Genoprotective effect of aspirin and ibuprofen in human lymphocyte cells

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Genoprotective effect of aspirin and ibuprofen in human lymphocyte cells

Effect of nano and bulk forms of aspirin and ibuprofen on lymphocytes from breast cancer patients compared with those from healthy females

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Abstract

Various recent studies have suggested that regular intake of some non-steroidal anti-inflammatory drugs (NSAIDs) have a preventative effect against several types of tumours including breast cancer. The term nanotechnology refers to technology in which one-billionth of a meter is used as a scale for chemical particle size. This work aims to study the effect of both ibuprofen and aspirin on DNA damage using peripheral blood lymphocytes from breast cancer patients and comparing the results with those from healthy females as a control using the Comet and micronucleus assays. Western blot analysis (WBA) was used to investigate the effect of these drugs on XRCC3 and p53 proteins, whereas QPCR was to evaluate this effect on p53, cox1 and cox2 genes. Two hundred fifty ng/ml of ibuprofen (NP and bulk) and 500 ng/ml of aspirin (NP and bulk) were used to treat the lymphocytes. Both aspirin and ibuprofen caused a reduction in DNA damage and micronucleus formation. Aspirin, both forms, showed a reduction in DNA damage in the Comet and micronucleus assays. Ibuprofen both forms, by contrast, showed a statistically significant reduction in micronucleus frequency in the micronucleus assay, while its preventative effect with the Comet assay was weak or insignificant. NPs of both agents were more effective than bulk sizes. Using the Comet repair assay, aspirin and ibuprofen nano form catalysed DNA repair to a greater extent than their bulk forms. Also, both sizes showed better repair with NSAIDs compared to samples repaired without NSAIDs. In WBA aspirin increased the expression of XRCC3 protein in healthy cells. However, both NSAIDs decreased that expression in cells from BC patients. Furthermore, aspirin increased p53 expression in BC patients lymphocytes. With the QPCR method, results of both aspirin forms

increased the expression of the p53 gene in BC patient cells statistically significantly. Both drugs reduced cox1 expression in healthy volunteers and cancer patients lymphocytes. Moreover, cox2 reduction was only in lymphocytes from BC patients. The results of this work are consistent with the view that NSAIDs, particularly aspirin and ibuprofen, could have a promising role in cancer treatment including breast cancer.

Keywords: Breast cancer, lymphocyte, aspirin, ibuprofen, nanoform, Comet assay, DNA damage, in vitro.

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Table of Contents

List of figures

List of tables

Abbreviations

ALS	Alkali-labile site
ASP:	Aspirin
ATM	Ataxia telangiectasia
BC	Breast cancer
BER	Base excision repair
BLM	Bleomycin
BLMR	Bleomycin with allowing repair
CBMN	Cytokinesis Block Micronucleus assay
COX	Cyclooxygenases
CPD	Cyclobutane pyrimidine dimers
CPT	Camptothecin
CT	Threshold cycle
Cyto B	Cytochalasin-B
DDH$_2$O	Deionized distilled water
DMSO	Dimethyle sulfoxide
dNTPs	Deoxynucleoside triphosphate
DSB	DNA double strand break
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetrachloro acetic acid
EL	Erythrocyte lysis buffer
EMQN	European Molecular Genetics Quality Network
ER	Oestrogen receptors
FBS	Foetal bovine serum
GBM	Glioblastoma multiform
H_2O_2	Hydrogen peroxide
HBOC	Hereditary Breast and Ovarian Cancer Syndrome
HER2	Human epidermal growth factor
HO.	Hydroxyl radical
HR	Homologous recombination
Ibu	Ibuprofen
IDLs	insertion/deletion loops

LMP	Low melting point agarose
MAPK	Mitogen activated protein kinases
MMC	Mitomycin C
MMR	Mismatch repair
MNi	Micronucleus
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBUDs	Nuclear Buds
NDI	The nuclear division index
NER	Nucleotide excision repair
Ng	Nanogram
NHEJ	non-homologous end joining
NMP	Normal melting point agarose
NPBS	Nucleoplasmic bridges
NPs	Nanoparticles
NSAID	Nonsteroidal anti-inflammatory drugs
O2-	Superoxide radical
OTM	Olive tail moment
PC	Positive control
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PIP3	phosphatidylinositol-3,4,5-triphosphate
PTEN	Phosphatase and tensin homolog gene
PTMs	Posttranslational modifications
PVDF	Nitrocellulose and polyvinylidene fluoride
ROS	Reactive oxygen species
RT	Room temperature
SSB	Single strand break
STM	Scanning tunneling microscope
TCGA	The Cancer Genome Atlas
TEM	Transmission electron-microscope
Trx	Thioredoxin
WB	Western blot

µM	Micro molar

Chapter 1- Introduction

1. Introduction

1.1 Genotoxicity

Genotoxicity can be defined as any alteration or damage by a toxicant in the genome, which under some conditions correlates to mutagenicity. Hence, genotoxicity can be divided into different gene alterations (insertion, deletion and point mutation), clastogenic impacts (breakage in DNA strands leading to alterations in chromosomes structures), and aneugenic defects (numeral chromosomal aberrations leading to the development of aneuploidy or polyploidy) (Eastmond et al., 2009). Genetic mutation and DNA damage can be introduced by different factors having genotoxic potential such as chemical substances and physical agents (UV and X radiation). This genetic alteration could be detrimental where it increases the chance of cancer, congenital defects, and inflammation. Consequently, genotoxicology research becomes an essential part of biomedical science that enable scientists to study and discover the impact of other chemicals, and physical agents.

1.2 Cancer

The term cancer refers to diseases in which cells divide and proliferate abnormally without any control and have the ability to invade other parts of the body. Some types of cancer have the capacity to spread to other tissues of the body through the blood and lymph systems or through direct invasion (Hanahan and Weinberg, 2000, 2011). There are more than 100 different types of cancer. Most of them are classified depending on pathological criteria, where cancers are named according to the organ or type of cell in which they originate - for instance, cancer that develops in the breast is called breast cancer; cancer that develops in melanocyte

cells of the skin is called melanoma (Hoadley et al., 2014). However, extensive-scale genomics is providing at the present detailed molecular descriptions of thousands of cancers, producing a systematic molecular-based classification of tumours. Indeed, The Cancer Genome Atlas (TCGA) Project Network completed genome-wide studies of ten different malignancies: the glioblastoma multiform (GBM) (Hoadley et al., 2014; McLendon et al., 2008).

1.3 Breast cancer

Breast cancer (BC) has been found as the most common type of cancer affecting females worldwide. It accounts for almost 1 in 4 female malignancy cases globally. In 2008, 1.38 million patients were diagnosed with breast cancer and this number has been estimated to reach 1.7 million by 2020 (Bhikoo et al., 2011). Despite the fact that BC is more common in the developed countries compared to developing countries, the overall survival from BC is increasing but survival remains poorer in developing nations. This was found to be due to reasons such as adaptation to western life styles by developing nations, less BC screening and also poorer health care services (Bhikoo et al., 2011; Osaro, 2016). Various aspects of BC epidemiology have demonstrated that sex steroid hormones play a crucial role in carcinogenesis (Henderson and Feigelson, 2000; Hormones and Group, 2013). It has been shown that the frequent exposure to some risk factors such as oestrogens lead to an increase in breast cell proliferation. However protective effects have been noticed when there was a reduction in exposure to female sex hormones to the same cell types (Cuzick, 2003; Musgrove, 2013). The cumulative exposure of women to steroid hormones induces breast cell proliferation and this in turn increases the proportion of genetic mutation which is essential to cancer

growth and development (Jung et al., 2015). Some studies proved that many factors including sex hormones are participating in the emergence of cancer in the breast, for instance external environmental contaminants, oncogenic viruses, and inflammatory factors (Chace, 2015; Glaser et al., 2004).

1.3.1 Classification of breast cancer

BC is categorised into two main types which are: 1) carcinoma *in situ* breast cancer and 2) invasive carcinoma breast cancer. The *In situ* carcinoma is subdivided into ductal or lobular carcinoma *in situ* according to the tumour origin. In both subtypes, there is no invasion and the tumour cells remain localised and hence there is only a small chance for metastasis. However, invasive breast carcinoma is associated with metastasis and it occurs when *in situ* ductal or lobular tumour cells migrate over their natural boundaries, namely through their basement membrane with the consequence of spreading to the neighbouring tissues (Richie and Swanson, 2003; Ward et al., 2015).

1.3.2 Invasion and metastasis

Breast cancer has the ability to invade the adjacent tissue and this is the main fatal aspect of this tumour type (Figure 1). The new tumour may relocate to and grow in other, secondary sites of the body distal to the primary breast tumour (Talmadge and Fidler, 2010). The capacity of BC cells to migrate and invade surrounding tissues, and the process of reduced adherence to the main tumour are demonstrated and well known at the cellular level (Talmadge and Fidler, 2010). However, at the molecular level these processes are yet poorly understood. The invasion process begins when the cancerous cells head to the lymphatic system and blood vessels, then into the blood stream, and eventually to a new healthy

tissue where they settle down and grow to create secondary tumour sites in a process called metastasis. The secondary tumours have properties that are often similar to the original tumour. The majority of uncured tumours have a high potential to invade and metastasise. Nevertheless, some other cancer types have very low tendency to metastasis, for instance basal and glioma carcinoma cells. Metastases from BC have been seen decades after the primary tumour treatment. The majority of cancer deaths are caused by cancer metastasis and not the primary tumour. Cancer metastasis is the main cause of morbidity and mortality and accountable for about 90% of cancer deaths (Guan, 2015; Karrison et al., 1999). Tumour cells can be present in three different states at secondary malignant sites: solitary cells in passivity, active pre-angiogenic, and micro metastasis. This means that there is no increase in tumour size because apoptosis is balanced with cell proliferation, so could be small and clinically unidentifiable, or could be large and detectable by existing technology (Demicheli, 2001; van Zijl et al., 2011).

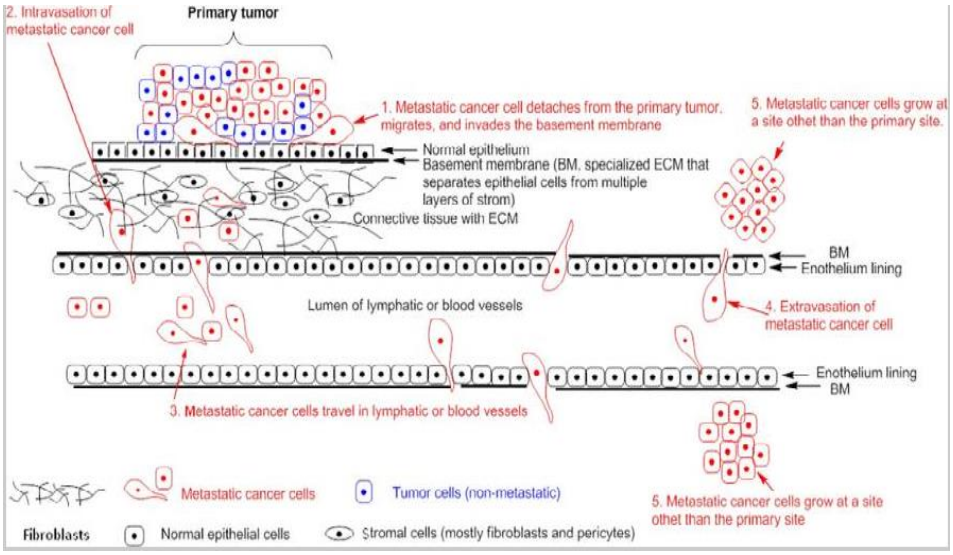

Figure 1 Metastatic cascade. Metastatic cells detach from the primary tumor site, migrate and invade through the BM and ECM, enter the blood or lymphatic vessels (intravasation), travel in the blood/or lymphatic vessels, leave the blood orlymphatic vessels (Guan, 2015).

1.3.3 Hereditary breast cancer

The association between breast cancer and family history was first described in 1866 by French pathologist Paul Broca. This was when Broca's wife was suffering from early onset BC when she was in the early stage of her life and Broca followed a history of her family and faound that the BC could be traced back through four generations (Broca, 1866; van der Groep et al., 2011). Broca's observation was considered as the first description that a predisposition to getting BC could be inherited and thereby passed down through generations. Since then the contribution of the family history and BC was established and considered as one risk factor for the pathology of the BC disease (Claus et al., 1998; van der Groep et al., 2011).

1.3.4 The molecular genetics of breast cancer

Breast cancer arises as a result of complicated genetic alteration and epigenetic formation, and these events take place during the tumourigenesis of breast cells. The consequence of genetic alteration or mutation can be the changing of nucleotide base pairs in specific places of genes, or complete gene alteration or gene loss, which in turn leads to aberrant RNA, protein, and loss of gene function (Sadikovic et al., 2008). Epigenetic changes can also be involved in the aetiology of BC where it prevents the transcription of some genes without any changes in base pair sequence (Buchholz and Wazer, 2002). The somatic mutation theory which was presented by Boveri in 1914 became the principal genetic guide in the twentieth century (Boveri, 1914; Suter and Marcum, 2007). This theory suggests that cancer is the consequence of hereditary and/or sporadic genetic mutations in germinal and somatic cells, respectively. Many cellular pathways are affected by

such mutations for instance, mitogen-activated protein kinases (MAPK), RB/E2F that arrest cells in G1, intracellular apoptosis pathway (P3K/AKT/mTOR), and TP53 pathways, which function as tumour suppressors. These pathways are essential in controlling cell growth and proliferation and hence disturbance in their roles due to mutation can cause cancer particularly breast cancer (Suter and Marcum, 2007; Zhang et al., 2015). BC is considered as a complicated molecular disease, where the mutations take place in genes, which are responsible for growth and proliferation. Sporadic formation of BC is the most prevalent and the oncogenes, which are initially mutated, in turn lead to dysregulation of cell proliferation and performance, which eventually lead to malignancy. Genes that have inheritable mutation tendencies are more responsible for familial BC, which is responsible for only 5%-10% of all breast cancer cases (Suter and Marcum, 2007).

1.4 Oestrogen and its receptor

The oestrogen hormone is an effective mitogen which induces cell proliferation in normal breast epithelial cells and ductal morphogenesis but also plays an essential role in carcinogenesis of BC through the over-expression of the oestrogen receptor (ER). However, scientists still debate the role of oestrogen in BC (Russo and Russo, 2006; Simoes et al., 2015). Epidemiological research show evidence that BC risk is positively correlated with post-menopausal levels of oestrogen. The impact of oestrogen mostly presented through connecting to two nuclear ligand to activate transcription factors, the ERs ERα and ERβ, which finally bind to oestrogen-responsive elements in the DNA sequence to regulate the transcription of specific genes (Simoes et al., 2015). In BC tissue the ERα isoform considered as an essential agent in mitogenic processes. This isoform is highly expressed in

the early stage of BC (Hewitt et al., 2005). Higher levels of ER are expressed in nearly 66% of BC tissues compared to normal breast tissues. A stable receptor dimer is formed by binding oestrogen to ERα and this phosphorylated dimer activates conformational change (Butt et al., 2005; Suter and Marcum, 2007). In BC the mitogenic processes can also be activated by binding growth factors with ER, for instance TGFα, EGF, and IGF-I (Butt et al., 2005; Simoes et al., 2015). The way that ERβ participates in the development of BC is still unclear, as well as, the ratio of ERβ to ERα that is essential in BC. Many studies found that the level of ERα-positive cells in the Oestrogen-dependent breast cancers is higher than it is proportion in normal breast tissue, however, the expression of ERβ is reduced, demonstrating an antagonistic relationship between ERα and ERβ (Renoir et al., 2013). This finding supports the theory that ERα play an important role in malignancies, and upregulation of ERβ may inhibit the promotion of breast tumour (Chen et al., 2014). Eventually, both ER-negative and ER-positive tumours can be found in breast tissue. A unique molecular subgroup can be formed by ER-positive tumours, whereas, the highly aggressive form of BC is often associated with ER-negativity and a poor prognosis (Suter and Marcum, 2007).

1.5 BRCA1 and BRCA2

Around 10,000 to 20,000 women yearly are diagnosed with breast cancer that shows a family history of the disease. This suggests the probability of a germline mutation having occurred. In the 1900s, genetic studies using lymphocytes from these families led to the discovery of two genes BRCA1 and BRCA2. These genes show germline mutations in 65% of family malignant breast cases. BRCA1 and BRCA2 are identified tumour suppressor genes. BRCA1 and BRCA2 are the most

important and predisposition genes, inhibiting risks of breast and ovarian cancer for a lifetime (Pelttari et al., 2015). The proteins that are encoded by these genes have a role in the elimination of DNA damage and genomic integrity. These two genes are located on different chromosomes and have dissimilar genetic sequences; however, they share many similar functional qualities. BRCA1 and BRCA2 encode proteins which form a protein complex with Rad51 which is very important in the recognition and repair of DNA double strand breakage. But mutated BRCA1 and BRCA2 can function adversely and inhibit the repair of double strand DNA breakage (Cunningham et al., 2014; Zhong et al., 1999). Eventually, the BRCA1 and BRCA2 encoded proteins are expressed through cell division in G1/S and G2/M phases. Additionally, the expression of wild-type BRCA1 is high in proliferating cells. BRCA1 has an essential role in a checkpoint response (Buchholz and Wazer, 2002). The cellular disorders in DNA double-strand repair increases the proportion of breast cancer formation in female with mutated BRCA genes in germline cells. The unrepaired damaged DNA in turn lead to additional mutations (Buchholz and Wazer, 2002; Zhong et al., 1999). Additionally, both proteins BRCA1 and BRCA2 are functioning in homologous recombination (HR), an essential DNA repair procedure that uses the undamaged sister chromatid to perform high-fidelity repair of mainly replication-associated with DNA double-strand breaks (DSB). HR appears to be the significant pathway responsible for protecting the integrity of the genome throughout proliferation, whereas, other DSB repair mechanisms are error-prone and may generate chromosome disorders such as deletions and translocations (Schlacher et al., 2011). Interestingly, in the hereditary breast and ovarian cancer syndrome (HBOC) the BRCA1 associated with BC is

predominantly oestrogen-receptor (ER) negative, however BRCA2 correlated with BC have similar distribution of cancer subtypes as found sporadically (Roy et al., 2012).

1.6 TP53 tumour suppressor

P53 is considered as a tumour suppressor and confirmed to be 'the guardian of the genome' due to its contribution in a variety of cellular pathways (Figure 2) such as DNA repair, apoptosis, cell cycle arrest, and senescence (Kim et al., 2015).The significance of p53 in cancer suppression is clear, where it is inactive in more than 50% of all sporadic human tumours (Bieging et al., 2014).

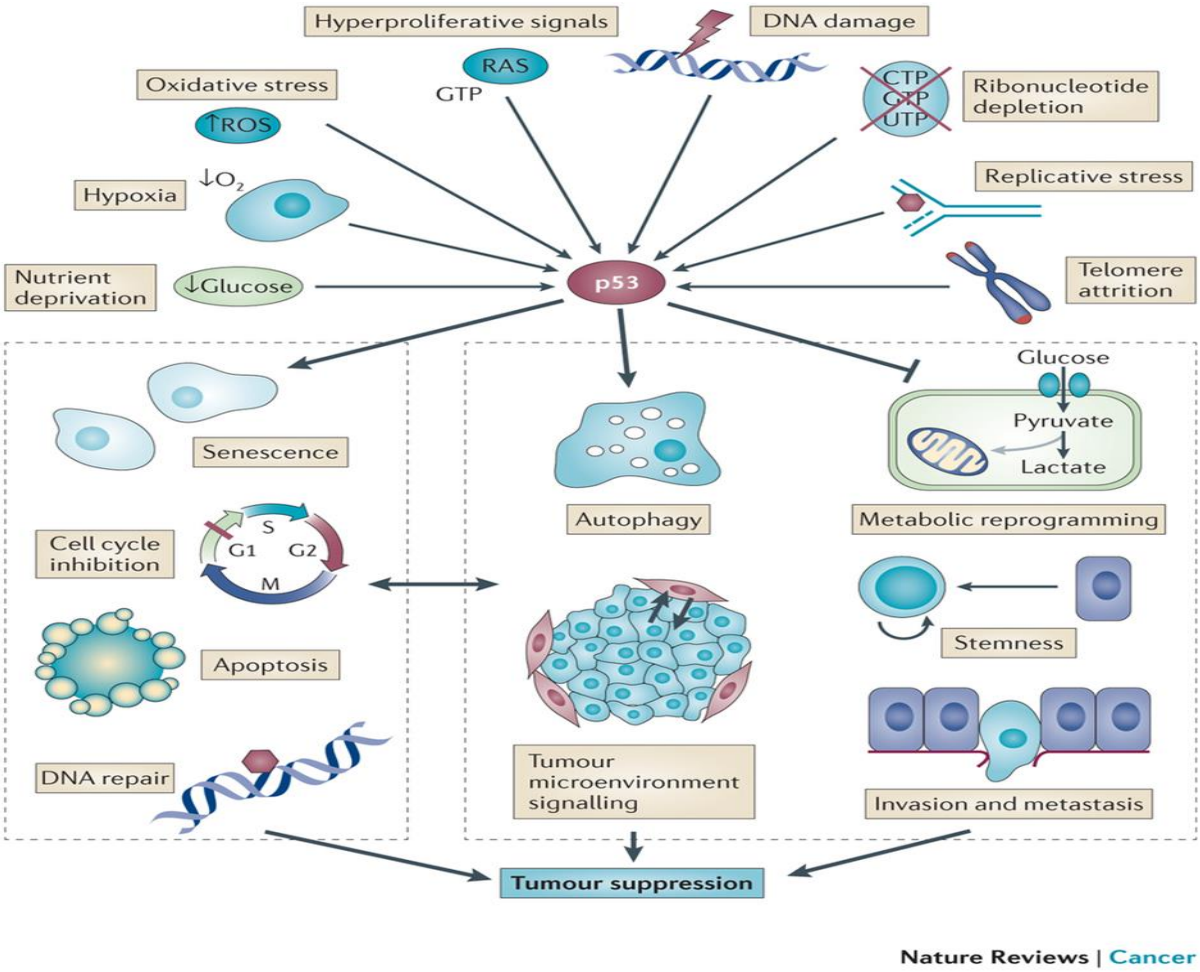

Figure 2 shows p53 role in different cellular pathways (Bieging et al., 2014).

