functions and angiogenesis through CXCR1/2 receptors, contributing to oxidative stress pathways in various physiological and pathological contexts[287]. Increased levels of both have been seen in different cancer physiologies including CRC. The relationship observed here could suggest that IL-8 levels and their corresponding lymphocyte MN% in lymphocytes could be used as a marker of inflammation and increased risk of OAC development.

Exposure of TK6 cells to patient plasma

The overall ability of plasma to induce DNA damage in cells was tested *in vitro* in **Chapter 6** through the exposure of the lymphoblastoid cell line TK6 to supplemented media made up of 10% patient plasma. The results showed both a significant increase in induction in TK6 cells (measured by fold change) in patients with OAC compared to HV, and a moderate positive correlation between MN induction and baseline MN of lymphocytes. The potentially genotoxic effect of plasma was also shown when treating the OAC cell line OE33 in a study by Naser *et al*. (2024). They treated OE33 cells with plasma from those with GORD, BO, OAC and HVs. They observed that while the average increase in MN% was only slightly higher in cells treated with plasma from cancer patients, there was a notable decrease in the proportion of cells in the S phase following direct plasma treatment at 24 hours[288]. Maffei *et al*. (2011) also saw the

ability of plasma from CRC patients, polyp patients, and those with positive faecal occult blood tests to damage lymphocyte DNA, as measured by MN induction[252].

As well as inducing DNA damage, some individual's plasma appeared to have a protective effect on the TK6 cells, with a reduction observed in fold-change compared to the untreated control. One potential explanation for this could stem from the overall lifestyle of the individual, including their diet and exposures. As shown in **Chapter 3**, diet appears to have an overall effect on the MN% of endoscopy patients, with higher dietary quality corresponding with lower MN%. This finding was supported by Fenech *et al*, (1994), whereby the relationship between micronuclei frequencies and levels of vitamins (C, E, B12, folic acid) in plasma or serum was investigated[232], showing an inverse relationship. BMI was found to minimally affect lymphocyte MN% in obese individuals, albeit non-significantly. Obesity-associated inflammation has also previously been demonstrated elevated levels of inflammatory markers such as CRP, IL-6, and TNF-α in plasma[289, 290]. These findings could suggest that both dietary factors, such as increased antioxidants, and inflammatory status in plasma may play crucial roles in modulating DNA damage and protective mechanisms observed in TK6 cells when exposed to individual plasma samples.

7.3.2 Inflammation and cGAS-STING and MN

Exploring the relationship between cGAS-STING and MN *ex vivo*

The cGAS-STING pathway plays a crucial role in cancer development and response to therapy through its ability to detect cytosolic DNA, including DNA from ruptured micronuclei. cGAMP can move through gap junctions in epithelial cells and via viral particles, signalling nearby cells and causing STING activation in neighbouring non-cancer cells[256]. While cGAMP cannot passively cross cell membranes, it is exported and degraded by the extracellular enzyme ENPP1, present in both membrane-bound and soluble forms in serum. Additionally, its extracellular presence is facilitated by transport through SLC19A1[256]. The investigation into the cGAS-STING pathway in **Chapter 6** found elevated levels of 2'3'-cGAMP, IFNβ, and IL-6 in the plasma of cancer patients compared to healthy volunteers, all key components of the cGAS-STING pathway. Correlation analysis revealed a link between plasma cGAMP levels and MN% in lymphocytes, suggestive of a link to MN formation. Currently, literature lacks studies specifically measuring cGAMP in patient plasma, focussing instead on

intracellular and extracellular levels in cancer and ENPP1 activity. Li *et al.,* (2021) demonstrated in a mouse colon cancer model, that exogenous cGAMP inhibits tumour growth dose-dependently. They showed increased ENPP1 activity promoted metastasis by degrading extracellular cGAMP, suppressing immune responses. ENPP1 loss reduced metastasis, and improved response to immune therapies via cGAS and STING, whilst ENPP1 overexpression increased metastasis and immunotherapy resistance[291]. Carozza *et al.,* (2020) reported that depleting extracellular cGAMP in mouse tumours decreased tumour-associated immune cells and negated the curative effects of IR. They saw that boosting extracellular cGAMP with ENPP1 inhibitors synergizes with IR to delay tumour growth[256]. Whilst the data shown in **Chapter 6** indicated elevated levels of 2'3'-cGAMP and other key components of the cGAS-STING pathway in the plasma of cancer patients, suggesting a potential association with MN formation, it is important to recognise that these findings do not directly imply poor outcomes or increased risk of cancer. The literature presents a complex picture of cGAMP's role, with studies highlighting its potential to inhibit tumour growth and enhance therapy efficacy when modulated. Therefore, further research is needed to elucidate how plasma cGAMP levels relate to specific aspects of cancer progression and treatment response, and to better understand the contextual factors influencing these relationships.

Exploring the relationship between cGAS-sting and MN *in vitro*

The moderate correlation of cGAMP with lymphocyte MN raised the possibility that activation of this pathway in tumour or oesophageal tissue, followed by measurement in plasma, reflects the state of genomic instability in these tissues. These findings also led to a hypothesis: MN activate the cGAS-STING pathway, leading to the upregulation of proinflammatory cytokines and type one interferons. This triggers an inflammatory response, whereby more MN are potentially induced due to increased inflammation driven oxidative stress. To test this hypothesis an *in vitro* experiment was designed. Following confirmation that this pathway can be successfully activated in TK6 cells using a PCR array, cells were treated with a STING inhibitor prior to IR to induce MN. Following this, it appeared that the MN% was slightly reduced in the cells treated with the STING inhibitor. This feedback loop suggests the cGAS-STING pathway's role in linking DNA damage and inflammatory responses, suggesting that it could be a crucial mediator of genomic instability in cancer. Whilst this was an

interesting finding for a preliminary experiment, there are lots of conflicting reports in the literature about the ability of MN to activate this pathway([66, 67, 263, 264, 292]), and further experiments should be designed to explore this in patient lymphocytes.

7.4. Confounding factors and study limitations

Despite the significant findings reported here, several confounding factors and limitations must be acknowledged. These include sample size limitations, preanalytical and analytical factors affecting cytokine quantification, and the complexity of interactions between various biomarkers and physiological processes. Additionally, the study's observational nature precludes causal inference, necessitating further experimental validation and replication.

Patient recruitment

Throughout all four data chapters, a central discussion point is the factors limiting this study. Patient recruitment posed several challenges, primarily managed independently with initial support from Dr. Rachel Lawrence in the first year and later assistance from Dr. Hamsa Naser towards the end. Beginning my PhD in September 2020, COVID-19 remained a concern, impacting patient willingness to engage with the study and complicating blood sample acquisition due to lengthy endoscopy lists. Managing oncology patient follow-ups independently further complicated matters, relying on patient-initiated contact to minimise additional blood draws. Recruitment pauses also occurred due to required ethics updates and changes in study leadership in both endoscopy and oncology departments, reducing the overall number of samples that could contribute to this study. Additionally, the volume of blood required occasionally posed challenges, especially for CBMN experiments which required larger quantities. The variation in PBMCs isolated from different volumes of blood meant that for one single patient, that not all biological endpoints of interest could be analysed and repeated experimental use was not a possibility.

Future involvement of a research nurse or clinician could enhance workflow and sample viability, while increased patient recruitment would improve statistical power and enable firmer conclusions to be drawn.

Biological variations

Biological variations in the cohort such as age, diet, BMI, gender, medications, and occupational or environmental exposures exert significant influence on the DNA damage endpoints measured in blood in this study. The questionnaire distributed to endoscopy and cancer patients aimed to account for these factors, but challenges in data collection emerged, including incomplete responses due to participant reluctance and uncertainties related to occupational exposures. Relying solely on self-reported data also has limitations, especially since healthy volunteers did not undergo comprehensive questionnaire assessments. Notably, when analysing overall markers like MN or *PIG-A* and considering demographic or lifestyle factors, cancer patients emerged as a significant confounder and were consequently excluded from the analysis. Moreover, ensuring the health status of volunteers labelled as "healthy" remained crucial for accurate interpretation of study outcomes.