The p53 role in the aging status is clearly understood *in vivo*. In a transgenic mouse model, a truncated form of p53 that enhances wild-type p53 activity augmented resistance to spontaneous cancer development. Recent researchers reported that the expression of p53 decreased in neural progenitor cells and mesenchymal stem cells depend on an age-dependent manner (Wilson et al., 2010) and that p53 efficiency decreases due to age at the organismal level (Feng et al., 2007). In general, these outcomes suggest that the decrease or loss of p53 expression throughout aging may involve in the development of cancer (Kim et al., 2015). P53 tumour suppressor gene has also been found as one of the most mutated genes in breast cancer and is found in approximately of 20-40% of the cases. This consequently results in the loss of its growth suppressive character and eventually leads to uncontrolled cell proliferation (Christgen et al., 2012; Ergul and Sazci, 2001). In addition, individuals with mutations in the genes BRCA1 and BRCA2 have a high possibility for development of p53 mutations (Roy et al., 2012). Furthermore, change in the genes involved in the p53 pathway are also linked to the development of breast cancer, for instance: G1 cell cycle checkpoint kinase is one of the p53 gene pathway and its mutation results in breast cancer due to destabilisation of p53, which ultimately results in proliferation of cells with damaged DNA (Bieging et al., 2014; Suter and Marcum, 2007).

1.7 Ataxia telangiectasia gene (ATM)

This gene phosphorylates various key proteins which activate the DNA damage checkpoint, initiates cell cycle arrest, DNA repair or apoptosis. The mutation of this gene results in a deficiency in the DNA repair system as well as a cell-cycle checkpoint with a net effect of evolving into breast cancer. The mutation of this

gene has been observed to increase the chance for getting breast cancer by 100-200 fold compared to people with the normal ATM gene. Waha (1998) investigated the level of ATM transcripts in some breast cancer patients, benign breast cancer and normal individuals. They found that the ATM transcript level was the lowest in the breast cancer patients and the highest levels were found in the normal individuals. However, benign breast cancer cases showed moderate levels between breast cancer and normal individuals (Ergul and Sazci, 2001). Recently some studies suggested the ATM gene as risk marker in breast cancer, where ATMmvp2a hypermethylation can be detectable in blood previous to disease onset, and this hypermethylation is associated with increased breast cancer risk (OR=1.89). However, the mechanism of increased risk is not clearly understood, nor possible causes of increased hypermethylation at this region (Brennan et al., 2013).

1.8 Phosphatase and tensin homolog gene (PTEN)

Phosphatase and tensin homolog gene (PTEN) is classified as a phosphatase; however, it differs from phosphatase as it targets fatty acids instead of proteins. Its target is called phosphatidylinositol-3,4,5-triphosphate (PIP3) and PTEN has crucial roles in mediating cell proliferation as well as cell death and such properties make it dual functional in respect of cell growth (Leslie and Foti, 2011; White et al., 2006). However, when this gene is mutated as in the case of breast cancer, it causes a disruption in the balance between cell survival and death towards cell proliferation with the overall result of production of uncontrolled cell growth. However, the contribution of this gene to breast cancer pathology remains small

and has been found to be responsible for about only 6% of breast cancer cases (Ergul and Sazci, 2001; Suter and Marcum, 2007).

1.9 XRCC3

XRCC3 is considered as one of the five RAD51 paralogs (i.e. XRCC2, XRCC3, RAD51L1/RAD51B, RAD51L2/RAD51C and RAD51L3/RAD51D) that have an essential role in the repair of double strand DNA breakage by homologous recombination (HR) (Thacker, 2005) in cells, by interacting with its binding supplementing Rad51C (Girard et al., 2013; Masson et al., 2001). XRCC3 deficiency or mutation exhibit to a number of impaired DNA damage repair which, induced by RAD51 foci formation, increased chromosome aberrations and elevated sensitivity to camptothecin (CPT), various DNA cross-linking agents, and impaired HR (Girard et al., 2013). Human XRCC3 (hXRCC3) has 8 cysteine residues, and was identified from a cosmid library across its ability to complement the mutagen-sensitivity of Chinese hamster irs1SF cells (Girard et al., 2013). Various association studies of XRCC3 have demonstrated debated consequences, however a meta-analysis by He et al. illustrated a correlation between common XRCC3 polymorphisms and BC risk (He et al., 2012). A prospective lethal missense mutation in the XRCC3 gene has been recognised in one breast and one ovarian cancer (Golmard et al., 2013).

1.10 DNA Repair

DNA is complex chemical structure that serves as the source and repository of genetic information in both prokaryotic and eukaryotic cells (Davis, 2012). Modifications to this complex structure caused by exposure to different elements from the environment and within a biological system could result in mutations that

related to the pathogenesis of several diseases (Boiteux and Jinks-Robertson, 2013; Clancy, 2008) Hence, DNA stability and integrity are prerequisites to normal physiological function and life (Davis, 2012). The pathogenesis of cancer has been associated with many toxic environmental factors. For instance, skin cancer is majorly associated with environmental induced DNA damage, which is mainly due to by excessive exposure to UV radiation. Similarly, tobacco smoking leads to damaged DNA in epithelial cells, a critical process of lung cancer pathogenesis (Lord and Ashworth, 2012). In addition to exogenous substances that can cause DNA damage, by-products of cell metabolism lipid peroxidation have the potential to interact with and damage DNA (Kryston et al., 2011). Indeed, the free radicals by-products of cellular metabolism has been shown to cause oxidative stress through increase in reactive oxygen species such as, superoxide anions, hydrogen peroxide, and hydroxyl radicals. Interestingly, several research studies have shown that each cell can experience more than one million DNA lesions per day (Lodish et al., 2000). Therefore, the importance of well-regulated DNA repair mechanisms is crucial in resolving this magnitude of damage in order to maintain normal cell function (Clancy, 2008). Processes of DNA repair exist in both prokaryotic and eukaryotic cells, and many proteins involved have been discovered throughout the study of DNA repair mechanisms. Depending on the type of damage which can occur to DNA, cells are using various mechanisms to detect and repair DNA damage, regardless the damage accrued by the environment or inaccurate replication (Abbotts et al., 2014; Clancy, 2008). Because DNA is responsible for protection and transference of genetic traits it also plays a crucial role in cell division. Additionally, DNA repair is strongly correlated to the regulation of the cell

cycle. Throughout the cell cycle, checkpoint processes ensure that a cell's DNA is intact before allowing DNA replication and cell division to occur. However, suggest failure of these checkpoints mechanisms can lead to an accumulation of DNA damage, which in turn leads to mutations and fatal diseases (Clancy, 2008; Goodarzi and Jeggo, 2012)

Depending on different types of DNA damage that might occur, cells have developed several repair pathways for their different types of damages. These repair mechanisms (Figure 3) are commonly divided into five major types: base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), and double-strand break repair, which includes both homologous recombination (HR) and non-homologous end joining (NHEJ)(Houtgraaf et al., 2006; Klapacz et al., 2015).

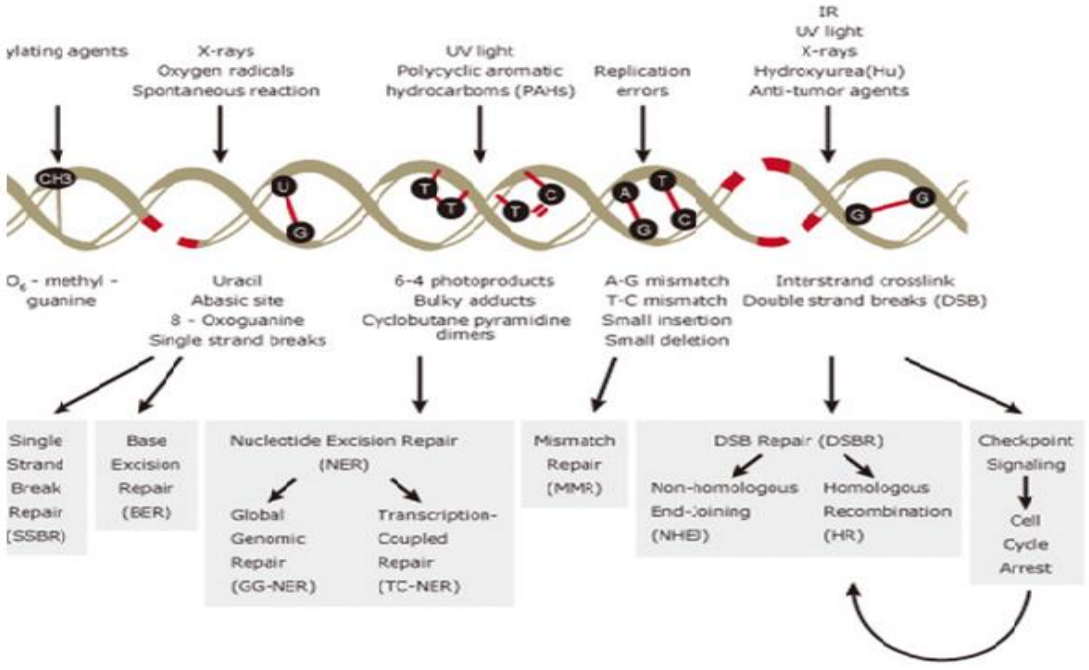

Figure 3 DNA several repair pathways

http://www.bio-connect.nl/uploads/websiteimages/1%20Lev%20BC/GeneTex/DNA-repairs.gif.

1.10.1 Base Excision Repair

Oxidative stress is considered as the main cause of endogenous DNA damage. The hydrolysis and deamination are eliminated by the base excision repair (BER) mechanism. BER involves the sequential action of five DNA-modifying stages: releasing nitrogenous bases from deoxyribose by DNA N-glycosylase enzyme, an endonuclease enzyme that cleaves the DNA backbone at the resulting apyrimidinic/apurinic (AP) site, a 39- or 59-phosphodiesterase enzyme that sweeps the remaining deoxyribose phosphate residue, a DNA polymerase enzyme that fills the cavity thus created, and a DNA ligase enzyme to seal the remaining incision (Boiteux and Jinks-Robertson, 2013; Hoeijmakers, 2001).

1.10.2 Nucleotide Excision Repair (NER)

Nucleotide excision repair (NER) is described as the essential repair mechanism from prokaryotes to higher eukaryotes (Morita et al., 2010). The most important advantage of the NER system is its extensive substrate specificity: NER can mend DNA lesions such as UV-induced pyrimidine dimers or further bulky adducts (Truglio et al., 2006). NER is a highly versatile and multipurpose used repair pathway that can identify and repair a variety of bulky, helix-distorting damage in DNA. Such damage results in pyrimidine dimers, for instance cyclobutane pyrimidine dimers (CPD) and 6–4 photoproducts, which are induced by the UV light after exposure to sun. An alternative notable substrate of NER is cisplatin-DNA intrastrand crosslinks. NER is mediated by the sequential functioning of repair proteins at the location of the DNA lesion. Despite similarities to BER, the NER pathway is more complicated, where it requires thirty different proteins to achieve a multi-stride ‘cut-and-patch’-like mechanism. These steps include DNA lesion

recognition, local unwinding of the DNA helix near the lesion site, removal of a short single-strand section of DNA containing the lesion, and sequential repair synthesis and strand ligation (Shuck et al., 2008). The biological significance of NER is supported by the fact that defects in the NER pathway can cause several human genetic diseases, such as xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy, which all show sun sensitivity. Furthermore, these diseases show overlapping symptoms and clinical signs including cancer, developmental delay, neurodegeneration, immunological disorders, and premature aging (Cleaver et al., 2009). The NER pathway divided to two linked sub-pathways, which are termed global genome NER (GG-NER) and transcription-coupled NER (TC-NER). As the names indicate, GG-NER repairs DNA lesions throughout the genome and cell cycle, whereas, TC-NER is especially responsible for eliminating DNA lesions located on the coding strand site expressed active genes. Both sub-pathways are acting mechanistically in the same way, apart from the initial DNA damage recognition step (Sugasawa, 2010).

1.10.3 Mismatch Repair (MMR)

The MMR pathway plays a crucial role in post-replication repair of misincorporated bases that have avoided the checkpoint processes of replication polymerases. In addition, MMR proteins also involved in accurate insertion/deletion loops (IDLs) that consequence from polymerase slippage through replication of repetitive DNA sequences. The importance of this pathway is established by the fact that deficiency in the MMR pathway leads to a mutator phenotype in cells, which is distinguished by constant microsatellite instability and an increased mutation frequency. Moreover, germline mutations in MMR genes are correlated to a variety

of cancers, such as hereditary non-polyposis colon cancer, also known as Lynch syndrome (Fukui, 2010; Larrea et al., 2010). The MMR pathway can be divided into three sub-type pathways: a recognition stage where mis-paired bases are identified, an excision stage where the strand mis-paired region is removed including oligonucleotides resulting in a gap, and a repair synthesis stage, where the cavity is filled by the DNA resynthesis (Larrea et al., 2010).

1.10.4 Double-Strand Break Repair

Double-strand breaks (DSBs) is one of the most biologically dangerous types of DNA damage (Dexheimer, 2013). For instance, a single unrepaired double strand break is sufficient to lead to cell death. Furthermore, defective repair can cause deletions or chromosomal aberrations, which are linked with the progress of cancer or other abnormal genomic syndromes. Therefore, the repair of DSBs is essential for both cell survival and maintenance of genome integrity (Dexheimer, 2013; Khanna and Jackson, 2001; van Gent et al., 2001). Mammalian cells are use two different mechanisms for DSBs repair which are homologous recombination (HR) and non-homologous end-joining (NHEJ). These two repair pathways have different requirements for a homologous template DNA and in the precision of DSB repair. HR-directed repair is largely an error-free mechanism as it uses the genetic information available in the undamaged sister chromatid as a copy template (Dexheimer, 2013; Li and Heyer, 2008). However, NHEJ is typically error-prone and repairing the DSBs by direct ligation of the broken ends (Lieber, 2010). NHEJ considered as the predominant and fast repairing pathway in mammalian cells, which is using in all phases of the cell cycle, whereas, homologous recombination (HR) is limited to the late-S and G2 phases (Dexheimer, 2013).

The biological importance of DNA repair pathways is emphasized by the fact that the deregulation of these mechanisms can contribute to the inception and progression of tumours. By contrast, DNA repair can cause resistance to front line cancer treatments such as chemotherapy and radiation, which act to cause DNA damage to kill tumour cells. Thus, the sensitivity of tumour cells to DNA damaging agents is almost likely correlated to intrinsic disorders in DNA repair pathways. The capacity of tumour cells to distinguish DNA damage and introduce DNA repair is a key mechanism for therapeutic resistance (Abbotts et al., 2014; Dexheimer, 2013).

1.11 Nanotechnology

The term nanotechnology refers to technology in which one-billionth of a meter is used as the scale. It involves design, manufacturing, identification and use of substances by making their sizes at the nanometer scale. Once the size of the material is reduced to nano-size it shows new characteristics such as enhancing the physicochemical and ultimately biological properties compared to bulky materials (Ochekpe et al., 2009). For decades, nature has been a good source, which inspired mankind to make tremendous progress in innovations, design, discovery, and inventions. Scientists have long been promoting the idea of utlising the level of atomic or molecular structures and systems that have new properties, characteristics, and functions that led to a revolution named "nanotechnology" (Sharma et al., 2009). The oldest scientific illustration of nanomaterials and optical properties was given by Michael Faraday in 1857, and the title of that paper was “Experimental relations of gold (and other metals) to light” (Wilcoxon, 2009). The first idea of nanotechnology was described by American physicist Richard

Feynman in December 1959 at the American Physical Society. Feynman explained a procedure by which smaller groups of molecules and atoms can be formed through the manipulation of larger ones (Pierotti et al., 2008). Furthermore, in 1974 Professor Norio Taniguchi demonstrated the term nanotechnology as the procedure of separation, integration, and deformation of materials by one molecule or atom (Khawaja, 2011). The real start of the nanotechnology and nanoscience was in the early 1980s, and as a consequence of this technology the scanning tunneling microscope (STM) was invented. The fullerenes and carbon nanotubes were discovered in 1985 as a result of this development. The United States National Nanotechnology Society was established in the year 2000 to organize nanotechnology research and its progress (Guzman et al., 2006; Tinkle, 2010). Nanoparticles (NP) are considered to be the stage between the bulk and atomic structure of materials. Substances have stable and known chemical and physical properties in their normal size; however, these properties can be changed to completely different ones at the nano stage (Ehrman, 1999; Hutter and Maysinger, 2011; Perrier et al., 2010). Agglomeration or aggregation is another physio-chemical advantage of NPs; because NPs are held together by soft and strong forces, as well as Vander Waals is electrostatic forces and sintered bonds. In addition, the solubility of nano-materials can be affected by the binding force aggregation under several different conditions (Allouni et al., 2009; Poizot et al., 2000; Wokovich et al., 2009). The 21st century can be called the "nano century". As a result of the revolution in nanotechnology, most aspects of human life have been touched by nanotechnology development. This intervention was obviously seen in many sectors, including industries, consumer products, drug delivery,

optical devices, cosmetics, and sport accessories (Balshaw et al., 2005; Kango et al., 2013; Lee et al., 2008; Sharma et al., 2009). The essential reason behind the popularity of nanotechnology in the technological world at present can be attributed to the considerable increase in proportion between surface area and volume at the nanoscale, which brings quantum mechanics into display providing evolution of novel and improved mechanical, optical and electronic properties to nanomaterials (Oberdorster et al., 2005). However, these novel properties featured in the materials at the nano-range enable them to interact with biological systems, and this can lead to unpredictable outcomes. This has been proved recently by a several studies concerning the undesirable consequences of nanoparticle exposure (Brumfiel, 2003; Goldston, 2007; Magdolenova et al., 2014; Nel et al., 2006). Different methods have been used to synthesise a variety of NPs with disparate chemical composition, differing in shape, surface coating, size, etc. These NPs have been confirmed to be cytotoxic and genotoxic to mammalian cells (Dhawan et al., 2006; Isakovic et al., 2006; Lewinski et al., 2008; Magdolenova et al., 2014; Papageorgiou et al., 2007; Singh et al., 2007; Wang et al., 2007). In the last 4 years the Health and Environmental Safety Department of Nanotechnology has shown an increasing attentiveness, after the first report which was published in 2004 by the Royal Society and Royal Academy of Engineering highlighting the lack of required information that illustrates the impact of engineered nanomaterials on human health and the environment (Singh et al., 2009; Society and Engineering, 2006). The mechanism behind NPs, toxicity is yet to be demonstrated (Singh et al., 2014). Many studies have illustrated that NPs have the potential to induce DNA damage, apoptosis, cell membrane disruption through oxidative stress and lipid

peroxidation (Hsin et al., 2008; Li et al., 2008; Magdolenova et al., 2014; Sharma et al., 2009). Nanotechnology is also used as a means for drug delivery to its target tissue or organ. In doing so nanotechnology is used to overcome some problems associated with some drugs such as improving bioavailability, drug solubility and minimising or reducing side effects. Moreover, the presence of drug in nano-size gives better delivery properties including better membrane entrance or permeability and hence better intracellular drug concentration (Ochekpe et al., 2009). Recently, drug delivery based on nanotechnology has been developed as a dynamic strategy by overcoming various biological, biophysical and biomedical obstacles that emerge in the body against successful delivery of chemotherapy drugs to cancer tissues. The constant, organized and aimed delivery of anticancer drugs in combination with a nanotechnology approach enhances the influence of cancer therapeutic and elimination of side effects associated with cancer drugs (Parhi et al., 2012).

1.12 Ibuprofen

In Europe, non-steroidal anti-inflammatory drugs (NSAIDs) including ibuprofen are consumed in amounts of over 100 tablets yearly (Heckmann et al., 2007). The discovery of ibuprofen (Figure 4) goes back to more than four decades when Dr Stewart Adams and his colleagues observed the anti-inflammatory effect of this agent on guinea pigs after its synthesis by Dr John Nicholson, as an attempt to find safer anti-inflammatory agents than ‘’ulcerogenic’’ aspirin. It has been found that ibuprofen is less irritating to the gut compared to aspirin with remarkable anti-inflammatory effects. In 1970, the action of prostaglandin in mediating some inflammation and pain was established and ibuprofen was found as a good

inhibitor for the production of this compound (Rainsford, 2011). Consequently, it has been classified as one of the non-steroidal anti-inflammatory drugs (NSAIDs). Ibuprofen has many therapeutic applications such as being anti-inflammatory, antipyretic and analgesic. In addition, ibuprofen has also been used as one of the medications for treatment of various joint and bone inflammations; for instance, osteoarthritis, rheumatoid arthritis and mild to moderate pain. (Potta et al., 2011).

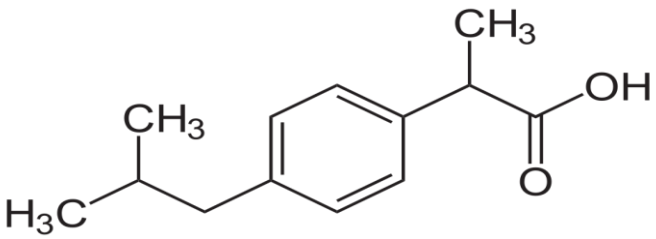

Figure 4 Ibuprofen structural formula; taken from **http://pubchem.ncbi.nlm.nih.gov**

1.12.1 Mechanism of action of ibuprofen

Ibuprofen works by inhibiting both cyclooxygenases I and II (COX-1 and COX-2) non-selectively (Figure 5). These enzymes are responsible for the biosynthesis of inflammatory mediators, namely prostaglandins. Ultimately, inhibition of such enzymes causes lowering of the levels of prostaglandins which in turn leads to inhibition of the various effects related to prostaglandins such as reducing pain, inhibition of the inflammatory process and also reduction of body temperature (Bushra and Aslam, 2010).

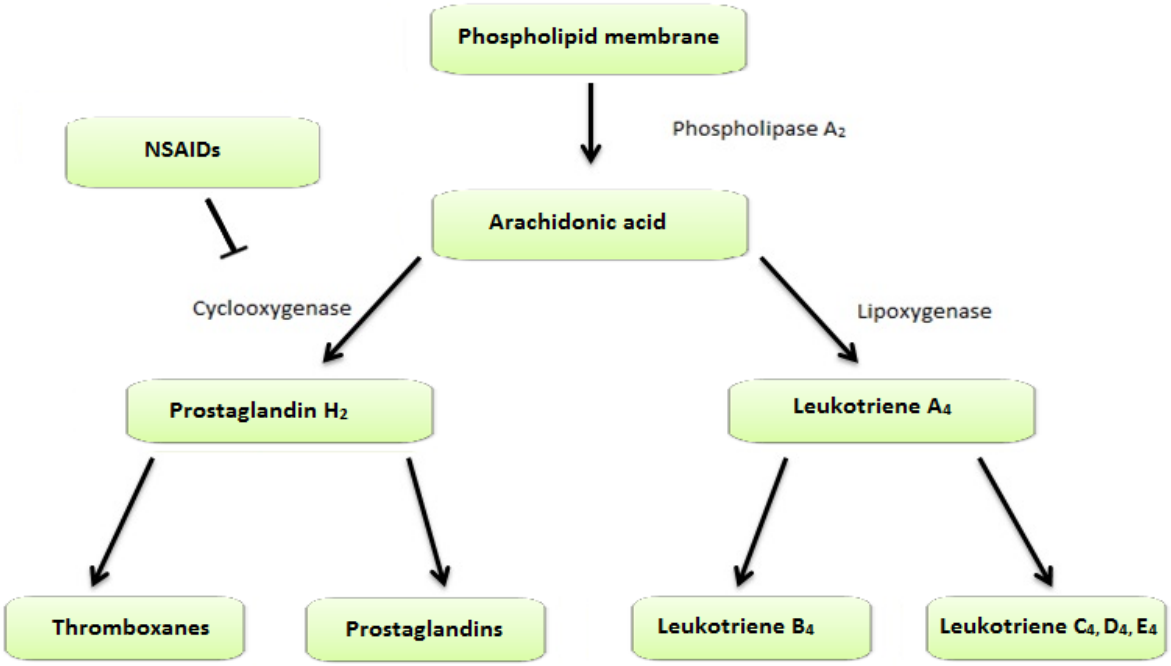

Figure 5 Biosythesis of prostaglandin and leukotrienes. NSAIDs block cyclooxygenase (Pardutz and Schoenen, 2010).

1.12.2 Genotoxicity of Ibuprofen

Despite the fact that ibuprofen is available as a non-prescription medication it can cause some toxic effects particularly it can act as a genotoxin. The genotoxicity induced by ibuprofen has been debated in recent years, for instance, the genotoxicity of ibuprofen on *Salmonella* strains did not produce conclusive results (Tripathi et al., 2012). Philipose et al. (1997) on the other hand, demonstrated the genotoxic effect of ibuprofen in mice where it caused a weak genotoxic impact on sister chromatid exchange. Moreover, also adverse results were found when it was reported that ibuprofen has no genotoxic effect after a short term two weeks period treatment (Ghosh et al., 2010). Conversely, Tripathi et al. (2012) conducted a similar study to investigate the genotoxic effect of ibuprofen on bone marrow cells of mice. Ibuprofen was administrated orally at different doses which were 10, 20, 40, and 60 mg/kg body weight. Chromosomal aberrations, were used as a

parameter to assess the genotoxicity. It was observed that Ibuprofen led to an increase in the number of chromosomal aberrations that was more pronounced at 40 and 60 mg/kg doses.

1.12.3 Ibuprofen and breast cancer

There are limited studies on the relationship between ibuprofen and breast cancer. Harris et al. (1999) performed a study to compare the influence of some NSAIDs including ibuprofen on breast cancer patients. It was found that regular ibuprofen consumption caused a reduction of breast cancer rate by about 50%. As a result of this it was suggested that NSAIDs including ibuprofen might be useful as a means for prevention of the occurrence of breast cancer(Bushra and Aslam, 2010; Harris et al., 1999). Sun et al. (2012) evaluated the anti-breast-cancer efficacy of phosphor- ibuprofen (P-I) 400 mg/kg/day on (MCF-7) and (MDA-MB231) breast cancer cell lines comparing with control, where, the phosphor-ibuprofen inhibited growth of MCF-7 and MDA-MB231 cell lines 51% and 26.6% respectively. They suggested that P-I induced oxidative stress, which disrupted the thioredoxin system by inhibiting the expression of oxidized Trx-1 and suppressing thioredoxin reductase activity.

1.13 Aspirin

The history of aspirin (Figure 6) started a long time ago; its discovery came after the optimisation of salicin which was obtained from the bark of willow trees (Thorat and Cuzick, 2015). In 1853, Charles Gerhardt made a modified form of salicin, namely acetylsalicylic acid, for first time. However, its therapeutic application was discovered later in 1876 when some physicians like Thomas Maclagan started using it for both alleviating pain as well as in the treatment of rheumatism. In 1897,

a German scientist Felix Hoffman managed to create acetylsalicylic acid in the lab and after which acetylsalicylic acid was named as aspirin (Miner and Hoffhines, 2007; Thorat and Cuzick, 2013; Vane and Botting, 2003; Wood, 2015).

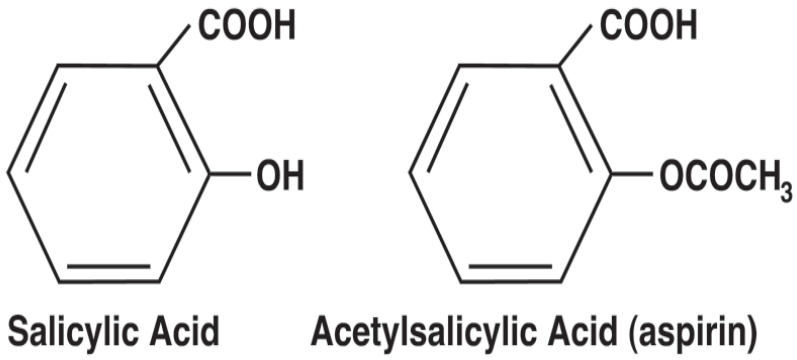

Figure 6 Salicylic Acid and Aspirin structural formula taken from http://www.theodora.com

1.13.1 Mechanism of Action of Aspirin

Although aspirin is an old drug, its mechanism of action became known only in 1971 when John Vane found that aspirin blocks the COX enzyme (Figure 7,8) by adding functional group to it. As mentioned earlier, COX enzyme is a very crucial enzyme playing a role in the biosynthesis of various inflammatory mediators, most importantly prostaglandins. As a consequence of this effect on cyclooxygenase enzyme, prostaglandins and some other inflammatory mediators cannot any longer be produced from their bioprecursor arachidonic acid, as long as aspirin is present. Therefore, the inhibition of cyclooxygenase enzyme by aspirin results in numerous effects such as inhibition of inflammation and its associated oedema (Wood, 2015).

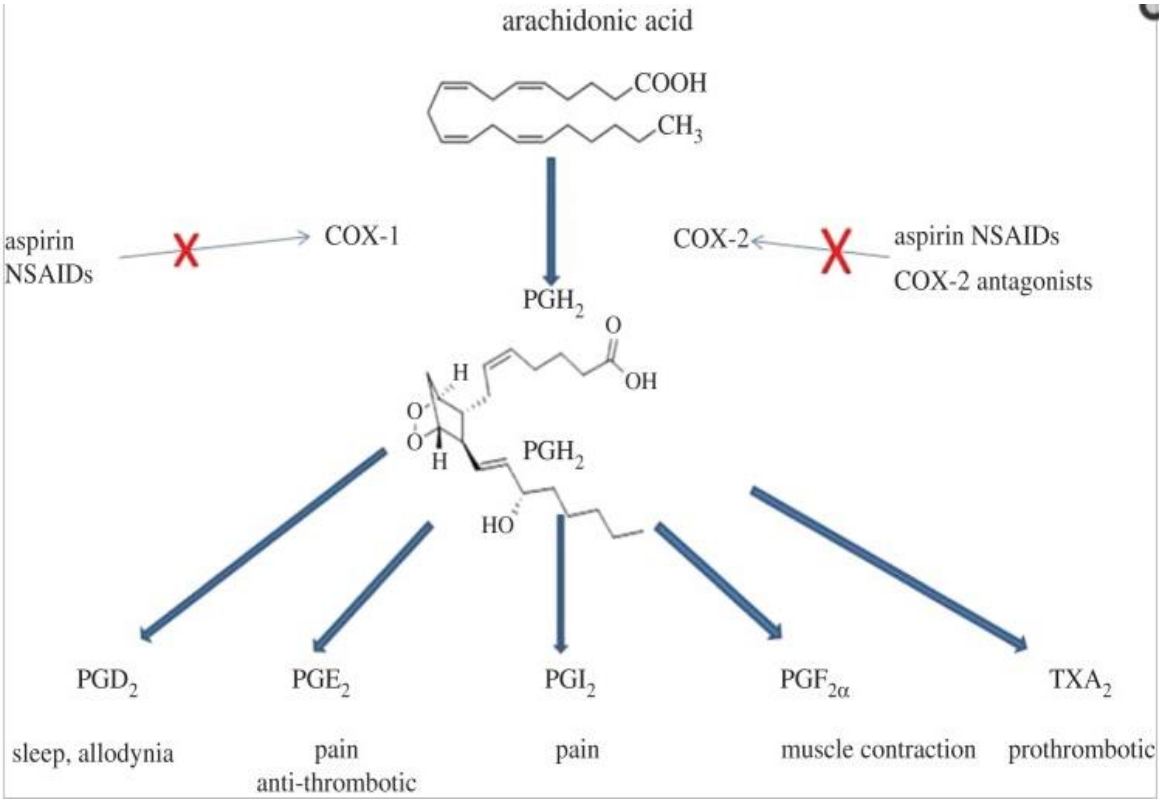

Figure 7 The role of aspirin inhibiting inflammatory mediators (Wood, 2015)

In addition, aspirin also causes inhibition of other inflammatory mediators other than prostaglandins such as the leukotrine derivative 12-HETE. This compound is important in mediating the recruitment of immune cells such as eosinophils and neutrophils in the inflammation areas with the consequence of releasing some inflammatory mediators from these cells and causing tissue destruction and hence inflammation. Thromboxine is another important bio-mediator and is similar to prostaglandin in that it is produced from the same bio-precursor under effect of cyclo-oxygenase enzyme. However, thromboxane possesses a blood clotting effect and thus the inhibition of its biosynthesis by aspirin results in an anticoagulant effect (Fuster and Sweeny, 2011; Koester, 1993; Peixoto and Silva, 2014; Vane, 1971).

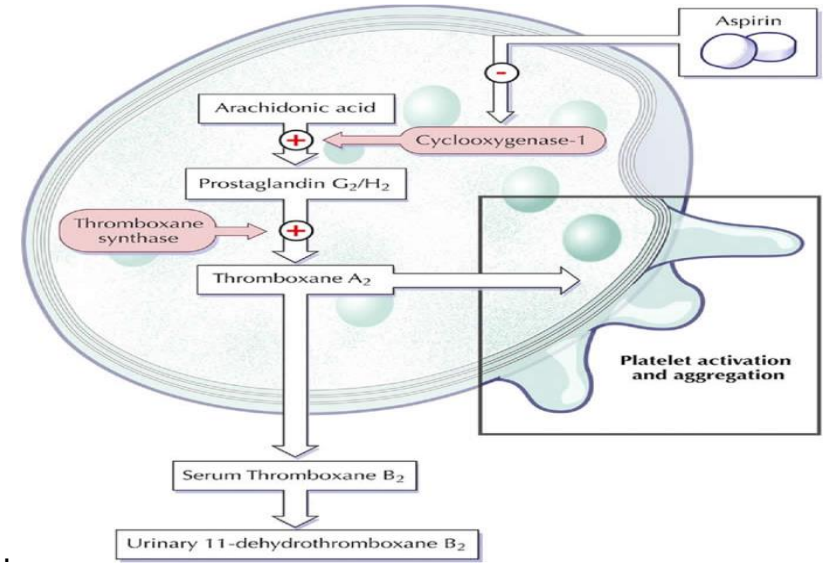

Figure 8 Aspirin mechanism of action (Gasparyan et al., 2008).

1.13.2 Genotoxicity of aspirin

Although aspirin itself does not exhibit a gentoxic effect, its protective potential was observed when it was combined in a treatment with the genotoxic anticancer agent mitomycin C (MMC). This was concluded when aspirin was used in a mouse study at doses of 0.5, 5 and 50 mg/kg combined with 2 mg/kg dose of MMC, it has been observed that aspirin led to a reduction in the genotoxocity produced by MMC in various organs such as liver and spleen and the reduction was in dose-dependent fashion (Baron and Sandler, 2000; Niikawa et al., 2008)

1.13.3 Aspirin and breast cancer

The use of aspirin in prevention of breast cancer is still a new subject and there are only a few studies in this regard. The effect of aspirin to protect against breast cancer was observed in some patients who are on regular intake of aspirin such as patients with cardiac disease who take aspirin (100mg) on a daily basis (Lotrionte et al., 2016). The reduction of breast cancer incidence by aspirin was linked to its

inhibition of the cyclo-oxygenases COX-1 and COX-2 (Fig 5 and 8) with the former being inhibited more strongly than the latter. This results in inhibition of prostaglandin H2 production from its bioprecursor arachidonic acid which in turn leads to inhibition of further prostaglandin production from its biosource prostaglandin H2 (Cazzaniga and Bonanni, 2012; Lazzeroni et al., 2013). Ultimately, the inhibition of prostaglandin production leads to the inhibition of their physiological roles such as inhibition of cell growth and angiogenesis which contributes to its antitumour effect. In addition, aspirin also causes stimulation of AMPK signalling pathway which also cause inhibition of cancer cell growth. Prostaglandin E2 has been found to elevate the level of expression of the aromatase gene which in turn is responsible for the biosynthesis of oestrogen. It has been observed that there is a direct correlation between the level of expression of COX-1, COX-2 and aromatase stimulation and hence oestrogen production and this eventually means aspirin indirectly inhibits oestrogen production and its associated breast cancer growth. Despite such effects of aspirin, it is too early for using it as a single agent in chemoprevention of breast cancer due to limited available results on its effect for protection against breast cancer and it consequently should be used in combination with other breast cancer therapies (Cazzaniga and Bonanni, 2012; Lazzeroni et al., 2013).

1.14 Bleomycin

Bleomycin (BLM) is a drug extracted from *Steptomyces verticillus*, and classified as an anti-cancer therapy drug which belongs to a group of natural glycopeptides. The drug was described for the first time in Japan by Umezawa in 1976. BLM has a unique structure and pathway of action among chemotherapy drugs (Sikic et al.,

1985). Bleomycin is used to cure several types of cancers such as head and neck cancer, lymphomas and testicular cancer. BLM is used for therapeutic purpose because of it has the potential to cause DNA damage like single and double strand breaks (SSB, DSB) in the presence of cofactors O_2, Fe (II), and a one-electron reduction (Chen et al., 2008). The actual mechanism behind DNA strand scission is still unclear. However, a hypothesis suggested that BLM has the ability to prevent the integration of thymidine into DNA. Furthermore, BLM may react with metal ions producing metal-bleomycin which in turn react with O_2 to generate free radicals hydroxide and superoxide that break DNA strands. An alternative study proposed that BLM may link at particular locations in the DNA strand and induce cleavage by inhibiting the hydrogen atom from the nitrogen base to generate strand breakage as the base show a Criegee-type rearrangement (Chen et al., 2008; Huls and ten Bokkel Huinink, 2003). Sikic et al. (1985) demonstrated that BLMs binds to DNA and worked as mini enzyme after forming a complex with iron and O_2 that formed free radical formation and finally leads to DNA strand breakage. Some studies stated that BLM's capacity to induced chromosomal breaks were significantly increased in the cells of both familial and sporadic breast cancer patients compared to the healthy controls (Hu et al., 2013).

1.15 Reactive oxygen species (ROS)

ROS receive considerable attention in genotoxicology science. They are defined as short-lived molecules that are capable of releasing oxygen atoms. In general they can be found in micromolar or picomolar different concentrations in natural systems (Burns et al., 2012). In environmental systems, O_2, $^{\cdot}OOH$, $^{\cdot}OH$, H_2O_2, and CO_3^{-} have the ability to oxidise a varieties of molecules including biomolecules

(Burns et al., 2012; Canonica et al., 2005). It is well-known that increasing levels of oxidative stress leads to damage to sensitive biological components for instance DNA, membrane lipids and proteins, and such negative influence may contribute in the aetiology of various fatal diseases such as cancer (Naveenkumar et al., 2013). ROS are commonly produced by photolysis, electron transfer or energy transfer reactions (Bartosz, 2006). Free superoxide & hydroxyl radicals can be created by reduction of oxygen (Gutteridge, 1994). The superoxide radical (O^{-}_{2}) is produced during the activity of enzymes such as NADPH oxidase and xanthine oxidase. Superoxide ($^{\cdot}O^{-}_{2}$) is considered the essential form of the free oxygen radical (Dohi et al., 2010; Floyd and Carney, 1992), and it acts as a reductant or an oxidant (Imlay et al., 1988). The superoxide radical, which is formed by organic agents has a high reactivity and is more reactive than any ($^{\cdot}O^{-}_{2}$) generated in interior biological processes and might cause significant damage to DNA (Gutteridge, 1994). Hydrogen peroxide (H_2O_2) is an active form, which is enzymatically generated from the superoxide radical. A more effective and aggressive form of oxygen radical, the hydroxyl radical ($^{\cdot}OH$) can be created by the combination of ($^{\cdot}O^{-}_{2}$) and H_2O_2 (Ben-Shaul et al., 2001; Sewerynek et al., 1996). The most reactive oxygen radical known to chemistry is the hydroxyl radical; it has a massive potential to cause biological damage once it comes in to contact with all biological molecules. All DNA components including pyrimidine and purine bases can be damaged by the hydroxyl radical (Gutteridge, 1994). Mitochondrial DNA is more sensitive than nuclear DNA to reactive oxygen species induced damage, and delayed treatment leads to constant mitochondrial DNA damage and impaired of mitochondrial function (Naveenkumar et al., 2013).

1.16 The Comet assay

Methodologies, that can recognize and evaluate DNA damage, have been developed in the last two decades. The first expression of DNA damage in a single cell was developed by Rydberg and Johanson in1978, by lysing and inserting cells in agarose on slides in the presence of an alkaline pH ≥ 13, allowing the partial unwinding of DNA. After neutralisation, cells were dyed with acridine orange and the amount of DNA damage was indicated by measuring the extent of green (equivalent to double-stranded DNA) and red (equivalent to single-stranded DNA) fluorescence using a photometer. A microgel electrophoresis method, that is known as the Comet assay, was first developed by Ostling and Johanson, to increase the sensitivity of these experiments for detecting DNA damage in individual cells (Collins, 2004; Ostling and Johanson, 1984; Rojas et al., 1999; Singh et al., 1988; Tice et al., 2000). The Comet assay is a term referring to single cell gel electrophoresis. The Comet assay has been specified to be a sensitive and accurate technique. It can determine DNA damage and detect DNA integrity (Anderson et al., 2013; Tice et al., 2000). As a result, the Comet assay is typically used to determine genotoxicity, and consequently used to evaluate the effectiveness of chemotherapy compounds on tumour cells and their effects on germ and somatic cells (Anderson et al., 1997). The Comet assay can be carried out in two versions (Figure 9), the neutral Comet assay, which mainly detects double strand DNA breakage and the alkaline Comet assay, which additionally assesses single-strand damage and alkaline labile sites (Collins, 2004; Singh et al., 1988). In this technique, cells were embedded in agarose. High salt concentration and a detergent were used to lyse the cells. Under natural conditions, the released DNA was electrophoresed. Increased rates of DNA double

strand break (DSB) in cells increased the migration of DNA towards the anode. The DNA was stained using ethidium bromide. The damage was evaluated by measuring the ratio of intensity of fluorescence between two fixed points and this was throughout the migration step by utilising a florescence microscope, camera and capture system; software Komet 6 . The neutral pH, which was used in this assay led to the lack of efficiency of this procedure (Collins, 2004; Tice et al., 2000).

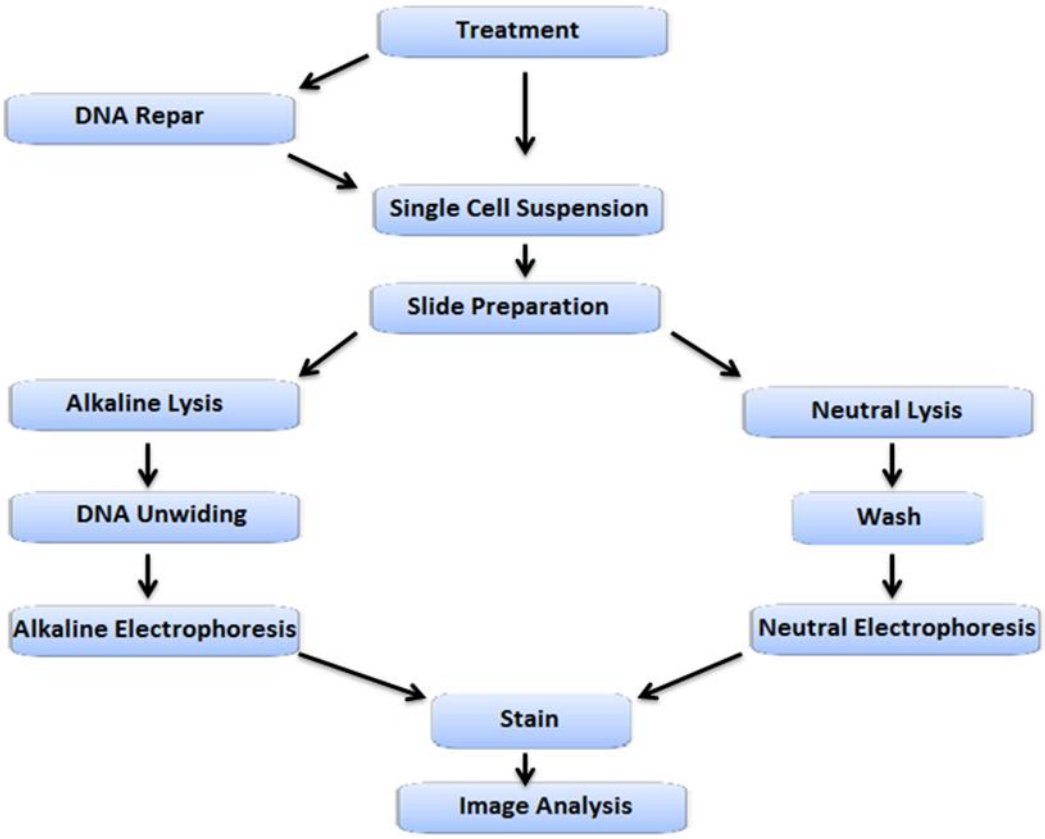

Figure 9 A general protocol for alkaline and neutral Comet assay

As a result, using the Comet assay under alkaline pH (pH>13), it became possible to identify the single-strand DNA breakage (Singh et al., 1988). At pH>13 the migration of DNA depends on the increase in the level of DNA single-strand breakage (SSB), which is associated with inadequate excision repair process at

damaged sites and alkali-labile site (ALS). Most of the genotoxic agents induce SSB and ALS rather than DSB; the alkaline Comet assay provided additional sensitivity in recognition of more genotoxic agents. This single cell gel electrophoresis assay is the most beneficial method in genotoxic research (Collins, 2004; Singh et al., 1988; Tice et al., 2000). In the Comet assay the image that is seen under the fluorescence microscope, is similar to a comet with a prominent head and tail; the head consists of integrated undamaged DNA and the tail of migrated DNA fragments (Collins, 2004; Kumaravel and Jha, 2006). Consequently, the DNA amount in the tail of the comet would reflect an estimated percentage of damaged DNA. Subsequently, DSB, SSB and oxidative damage of DNA base pairs can be successfully detected by the Comet assay (Tice et al., 2000). The Comet assay has several advantages such as the collected data are from single cells, providing more pronounced statistical analysis; it needs a small number of cells per sample (less than 10,000), it is a highly sensitive method for detection of DNA cross links and a convenient assay for any eukaryotic individual cell (Dixon et al., 2002; Lee and Steinert, 2003).