Limitations in experiments

Various challenges arose in terms of experimental work. Contamination presented issues and resulted in the loss of precious patient samples. Equipment breakdowns raised problems. For instance, there was a significant setback when the flow cytometer used to measure *PIG-A* MF stopped working, leaving us unable to proceed with measurements for several weeks

7.5 Future work

To advance our understanding of the increased MN% in lymphocytes and its implications for OAC progression, several avenues of future research can be explored. The addition of the buccal cell MN assay, as well as the measurement of NPBs in lymphocytes could provide further information regarding exposures, underlying genetic instability or mechanisms behind increase MN in these non-cancerous cells.

Conducting comparative studies between MN% in lymphocytes and oesophageal cell MN would be pivotal. Establishing this correlation could provide deeper insights into how systemic DNA damage manifests locally in the oesophagus. Culturing organoids derived from BO and OAC patients represents one approach. These 3D cell models better mimic the complex tissue architecture and cellular interactions found in vivo[293]. Organoids can be exposed to DNA-damaging agents such as radiation or genotoxic

chemicals to simulate environmental stresses and therapeutic treatments. By monitoring DNA repair pathways and genomic stability in these organoids, variations in DNA repair efficiency across normal, precancerous, and cancerous tissues could be measured. This could help elucidate how systemic DNA damage reflected in lymphocytes correlates with tissue-specific responses in the oesophagus.

Additionally, exploring somatic mutations in the *PIG-A* gene in oesophageal tissue could offer an insight into the increased *PIG-A* MF in the erythrocytes of those with OAC.

Moreover, investigating plasma constituents that modulate MN% in lymphocytes using mass spectrometry is crucial. Identifying genotoxic metabolites and inflammatory markers in patient plasma could help uncover mechanisms driving increased MN formation. For instance, assessing the role of ROS and specific inflammatory cytokines linked to OAC could clarify their contribution to DNA damage and carcinogenesis, as well as potentially guide the development of new therapies.

In terms of treatment response, future research should focus on comparing predictive monitoring methods, with traditional imaging techniques in a larger cohort of patients, with varying histology and treatment plans. Understanding how changes in MN%/*PIG-A* MF correlate with treatment outcomes and disease progression could refine prognostic assessments and optimise therapeutic interventions. Investigating the interplay between the cGAS-STING pathway activation and MN% in response to treatment also warrants further work. This pathway plays a pivotal role in immune response and DNA damage sensing. Exploring its dynamics in relation to MN% fluctuations during treatment could unveil novel therapeutic targets or predictive markers for personalised medicine approaches in OAC.

7.6 Conclusions

This PhD studied blood-based markers of DNA damage including lymphocyte MN% and *PIG-A* MF in erythrocytes. The investigation into histology and blood-based markers of DNA damage revealed a significant increase in *PIG-A* MF and MN% among patients with OAC compared to those with GORD, BO, and HVs, suggesting a potential link between disease progression and DNA damage in circulating blood cells. The induction of MN in lymphocytes and *PIG-A* MF in erythrocytes in response

to chemo and radiotherapy was explored. Whilst both MN% and *PIG-A* MF were significantly induced during cancer therapy, due to the low number of patients it was not possibly to properly assess the relationship of DNA damage with tumour response. A particular emphasis was placed on developing a mechanistic understanding of the increased baseline lymphocyte MN% in association with patient histology. This was carried out using challenge assays to assess individual susceptibility, measuring markers of inflammation in patient plasma, and beginning to explore a relationship with the cGAS-STING pathway. Across multiple chapters, several key findings emerged from the research conducted. Some patients with elevated MN% showed an adaptive response when treated with pro-oxidant chemicals *in vitro,* suggesting the role of oxidative stress. Moreover, plasma markers such as 2'3'-cGAMP and IL-8 showed moderate positive correlations with lymphocyte MN%, indicating their potential utility as blood-based markers of DNA damage and inflammation. In the future, integrating insights from organoid cultures, genetic analyses, plasma biomarker profiling, and treatment response assessments holds promise for advancing our understanding of these biomarkers in OAC.

Appendices

Appendix I. Participant information sheet for recruitment of healthy adult volunteers.

Swansea University Prifysgol Abertawe | Medical School Ysgol Feddygaeth

PARTICIPANT INFORMATION SHEET

The Research Use of Blood from Healthy Adult Volunteers

Before consenting to take part in this research, please read the following information.

What is the purpose of the research?

Blood from healthy adult donors is required for many aspects of medical research. These include improving our understanding of how cells function in health so that we can better understand disease, and the development of new products (e.g. medical devices) and processes (e.g. early stage drug testing) for therapeutic use. Your blood might be used for one or more research projects.

Who is carrying out the research?

The study is organised and carried out by numerous research teams at Swansea University. University Medical School and is overseen by Professor Cathy Thornton. *This research has been given a favourable ethical opinion by Swansea University Medical School (SUSM) Research Ethics committee (RESC), project reference 2022-0029.*

What happens if I agree to take part?

After you read this participant information sheet, we will record your consent to taking part in this study. Taking part in this study first involves answering a few questions about yourself (e.g., your age and ethnicity) and your health (e.g., smoking and any chronic disease). If you agree to help with this research, we will take some blood from you. The amount of blood we take from you will depend on the research study/studies for which it is to be used but it is likely to be around 1 – 8 tablespoonfuls (8 tablespoonfuls is about 120mls or around one-third of a standard carbonated drink can). You might be asked to donate repeatedly to a study but the total volume collected from you will not exceed 500mls in the 6 months since you give your first donation. Sometimes a much smaller sample of around half a teaspoonful might be collected by fingerpick.

The blood samples will be studied in the laboratory to measure the molecules and cells that circulate in your blood and find out how these react in various tests. Many of these tests will relate to (a) better understanding of immunity, inflammation, and blood clotting, (b) the development of tests for disease markers, or (c) the development and testing of medical devices. We will not study your DNA. In some instances samples of your blood might be kept

for ten years and be used for other related tests in the future. Your sample might be used in more than one research study and we will give you some information about these studies verbally at the time of your donation

Your blood sample will be transferred to the research laboratories of Swansea University Medical School for analysis and storage. At the end of the study, we will dispose of any unused samples.

Are there any risks associated with taking part?

The research has been approved by Swansea University Medical School Research Ethics Committee. There are no significant risks associated with participation. Taking the blood sample from you may cause some minor discomfort and bruising can develop at the site. If you have or are at risk of having a blood borne disease please do not donate.

Data Protection and Confidentiality

Your data will be processed in accordance with the Data Protection Act 2018 and the General Data Protection Regulation 2016 (GDPR). All personal information collected will be kept strictly confidential. You will be assigned a unique study number that will be used to label your samples and link to the laboratory data gathered using your samples. The personal information provided by you will only be viewed by Professor Cathy Thornton and her immediate research team. All collected identifiable data will be destroyed on or before 28th February 2027 when it is no longer required for these research endeavours.

What will happen to the information I provide?

An analysis of the information you provide will form part of our report at the end of the study and may be presented to interested parties and published in scientific journals and related media. All information presented in any reports or publications will be anonymous and unidentifiable.

Is participation voluntary and what if I wish to later withdraw?

Your participation is entirely voluntary – you do not have to participate if you do not want to. If you decide to participate, but later wish to withdraw from the study, then you are free to withdraw at any time, without giving a reason and without penalty. You can withdraw by contacting immunuology-studies@swansea.ac.uk.

Data Protection Privacy Notice

The data controller for this project will be Swansea University. The University Data Protection Officer provides oversight of university activities involving the processing of personal data and can be contacted at the Vice Chancellor's Office.

Your personal data will be processed for the purposes outlined in this information sheet.

Standard ethical procedures will involve you providing your consent to participate in this study by completing the consent form that has been provided to you.

The legal basis that we will rely on to process your personal data will be processing is necessary for the performance of a task carried out in the public interest. This public interest justification is approved by Swansea University Medical School Research Ethics Committee.

The legal basis that we will rely on to process special categories of data will be processing is necessary for archiving purposes in the public interest, scientific or historical research purposes or statistical purposes.

How long will your information be held?

Any identifiable data held about you such as your name will be destroyed on or before 28th February 2027 when it is no longer required by the researchers. The laboratory data that we generate using your blood will be held for 10 years after the study has finished. This is so that we can ensure all our findings from the study are published in scientific journals and comply with the regulations relating to this.

What are your rights?

You have a right to access your personal information, to object to the processing of your personal information, to rectify, to erase, to restrict and to port your personal information. To safeguard your rights, we will use the minimum personally identifiable information possible. Please visit the University Data Protection webpages for further information in relation to your rights.

Any requests or objections should be made in writing to the University Data Protection Officer:

University Compliance Officer (FOI/DP)
Vice-Chancellor's Office
Swansea University
Singleton Park
Swansea
SA2 8PP
Email: dataprotection@swansea.ac.uk

How to make a complaint

If you are unhappy with the way in which your personal data has been processed, you may in the first instance contact the University Data Protection Officer using the contact details above.

If you remain dissatisfied, then you have the right to apply directly to the Information Commissioner for a decision. The Information Commissioner can be contacted at:

Information Commissioner's Office
Wycliffe House
Water Lane
Wilmslow
Cheshire
SK9 5AF
www.ico.org.uk

What if I have other questions?

If you have further questions about this study, please do not hesitate to contact us:

immunology-studies@swansea.ac.uk

Appendix II. Participant health questionnaire for recruitment of healthy adult volunteers.

Swansea University Prifysgol Abertawe | Medical School Ysgol Feddygaeth

HEALTH QUESTIONNAIRE

The Research Use of Blood from Healthy Adult Volunteers

This research has been given a favourable ethical opinion by Swansea University Medical School (SUSM) Research Ethics committee (RESC), project reference 2022-0029.

Participation Identification Number for this study: _____

Date of first participation:

DOB:

Age: **Sex (F/M):**

Height (cm): **Weight (kg):** **BMI:** _____

Ethnicity:

Smoking status:

Notes (Number/day? Reduced? When did you quit?):

Chronic conditions (asthma, hayfever, Chron's, depression etc):

Autoimmune disease?

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Current medication:

FEMALES ONLY:

Have you ever been pregnant (Y/N)? Y

If yes, how many times?

If yes, how long since your last pregnancy?

How many times have you given birth?

SAMPLE COLLECTION DATA

SAMPLE ONE

Date: _____/_____/_____

Volume of blood collected:_____

Intended users:_____

Have you had any vaccines in the last two weeks (Y/N)? N

If so, what was this? _____

Any signs or symptoms of infection in the past week (Y/N)? N

Was this – gastrointestinal / respiratory / skin / other (circle as appropriate)?

SAMPLE TWO

Date: _____/_____/_____

Volume of blood collected:_____

Intended users:_____

Have you had any vaccines in the last two weeks (Y/N)? _____

If so, what was this? _____

Any signs or symptoms of infection in the past week (Y/N)? _____

Was this – gastrointestinal / respiratory / skin / other (circle as appropriate)?

SAMPLE THREE

Date: _____ /_____ /_____

Volume of blood collected:_____

Intended users:_____

Have you had any vaccines in the last two weeks (Y/N)? _____

If so, what was this? _____

Any signs or symptoms of infection in the past week (Y/N)? _____

Was this – gastrointestinal / respiratory / skin / other (circle as appropriate)?

SAMPLE FOUR

Date: _____ /_____ /_____

Volume of blood collected:_____

Intended users:_____

Have you had any vaccines in the last two weeks (Y/N)? _____

If so, what was this? _____

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Any signs or symptoms of infection in the past week (Y/N)? _____

Was this – gastrointestinal / respiratory / skin / other (circle as appropriate)?

***** Add further sample numbers as needed *****

***** Note that consent must be renewed annually *****

Appendix III. Participant consent form for recruitment of healthy adult volunteers.

Swansea University Prifysgol Abertawe | Medical School Ysgol Feddygaeth

PARTICIPANT CONSENT FORM

The Research Use of Blood from Healthy Adult Volunteers

Research Ethics Number: *2022-0029*

Name of Researcher: Professor Cathy Thornton

Participation Identification Number for this study: HAB_____

The researcher requests your consent for participation in the following study. By giving consent you agree to complete the data collection form and donate blood by venesection or finger prick for use in various research studies at Swansea University Medical School.

Your data will be anonymised, and participation is entirely voluntary.

By submitting this form, you declare that you have read the participant information sheet, understand the nature and purpose of the study and that you agree to the terms described.

If you have any further questions, please contact immunology-studies@swansea.ac.uk

This research has been given a favourable ethical opinion by Swansea University Medical School (SUSM) Research Ethics committee (RESC), project reference 2022-0029.

Thank you.

	Please initial boxes
I confirm that I have read the participant information sheet for the above study. I have had the opportunity to consider the information. I have had the opportunity to ask the researcher any questions regarding the study and have had these answered satisfactorily.	
I understand that my participation is entirely voluntary.	
I grant permission for the data generated using the data collection form and from donated samples to be used in future publications.	
I agree to take part in the above study.	

Name of participant	**Signature**	**Date**
Name of researcher taking consent	**Signature**	**Date**

Appendix IV. Patient information sheet for recruitment from endoscopy. Study title: Studies in the molecular and lifestyle differences of patients with Gastroesophageal reflux disease and Barrett's oesophagus.

Endoscopy Department,
Singleton Hospital
Sketty Lane
Sketty
Swansea
SA2 8QA

Patient Information Sheet– 26th October, Version 5.0

Information Sheet for Research Participants

Study title:

Studies in the molecular and lifestyle differences of patients with Gastro Oesophageal Reflux Disease & Barrett's Oesophagus

Principal Investigator: Professor Gareth Jenkins

Chief Investigator: Dr Haboubi

You are being invited to take part in a research study which is intended towards forming part of a PhD qualification though Swansea University. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and members of hospital staff. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part in the study.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. If you decide not to take part or to withdraw at any other time without explanation, your future care will not be affected by your decision. Thank you for reading this.

What is the purpose of the study?

You have symptoms which may be due to Gastro-Oesophageal Reflux Disease which is a condition where food and liquid leak up from your stomach into your food-pipe/gullet. This can sometimes cause discomfort in the upper part of your stomach, but can also lead to a different condition called Barrett's Oesophagus, which is where there is inflammation in your food-pipe/gullet caused by the acid has refluxed up from your stomach. It appears that there are differences between male and female patients in the occurrence of this condition. We would like to find out if this difference between men and women is due to hormones, lifestyle differences, or bile acid differences between the sexes.

We plan to investigate this by:

a) Analysing your biopsy samples from your planned endoscopy
b) Asking you to fill out a questionnaire
c) Taking a blood test from the intravenous line which will be inserted as a matter of routine (should you request sedation) for the endoscopy procedure. This will be approximately 5mls (approx. 1 teaspoon).

Why have I been chosen?

You have symptoms suggestive of reflux disease and may have "Barrett's Oesophagus" on your endoscopy.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive in the future.

What do I have to do?

You will be approached and informed about the study while attending the Endoscopy Department for your follow-up endoscopy, OR while attending your follow-up outpatient appointment in the main outpatients department. The research doctor will:

- Ask you about your health in general, and any previous operations
- Explain the purpose of this study in further details and answer your questions

Taking part in this study will not affect the standard treatment that you receive.

You can opt out of the study at any stage.

What will happen to me if I take part?

If you decide to take part in this study you will be asked to sign the necessary consent form, then you will have:

1) A questionnaire which will take no longer than 15-20 minutes to complete, which will ask you about your diet, lifestyle and medications. It will also ask you to give your weight and height measurements.
2) Your planned endoscopy where biopsy samples will be taken as per routine as well as suctioning of stomach juices. Your doctor will need to take samples for him/her to decide if you need any treatment. We will take a couple more biopsies during the procedure, which will last about 30 seconds to a minute longer than normal and should not hurt. We will use them to see if there are any changes in the levels of the hormones or other changes in the cells.
3) A blood test will also be offered to you, which you can accept or refuse. We hope to take this blood from the intravenous line that will

be inserted for your sedation (if you wish to have sedation), thereby preventing you from having more than one needle-stick, and ensuring your comfort is maintained.

What are the possible disadvantages and risks of taking part?

There are no disadvantages or major risks associated with taking part. The endoscopy is a planned procedure, and all we will be taking is a few more samples of the tissue. Staff who are trained and experienced in the field will perform the endoscopy procedure.

What are the possible benefits of taking part?

There are no additional benefits to your care in taking part in this study.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Your name and address will be removed from the information when it is shown to other medical staff outside the study.

Who is organising the research?

This study is being organised by the staff of the endoscopy departments of ABM University NHS Trust and Swansea University.

Are expenses covered within this study?

There is no policy for reimbursement of financial expenses for participation in this study.