1.17 The cytokinesis blood micronucleus (CBMN) assay

A micronucleus (MNi) is a small supplemental nucleus that is exhibited after the anaphase through nuclear division. MN is a remnant of a centric chromosome fragment or an entire chromosome. This chromosomal fragment is not incorporated in the daughter nuclei (Fenech, 2007). The first description of a micronucleus in an erythrocyte cytoplasm was by Howell and Jolly more than a century ago, and MNs have been called Howell-Jolly bodies by haematologists since that time (Kirsch-Volders et al., 2003). In 1969 the term micronucleus test proposed by Boller and

Schmid (1969). MN can be seen in bi-nucleated cells in humans or mammals, after using cytochalasin-B (a microfilament ring assembly inhibitor) to block cytokinesis (Fenech, 2000). Hence another name for the MN assay is the cytokinesis block micronucleus assay (CBMN). This assay is considered as the preferred method to score micronuclei (MNi) because it is restricted to binucleated cells. The CBMN assay is the commonly used method to track DNA damage in human lymphocytes after being insulted by genotoxic and cytotoxic agents (Fenech, 2007), in medicine prevention, and determination of chemical and pharmaceutical safety (Bonassi et al., 2007). The CBMN assay has many advantages compared to other cytogenetic methods. It is relatively quick, inexpensive, and it can be used easily to identify and score MNs in cells that have successfully completed one nuclear division. The complete nuclear division is essential for the appearance and recognition of MNi, nuclear buds and nucleoplasmic bridges (NPBs) (Fenech, 2007; Umegaki and Fenech, 2000). The CBMN assay can be carrid out in human lymphocytes or cell lines after the cells have been cultured. Cell division of lymphocytes in culture is stimulated by phytohaemagglutinin (PHA). Measurements must be carried out in deviding cells because scoring should be in binucleated BN cells that indicate cell division (Fenech, 2000; Lyall et al., 2007).

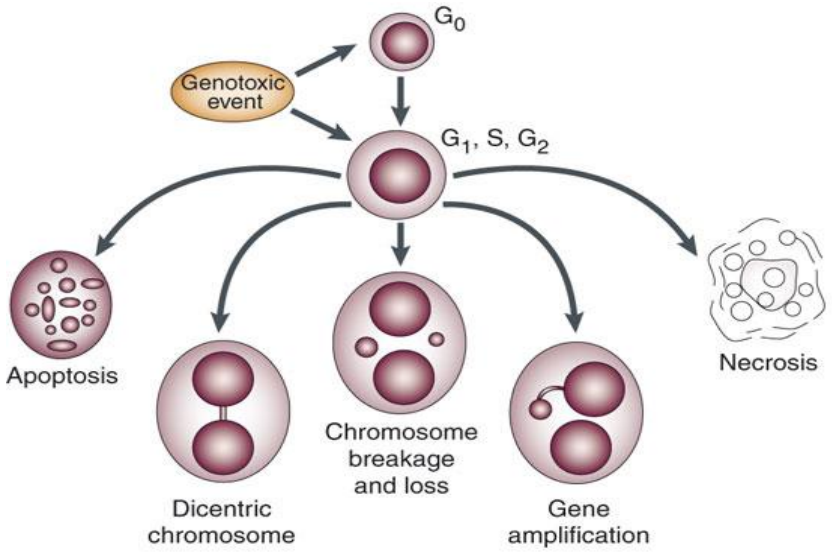

Figure 10 Several possible fates of cultured cells in the CBMN assay (Fenech, 2007).

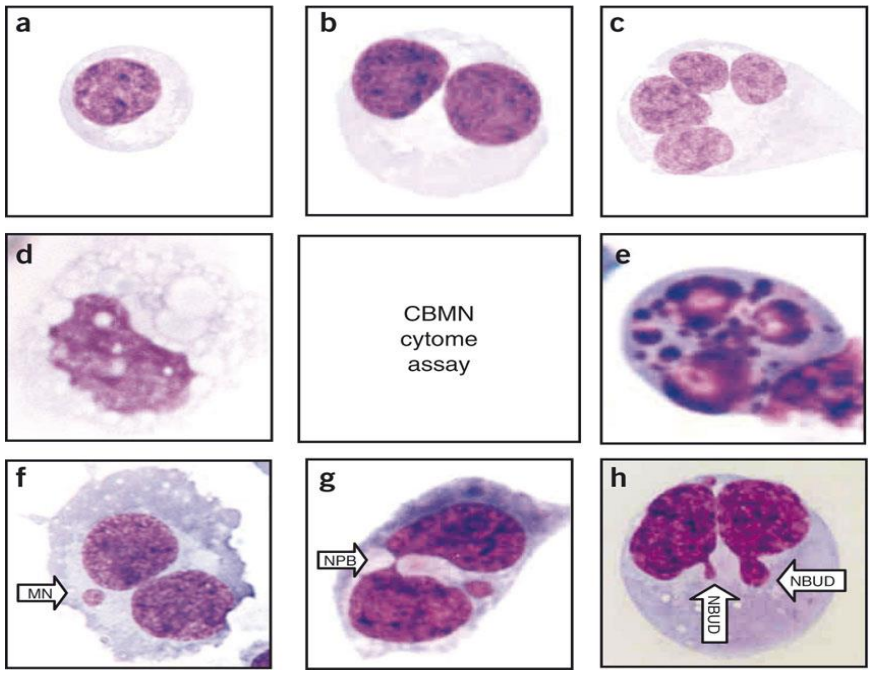

Figure 11 Photomicrographs of the cells scored in the CBMN assay. (a) mononucleated (MonoNC cell); (b) binucleated (BiNC) cell; (c) multinucleated (multiNC) cell; (d) early necrotic cell; (e) late apoptotic cell; (f) BN cell with one or more MNi; (g) BN with an NPB and a MN; (h) BN cell with NBUDs (Fenech, 2007).

In the CBMN assay (Figures 10 and 11), DNA damage deletion rate can be measured by scoring the MNi in BN cells, detecting cytogenetic damage such as chromosomes breaks or loss of whole chromosomes. MN and NPBs indicate DNA disrepair. Nuclear buds and chromosome rearrangement reflect amplified DNA repair complexes (Shimizu et al., 2000). The generation of MNi, NPBs and nuclear buds occurs at the anaphase stage of cell division, chromosome fragments or whole chromosome losses lead to the rise of MNi (Fenech, 2000; Schmid, 1975).

NPBs could result from di-centric chromosome or from chromatids that pulled toward the opposite end of the cell and lead to asymmetrical chromosome rearrangement or DNA strand break disrepair (Thomas et al., 2003); or telomere end fusion that occurs as a result of telomere shortening associated with the aging process (Blasco, 2005; Stewenius et al., 2005). Furthermore, nuclear buds indicate the possible gene amplification and are considered as evidence of genotoxic exposure, likewise the MNi and NPBs, and this could be an additional advantage of the CBMN assay (Fenech, 2002; Serrano-Garcia and Montero-Montoya, 2001).

1.18 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a molecular biology technique that is based on the cells' capacity to replicate new DNA strands complementary to the displayed original strands (Ngô and Van de Voorde, 2014). The American biochemist Kary Mullis developed the PCR in 1984 and later on in 1993 won the Nobel prizes (Bartlett and Stirling, 2003). The PCR technique soon became one of the most important methods used in molecular biology due to be simple, quick and inexpensive (Joshi and Deshpande, 2011). The PCR technique is a revolutionary invention in the history of molecular biology and medical sciences. The application

of PCR was a milestone in the field of molecular genetics research as well as animal and plant biotechnology, moreover, PCR has also demonstrated its importance and ingenious utility in various science fields such as forensic sciences, molecular epidemiology, archaeology, anthropology and evolutionary genetics (Singh et al., 2014). The principle of PCR amplifies one DNA molecule into two, then four, then eight and so forth. This incessant doubling process is accomplished by thermostable polymerases that are able to bind together DNA building blocks to procedure long molecular strands. These techniques, which produce more than one million specific DNA sequences also need primers which are small DNA fragments. Primers function by connecting the constructing blocks to form a longer DNA molecule to work as a template for building the new strand (Joshi and Deshpande, 2011). The basis of PCR depended on the certainly that at high denaturing temperatures approaching 95°C, the two strands in the target DNA molecule separated by breaking bonds between A-T and G-C. At the annealing temperatures in the range of 50-65°C, the selected forward and reverse primers bind at the 3' end of the specific regions in each of the separated strands in DNA molecule. In the extension stage, Taq polymerase enzyme extends the new DNA strands in the presence of deoxynucleoside triphosphate (dNTPs) and the single strands rebind themselves during the extension temperature 72°C. These stages are repeated a 35 times to produce multiple copies and finally amplify the target DNA molecule (figure 12). The European Molecular Genetics Quality Network (EMQN) provide the best practice guidelines, which, should be followed for best results (Müller, 2001).

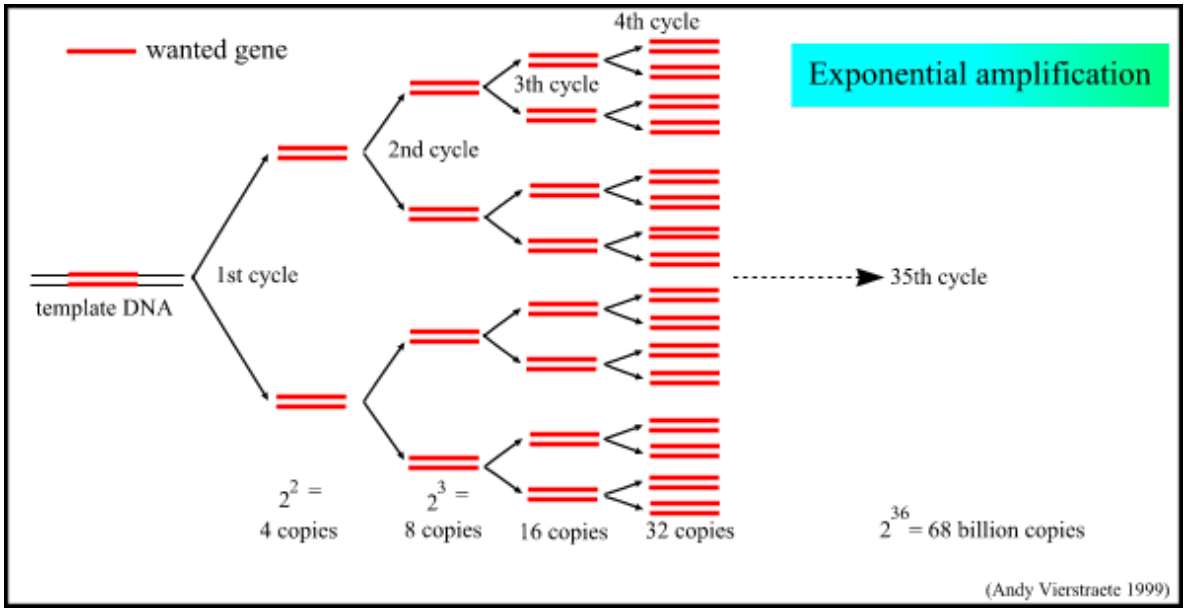

Figure 12 shows the exponential amplification of the gene in PCR (Singh et al., 2014)

1.18.1 Real time-PCR or quantitative PCR (qPCR)

The first description of qPCR was by Higuchi et al. (1992) and co-workers (Singh et al., 2014). The development of real-time (q-) PCR characterizes has a substantial advance in various molecular techniques including nucleic acids' analysis (Rodriguez-Lazaro and Hernandez, 2013). The qPCR method has the ability to detect a fluorescent DNA stain, such as SYBR Green and measuring the amplification of the target DNA at each cycle of PCR. During the log-linear phase of magnification, the fluorescence level increases to a measurable point which is called threshold cycle (CT). Therefore, the qPCR results consist of amplification; standard curves log concentration vs CT, which is plotted by using serial dilutions of a known magnitude of standard DNA, the amount of DNA or complementary DNA (cDNA) in an unknown specimen can be detected as CT value by this standard curve (Singh et al., 2014). Major advantages of qPCR are combining the amplification and detection into one step therefore eliminating any requirement for post amplification processing of the targeted DNA (Mackay, 2004). Furthermore,

qPCR is a technique with a high level of sensitivity, accuracy, speed of analysis and real time detection of measuring progress (Gachon et al., 2004). This is due to the availability of either fluorescent materials or fluorescently-attached oligonucleotide probes whose intensity reflects the amount of targeting DNA formed (Wong and Medrano, 2005). Various categories of polymerases are utilised to assist different types of qPCR reactions, such as high precision, hot start and enzymes with high and fast activity. Hence, qPCR machines are designed to perform these reactions that contain a thermic cycler needed for DNA amplification, an optical environment to stimulate fluorophores and capture generated fluorescence from the detection chemistry, and using special software to gather and investigate the quantitative data emitted. The most essential advantage of qPCR is that it has a low proportion of contamination caused by PCR products such as cDNA and RNA (Singh et al., 2014). However, qPCR has some drawback including expensive reagents. This technique is a very sensitive procedure and understanding the experimental design of this method is essential for accurate and valuable results (Wong and Medrano, 2005). qPCR has become the preferred choice in PCR techniques for diagnostic applications for several reasons. It is more sensitive and can be more controlled compared to ordinary PCR and these are important characteristics for diagnostic utilizations. qPCR additionally is preferred than end point PCR due to several reasons, including the amplification of short DNA fragments, the ability of fluorescent detection, which allow the detection of minor amounts of amplified products, and enhanced tolerance to inhibit materials interfering with DNA purification compared to conventional PCR process. Moreover, working as one process in qPCR instruments helps to avoid post PCR

contamination which could lead to false positive results. Finally, the absence of gel electrophoreses step in real time PCR makes the method easy to automate and suitable for fast throughout (Ravnikar et al., 2016).

1.18.2 RT-PCR/qPCR combined

The (RT-PCR) polymerase chain reaction technique is used when measuring the qualitative expression of RNA by converting the RNA template to cDNA, however, both RT-PCR and qPCR techniques are used for quantitative detection of RNA expression, and this merger technique is named qRT-PCR/ quantitative RT-PCR or RT-qPCR (Singh et al., 2014).

1.19 Western blot (WB)

Western blotting (WB) has become the most important technique that has been used in cell and molecular biology, which is often utilized in protein diffusion and detection. Since 1979, protein blotting has been developed significantly. WB allows researchers to measure and detect specific proteins from a relative quantities of individual proteins isolated from cells (Gürtler et al., 2013; Kurien and Scofield, 2006; Mahmood and Yang, 2012). The principle of WB can be shortened to five major steps: 1. diffusion of the proteins depend on their sizes by using polyacrylamide gel electrophoresis (SDS-gel); 2. Transfer and relocate the protein from SDS-gel to artificial membrane such as nitrocellulose and polyvinylidene fluoride PVDF (Electroelution); 3. Use specific primary antibody to bind the protein attached to the membrane 4. Use the enzyme conjugate secondary antibody to bind the protein-primary antibody complex, such as, horseradish peroxidase (HRP); 5. The isolated protein bands can be imaged in the presence of ECL substrate and dye (Liu et al., 2014; Mahmood and Yang, 2012). WB is considered

to be the preferred technique among immunoassay methods that studying protein expression due to its accuracy, easy of analysis, translation of results, and reasonable cost (Westermeier and Marouga, 2005). The density of the bands is proportional to the concentration of the protein of interest (Mahmood and Yang, 2012). WB also possesses excessive ability to indicate the presence of such protein by size or through the binding of an antibody, which increases the capability of the WB method to follow protein fractions throughout the protein purification procedure. This allows observing protein expression from different cells or the response of specific proteins after treatment by a particular drug. WB has been used to study the effect of the chemotherapeutic agent Adriamycin (doxorubicin) on BRCA1 and PARP-1 expression in MCF-7 breast cancer cells, thus proteins are playing essential roles in DNA repairing pathways, and this study allowed them to follow the drug impact and if there is a resistance to doxorubicin (Wang et al., 2014). WB is a lengthy procedure with several manual phases including protein assay, gel electrophoresis [SDS–PAGE], protein transfer, and detection, which might increase the possibility of a higher error rate(Gibbons, 2014; Mahmood and Yang, 2012). The WB technique was introduced recently to detect the genotoxicity of nanoparticles (Kumar et al., 2014).

1.20 Aim

The main aim of this work is to study the DNA protective effect of two well-known NSAIDs namely ibuprofen and aspirin in bulk form and as nanoparticles. Some previous studies have demonstrated that regular intake of either drugs results in protection against certain tumours such as colorectal, lung and breast cancer. However, these studies did not investigate the effect of these agents at the DNA

level. Consequently, this work aims to examine the effect of both agents on DNA damage using lymphocytes obtained from breast cancer patients and comparing the result with healthy females as controls. Two different particle sizes were selected and used in this study namely NPs and bulk forms to analyse the effect of size difference on DNA damage. Comet and micronucleus assays were used as techniques to assess the extent of DNA damage. The Comet repair assay was also chosen for monitoring DNA damage repair, after challenging lymphocytes using a damaging agent bleomycin and allows the DNA repair in the presence and absence of aspirin and ibuprofen. Furthermore, the role of both NSAIDs on the expression of XRCC3 and p53 proteins was studied by WB. Finally, the influence of aspirin and ibuprofen in both nano and bulk forms on p53, COX1 and COX2 genes expression in lymphocytes from BC patients compared to the those from healthy females volunteers using real-time PCR were studied.

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Chapter 2 - Material and Methods

2. Material and Methods

2.1 Material

The chemicals and reagents that were used in this work, as well as their sources and CAS numbers are summarised in the following Table (1, 2 and 3).

Chemicals	Supplier	Product or CAS number
Bis-acrylamide, 30% solution	Sigma, UK	110-26-9.
Aspirin (SLS)	Sigma-Aldrich UK	50-78-2
Bleomycin sulfate	Sigma UK	9041-93-4
Western ECL Substrate, 200 ml	Bio-Rad	1705060
Cytochalasin-B	Sigma-Aldrich UK	14930-96-2
DMSO	BDH, UK	67-68-5
Ethanol	Sigma, UK	64-17-5
Ethidium bromide	Sigma, UK	1239-45-8
Fast SYBR® Green Master Mix	Life Technologies	4385610
Fetal Bovine serum	Sigma, UK	n/a
Foetal Bovine Serum	GIBCO Invitrogen UK	10270
Hydrogen peroxide	Sigma, UK	7722-84-1
Ibuprofen USP	Albermarle sprt	15687-27-1
Reverse Transcription System	Promega	A3800
Low melting point (NMP) agarose	Invitrogen, UK	39346-81-1
Lymphoprep	Axis-Shield Norway	66720-17-0
Mitomycin C	Sigma-Aldrich UK	50-07-7
$Na_2EDTA{\cdot}2H_2O$	Sigma, UK	6381-92-6
NaCl	Sigma, UK	7647-14-5
NaOH	BDH, UK	1310-73-2
Normal melting point (NMP)agarose	Invitrogen, UK	9012-36-6
PBS phosphate buffered saline	Sigma, UK	n/a
Phytohaemagglutinin , Liquid	GIBCO Invitrogen UK	9008-97-3
QIAamp® RNA Blood Mini	Qiagen	52304
Bradford Protein Assay Kit 1	Bio-Rad	5000201
RPMI-1640 Medium	Sigma-Aldrich UK	n/a
Trition X-100	Sigma, UK	9002-93-1
Trizma Base	Sigma, UK	77-86-1
Trypan blue	Sigma, UK	72-57-1

Table 1 Chemicals with their corresponding sources and CAS numbers.

Primer	Company	Sequince or ID
P53 primer	Qiagen	QT00050785
COX1 primer	Qiagen	QT00210280
COX2 primer	Qiagen	QT00040586

Table 2 List of primers

Antibodies	Source	Company
Anti- p53 antibody	ABCAM	ab1431
Anti-beta Actin antibody	ABCAM	ab189073
Anti-XRCC3 antibody	ABCAM	ab58467
Donkey Anti-Rabbit IgG H&L	ABCAM	ab6802

Table 3 primary and secondary antibodies

2.2 Blood samples

Whole blood was collected by venepuncture after receiving the consent from both healthy non-smoking female volunteers and breast cancer patients. The breast cancer samples were provided by the Ethical Tissue Bank using licence12191. The blood was collected in labelled lithium heparin coated tubes. Ethical approval was granted by the University of Bradford's Sub-Committee for Ethics in Research involving Human Subjects (Reference no.: 0405/8). Control samples were also taken under IRAS/NRES application 12/YH/0464. The Research Support and Governance Office Bradford Teaching Hospital NHS Foundation granted the Re

DA number: 1202. Blood was collected in labelled lithium heparin coated tubes. Samples were diluted 1:1 with RPMI-1640 medium and then 10% DMSO was added. The diluted blood samples were divided and transferred to labelled Eppendorf® tubes, which were tightly closed and stored in a -80°C freezer. However, fresh blood samples were used in the micronucleus assay, qPCR, and western blot analysis. The data shown in the appendix 2 and the confound are discussed following it.