Who has reviewed the study?

The Research Ethics Committee of South West Wales has reviewed this study.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect, or about the way you have been treated during the course of this study then you should immediately inform Dr Hasan Haboubi or (see contact details below). The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the ABM NHS Trust University Joint Research Office.

ABMU Health Board is the sponsor for this study based in the United Kingdom. We will be using information from you in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. ABMU health board will keep identifiable information about you for x years.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information at URL or by contacting the chief/principle investigators named below.

The NHS will collect information from you for this research study in accordance with our instructions.

We will use your name, NHS number and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from ABMU health board and regulatory organisations may look at your medical and research records to check the accuracy of the research study. ABMU will pass these details to Swansea University along with the information collected from you. The only people in Swansea University who will have access to information that identifies you will be people who need to contact you to audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, hospital number or contact details.

ABMU health board will keep identifiable information about you from this study for x years.

Contact for Further Information

If you want more information, before or after you return your form, you can phone (9am-5pm Mon-Fri)

Dr Hasan Haboubi

Email: [redacted]

Professor Gareth Jenkins on [redacted]

The local Research Ethics Committee has approved the above statement.

Appendix V. Patient questionnaire. Study title: Studies in the molecular and lifestyle differences of patients with Gastroesophageal reflux disease and Barrett's oesophagus.

BARRETT'S OESOPHAGUS SYMPTOMS & LIFESTYLE SURVEY

This questionnaire will take approximately 10-15 mins to complete.

Some questions are personal, but we guarantee that all information will be treated with absolute confidentiality and will be used only for medical research.

To help us read your answers, please write as clearly as possible with a black pen and complete the questionnaire by circling the number corresponding to your answer or putting a cross in the appropriate box(es).

SECTION A:

DEMOGRAPHICS & PATIENT INFORMATION

ID Number: _____(PLEASE LEAVE BLANK)
To be completed by Research Office

Age: _____years old
Gender: **Male ☐ Female ☐**

Height (if known): _____
Weight (if known): _____
Waist Circumference (if known): _____

SECTION B:

YOUR VIEWS ABOUT YOUR ILLNESS

We are interested in your own personal views of how you now see your current illness.

For the following questions, please circle the number that best corresponds to your views:

Q1. How much does your illness affect your life?

0 1 2 3 4 5 6 7 8 9 10

No effect at all — Severely affects my life

Q2. How long do you think your illness will continue?

0 1 2 3 4 5 6 7 8 9 10

A very short time — Forever

Q3. How much control do you feel you have over your illness?

0 1 2 3 4 5 6 7 8 9 10

Absolutely no control — Severe level of control

Q4. How much do you think your treatment can help your illness?

0 1 2 3 4 5 6 7 8 9 10

Not at all — Easily treatable

Q5. How much do you experience symptoms from your illness?

0 1 2 3 4 5 6 7 8 9 10

No symptoms at all — Many severe symptoms

Q6. How well do you feel you understand your illness?

0 1 2 3 4 5 6 7 8 9 10

Don't understand at all — Fully Understand

Q7. How much does your illness affect you emotionally? (e.g. does it make you angry, scared, upset or depressed?)

0 1 2 3 4 5 6 7 8 9 10

No effect at all — Severely affects me emotionally

SECTION C:

CAUSES OF MY ILLNESS

We are interested in what you consider may have been the cause of your illness. As people are very different, there is no correct answer for this question.

Below is a list of possible causes for your illness. Please indicate how much you agree or disagree that they were causes for you by ticking the appropriate box.

POSSIBLE CAUSES	Strongly Disagree	Disagree	Neither Agree Nor Disagree	Agree	Strongly Agree
Stress or worry					
Overwork					
My emotional state e.g. feeling down, lonely, anxious, empty					
Ageing					
Accident or injury					
My personality					
Altered immunity					
My own behavior					
My mental attitude e.g. thinking about life negatively					
Diet or eating habits					
Chance or bad luck					
Alcohol					
Smoking					

In the table below, please list in rank-order the three most important factors that you now believe caused YOUR ILLNESS. You may use any of the items from the box above, or you may have additional ideas of your own.

The most important causes for me:

1. _____
2. _____
3. _____

SECTION D:

MEDICAL CARE.

We are interested in the type of healthcare you have received

1. please tick one answer for each question:

	Never	Almost Never	Some times	Fairly often	Always
When you **visit your doctor,** how often do you do the following					
Prepare a list of questions for your doctor					
Ask questions about the things you want to know and things you don't understand about your treatment					
Discuss any personal problems that may be related to your illness					

2. In the past 6 months, how many times did you visit your GP?
_____ visits

3. In the past 6 months, how many times did you attend hospital Gastroenterology Outpatients?
_____ visits

4. In the past 6 months, how many times did you go to a hospital emergency department?
_____ times

5. In the past 6 months, how many TIMES were you hospitalized for one night or longer? _____ times

5b. How many total NIGHTS did you spend in the hospital in the past 6 months? _____ nights

SECTION E:

REFLUX SYMPTOMS.

We are interested in the types of symptoms that you get, and their frequency.

1. Please tick one answer for each question.

Question	Number of days per week you get your symptoms			
	0 times per week	Once a week	2-3 times per week	4-7 times per week
Days per week with burning feeling behind the breastbone (heartburn)?				
Days per week with stomach contents moving up to the throat or mouth (regurgitation)?				
Days per week with pain in the middle of the upper stomach area?				
Days per week with nausea?				
Days per week with trouble getting a good night's sleep because of heartburn or regurgitation?				
Days per week with the need for over-the-counter medicine for heartburn or regurgitation (such as Rennie's/ Gaviscon/ Peptac), in addition to the medicine your doctor prescribed?				

2. You are prescribed an anti-acid medication (Omeprazole/Lansoprazole/Pantoprazole, etc.)

	Yes	No
Do you ever forget to take your medicine?		
Are you careless at times about taking your medicine?		
When you feel better do you sometimes stop taking your medicine?		
Sometimes if you feel worse when you take the medicine, do you stop taking it?		

SECTION F:

DIET.

We are interested in the type of foods you eat and how frequently you eat them.

Questions about your diet

1. Do you eat meat? **Yes ☐ No ☐**

If **Yes**, how many times a week do you eat meat?
_____times a week

If **No**, how old were you when you last ate meat?
_____years old

2. Do you eat any fish? **Yes ☐ No ☐**

If **Yes**, how many times a month do you eat the following?
(put '0' if less than once a month)

Fatty fish ____times a month (including sardines, salmon, mackerel, herring)

Other fish ____times a month (including Cod, tuna, haddock)

If **No**, how old were you when you last ate fish?
_____years old

3. Do you eat any dairy products? **Yes ☐ No ☐**
(including milk, cheese, butter, yoghurt)

If **No**, how old were you when you last ate dairy products?
_____years old

4. **Do you eat any eggs?** **Yes ☐ No ☐**
(including eggs in cakes and other baked foods)

If **Yes**, how many eggs do you eat each week?
_____eggs each week
Put "0" if eaten less than once a week

If **No**, how old were you when you last ate eggs?
_____years old

5. What type of milk do you use most often?

Full cream ☐
Semi-skimmed ☐
Skimmed/fat free ☐
None ☐
Soya milk fortified with calcium ☐
Soya milk not fortified with calcium ☐
Other ☐

How much milk do you drink each day, including milk with tea, coffee, cereals, etc.

Less than a quarter of a pint (<150ml) ☐
Quarter of a pint (150ml) ☐
Half a pint (300ml) ☐
Three quarters of a pint (450ml) ☐
One pint (600ml) ☐
More than one pint (>600ml) ☐

6. What type of spread do you use most often on bread, crispbreads, vegetables, etc.?

Butter	☐	Hard margarine (in wrapper not tub)	☐
Dairy spread e.g. Clover	☐	Soya margarine or other milk free margarine	☐
Low or reduced fat spread	☐	Cholesterol lowering spread e.g.benecol/Flora pro active	☐
Olive based spread e.g. Olivio	☐	Other margarine	☐
Polyunsaturated margarine e.g. Flora	☐	None	☐

How thickly do you spread it?

Thick ☐ Medium ☐ Thin ☐

Do you add it to potatoes? **Yes** ☐ **No** ☐

Do you add it to other vegetables? **Yes** ☐ **No** ☐

7. What type of fat do you use most often for cooking?

Butter	☐	Lard	☐
Soft margarine	☐	Olive oil	☐
Hard margarine	☐	Other vegetable oil	☐
Solid vegetable fat e.g. White flora	☐	None	☐

8. Do you eat organic food?

Never ☐
Sometimes ☐
Usually ☐
Always ☐

9. How much bread, crispbread, etc. do you normally eat each day?

	(Put '0' if none)		(Put '0' if none)
White bread	____ (slices per day)	Crispbread	____ (biscuits per day)
Brown bread	____ (slices per day)	Sweet biscuits	____ (biscuits per day)
Wholemeal bread	____ (slices per day)		

10. What type of beakfast cereal do you eat most often?

Bran cereal e.g. Branflakes	☐	Muesli, oat clusters, etc	☐
Wholemeal cereal e.g. Weetabix	☐	Other e.g. cornflakes, Rice krispies	☐
Porridge, hot oat cereal	☐	None	☐

How many bowls of cereal do you eat each week?

_____bowls a week

Put '0' if none

11. How much of the following do you drink each day?

Put '0' if none

Tea	____ cups daily	Pure fruit juice	____ glasses daily
Herb tea	____ cups daily	fruit drinks, squash	____ glasses daily
Coffee	____ cups daily	"Diet" fizzy soft drinks	____ glasses/cans daily
Water	____ glasses daily	Fizzy soft drinks	____ glasses/cans daily

12. How many teaspoons of sugar, in total, do you add to your tea, coffee, cereal, fruit, etc. each day

____teaspoons each day

Put '0' if none

13. At present, about how many alcoholic drinks do you have each week?

Put '0' if none

Beer, lager or cider	____pints each week	Sherry or fortified wine	____glasses each week
Red wine	____glasses each week	Spirits – whisky, gin, brandy	____glasses each week
White wine	____glasses each week		

13. How often do you eat the following?

Please cross one box for each item

	Never	Seldom	Once a week	2-4 times a week	5-6 times a week	Once or more a day
Fresh fruit						
Dried fruit (raw)						
Stewed fruit, tinned fruit						
Pasta, e.g. spaghetti						
Rice						
Pizza						
Chips						
Other potatoes						
Peas						
Baked beans						
Lentils, dried beans						
Tomatoes						
Carrots						
Green vegetables						
Other cooked vegetables						
Salad/raw vegetables						
Tofu						
Soya meat, burgers, TVP						

	Never	Seldom	Once a week	2-4 times a week	5-6 times a week	Once or more a day
Other veggie burgers						
Cheese						
Cottage cheese						
Soya cheese						
Yoghurt, dairy desserts						
Soya, yoghurt, soya desserts						
Cream, ice cream						
Soya cream, ice cream						
Cakes, puddings, pies, buns, etc						
Chocolate, any type						
Other sweets, e.g. boiled sweets						
Crisps, Hula hoops, etc						
Peanut butter, salted nuts						
Other nuts and seeds not in muesli						
Jam, marmalade						
Yeast extract, Marmite						

SECTION G:

LIFESTYLE.

We are interested in the type of activities you undertake.

Questions about your lifestyle

1. Have you ever smoked cigarettes **Yes ☐ No ☐**

If you have stopped smoking cigarettes, how old were you when you gave up? _____years old

If you smoke now, how many cigarettes, do you usually smoke each day? _____cigarettes a day

2. Do you smoke cigars? **Yes ☐ No ☐**

3. Do you smoke a pipe? **Yes ☐ No ☐**

4. Do you have a paid job at present?

Yes, full-time ☐ Yes, part-time ☐ No ☐

If **Yes**, we would like to know the type and amount of physical activity involved in your work.
Please put a cross in the appropriate box.

Sedentary occupation ☐
You spend most of your time sitting (such as in an office)

Standing occupation ☐
You spend most of your time standing or walking, but your work does not require intense physical effort (e.g. shop assistant, hairdresser, guard)

Manual work ☐
This involves some physical effort including handling of heavy objects and use of tools (e.g. plumber, electrician, carpenter)

Heavy manual work ☐
This involves very vigorous physical activity including handling very heavy objects (e.g. docker, miner, bricklayer, construction worker)

5. In a typical week during the past year, how many hours did you spend per week on the following activities? Put '0' if none

	In Summer	In Winter
Walking, including to work, shopping and leisure time	_____hours per week	_____hours per week
Cycling, including cycling to work, and during leisure time	_____hours per week	_____hours per week
Gardening	_____hours per week	_____hours per week
Do-It-Yourself	_____hours per week	_____hours per week

	In Summer	In Winter
Physical exercise such as keep-fit/aerobics, swimming, jogging, tennis, etc.	_____hours per week	_____hours per week
Housework, such as cleaning, washing, cooking and childcare	_____hours per week	_____hours per week

6. In a typical week during the past 12 months, did you practice any of these activities vigorously enough to cause sweating or a faster heartbeat?

Yes ☐ No ☐

If **Yes**, for how many hours per week in total did you practice such vigorus activity? _____hours per week

QUESTIONS FOR WOMEN ONLY

8. Have you had your menopause (stopped having periods)?
Yes ☐ No ☐ Not sure ☐
(because taking HRT, irregular periods, etc)

If **Yes**, how old were you when you stopped having periods?
_____years old

9. How many periods have you had in the last 12 months?
_____periods (Put '0' if no periods)

10. Have you ever taken the contraceptive pill?
Yes ☐ No ☐
If **Yes**, for how long altogether have you used the pill? _____years
(Put '0' if less than one year)

Are you currently taking the contraceptive pill? **Yes ☐ No ☐**
If **No**, at what age did you stop? _____years old

11. Have you ever taken Hormone Replacement Therapy (HRT)? Yes ☐ No ☐

If **Yes**, for how long altogether have you used HRT? ____years
(Put '0' if less than one year)

Are you currently taking HRT? **Yes ☐ No ☐**
If **No**, at what age did you stop? ____years old

12. During the last six years, have you had any children?
Yes ☐ No ☐

If **Yes**, please enter the years of birth and sex below:
1. _____(YEAR) Boy ☐ Girl ☐
2. _____(YEAR) Boy ☐ Girl ☐
3. _____(YEAR) Boy ☐ Girl ☐

13. Have you ever had a hysterectomy (womb removed)?
Yes ☐ No ☐
If Yes, at what age _____years old

14. Have you ever had an operation to remove one or both ovaries? Yes ☐ No ☐ Don't know ☐

If **Yes**, were one or both ovaries removed?
One ☐ Both ☐ Don't know ☐

At what age? ____years old

15. Have you ever had breast screening by mammography (x-ray)? Yes ☐ No ☐

If **Yes**, how many times in the last ten years? ____times

When did you last have a breast screen? ____*YEAR*

16. Have you ever had a cervical smear test?

Yes ☐ No ☐

If **Yes**, how many times in the last ten years? ____times

When did you last have a cervical smear? ____*YEAR*

Thank you for taking the time to complete this questionnaire.

Please return to Dr H. Haboubi, Endoscopy Department, Morriston Hospital, Heol Maes Eglwys, Swansea, SA6 1NL

OR

Bring this questionnaire with you on the day of your endoscopy

Appendix VI. Patient consent form. Study title: Studies in the molecular and lifestyle differences of patients with Gastroesophageal reflux disease and Barrett's oesophagus.

Endoscopy Department,
Singleton Hospital
Sketty Lane
Sketty
Swansea
SA2 8QA

ABM
Ymddiriedolaeth Prifysgol GIG
Abertawe Bro Morgannwg
University NHS Trust

Swansea University
Prifysgol Abertawe

Participant Consent Form Version 5, 26th October 2022

Title of research "Sex Differences in Barrett's Oesophagus"

PI: Professor Gareth Jenkins

CI: Dr Hasan Haboubi

Participants should complete the whole of this sheet themselves.
(Please put your initials in the box by each statement if it applies to you)

I have read the Information Sheet for Patients V5. Dated 26th October 2022	☐
I have received enough information about the study, have been given the opportunity to ask questions and discuss the project and have received satisfactory answers to all my questions.	☐
I understand that relevant sections of my medical notes may be accessed by members of the research responsible individuals from Swansea University, the Trust or from regulatory authorities for the purposes of monitoring or auditing	☐
The study, including the risks and benefits, has been explained to me by a member of the clinical research team	☐
I consent to the research team taking additional biopsy samples during the endoscopy	☐
I consent to having a blood sample taken for analysis by the research team	☐
I agree to completing the questionnaire associated with this study	☐
I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting my future medical care.	☐

Signed..... Date.....