2.3 Aspirin and ibuprofen

2.3.1 Preparation of aspirin and ibuprofen nanoparticles

Aspirin and Ibuprofen were suspended 3% and 4% (w/w) respectively with solid loads in special suspension media, which was prepared from melting polyvinylpyrrolidone K-30 (0.5% w/w), hydroxypropyl methylcellulose (HPMC) (0.5% w/w), and sodium lauryl sulphate (0.1% w/w) in deionised water. The Lena nanoceutics technology DM-100 machine was utilised for milling the suspensions. 150 ml of 0.2 mm yttrium was used to mill 250 ml of each suspension in the presence of stabilised zirconium beads (Glen mills, USA). Recycling the suspension in the milling machine took 60 min before being discharged and transferred to an impervious glass bottle. The suspensions were stored in a 4°C fridge.

2.3.2 Measuring of nano-suspension particle size

The size of aspirin and ibuprofen particles was regularly measured to confirm no significant alteration in particle size occurred for the duration of the Comet assay, MN, qPCR, and western blot experiments. The particle size distribution of aspirin

and ibuprofen nano-suspensions were measured using the dynamic light scattering technique of the Zetasizer Nano ZS (Malvern instruments, UK). Both aspirin and ibuprofen particles were measured at room temperature 25 °C using 2 ml unused cuvettes. All measurements were done in triplicate. However, the bulk particle sizes of aspirin and ibuprofen powder were measured utilising the laser diffraction technique (Sympatec Helos, UK). For each drug 20 mg were transmitted into the sample vial and 4 bar primary pressure was selected while the feeder speed was adjusted to 40mm/s. Three samples of each compound were determined using an R2 lens (0.25/0.45, 87.5 µm).

Images were captured using a transmission electron-microscope (TEM) (Estop et al., 1993). Specimens were prepared by adding a drop of the drug suspension (aspirin or ibuprofen) on the TEM carbon grid. After 10 min the grid was rinsed by immersion in water. A drop of uranyl acetate was floated on the grid for 10 min then rinsing again in water. After drying, the grid was placed in the TEM and 100, 000x magnification power was used, and the 60 kV and 40 kV beam intensity were chosen for aspirin and ibuprofen sample respectively.

2.3.3 Zeta potential analysis of nano suspensions

The zeta potential for the suspensions was determined using Zetasizer Nano ZS (Malvern instruments, UK). The suspensions were diluted 1:100 using deionised water and measured at room temperature. Disposable zeta cells were used. Measurement extent was set as automatic between 10 and 100 runs maximum. All measurements were repeated three times.

2.3.4 Aspirin and Ibuprofen Doses

In this project two different sizes of ibuprofen and aspirin were used (NPs and Bulk) using the same concentrations of both NPs and Bulk sizes. The ibuprofen concentration used was 250 µg/ml, whereas, 500 µg/ml was chosen for aspirin. These doses were selected according to the differences in properties between the two drugs and appropriate doses were used so that they did not lead to cytotoxicity and cell apoptosis.

2.4 Lymphocyte isolation from whole blood

Whole blood was mixed 1:1 in Falcon tubes with saline (0.9% NaCl), which was prepared by dissolving 4.5 g of NaCl in 500 ml of distilled water. In a Falcon tube, 6 ml of diluted blood were carefully overlaid on 3 ml of Lymphoprep which has the same density as lymphocyte cells, then transferred to centrifuge at room temperature (RT) for 20 min at 600 g. After the centrifugation, the white layer of lymphocytes was obtained from the top of the Lymphoprep within the plasma interface of the sample and washed with saline in an universal tube. Then, the suspension was centrifuged at 375 g for 15 min at RT, and the centrifugation the supernatant was treated with 2% virkon, then discarded. The pellet was resuspended in 1800 µl of foetal bovine serum (FBS), then, 450 µl of the suspension transferred to each of 4 Eppendorf tubes, which contained each 50 µl of dimethyl sulfoxide (DMSO) 100%. The tubes were tightly closed and gently mixed and stored overnight at -20 °C before being transferred into -80 °C freezer for prolonged storage.

2.5 Comet assay

2.5.1 Cell treatment

The stored blood samples were allowed to thaw at room temperature. After that, 100 µl of blood suspension was added to 890 µl RPMI-1640 media in Eppendorf® tubes, which contained 10µl of NC (control negative) (chemical solvent), PC (control positive) (50 µM H_2O_2) and test agents. In this project, chemicals examined were ibuprofen and aspirin in NPs and bulk sizes. The final volume in the Eppendorf® tubes was 1000 µl. The cells were incubated for 30 min at 37°C. The tubes were centrifuged for 5 min at 705 g. After the centrifugation, 900 µl of the supernatant was discarded, and 100 µl of 0.5% low melting point agarose (LMP) was added to the cell pellet and re-suspended. After that 100 µl of cell suspension was layered onto 2 duplicate slides pre-coated with 1% of normal melting point agarose (NMP). The slides were covered with cover-slips and transferred on to an ice tray for 5 min.

2.5.2 Cell lysis

In this step, after removal of the cover slips, the slides were immersed overnight in cold lysing solution at 4°C, which was always freshly prepared. The lysis solution consisted of 100 mM EDTA, 2.5 M NaCl, 10 mM Trizma base, 10% DMSO and 1% Triton X-100, pH 10. The lysis solution contained a high concentration of salt and a detergent. Proteins and their bonding and RNA were disrupted by aqueous salts. The cell membrane was dissolved by the detergent.

2.5.3 Electrophoresis

After cell lysis, the slides were placed horizontally in a gel electrophoresis tank with a fresh alkaline buffer (10M NaOH and 200mM EDTA, pH >13). The slides remained for 30 min at 4°C to allow unwinding of DNA before starting the electrophoresis. The electrophoresis was then run for 30 min at 25 volts/32 cm (0.78 v/cm) and 300 mA using a compact power supply.

2.5.4 Neutralization

The slides were removed from the electrophoresis tank and placed in a horizontal position, then covered with neutralising buffer (0.4 M Tris-HCl, pH 7.5) three times for 5 min each. For long term storage slides can be run through a dehydration process, by immersing them in methanol for 30 min at 4°C. The dehydrated slides can be rehydrated by placing them in chilled water for 30 min in order to rehydrate the gel, and the cells can be stained.

2.5.5 Staining and comet scoring

Ethidium bromide (20 µg/ml) stain was used for DNA staining; 60 µl of the dye was added on the top of each slide and covered with a cover slip. To avoid the drying of cells, they were kept in a damp and closed container. The cells were scored by using a fluorescence microscope equipped with CCD camera and connected with image computer software. Objectives of 200 x magnification were utilised in the evaluation of each dose. 100 cells were selected randomly and analysed from each slide. The mean of each set of the experiment's data was utilised in the statistical analysis. A significance level of $p<0.05$ was considered as statistically significant. Two comet data parameters Olive tail moment (OTM) and % tail DNA, were used to quantify DNA damage. OTM (the fraction of DNA in the tail multiplied

by the tail length) and % tail DNA (the percentage of DNA in the tail) are recommended to be the most reliable comet measurements with OTM being the most statistically significant (Kumaravel and Jha, 2006). of these two parameters, OTM is the more commonly used to measure DNA damage, however, it is recommended to provide in parallel with % tail DNA (Tice et al., 2000). Additionally, OTM and % tail DNA clearly indicate the comet demonstrating a linear relationship with the DNA break frequency over a wide range of levels of damage. Both OTM and % tail DNA can be used for scientific purposes. However, OTM values can change extensively among laboratories with different software packages; furthermore, for all studies that use different electrophoresis runs. It is advisable that % Tail DNA be used to eliminate the variability in the results. % Tail DNA is considered as appropriate for regulatory or inter-laboratory comparison studies (Hartmann et al., 2003; Kumaravel et al., 2009).

2.6 Micronucleus assay

2.6.1 Preparation of basic culture medium

Basic culture medium was prepared in the extraction laminar flow hood under sterile conditions. 15% of foetal bovine serum (FBS) and 1% of penicillin-streptomycin solution were added to the Roswell Park Memorial Institute medium (RPMI-1640) that contained l-Glutamine amino acid and 25 mM HEPES buffer. 4.5 ml of prepared medium was transferred into each of 25 cm^3 Corning culture flasks. The flasks were then stored in a -20 °C freezer.

2.6.2 Lymphocyte cell culture

Before starting the culture the T25 cm^3 flasks containing 4.5 ml of frozen prepared media were placed in a 37°C incubator (5% CO_2) for 30 min to adjust for temperature. Sterile materials and solutions were used when performing lymphocyte culture which lasted for 72h.

2.6.3 Culture start

At 0 h 500 µl of fresh blood obtained from healthy volunteers or from breast cancer patients were added to each flask containing 4.5 ml of basic medium. Then, 100 µl of phytohaemagglutinin (PHA final concentration 2%) were added to each flask. After 24h incubation 50 µl of 40 µM mitomycin C (end concentration 0.4 µM) was added to the positive control flask, 250 ng/ml ibuprofen and 500 ng/ml aspirin were added to different lablled flasks (Table 4). Then the cultures were incubated at 37°C in the presence of 5% CO_2.

Flasks No.	Type	Blood	PHA	compound	RPMI
Flask 1	Untreated cells	500 µl	100 µl	50 µl	4350 µl
Flask 2	PC	500 µl	100 µl	50 µl	4350 µl
Flask 3	Ibuprofen NPs	500 µl	100 µl	62.5 µl	4337.5 µl
Flask 4	Ibuprofen bulk	500 µl	100 µl	50 µl	4350 µl
Flask 5	Aspirin NPs	500 µl	100 µl	83 µl	4317 µl
Flask 6	Aspirin bulk	500 µl	100 µl	50 µl	4350 µl

Table 4 Six different treatments which were used in the CBMN assay

***Maximum volume of treating stock added was 1% of culture volume i.e. 50 µl**

2.6.4 Cytokinesis block

After 44 h of incubation, 30 µl of 6 µg/ml Cytochalasin B were added to each flask. This step is essential in this assay as the Cyto B inhibits the formation of actin

filaments that network strongly and this in turn leads to inhibition of cytoplasmic division and therefore, halts the cells in the bi-nucleated form.

2.6.5 End of cell culture

After 72 h the flasks were removed from the incubator and the flask content transferred to labelled 15 ml Falcon tubes.

2.6.6 Cell hypotonic shock treatment

The Falcon tubes containing the culture were placed in a centrifuge and centrifuged for 8 minutes at 107 g. After that a vacuum pump was used to remove the supernatant with retention of 500 µl. The tubes were agitated gently to re-suspend the pellet. To allow hypotonic treatment of cells 5 ml of cold 110 mM potassium chloride was added to each tube, and then the tubes were stored for 15 minutes in the refrigerator (4°C). Again the tubes were centrifuged for 8 minutes at 107 g and a vacuum pump was used to discard the supernatant with retention of 500 µl. Tubes were agitated gently to re-suspend the cell pellets.

2.6.7 Fixation of cells

Carnoy's solution (3 parts of methanol and 1 part of glacial acetic acid) and 38% formaldehyde were used 3 times for fixation of the cells.

2.6.7.1 Fixation 1

In this step 5 ml of Carnoy's solution (freshly prepared) was added to each tube gently and drop wise over the vortex followed by 3 drops of formaldehyde (38 %). The Falcon tubes were placed in a centrifuge and centrifuged for 8 minutes at 107 g. The vacuum pump was utilised to discard the supernatant with retention of 500 µl. Tubes were agitated gently to re-suspend the pellet.

2.6.7.2 Fixation 2 & 3

The fixation 1 step was repeated twice except for the addition of formaldehyde 38%.

2.6.8 Preparation, staining and slide fixation

After centrifugation the supernatant was discarded while retaining 100 µl. Depending on the amount of cells around 300-600 µl of freshly prepared Carnoy's solution was added and the pellet re-suspended. 20 µl of cell suspension was transferred twice onto new and clean slides in centre right and centre left. The slides were left to dry overnight. Two slides were prepared for each tube. The cells were stained in Giemsa stain for 20 minutes. Altered 5% Giemsa solution in Sorenson buffer (51 ml 0.2 M NaH_2PO_4 with 49 ml 0.2 M Na_2HPO_4). The slides were washed and left to dry and were fixed by adding DPX. Finally 24 mm x 50 mm coverslips were added on the top.

2.7 Comet assay on BLM with and without recovery

2.7.1 Reagents

The reagents were purchased including aspirin and ibuprofen as described in section 2.1. Bleomycin sulfate was purchased from Enzo Life Sciences Ltd, Exeter, United Kingdom. The stock solutions of BLM was prepared using deionized distilled water (ddH_2O) and stored in a freezer at -20°C. Working solutions (different concentrations) were also prepared using ddH_2O and stored at 4°C.

2.7.2 Cell treatment of Whole blood cells

Seven Eppendorf® tubes were chosen and labelled for the Comet repair assay; one negative control (untreated cells), two positive controls: BLM without allowing repair (BLM) and BLM with allowing repair (BLMR) which contained only bleomycin

and four treatments challenged with bleomycin and allowing repair individually in the presence of aspirin bulk, aspirin nano, ibuprofen bulk or ibuprofen nano. This was to demonstrate the effects of aspirin and ibuprofen (both forms) on DNA repair. Bleomycin was utilised as a genotoxic agent to induce DNA damage. Cells in each Eppendorf® tube were treated for 30 minutes in a first incubation at 37°C and then the cells were left for a further 30 minutes incubation at 37°C except the positive control 1 (BLM). The cells in BLM treatment were challenged for 30 minutes incubation at 37°C before being embedded in 5% low milting point (LMP) agarose on slides pre-coated with agarose, no time for repair being allowed. The BLMR was treated in the same manner as the BLM, but the cells were left for the second incubation (30 minutes) without any treatment with NSAIDs to allow DNA self-repair.

2.7.3 Treatment over duration of 1h

Time point 0 hrs: 100 µl of whole blood cells were added to each tube in the presence of 890 µl RPMI medium. 10 µl of 0.75 mg/ml bleomycin (final concentration 7.5 µg/ml) were added to each tube except the negative control, where 100 µl of whole blood cells added to 900 µl RPMI medium. All 7 tubes were incubated for 30 min at 37°C. After the incubation all tubes were removed from the incubator and centrifuged at 110 *g* for 3 min.

For BLM treatment, 900 µl of supernatant was removed, 100 µl of 0.5 % LMP agarose was added to cell pellet and mixed gently. The suspension was immediately embedded on two pre-coated slides with 1% normal melting point (NMP), 100 µl each and covered with cover-slips then transferred on an ice tray for 5 min. The slides were transferred in lysis buffer after removing cover slips.

2.7.4 Supplementation with NSAIDs during recovery

For the negative control, BLMR, and the other four treatment concentrations, 900 µl of supernatant was discarded and cell pellet were washed three times with 900 µl of fresh 1640-RPMI medium. Cell pellets were very gently mixed and centrifuged at 110 g for each wash.

For the negative control and BLMR tubes, after the centrifugation 900 µl of supernatant was discarded and 900 µl of fresh RPMI was added to the washed cell pellet of each tube and gently mixed. To the remaining 4 tubes after last washing 900 µl of supernatant was discarded and treated as showed in the table 5,

	RPMI	aspirin bulk	aspirin nano	ibuprofen bulk	ibuprofen nano	End conc of NSAIDs
Tube 1	890 µl	10 µl	0 µl	0 µl	0 µl	500 µg/ml
Tube 2	883.4µl	0 µl	16.6 µl	0 µl	0 µl	500 µg/ml
Tube 3	890 µl	0 µl	0 µl	10 µl	0 µl	250 µg/ml
Tube 4	887.5 µl	0 µl	0 µl	0 µl	12.5 µl	250 µg/ml

Table 5 Second incubation for all 6 tubes incubated for 30 min at 37 °C**.**

2.7.5 Embedding of cells in agarose

After the second incubation allowing repair, all the Eppendorf® tubes were centrifuged for 5 min at 110 g and 900 µl supernatant was discarded. 100 µl of agarose LMP was added to the remaining cell pellet and mixed gently. The cell suspension was layered on slides pre-coated with NMP agarose, two slides for each treatment. The slides were transferred onto a cold surface for 5 min. Then the coverslips were removed and the slides were placed overnight in a jar of cold lysis solution to remove cell membranes and denatured protein. The freshly prepared lysis solution consisted of 100 mM EDTA, 2.5 M NaCl, 10 mM Trizma base, 10% DMSO and 1% Triton X-100, pH 10.

2.7.6 DNA unwinding and electrophoresis

Following the lysis step, the slides were placed horizontally in electrophoresis tank in presence of electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH > 13). Slides remained in a tank for 30 min at 4°C for DNA unwinding, then electrophoresis was started for 30 min at 290-300 mA, at a constant voltage of 25 V (0.78v/cm).

2.7.7 Neutralisation

The cells and below were then neutralised three times for 5 min each using a neutralising buffer (0.4 M Tris-Hcl, pH 7.5). Cells were then stained with 20 µg/ml ethidium bromide reagent and covered with cover-slips in a dark place.

2.7.8 Scoring of Slides

Before scoring, all slides were double blinded and 100 cells were evaluated on each slide using a fluorescence microscope connected to a CCD camera and Computer system (Andor Technology Ltd, Belfast, UK). Data were analyzed to measure olive tail moment and % Tail DNA using kinetic imaging software (Andor Technology Ltd, Belfast, UK). Experiments were repeated for 15 healthy individual's samples and for 15 BC female patients' samples.

2.8 PCR

2.8.1 RNA Extraction

1ml of human whole blood was mixed with 5ml of Buffer EL (erythrocyte lysis buffer) in a 15 ml Falcon tube. The mixture was incubated for 15 min on ice and vortexed briefly 3 times throughout the incubation. After the incubation, the mixture was centrifuged at 400 x g for 10 min at 4°C, and the supernatant completely removed and discarded. 2 ml of Buffer EL were added to the pellet which was then

re-suspended by brief vortexing. The suspension was again centrifuged at 400 x g for 10 min at 4°C, and the supernatant removed completely and discarded. 350 µl of lysis Buffer (10 µl ß-mercaptoethanol per 1 ml Buffer RLT) was added to the pellet while vortexing. The lysate was directly pipetted in a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 min at full speed to homogenize. After the centrifugation the QIAshredder spin column was discarded and the collection tube containing homogenized lysate saved. The homogenized lysate was mixed with 350 µl 70% ethanol and carefully pipetted with any formed precipitate in a new QIAamp spin column placed in a 2 ml collection tube without wetting the rim, and was centrifuged for 15 s at 8000 x g. The QIAamp spin column was transferred into a new 2 ml collection tube. 700 µl of Buffer RW1 (a guanidine salt and ethanol) was applied to the QIAamp spin column and centrifuged for 15 s at 8000 G for washing. The QIAamp spin column placed in a new 2 ml collection tube. In this step 500 µl of Buffer RPE was pipetted into the QIAamp spin column and centrifuged for 15 s at 8000 x g. An additional 500 µl of buffer RPE was added and centrifuged again at maximum speed for 3 min. In the final step the QIAamp spin column was transferred into a 1.5 ml microcentrifuge tube and 50 µl of RNase-free water was directly pipetted in the middle of QIAamp membrane and centrifuged for 1 min at 8000 G to elute. The extracted RNA was stored at -80°C

2.8.2 Preparation of cDNA

The cDNA was prepared by the following steps. Mixture 1 was generated by mixing 2 µl of random hexamer primers with 8 µl of isolated RNA and heated by using PCR machine at 70°C for 5 min to denature the RNA. Meanwhile, mixture 2 was prepared by mixing 4 µl of Improm- IITM Reaction buffer, 2.4 µl of $MgCl_2$ (25 mM),

1 µl of dNTP (10 mM), 1.1 µl nuclease-free water, 0.5 µl of Recombinant RNasin (ribonuclease inhibitor), and 1 µl of Improm II RT (reverse transcriptase). In the final step, mixture 2 was added to the mixture 1 and transferred to a PCR machine. and incubated for 5 min in 25 °C then for 60 min at 42 °C and finally for 15min at 70 °C. After finishing the incubation the prepared cDNA was stored at - 20 °C.

2.8.3 Quantitative real time PCR analysis

The primers were optimised for their annealing temperature. For each reaction, ten µl of Fast SYBR® Green Master Mix, two µl of primer, 0.5 µl of cDNA and 7.5 µl of UltraPure™ DNase/RNase-Free distilled Water were mixed. The mixture was placed in a 96 well PCR reaction plate and sealed using a MicroAmp® Optical 8-Cap Strips; the mixture was directly vortexed then twirl down using a plate spinner to accumulate the solution at the bottom of the plate well ensuring no air bubbles were present. The reaction plate was placed into a quantitative real-time PCR machine, and the intended protocol was run. The reaction was done in triplicate wells. For each experimental condition, β actin was used as a housekeeping gene where levels were unaltered at different conditions. The CT value was generated for the reaction as soon as the PCR had run. From the CT values generated, a ΔCT value was calculated from the housekeeping gene (the expression of the gene of interest normalised against β actin); from that a ΔΔCT was further created to ensure that the expression could be obtained. For consistent purposes, expression was normalised against β actin. In the comparative or ΔΔCt method of qPCR data analysis, the Ct values from two different experimental RNA samples are directly normalised to a housekeeping gene and then compared. First, the difference between the Ct values (ΔCt) of the gene of interest and the

housekeeping gene is calculated for each sample. Then, the variance of the ΔCt values between the experimental and control samples ΔΔCt is calculated.