(NAME IN BLOCK CAPITALS).....

Investigator's signature..... Date: .....

(NAME IN BLOCK CAPITALS).....

Patient consent form, Sex Differences in Barrett's Oesophagus V5 26th October 2022

Appendix VII. Patient information sheet for recruitment of patients from oncology. Study title: A novel blood blood-based approach to identify chemotherapeutic response in upper GI tract cancer patients

Dept. of Oncology
Singleton Hospital
Swansea
SA2 8QA
01792 285332

Patient Information Sheet– 20th October 2022, Version 4.0

Information Sheet for Research Participants

Study title:

A novel blood-based approach to identify chemotherapeutic response in upper GI tract cancer patients.

Chief Investigator: Professor Gareth Jenkins
Principal Investigator: Professor Gareth Jenkins
Clinical lead: Dr Sarah Gwynne.

You are being invited to take part in a research study led by Swansea University Medical School. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and members of hospital staff. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part in the study.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. If you decide not to take part or to withdraw at any other time without explanation, your future care will not be affected by your decision. Thank you for reading this.

What is the purpose of the study?

You may undergo treatment for an upper GI tract cancer. We would like to develop blood-based tests so that in the future we can monitor tumour response to

1) A questionnaire which will take no longer than 15 - 20minutes to complete, which will ask you about your diet, lifestyle and medications. It will also ask you to give your weight and height measurements.
2) A blood test will also be offered to you, which you can accept or refuse. We will take this blood using a standard blood test prior to treatment.
3) Subsequent blood tests will also be offered at following treatment appointments, which you will also have the chance to accept or refuse. Approximately 4 blood tests may be taken in total over the course of your participation of the study.
4) You may be contacted by the Chief investigator (Professor Gareth Jenkins) to coordinate blood test appointments.

What will happen to my samples?

Any samples taken will be analysed in the Swansea University laboratories immediately and then destroyed- no samples will be stored.

What are the possible disadvantages and risks of taking part?

There are no disadvantages or major risks associated with taking part. The blood test will be taken by trained staff, although some minimal discomfort may be experienced, as per all blood tests.

What are the possible benefits of taking part?

There will not be immediate benefits from taking part in this study. The study will help develop a novel blood test that may be beneficial to patients in the future.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Your name and address will be removed from the information when it is shown to other medical staff outside the study.

Who is organising the research?

This study is being organised by the staff of the oncology department of Swansea Bay University Health Board and Swansea University.

Who has reviewed the study?

chemotherapy/ radiotherapy more easily. In order to do so we require donated blood samples from patients with a range of upper GI tract cancers. We will compare the results of our test in patients with different types of disease and treatments. We then can see if our newly developed test can (in the future) predict tumour response to chemotherapy.

We plan to investigate this by:

a) Asking you to fill out a questionnaire about your lifestyle
b) Taking a blood test from the intravenous line which will be inserted as a matter of routine prior to treatment and at following treatment appointments (approximately 4 in total). This will be approximately 20mls (1 and a half tablespoons).

Why have I been chosen?

You may undergo chemotherapy and/or radiotherapy as treatment for an upper GI tract cancer.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive in the future.

What do I have to do?

You will be approached and informed about the study while attending the Department for your follow-up clinic appointment, OR while attending your follow-up outpatient appointment in the main outpatient's department. The researcher will:

- Ask you about your health in general, and any previous operations
- Explain the purpose of this study in further details and answer your questions

You do NOT have to attend any other appointments other than your routine treatment appointments that you would be attending anyway.

Taking part in this study will not affect the standard treatment that you receive.

You can opt out of the study at any stage.

What will happen to me if I take part?

If you decide to take part in this study you will be asked to sign the necessary consent form, then you will have:

The study has been reviewed by HSC Research Ethics Committee A.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect, or about the way you have been treated during the course of this study then you should immediately inform Dr Sarah Gwynne (Dept Oncology, Singleton hospital, 01792 285332). The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the SBU NHS Health Board University Joint Research Office.

Contact for Further Information

If you want more information, before or after you return your form, you can phone (9am-5pm Mon-Fri)

Professor Gareth Jenkins on [redacted]

Dr Sarah Gywnne on [redacted]

The study has been reviewed by HSC REC A.

Appendix VIII. Patient questionnaire. Study title: A novel blood blood-based approach to identify chemotherapeutic response in upper GI tract cancer patients.

UPPER GI TRACT CANCER LIFESTYLE SURVEY

This questionnaire will take approximately 10mins to complete.

Some questions are personal, but we guarantee that all information will be treated with absolute confidentiality and will be used only for medical research.

To help us read your answers, please write as clearly as possible with a black pen and complete the questionnaire by circling the number corresponding to your answer or putting a cross in the appropriate box(es).

DEMOGRAPHICS & PATIENT INFORMATION

ID Number: _____(PLEASE LEAVE BLANK)
To be completed by Research Office

Age: _____years old

Height (if known): _____

Weight (if known): _____

Waist Circumference (if known): _____

Please indicate your marital status

Single ☐ Separated ☐

Married or living as married ☐ Divorced ☐

Widowed ☐

DIET.

We are interested in the type of foods you eat and how frequently you eat them.

Questions about your diet

1. Do you eat meat? **Yes** ☐ **No** ☐

If **Yes**, how many times a week do you eat meat?
_____times a week

If **No**, how old were you when you last ate meat?
_____years old

2. Do you eat any fish? **Yes** ☐ **No** ☐

If **Yes**, how many times a month do you eat the following?
(put '0' if less than once a month)

Fatty fish ____times a month (including sardines, salmon, mackerel, herring)

Other fish ____times a month (including Cod, tuna, haddock)

If **No**, how old were you when you last ate fish?
_____years old

3. Do you eat any dairy products? **Yes** ☐ **No** ☐
(including milk, cheese, butter, yoghurt)

If **No**, how old were you when you last ate dairy products?
_____years old

4. **Do you eat any eggs?** **Yes** ☐ **No** ☐
(including eggs in cakes and other baked foods)

If **Yes**, how many eggs do you eat each week?
_____eggs each week
Put "0" if eaten less than once a week

If **No**, how old were you when you last ate eggs?
_____years old

5. What type of milk do you use most often?

Full cream ☐ Soya milk fortified with calcium ☐
Semi-skimmed ☐ Soya milk not fortified with calcium ☐
Skimmed/fat free ☐ Other ☐
None ☐

How much milk do you drink each day, including milk with tea, coffee, cereals, etc.

Less than a quarter of a pint (<150ml) ☐ Three quarters of a pint (450ml) ☐
Quarter of a pint (150ml) ☐ One pint (600ml) ☐
Half a pint (300ml) ☐ More than one pint (>600ml) ☐

6. What type of spread do you use most often on bread, crispbreads, vegetables, etc.?

Butter ☐ Hard margarine (in wrapper not tub) ☐

Dairy spread e.g. Clover ☐ Soya margarine or ☐

other milk free margarine

Low or reduced fat spread ☐ Cholesterol lowering spread e.g.benecol/Flora pro active ☐

Olive based spread e.g. Olivio ☐ Other margarine ☐

Polyunsaturated margarine e.g. Flora ☐ None ☐

How thickly do you spread it?

Thick ☐ Medium ☐ Thin ☐

Do you add it to potatoes? **Yes** ☐ **No** ☐

Do you add it to other vegetables? **Yes** ☐ **No** ☐

7. What type of fat do you use most often for cooking?

Butter ☐ Lard ☐

Soft margarine ☐ Olive oil ☐

Hard margarine ☐ Other vegetable oil ☐

Solid vegetable fat e.g. White flora ☐ None ☐

8. Do you eat organic food?

Never ☐

Sometimes ☐

Usually ☐

Always ☐

9. How much bread, crispbread, etc. do you normally eat each day?

White bread ____ (slices per day) (Put '0' if none)

Brown bread ____ (slices per day)

Wholemeal bread ____ (slices per day)

Crispbread ____ (biscuits per day) (Put '0' if none)

Sweet biscuits ____ (biscuits per day)

10. What type of breakfast cereal do you eat most often?

Bran cereal e.g. Branflakes ☐

Wholemeal cereal e.g. Weetabix ☐

Porridge, hot oat cereal ☐

Muesli, oat clusters, etc ☐

Other e.g. cornflakes, Rice krispies ☐

None ☐

How many bowls of cereal do you eat each week?

_____bowls a week

Put '0' if none

11. How much of the following do you drink each day?

Put '0' if none

Tea	____ cups daily	Pure fruit juice	____ glasses daily
Herb tea	____ cups daily	fruit drinks, squash	____ glasses daily
Coffee	____ cups daily	"Diet" fizzy soft drinks	____ glasses/cans daily
Water	____ glasses daily	Fizzy soft drinks	____ glasses/cans daily

12. How many teaspoons of sugar, in total, do you add to your tea, coffee, cereal, fruit, etc. each day

____teaspoons each day

Put '0' if none

13. At present, about how many alcoholic drinks do you have each week?

Put '0' if none

Beer, lager or cider ____pints each week

Red wine ____glasses each week

White wine ____glasses each week

Sherry or fortified wine ____glasses each week

Spirits – whisky, gin, brandy ____glasses each week

13. How often do you eat the following?

Please cross one box for each item

	Never	Seldom	Once a week	2-4 times a week	5-6 times a week	Once or more a day
Fruit						
Bread						
Potatoes / pasta						
Pasta, e.g. spaghetti						
Dairy / Cheese						

LIFESTYLE.

We are interested in the type of activities you undertake.

Questions about your lifestyle

1. Have you ever smoked cigarettes **Yes** ☐ **No** ☐

If you have stopped smoking cigarettes, how old were you when you gave up? ____years old

If you smoke now, how many cigarettes, do you usually smoke each day? ____cigarettes a day

2. Do you smoke cigars? **Yes ☐ No ☐**

3. Do you smoke a pipe? **Yes ☐ No ☐**

4. Do you have a paid job at present?

Yes, full-time ☐ Yes, part-time ☐ No ☐

If **Yes**, we would like to know the type and amount of physical activity involved in your work.
Please put a cross in the appropriate box.

Sedentary occupation ☐
You spend most of your time sitting (such as in an office)

Standing occupation ☐
You spend most of your time standing or walking, but your work does not require intense physical effort (e.g. shop assistant, hairdresser, guard)

Manual work ☐
This involves some physical effort including handling of heavy objects and use of tools (e.g. plumber, electrician, carpenter)

Heavy manual work ☐
This involves very vigorous physical activity including handling very heavy objects (e.g. docker, miner, bricklayer, construction worker)

5. In a typical week during the past year, how many hours did you spend per week on the following activities? Put '0' if none

	In Summer	In Winter
Walking, including to work, shopping and leisure time	____hours per week	____hours per week
Cycling, including cycling to work, and during leisure time	____hours per week	____hours per week
Gardening	____hours per week	____hours per week
Do-It-Yourself	____hours per week	____hours per week

	In Summer	In Winter
Physical exercise such as keep-fit/aerobics, swimming, jogging, tennis, etc.	____hours per week	____hours per week
Housework, such as cleaning, washing, cooking and childcare	____hours per week	____hours per week

6. In a typical week during the past 12 months, did you practice any of these activities vigorously enough to cause sweating or a faster heartbeat?

Yes ☐ No ☐

If **Yes**, for how many hours per week in total did you practice such vigorus activity? _____hours per week

HEALTH.

We are interested in your past medical history and your current state of health.

Questions about your health

1. In the past six years, have you had any broken/fractured bones?

Yes ☐ No ☐

If **Yes**, please give details:

2. In the last six years, has your doctor told you that you that you had any of the following?

	Yes	Year first diagnosed	No
Cancer			
Type of cancer:			
Polyps in large intestine			
Enlarged prostate (men only)			
High blood pressure			
High blood cholesterol			
Angina			
Stroke			

Heart Attack			
Cardiac arrhythmias/ palpatations/ irregular heartbeat			
Blood clot in leg			
Blood clot in lung or elsewhere			
Diabetes			
Thyroid disease			
Cataract in eye			
Stomach ulcer			
Duodenal ulcer			
Gallstones			
Have you had your gallbladder removed?			
Crohn's disease			
Ulcerative colitis			
Asthma			
Bronchitis/emphysema			
Rheumatoid Arthritis			
	Yes	Year first diagnosed	No
Osteoarthritis			
Depression requiring treatment			
Other significant Illnesses or operations, please give details			

3. Do you regularly take any vitamins, minerals or other supplements?

Yes ☐ No ☐

If **Yes**, do you take: (you can cross more than one box)

Multivitamins (with minerals)	☐ Vitamin A ☐
Multivitamins (without minerals)	☐ Vitamin B ☐
Fish oil (including cod liver oil)	☐ Vitamin C ☐
Evening primrose oil	☐ Vitamin D ☐
Garlic	☐ Vitamin E ☐
Iron	☐ Other ☐ _____
Zinc	☐ (name and brand)
Calcium	☐

4. Have you taken any medications for most of the last 4 weeks?

Yes ☐ No ☐

If **Yes**, please list them below:

_____

_____

_____

_____

_____

_____

5. About how many bowel movements do you have each week?

____a week

How often do you take laxatives? (Put '0' if never)

____times a month

6. How would you describe your health now?

Excellent ☐ Good ☐ Fair ☐ Poor ☐

Thank you for taking the time to complete this questionnaire.

Please bring this questionnaire with you on the day of your treatment

OR

Send to: Kathryn Munn, Swansea University Medical School, Swansea, SA2 8PP

Appendix IX. Patient consent form. Study title: A novel blood blood-based approach to identify chemotherapeutic response in upper GI tract cancer patients

Oncology Department,
Singleton Hospital
Sketty Lane
Sketty
Swansea
SA2 8QA

Participant Consent Form Version 5, 20th October 2022

Title of research "Chemotherapy response monitoring in GI tract cancer patients"

PI: Professor Gareth Jenkins

CI: Professor Gareth Jenkins

Participants should complete the whole of this sheet themselves.

(Please initial each statement if it applies to you)

I have read the Information Sheet for Patients V4. Dated 20th October 2022	☐
I have received enough information about the study, have been given the opportunity to ask questions and discuss the project and have received satisfactory answers to all my questions.	☐
I understand that relevant sections of my medical notes may be accessed by the research team.	☐
The study, including the risks and benefits, has been explained to me by a member of the clinical research team	☐
I consent to having a blood sample taken for analysis by the research team	☐
I agree to completing the questionnaire associated with this study	☐
I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting my future medical care.	☐
I understand should I withdraw from the study that any samples I have previously given to the study may still be used but no new samples will be taken.	☐

Signed..... Date.....

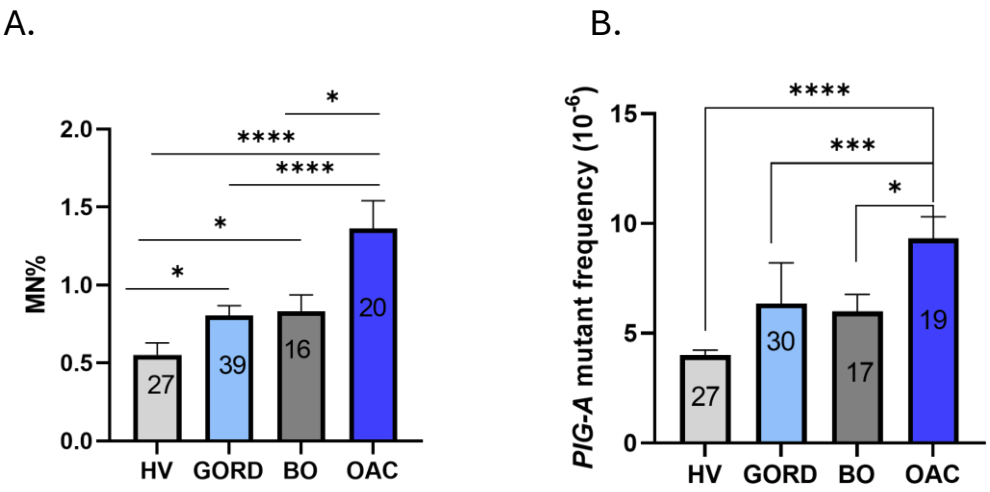
(NAME IN BLOCK CAPITALS).....