2.9 Western blot

2.9.1 Cell culture and protein isolation

After lymphocyte isolation from whole blood, the cells were counted and cultured in two of 6 well cell culture plates. Each well contained 5 ml of prepared RPMI and 70 µl of PHA (10^6cells/well) for 24 h. After 24 h the cells were treated with aspirin and ibuprofen (both forms) individually. Plate 1 was treated for 8 hrs and plate 2 for 24 hrs. Once the treatment time finished the medium was pipetted from the wells, and each well washed twice with 5ml sterile PBS. Then, 2 ml of lysis buffer were added to lyse and de-attach cells using a plastic scrubber. The isolated protein was transferred to Eppendorf tubes. The protein was heated at 90°C in a water bath for 10 min and stored in the - 20 °C freezer. The Bradford protein assay was used to measure the total protein in each sample (Bradford, 1976; Ernst and Zor, 2010).

2.9.2 Total protein evaluation

The protein concentration of samples was evaluated with the Bio-Rad Bradford assay kit following the manufacture's guidelines. The Bradford protein assay principle is based on colorimetric alterations resulting in binding of Coomassie Brilliant Blue G-250 dye to proteins. The unbound reagent is in the protonated red cationic form which is converted to an unprotonated blue coulor when bound to proteins. Five different standard concentrations were used (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml). 5 µl of each standard and sample (in triplicate) were pipetted into separate wells of 96 well plates, and 250 µl of diluted Bio-Rad dye reagent were added to each well. The mixtures were incubated at 25°C for 5 min

and absorbance evaluated spectrophotometrically at 595 nm with a microplate reader. Concentrations were calculated depending on standards concentrations.

2.9.3 SDS-PAGE gel preparing and electrophoresis

Cassettes and glasses were washed with water and dried, then fixed together**.** Each cassette was covered by a glass plate and fitted into a casting frame, and secured by turning the hinge around and placed in the Perspex rack holder. Five ml of spreading gel (5 ml ddH2O, 6.67 ml 30% acrylamide, 4 ml 1.5 M Tris pH 8.8, 160 µl 10% SDS, 160 µl 10% APS and 16 µl TEMED) were pipetted between cassettes and plates, by utilizing 1 ml pipette until brought to the green lane. The spreading gel was left to solidify completely, then a 1 ml pipette was used to add the stacking gel (5.3 ml ddH2O, 2 ml 30% acrylamide, 2.5 ml 1.5 M Tris pH 8.8, 100 µl 10% SDS, 100 µl 10% APS and 10 µl TEMED) to the top of each plate. A comb was introduced to each plate and this was done promptly before the gel sets. Cassettes filled with gel were removed from the plate holder and transferred to the electrophoresis plate holding rack. Cassettes held by the holding rack were placed in the electrophoresis tank and the combs were removed. The tank was filled with running buffer (30.29 g Tris base 25 mM, 142.63 g glycine190 mM and 10.0 g SDS 0.1% in 1L distilled water) then 30 µl of each prepared sample was added into one of the wells. Samples were mixed with laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue and 0.125 M Tris-HC) at a rate dependent on the concentration of total protein in each sample.The electrophoreses was run at 200 V for 1 h.

2.9.4 Protein transfer from the gel to the membrane

The Polyvinylidene difluoride (PVDF) membrane was cut in the same gel size and activated by placing it in methanol for 30 seconds, then placed in transfer buffer (3.02 g Tris base 25 mM, 14,26 g glycine 190 mM, 200 ml methanol in 1L distilled water) for 5 minutes on a rotator at room temperature. The gel containing the electrophoresed protein was removed from the plate and assembled to a transferring cassette. Gel and membrane were assembled in the transfer sandwich. The air bubbles trapped in the sandwich were removed. The blot was on the cathode, and the gel was at the anode. Each component was soaked in transfer buffer for a few seconds before assembly to keep the membrane moist, this step was carried out in a tray containing transferring buffer. The cassette was closed and positioned in the tank already filled with transfer buffer and ice block, then run at 100 V for 1 h.

2.9.5 Antibody staining

After the transferring process the membrane was removed and transferred to a tray with Blocking buffer (NaCl 2g + KCl 50mg + Trisma Base 57mg were dissolved in 250 ml dH2O and the pH adjusted to 7.4 then added to 250 µl of Tween 20 and PSA 9g 4%) and placed on a rotator in a cold room for 1 h. The primary antibody was diluted in blocking buffer to the appropriate concentration depending on the antibody type, and then the membrane was transferred to a plate containing 10ml of diluted primary antibody and incubated overnight on a rotator in the cold room (4°C). After that the membrane was washed for three washes with washing buffer (PBS/0.05%-0.1% Tween-20), for 5 min each. The membrane was incubated with

the recommended dilution (1:2000) of a conjugated secondary antibody using blocking buffer at room temperature for 1 h under rotating at room temperature.

2.9.6 Signal development

Three washes were performed each for 5 minutes with washing buffer in a tray under rotation. Before the last wash was commenced, the equipment and reagents for signal development were prepared in the dark room following the kit (ECL) as recommended by Bio-rad. The membrane was transferred on plastic wrap and covered with ECL reagent. Images were captured utilizing darkroom development techniques for chemiluminescence, or normal image scanning methods for colorimetric examination.

2.9.7 Western blot membrane stripping for restaining

The membrane was incubated for 10 min with 35 ml of stripping buffer (15 g glycine 200 mM, 1g SDS 0.1% and 10 ml Tween20 1%, pH adjusted to 2.2 before bring the volume 1L). Then the membrane incubated twice with 35 ml of PBS for 10 min each. After that the membrane transferred in 35ml of TSBT solution (NaCl 2g + KCl 50mg + Trisma Base 57mg) were dissolved in 250 ml dH2O and the pH adjusted to 7.4 after which was added to 250 µl of Tween 20 and PSA 9g 4%) for 5 min, this step was repeated two time. After that the western blot steps started from the blocking step to the end as described earlier in western blot method.

2.10 Cell viability

The cell viability assessment was evaluated for the fresh lymphocyte cells after 30 min of treatment with ibuprofen (NPs, bulk) 250 ng/ml or aspirin (NPs, bulk) 500 ng/ml. The cells were centrifuged in the micro-centrifuge at 1250 g for five minutes at room temperature. Then 0.4% of Trypan blue solution was mixed with treated

cells 1:1, 100 cells were recorded. Trypan blue permeation into the cell occurs after the breakdown of the cell membrane, which is associated with cell death.

2.11 Statistical analysis

Twenty different samples of healthy individuals and 20 samples of BC patients were used in the Comet assay and 5 samples of healthy individuals with 5 samples of BC patients in CBMN assay. In Comet repair assay 15 samples were used from each group BC patients and healthy female individuals. However, 3 samples from each group for western blot and qPCR. The means of group data were calculated with standard errors. For qPCR the ΔΔCT method was used which is a convenient way to analyse the relative changes in gene expression from real-time quantitative PCR experiments. The normality of distribution of the data were tested through the Kolmogorov-Smirnov and Shapiro Wilk's test to select the appropriate parametric statistics which could be used. The normality of distribution of the data were evaluated using the One Way ANOVA test between healthy control and BC blood samples. Normality was violated so non-parametric statistics were considered for data analysis. The differences between groups were tested using the Kruskal-Wallis test and Mann-Whitney U-test. Dose response correlations were determined by Pearson's test for both NSAIDs. A value of $p \leq 0.05$ value was chosen as statistically significant. All analyses were generated using SPSS for windows statistical package (version 18.0). Graphpad prism 6 was used to draw graphs.

Chapter 3 – The Comet assay in peripheral blood lymphocytes after treatment with the NSAIDs, ibuprofen and aspirin.

3. Comet assay studies

3.1 Introduction

Many studies have been done to examine the relationship between the level of NSAID (ibuprofen, aspirin) intake and the incidence of various malignancies including the most prevalent types: lung, prostate, colon, and breast cancer (Agrawal and Fentiman, 2008; Harris et al., 2005; Neill et al., 2013). The concentration-response curve of those studies showed an inverse relationship between NSAIDs intake and cancer risk. Moreover, there was a significant exponential decline in the emergence of cancer, when compared with the increase of NSAIDs (primarily aspirin or ibuprofen). It has been found that the daily intake of NSAIDs, generally aspirin led to risk reductions of breast cancer (39%), 63% for colon cancer, and 39% for prostate cancer. After five years of use, the NSAIDs impact became apparent, and this effect was more evident with increasing duration of use (Harris et al., 2005; Langley et al., 2011; Neill et al., 2013). Most studies concentrated on the role of inflammation in breast cancer, specifically the role of the cyclooxygenase enzyme COX-1 and COX-2 with drugs that inhibit COX-2 in several cancer types including breast cancer. The anti-inflammatory drugs study showed promising findings in the treatment and reduction of colon and breast cancer. Aspirin and ibuprofen were among the NSAIDs included in these studies. COX-1 is present in healthy cells, whereas, the COX- 2 can be found in high concentrations in inflammatory processes. It has been found that COX-2 has a role in some cancers including breast cancer. COX-2 has a role in the production of prostaglandins which in turn leads to increase the production of oestrogen. The oestrogens stimulate breast cell proliferation and finally increase the breast cancer

risk (Komen, 2004; Neill et al., 2013). Methodologies that can identify and evaluate DNA damage have been developed in the last two decades. The Comet assay or single cell gel electrophoresis assay is a technique that was designed to follow and measure DNA damage in individual cells including lymphocytes and sperm, especially double-strand breaks (Wu and Jones, 2012). In this assay, lymphocytes are embedded in the agarose gel and are transferred to an electrophoresis tank in the presence of an alkaline condition (pH> 13). The DNA fragments with a negative charge move through the gel towards the anode (+ve). The pieces move much faster than undamaged DNA, and this gives the appearance a comet under florescence. The Comet assay is used to analyse DNA damage in lymphocytes in two ways using Olive tail moment and % of Tail DNA (Wu and Jones, 2012).

In these experiments, the effect the ibuprofen a non-steroidal anti-inflammatory drug and aspirin a non-opioid analgesic (acetylsalicylic acid) NSAIDs in both NPs and bulk sizes were assessed for DNA damage in human blood lymphocyte cells. There were obtained from healthy female volunteer and breast cancer patients using the single cell gel electrophoreses method. Here, the classic genotoxic compound hydrogen peroxide (H_2O_2) (50 µM) was used as a positive control. Different experiments were conducted with fixed doses of both compounds (ibuprofen 250 ng/ml and aspirin 500 ng/ml) in NPs and bulk states respectively. These doses are not toxic. After doing these experiments, the data were analysed, to help to determine the effect of these two agents on reducing DNA damage and DNA integrity.

3.2 Materials

The materials that were used in this project are listed and described in section **2.1**.

3.3 Methods

Blood collection and preparation including lymphocyte isolation have been described in section **2.2**. The alkaline Comet assay protocol and all its steps have been described in section **2.5**. NSAIDs (aspirin and ibuprofen) doses are described in section **2.3.4**.

3.4 Scoring and data statistical analysis

After finishing experiments, coded slides were scored using a florescence microscope connected to a camera and computer system equipped with Komet 6 software (Andor Technology Ltd, Belfast, UK). A Description can be found in section **2.5.5**. SPSS statistics (version 18) were used to determine significance and data distribution from Olive tail moment and % tail DNA values.

3.5 Results

3.5.1 Ibuprofen NP and bulk forms concentration responses on lymphocytes DNA from female healthy volunteers

Figures 13 and 14 illustrate the concentration response of both NP and bulk sizes of Ibuprofen on lymphocyte DNA from healthy volunteers using Olive tail moment and % Tail DNA. It was clear from the histogram in Figure 13 that the effect of Ibuprofen on lymphocyte DNA was non-significant compared to the untreated, similarly to % Tail DNA (Figure14). Using the ANOVA test when making a comparison of ibuprofen NP and Ibuprofen bulk against untreated cells showed non-significant (ns) effect on lymphocyte DNA. Whereas, H_2O_2 (positive control) (50 µM) showed high significance ***$p \leq 0.001$. It shows non-significance when comparing the nano with bulk form (ns†).

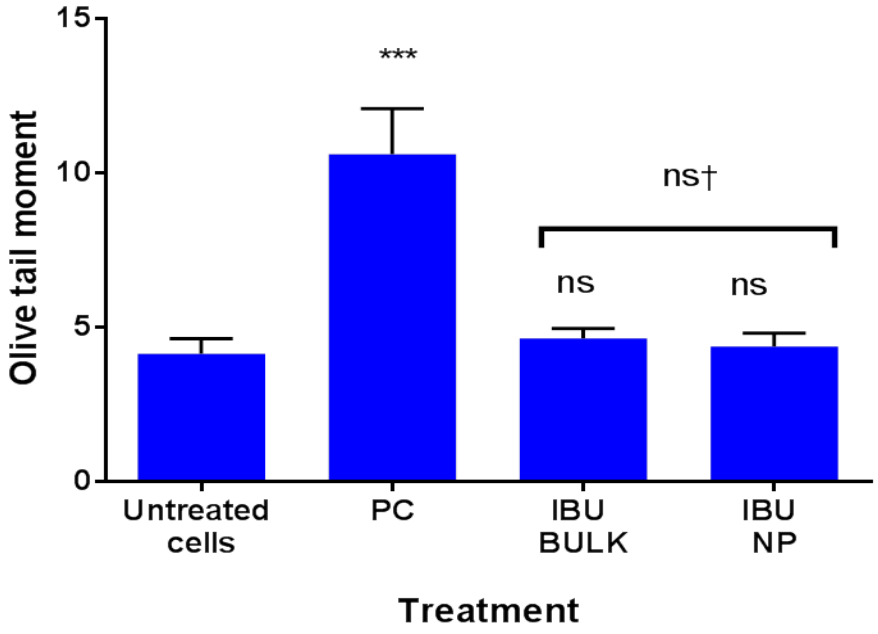

Figure 13 Concentration response of ibuprofen 250 ng/ml (NPs and bulk), SE, and significance on lymphocytes DNA from healthy volunteers using Olive tail moment. (N = 20)

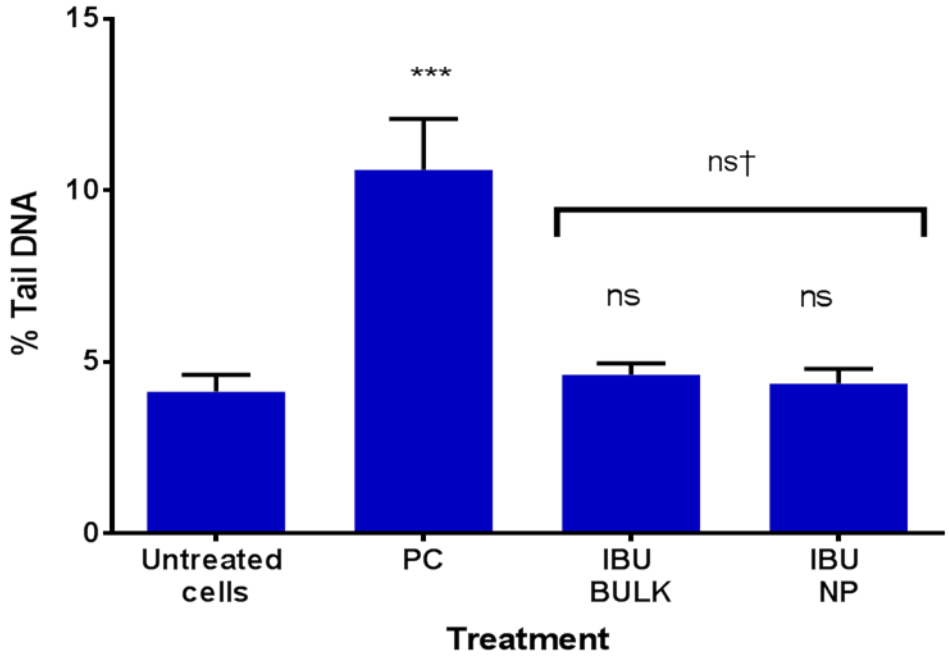

Figure 14 Concentration response of ibuprofen 250 ng/ml (NPs and bulk), SE, and significance on lymphocytes DNA from healthy volunteer using % Tail DNA. (N = 20)

3.5.2 Ibuprofen NP and bulk forms concentration response in lymphocytes DNA from BC patients

Figures 15 and 16 demonstrate the concentration response of both NP and bulk sizes of Ibuprofen on DNA in lymphocytes from BC patients using Olive tail moment and % Tail DNA. It was evident from the histograms in Figures 15 and 16 that Ibuprofen reduced the damage in lymphocyte DNA compared to the untreated cells. All controls and Ibuprofen were normally distributed. Using the ANOVA test represents the comparison of Ibuprofen NP and Ibuprofen bulk against untreated cells where there was no significant (ns) effect on lymphocyte DNA. Whereas, the positive control showed high significance ***$p \leq 0.001$. Additionally, there was no difference when compared the nano with bulk form (ns†).

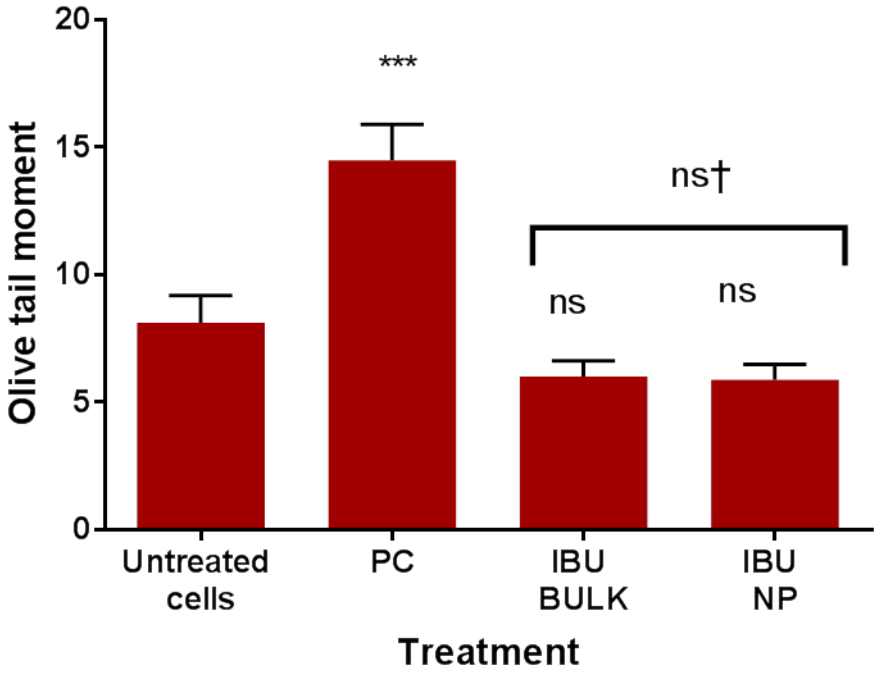

Figure 15 Concentration response of Ibuprofen (NPs and bulk), SE and significance on lymphocyte DNA from BC patients using Olive tail moment. (N = 20)

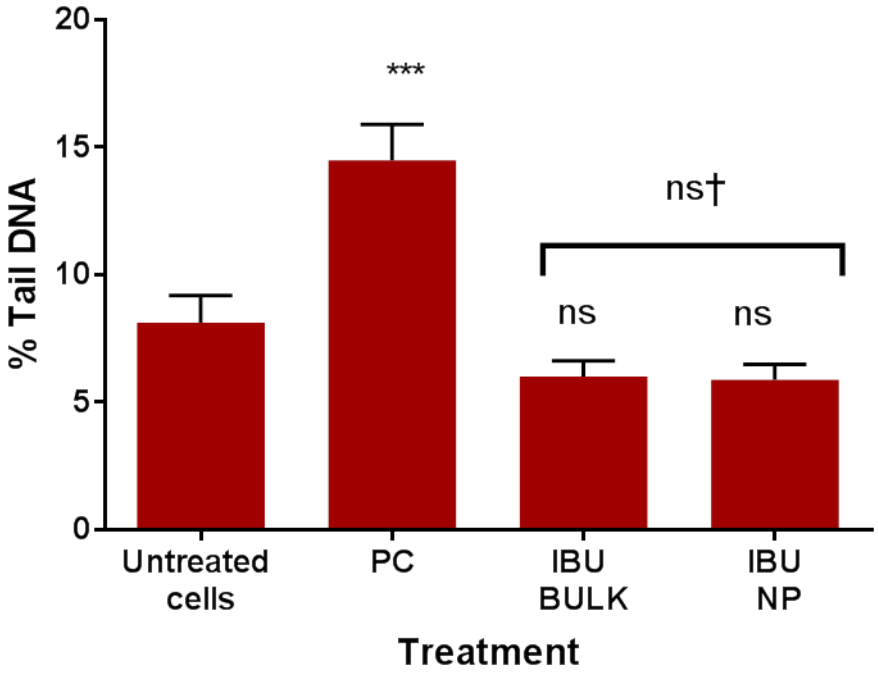

Figure 16 Concentration response of Ibuprofen (NPs and bulk), SE and significance on lymphocyte DNA from BC patients % Tail DNA. (N = 20)

3.5.3 Comparison of Ibuprofen NP and bulk forms for concentration responses on lymphocytes DNA from BC patients and healthy volunteers

Figure 17 and 18 showed the concentration response of both NP and bulk forms of ibuprofen in lymphocyte DNA from both BC patients and normal healthy volunteers using Olive tail moment and % Tail DNA. It was evident from the chart that ibuprofen reduced the damage in lymphocyte DNA from BC patients compared to untreated cells but the reduction was not significant (ns). The normal healthy cells showed a non-significant DNA damage increase compared to untreated cells. Both aspirin and ibuprofen showed non-significance increase when comparing the Bulk with Nano form (ns†).

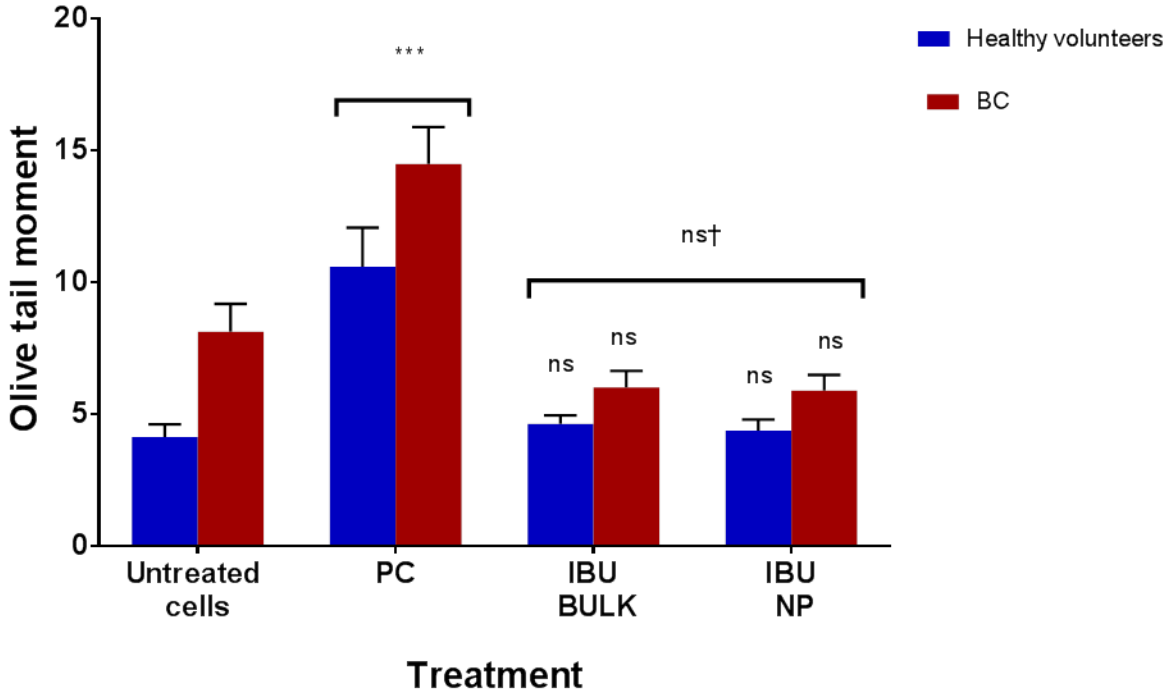

Figure 17 Comparison of ibuprofen concentration responses, SE, and significance on lymphocytes DNA from both healthy volunteers and BC patients using Olive tail moment. (N = 20)

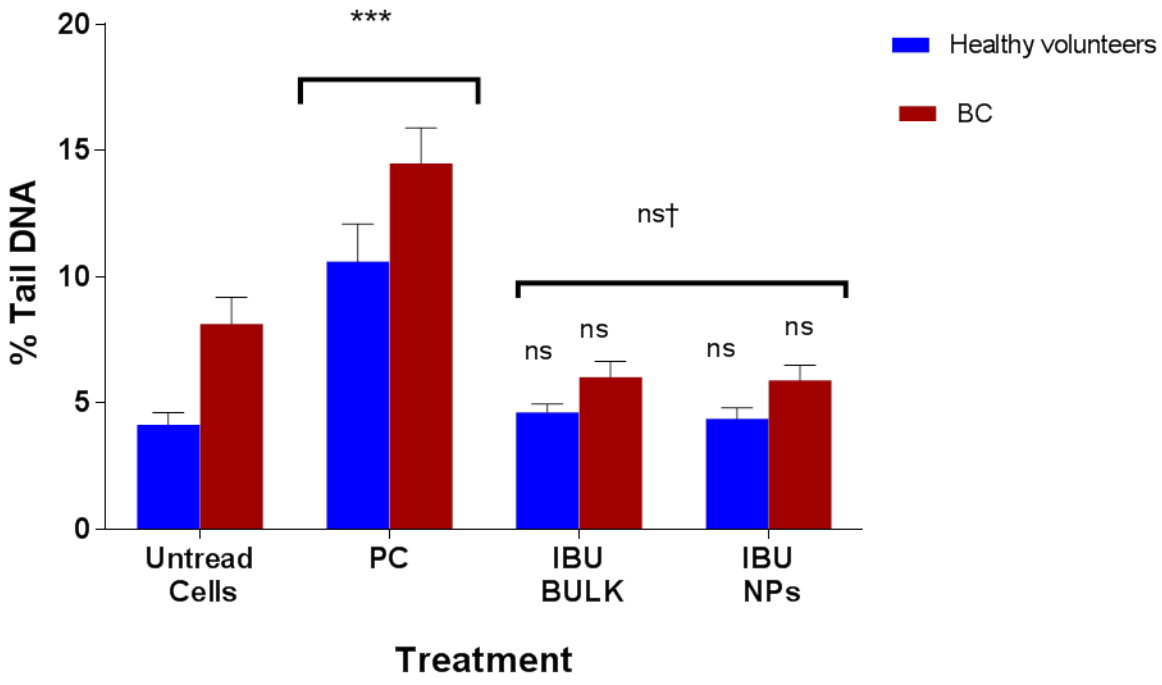

Figure 18 Comparison of ibuprofen concentration responses, SE, and significance on lymphocytes DNA from both healthy volunteers and BC patients using % Tail DNA. (N = 20)

3.5.4 Aspirin NP and bulk forms concentration responses on lymphocytes DNA from healthy volunteers

Figure 19 and 20 demonstrates the concentration responses of both NP and bulk forms of aspirin on lymphocyte DNA from healthy volunteers using Olive tail moment and % Tail DNA. It was clear from the histogram in Figure 19 and 20 that the effect of aspirin on lymphocyte DNA was weak and non-significant compared to untreated cells. Using the ANOVA test aspirin NP and bulk forms against untreated cells were compared and no significant (ns) impact on lymphocytes DNA was observed, whereas, H_2O_2 (50 µM) showed high significance ***$p \leq 0.001$. No significant difference in response was seen when the nano form was compared with the bulk ($ns^{\dagger}$).

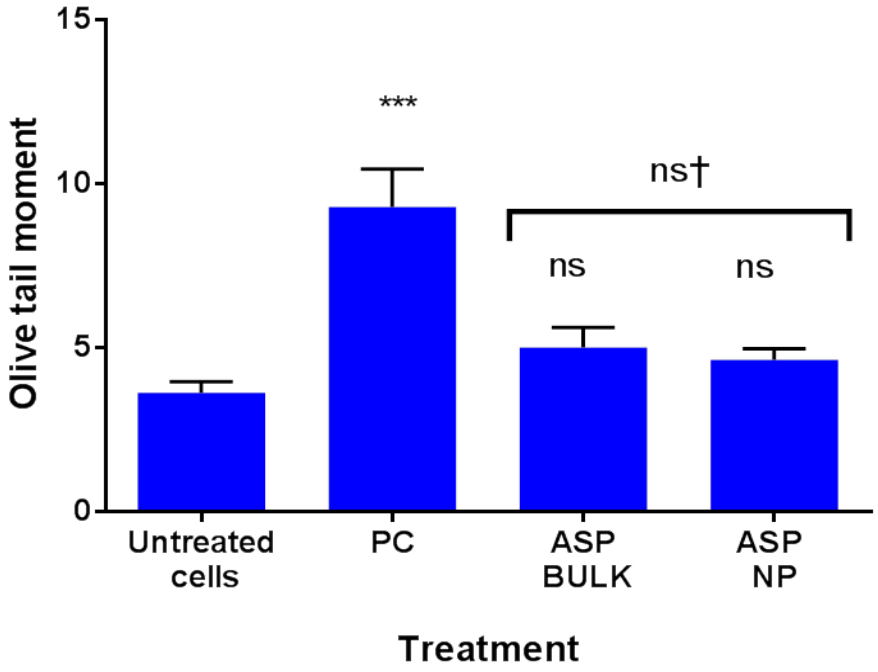

Figure 19 Concentration response to aspirin (NPs and bulk), SE, and significance in lymphocyte DNA from healthy volunteers using Olive tail moment. (N = 20)

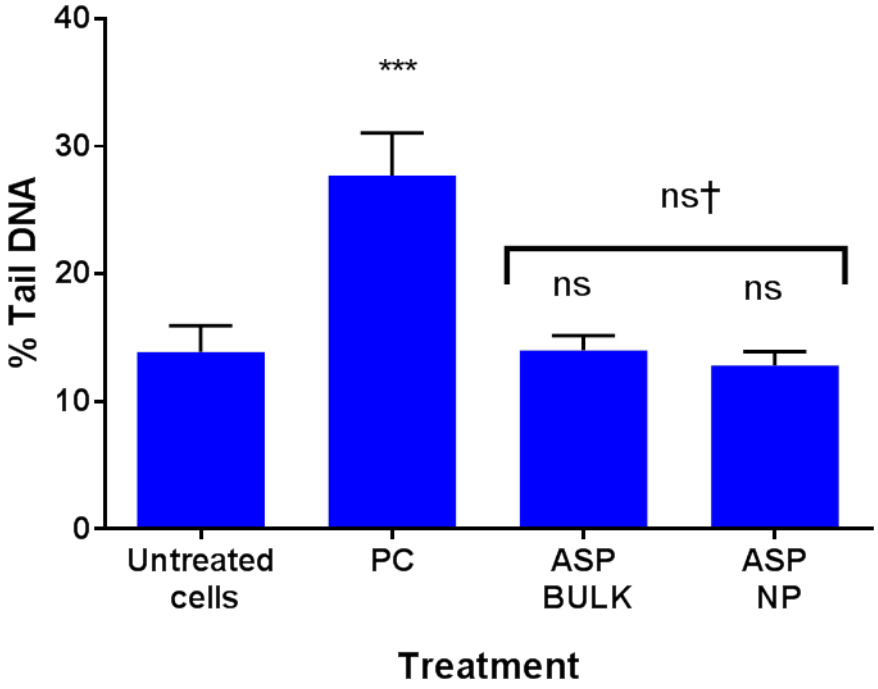

Figure 20 Concentration response to aspirin (NPs and bulk), SE, and significance in lymphocyte DNA from healthy volunteers using % Tail DNA. (N = 20)

3.5.5 Aspirin NP and bulk forms concentration responses in lymphocyte DNA from BC patients

Figure 21 and 22 illustrated the concentration response of both NPs and bulk forms of aspirin on lymphocyte DNA from BC patients using Olive tail moment and % Tail DNA. It was evident from the histogram that the aspirin reduced the damage on lymphocyte DNA compared to the untreated cells. All controls and aspirin insert data were normally distributed. By using the ANOVA test, aspirin NP and bulk against untreated cells showed significant reduction in lymphocyte DNA damage $^{**}p \leq 0.01$ and $^{*}p \leq 0.05$ respectively. H_2O_2 (positive control) (50 µM) showed a significance increase in DNA damage $^{***}p \leq 0.001$ when compared to untreated cells. There was a non-significant difference in effect when the nano-form was compared with the bulk form ($ns^{\dagger}$).

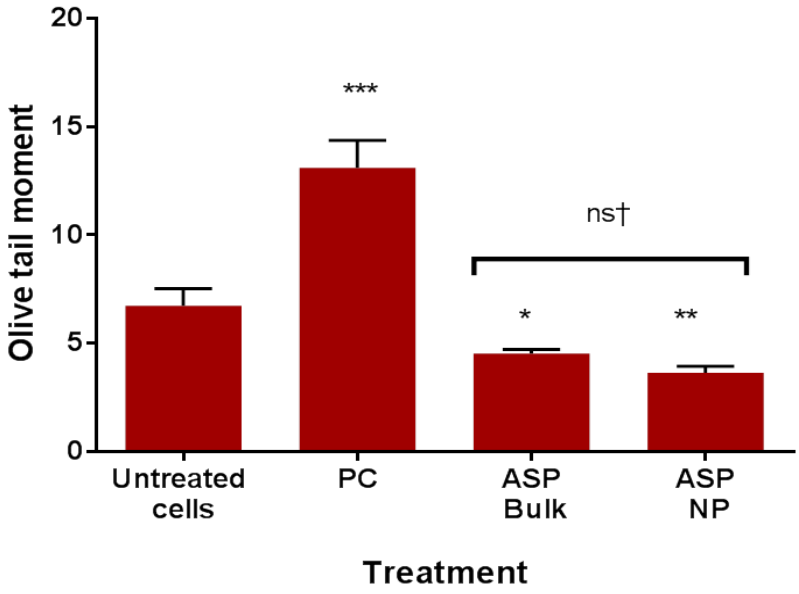

Figure 21 Concentration responses of aspirin (NP and bulk), SE and significance in lymphocyte DNA from breast cancer patients using Olive tail moment. (N = 20)

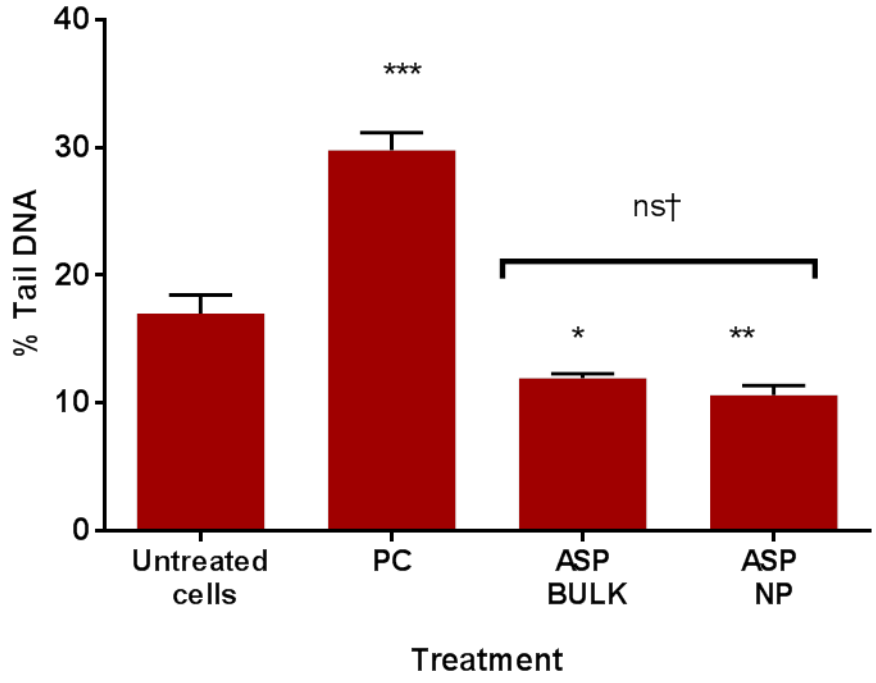

Figure 22 Concentration responses of aspirin (NP and bulk), SE and significance in lymphocyte DNA from breast cancer patients using % Tail DNA. (N = 20)

3.5.6 Comparing aspirin NP and bulk forms concentration responses in lymphocyte DNA from BC patients and healthy volunteers

Figure 23 and 24 demonstrate the concentration response of both NP and bulk forms of aspirin in lymphocyte DNA from BC patients and normal healthy volunteers using Olive tail moment and % Tail DNA. It was apparent from the histogram in Figure 23 and 24 that the both forms of aspirin significantly reduced the damage to lymphocyte DNA from BC patients compared to the untreated cells. However, normal healthy cells exhibited a weak and non-significant damage increase compared to the untreated cells. Both aspirin and ibuprofen showed non-significance when comparing the bulk with nano form (ns†).

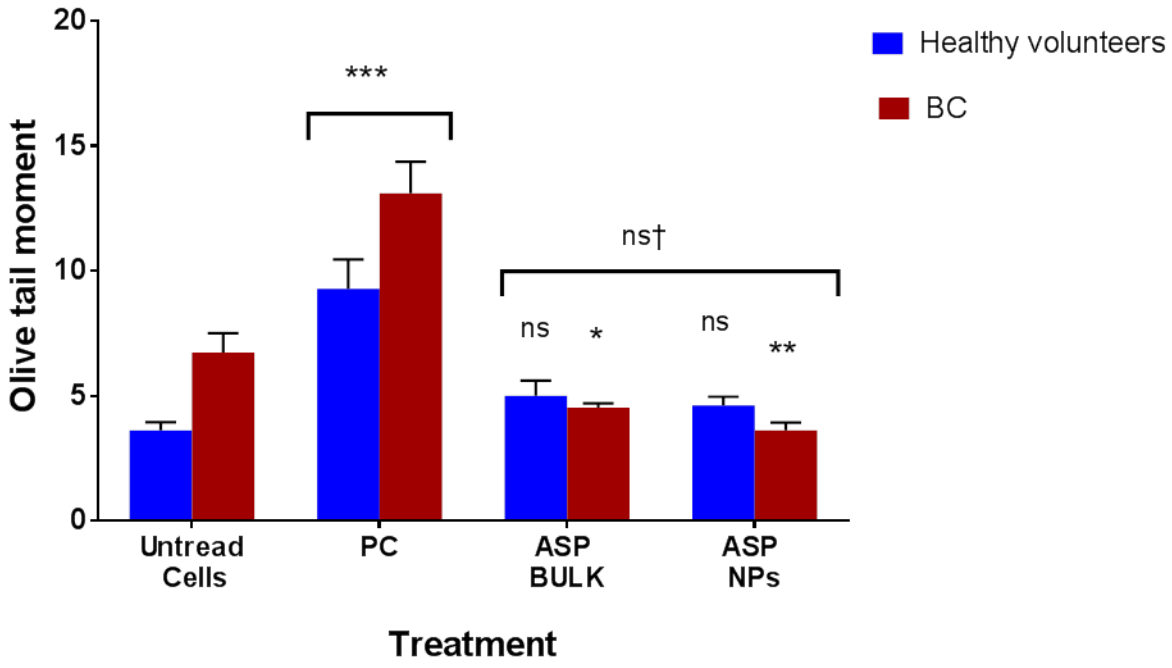

Figure 23 Comparison of the aspirin concentration responses, SE, and significance in lymphocyte DNA from both healthy volunteers and BC patients using Olive tail moment. (N = 20)

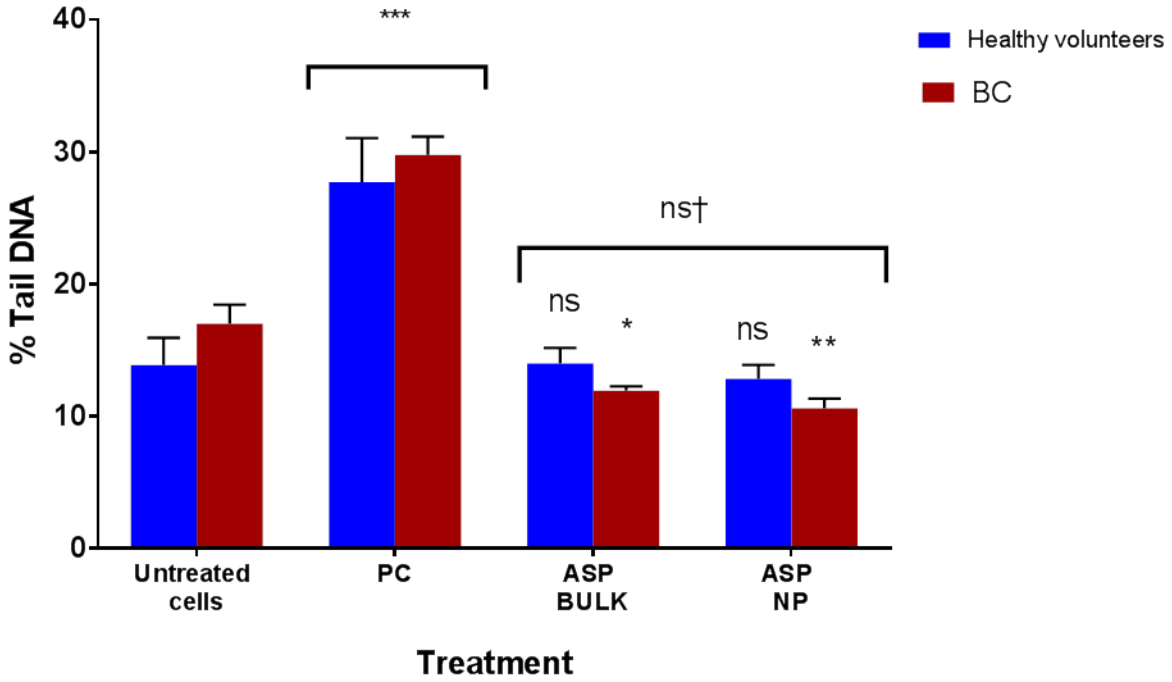

Figure 24 Comparison of the aspirin concentration responses, SE, and significance in lymphocyte DNA from both healthy volunteers and BC patients using % Tail DNA. (N = 20)

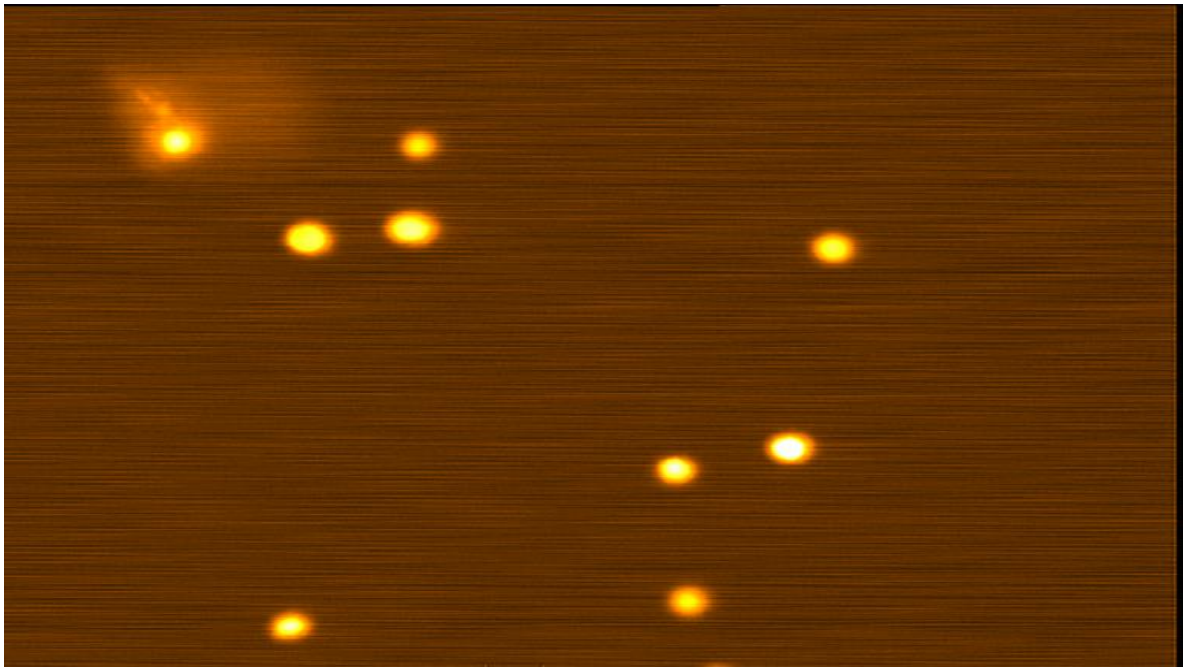

Figure 25 Typical comet image showing control lymphocyte cells (undamaged DNA)

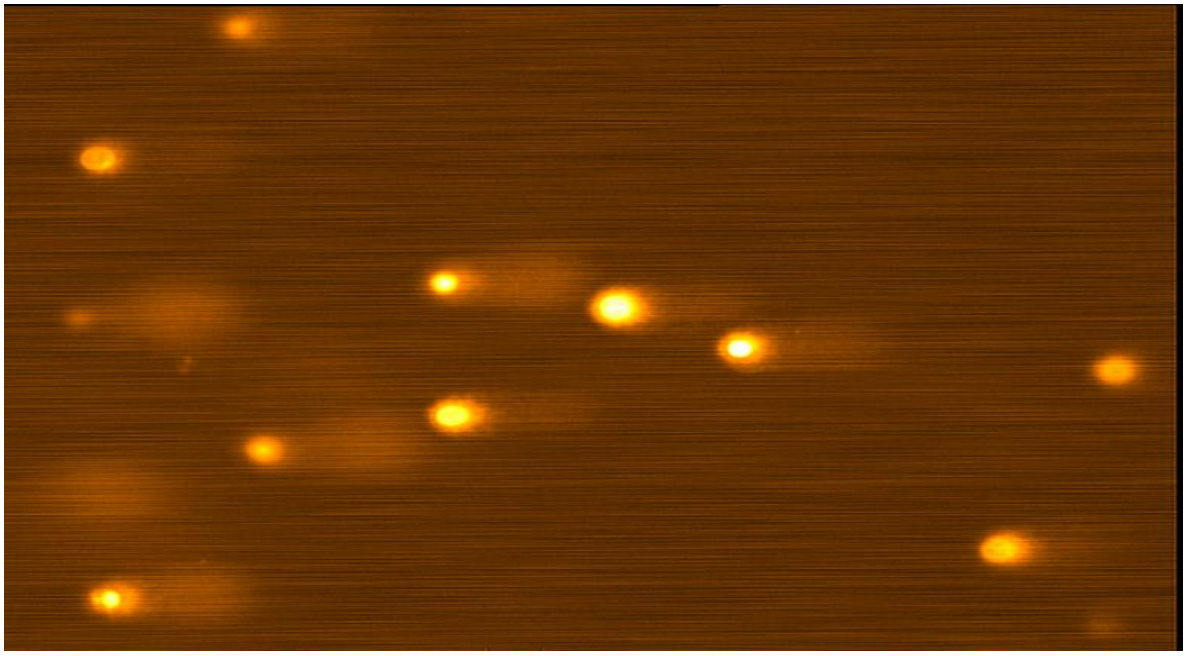

Figure 26 Typical comet image showing damage in lymphocytes cell DNA where the head represents undamaged DNA and tail reflects damaged DNA.