Investigator's signature..... Date: .....

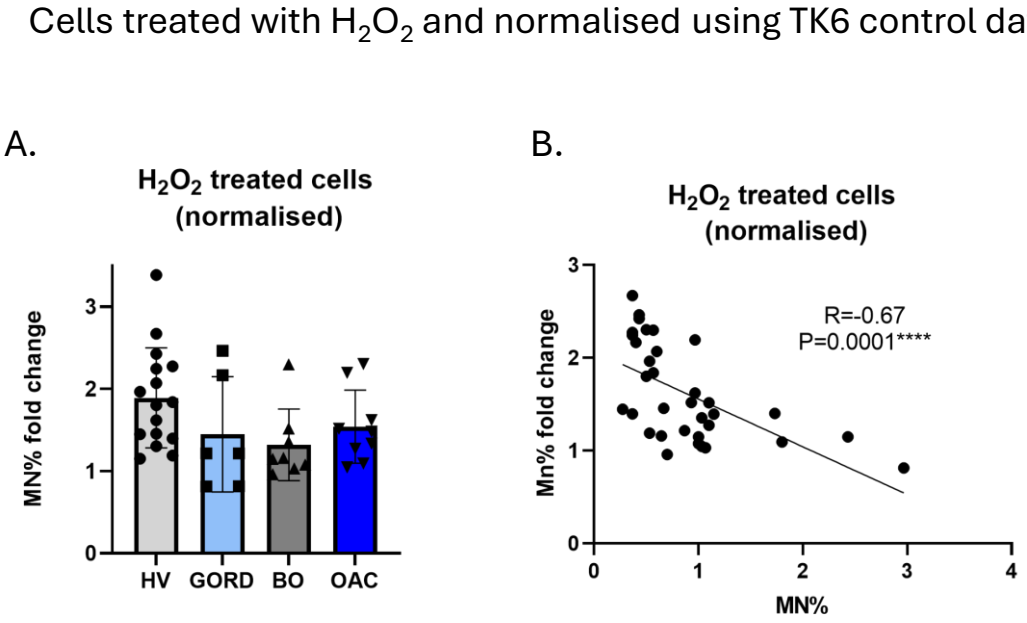
(NAME IN BLOCK CAPITALS).....

1 copy for patient, 1 copy for Principal Investigator, 1 copy for hospital notes

Appendix X. Comparison of DNA damage in histology groups, with the removal of BO patients who have co-existing cancers (excluding those of the oesophagus). (A.) Micronucleus frequency (MN%) with removal of 6 BO patients who had co-existing cancer. No change in significance observed. (B.) *PIG-A* mutant frequency (10^{-6}) with removal of 6 BO patients who had co-existing cancer. No change in significance observed compared to graphs in Chapter 3.



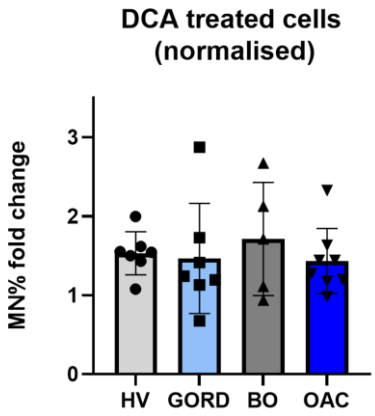
Appendix XI. Lymphocytes and TK6 cells were treated with 20µM hydrogen peroxide (H_2O_2) in parallel as part of the challenge assay. The TK6 cell MN fold change was used in order to normalise the lymphocyte MN data. (A.) The micronucleus frequency (MN%) fold change following treatment with 20µM H_2O_2 between different histology groups. No significant changes observed (p=0.7) (B). The baseline MN% of the untreated sample vs MN% fold change following treatment with H_2O_2. R=-0.67 suggesting strong negative correlation, p= <0.0001** n=34.**



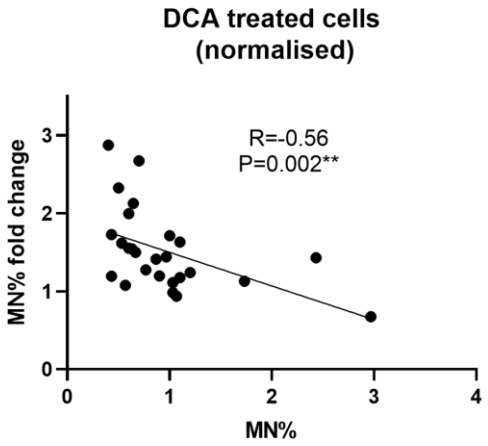
Appendix XII. Lymphocytes and TK6 cells were treated with 150µM deoxycholic acid (DCA) in parallel as part of the challenge assay. The TK6 cell MN fold change was used in order to normalise the lymphocyte MN data. (A.) The micronucleus frequency (MN%) fold change following treatment with 150µM DCA between different histology groups. No significant changes observed (p=0.8) (B). The baseline MN% of the untreated sample vs MN% fold change following treatment with DCA. R=-0.56 suggesting strong negative correlation, p= <0.002, n=27.**

Cells treated with DCA and normalised using TK6 control data

A.



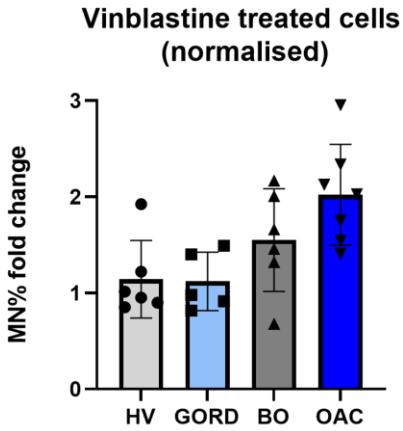
B.



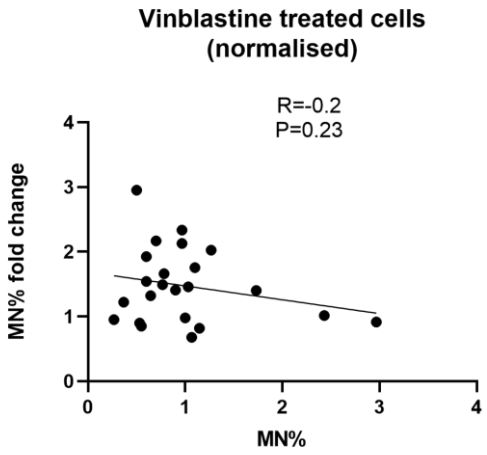
Appendix XIII. Lymphocytes and TK6 cells were treated with 0.9 nM vinblastine in parallel as part of the challenge assay. The TK6 cell MN fold change was used in order to normalise the lymphocyte MN data. (A.) The micronucleus frequency (MN%) fold change following treatment with 0.9 nM vinblastine between different histology groups. No significant changes observed (p=0.06) (B). The baseline MN% of the untreated sample vs MN% fold change following treatment with vinblastine. R=-0.2 suggesting weak negative correlation, non significant p= 0.23, n=23.

Cells treated with Vin and normalised using TK6 control data

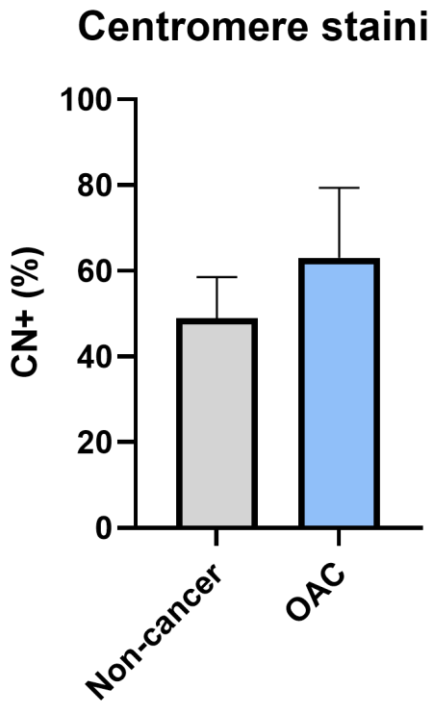
A.



B.



Appendix XIV. Percentage of centromere positive (CN+) micronuclei. Oesophageal adenocarcinoma (OAC) patients (n=4) have slightly higher percentage of chromosome positive micronuclei than non-cancer patients (n=3). P=0.4.



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