3.6 Discussion

The single cell gel electrophoresis assay (Comet assay) has become one of the most influential and standard assays to assess damage to DNA (Azqueta and Collins, 2013; Hoffmann et al., 2005; Vijaya Lakshmi et al., 1999). In studying DNA strand breakage, the Comet assay is already established to be the most sensitive technique available at present; it has many advantageous features including speed, ease and the fact that the DNA damage is evaluated at the level of a single cell (Collins et al., 1997; Wu and Jones, 2012). Lymphocytes obtained from human peripheral blood are used in different assays to assess genotoxicity. Lymphocytes are often utilised from workers who have been exposed to cytotoxic agents to follow chromosomal abnormality changes, sister chromatid exchanges, mutations, and after in vitro exposure (Anderson et al., 1991; Najafzadeh et al., 2012). Isolated and thawed lymphocytes obtained from both healthy volunteers and BC patients were treated in vitro separately, with ibuprofen NP 250 ng/ml, ibuprofen bulk 250 ng/ml, aspirin NP 500 ng/ml, and aspirin bulk 500 ng/ml individually for 30 min at 37°C. H_2O_2 (50 µM) was used as PC. After treatment, the lymphocytes were scored for Olive tail moment and % DNA tail. Healthy volunteer lymphocyte samples treated with ibuprofen and aspirin showed minor and non-significant damage in DNA. However in lymphocytes obtained from BC patients, both ibuprofen and aspirin (NP and bulk) displayed a decrease in DNA damage compared to the untreated cells. By using the ANOA test, these reductions were shown to be significant for aspirin NP and bulk $*p \leq 0.01$ and $*p \leq 0.05$ respectively, whereas, this decrease was non-significant when comparing the aspirin bulk with nano form. For both ibuprofen NP and bulk, the reduction was

non-significant compared to untreated cells. The Comet assay incubation time was 30 min, and this could be too short a time for ibuprofen to act and reduce DNA damage significantly. The aspirin outcomes demonstrated that the aspirin NP effect was stronger than the bulk form. When comparing the aspirin and ibuprofen results, it was shown that the aspirin response was faster and greater than that of ibuprofen.

Many studies suggest that NSAIDs such as ibuprofen and aspirin have the potential to reduce the probability of breast cancer development (Horn and Fentiman, 2010; Kwan et al., 2007). In this present study the Comet assay experimental outcomes corresponded to the previous suggestion that ibuprofen and aspirin have the ability to reduce DNA damage and therefore developing cancer (Baron and Sandler, 2000; Ghosh et al., 2010).

Chapter 4 – Anti genotoxic effects of ibuprofen and aspirin treatment in lymphocytes in the micronucleus assay

4. Micronucleus assay results

4.1 Introduction

The results from the ibuprofen (NPs and bulk) and aspirin (NPS and bulk) experiments in the previous chapter showed the potential of these compounds to reduce DNA damage. This was evident as seen by the significant decrease of Olive tail moment and % DNA tail using the Comet assay. Both ibuprofen and aspirin showed an ability to reduce the DNA damage; however, aspirin had a more significant impact. The Comet assay can demonstrate a compound's effect at a genomic level. From these findings, it is clear that another technique is needed, which can investigate the compounds' impact at a cytogenetic level. In this chapter results of the cytokinesis-block micronucleus (CBMN) are shown studying the effect of both agents in the micronucleus assay using somatic cells, i.e. lymphocytes. The definition of the cell growth cycle is the vital processes that allow the cell to proliferate into daughter cells by replicating their DNA and finally when becoming senescent reach apoptosis, also called programmed cell death, at the end of their lives. The cell cycle occurs over a 24 h period in eukaryotic cells and consists of four stages: Gap 1 phase (G1 phase), the synthesis of DNA (S phase), gap 2 phase (G 2 phase), and the mitotic phase (M phase) (Bertoli et al., 2013; Collins et al., 1997). In the first stage, G1 phase, the cell starts to prepare itself for progressing into S phase by synthesising protein required for DNA replication. In S phase, chromosomes are replicated to produce two identical copies of each chromosome. All components that are required for cell division and M phase are being prepared in G2 phase. The M phase is an essential stage in which nuclear division takes place and this stage can be divided in 5 different sub-

stages prophase, prometaphase, metaphase, anaphase, and telophase (Bertoli et al., 2013; Norbury, 1992). Eventually, cytokinesis occurs in which the cell divides into two daughter cells (Kim et al., 2012; Vermeulen et al., 2003). The CBMN technique utilises once divided cells; these cells are characterised by the presence of two nuclei in cells known as binucleated cells (BiNC). These binucleated cells are a result of a blocking cytokinesis with cytochalasin B (cyto B), which inhibits the formation of the actin filaments network. These filaments are crucial to complete the cytokinesis stage. During cell division, the chromosome structure can be changed and separation can be interfered with by so many chemicals and exogenous agents, and this may lead to a variety of genetic mutations (Fenech, 2007; Ionescu et al., 2011). The formation and presence of micronuclei (MNi) are a consequence of chromosomal breaks. These are fragments or whole chromosomes which can be scattered after the anaphase stage of mitosis. The presence of MNi in BiNC cells reflects precisely the cytogenetic damage. MNi can also be found in mononucleated (MonoNC) cells and multinucleated (MultiNC) cells; the presence of MNi in BiNCs exhibit the primary cytogenetic level; however, the MNi in MonoNC and MultiNC could be a result of different cell cycle kinetics (Fenech, 2006; Ionescu et al., 2011; Torres-Bugarín et al., 2014). Recently, research has demonstrated that the CBMN assay can as well measure nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) (Figure 10, 11). NPBs can be described as a small, very thin bridge between the two nuclei in BiNC. Occurrence of NPBs shows the presence of dicentric chromosomes which appear as a result of DNA mis-repair or telomere end-lesions. The importance of NPBs lies in its ability to measure some chromosomes rearrangements, which cannot be

shown by scoring the MNi alone (Fenech et al., 2011). Gene amplification can be detected via NBUDs as a biomarker. These NBUDs persist attached to the original nuclei throughout the nucleoplasm. NBUDs are similar to MNi, the only difference being that NBUDs are physically connected to the nucleus (Fenech, 2007; Luzhna et al., 2013). MNi induction in lymphocytes has been well recognised in the CBMN assay (Fenech, 2000). The CBMN assay was selected and performed to evaluate MNi induction following NSAIDs (aspirin and ibuprofen) exposure. Two doses, 500 µg/ml and 250 µg/ml, were chosen for aspirin and ibuprofen, respectively. As positive control 0.4 µM of mitomycin C was selected. MNi, NBPs, and NBUDs were scored in BiNC up to 1000 cells. One thousand other cells were scored to calculate the percentages of each type of cells' MonoNCs, BiNC, and MultiNC. After the scoring proportions of 3 cell types were used to calculate the nuclear division index (NDI). The NDI is essential to measure the rate of mitotic division as it reflects cytostatic effects (Fenech, 2007).

4.2 Materials and Methods

4.2.1 Materials

All materials that have been used in these experiments are mentioned and described in section **2.1**

4.2.2 Methods

The collection of whole blood samples was described in section **2.2**. The CBMN assay protocol was described in section **2.6**. Treatment groups, including MMC, ibuprofen (NPs and bulk), and aspirin (NPs and bulk) per each experiment were described in section **2.6.3.** (See Table 4).

4.2.3 Statistical Analysis

Slides were coded and scored using Fenech's protocols (Fenech, 2007). The determination of NDI was obtained by using NDI = (M1) + 2 (M2) + 3 (M3) /N formula, where, M1 = mononucleated cells, M2 = binucleated cells, M3 = multinucleated cells and N = the total number of viable cells scored. The ANOVA test was used to determine the significance of each group compared to the untreated cells.

4.3 Results

4.3.1 Results of CBMN Assay following ibuprofen and aspirin (NPs and bulk) exposure to lymphocytes

Table 6 and 7 showing various cytological scoring parameters, including cell mitotic status (BiNC, MonoNC, MultiNC), MNi frequency, NDI and chromosomal damage/instability parameters in the form of NPBs and NBUDs. Lymphocytes from 5 female volunteers and 5 BC patients were used. The cells were treated with 250 µg/ml of ibuprofen and 500 µg/ml of aspirin (NPs and bulk) individually. By using the ANOVA test. There were no differences in MNi frequency in lymphocytes from healthy volunteers compared to an treated cells. However, the positive control showed an increased the MNi frequency significantly ***$p \leq 0.001$ compared to the untreated cells. In lymphocyte from BC patients, the frequency of MNi in BiNC was reduced significantly after the treatment with ibuprofen and aspirin. Moreover, both forms of aspirin and ibuprofen showed ***$p \leq 0.001$, whereas, ibuprofen bulk showed **$p \leq 0.01$ compared to the untreated cells.

Treatment	Chemical concentration	NDI (mean)	% BiNC	MNi per 1000 BiNC mean	NPBs mean	NBUDs mean	%MonoNC	% MultiNC
untreated lymphocytes		1.9	67.4	3	1	1	21	11.6
PC	0.4 µM	2.0	73.8	24 ***	3	6	11.2	15
Ibuprofen NPs	250 ng/ml	1.8	70.1	2 (ns)	0	0	15.1	14.8
Ibuprofen bulk	250 ng/ml	2.0	60	3 (ns)	0	0	29.4	10.6
Aspirin NPs	500 ng/ml	2.0	69.4	2 (ns)	0	0	13	17.6
Aspirin bulk	500 ng/ml	1.9	74	2 (ns)	0	0	14.4	11.6

Table 6 Various cytological scoring parameters, including cell mitotic status (BiNC, MonoNC, MultiNC), NDI and chromosomal damage/instability parameters in the form of NPBs and NBUDs in lymphocytes from 5 healthy female volunteers following exposure to ibuprofen and aspirin (NPs and bulk). (N = 5)

NDI = the nuclear division index
BiNC = Binucleated cells, % BiNC, is % expressed out of all types of 1000 cells scored.
MultiNC = Multinucleated cells % MultiNC and MonoNC= Mono nucleated cells %MonoNC. The % expressed out of all types of 500 cells scored.
MNi = Micronuclei score/1000 cells each of BiNC
Where ***** is used to compare all groups to the untreated lymphocytes. ******* $p \leq 0.001$ and ns = not significant.

Treatment group	Chemical concentration	NDI (mean)	% BiNC	BiMNi per 1000 BiNC mean	BiNPB mean	BiBuds mean	%MonoNC	% MultiNC
NC		2.0	67.9	11.4	1.4	1	12.1	20
PC	0.4 µM	1.9	69.1	17.6 **	2.4	2.4	17.6	13.3
Ibuprofen NPs	250 ng/ml	1.8	67.5	5 ***	0	0	23.9	8.6
Ibuprofen bulk	250 ng/ml	1.8	65.3	6 **	0	0	28.3	6.4
Aspirin NPs	500 ng/ml	1.8	65.8	3.2 ***	0	0	26	8.2
Aspirin bulk	500 ng/ml	1.9	67.6	3.4 ***	0	0	23.1	9.3

Table 7 Various cytological scoring parameters, including cell mitotic status (BiNC, MonoNC, MultiNC), NDI and chromosomal damage/instability parameters in the form of NPBs and NBUDs in lymphocytes from 5 BC patients following exposure to ibuprofen and aspirin (NPs and bulk).

NDI = the nuclear division index
BiNC = Binucleated cells, % BiNC, is % expressed out of all types of 500 cells scored.
MultiNC = Multinucleated cells % MultiNC, is % expressed out of all types of 500 cells scored.
MNi = Micronuclei score/500 cells each of BiNC
Where ***** is used to compare all groups to the untreated lymphocytes. ****** $p \leq 0.01$ and ******* $p \leq 0.001$.

4.3.2 Ibuprofen and aspirin (NPs and bulk size) concentration response on MNi induction in lymphocytes from healthy female individuals.

Figure 27 illustrates the concentration response of both ibuprofen and aspirin (NP and bulk) on lymphocytes from healthy volunteers. Using the ANOVA test, both NSAIDs (nano and bulk) showed no significant (ns) change in lymphocyte MNi frequency compared to the untreated cells. Whereas, 0.4 µM MMC (PC) showed a significant increase in MNi frequency ***$p \leq 0.001$ compared to the untreated cells. There was no significant difference between both nano and bulk forms in either aspirin or ibuprofen (ns†).

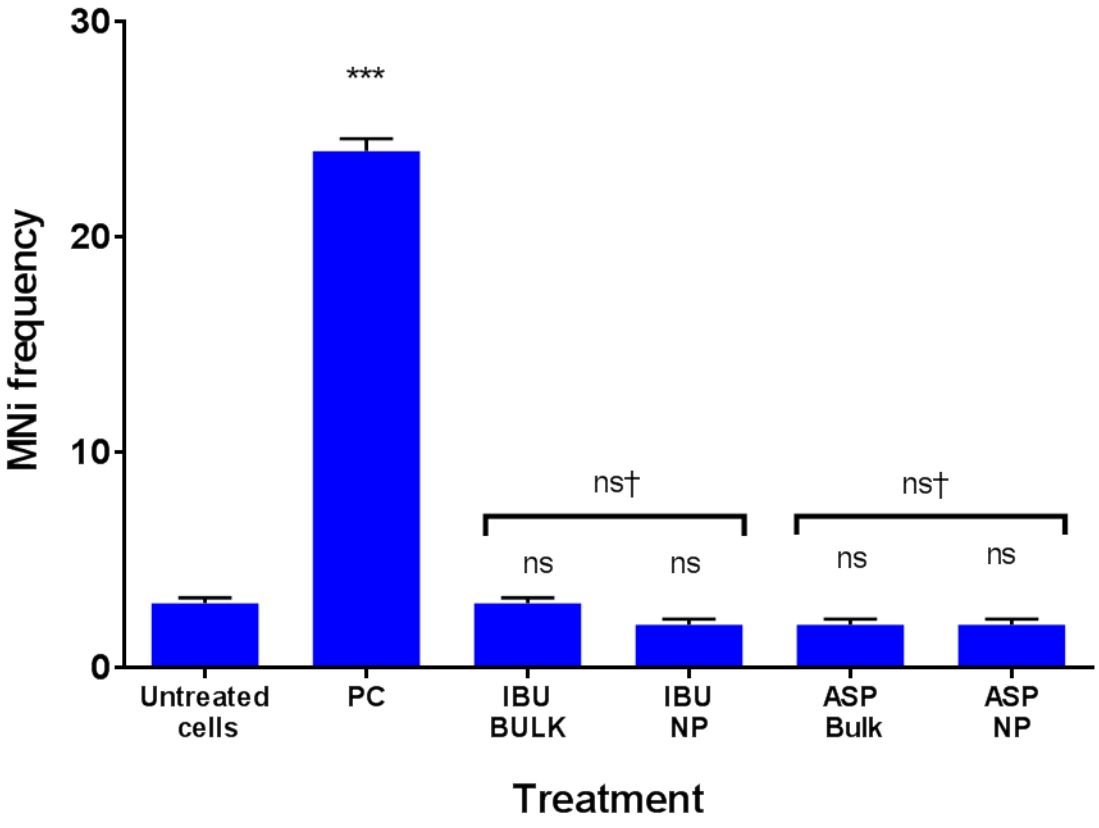

Figure 27 MN frequency in lymphocytes from healthy females exposed to ibuprofen and aspirin including SE and significance. (N=5)

4.3.3 Ibuprofen and aspirin (NPs and bulk size) concentration response on MNi induction in lymphocytes from BC patients

Figure 28 illustrates the concentration response of both ibuprofen and aspirin (NP and bulk) in lymphocytes from breast cancer patients to induce MNi in BiNC. The histogram in Figure 28 shows that the ibuprofen and aspirin significantly reduced the frequency of MNi in lymphocytes of BC patients in vitro when compared to untreated cells. Using the ANOVA test, results showed a significant decrease ***$p \leq 0.001$ in MNi for ibuprofen nano, aspirin nano and aspirin bulk. However, ibuprofen showed **$p \leq 0.01$ compared to the untreated cells. Furthermore, 0.4 µM MMC (PC) showed a significant increase in MNi ***$p \leq 0.001$ compared to the untreated cells. Both bulk and nano-forms of aspirin and ibuprofen had similar effects ns†.

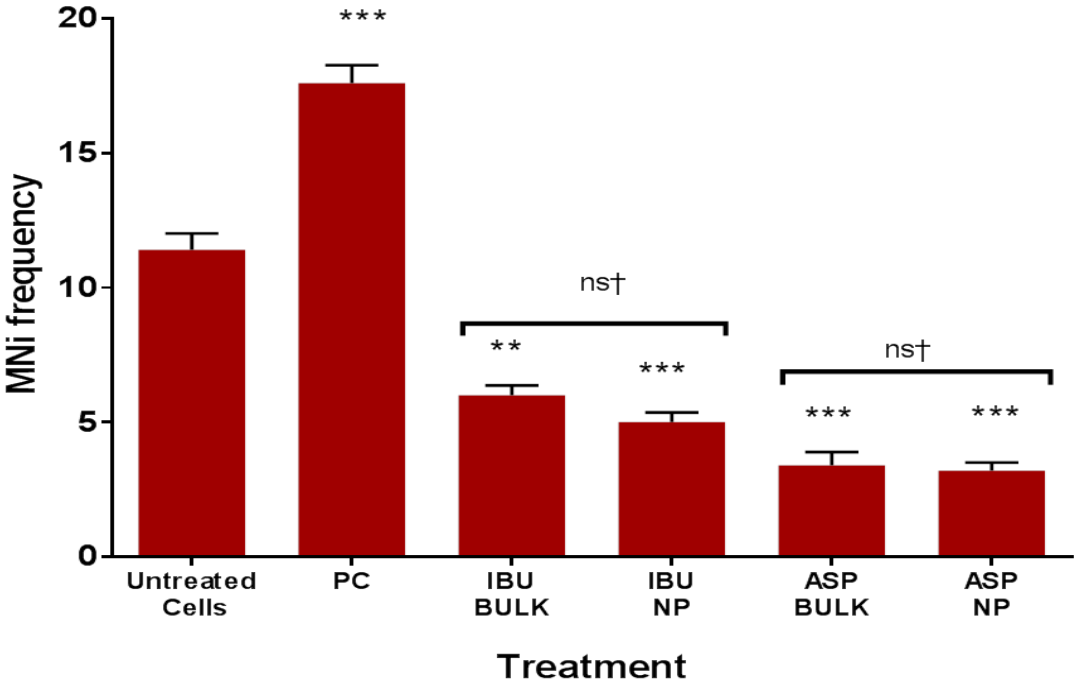

Figure 28 MN frequency in lymphocytes from BC patients exposed to ibuprofen and aspirin including SE and significance. (N=5)

4.3.4 Comparing ibuprofen and aspirin (NPs and bulk) concentration response on MNi frequencies in lymphocytes from BC patients and healthy females volunteers

Figure 29 demonstrates the dose response of both NPs and bulk sizes of aspirin on MNi frequencies in lymphocytes from breast cancer patients and normal healthy volunteers. The histogram in Figure 29 shows that the ibuprofen NP ***$p \leq 0.001$, ibuprofen bulk **$p \leq 0.01$, aspirin NP ***$p \leq 0.001$ and aspirin bulk ***$p \leq 0.001$ significantly reduced MNi numbers in lymphocyte from breast cancer patients compared to the high baseline frequency of MNi in the untreated cells; by contrast, in lymphocytes from healthy individuals both drugs had no effect in either form when compared to the relatively low MNi frequency in the untreated cells.

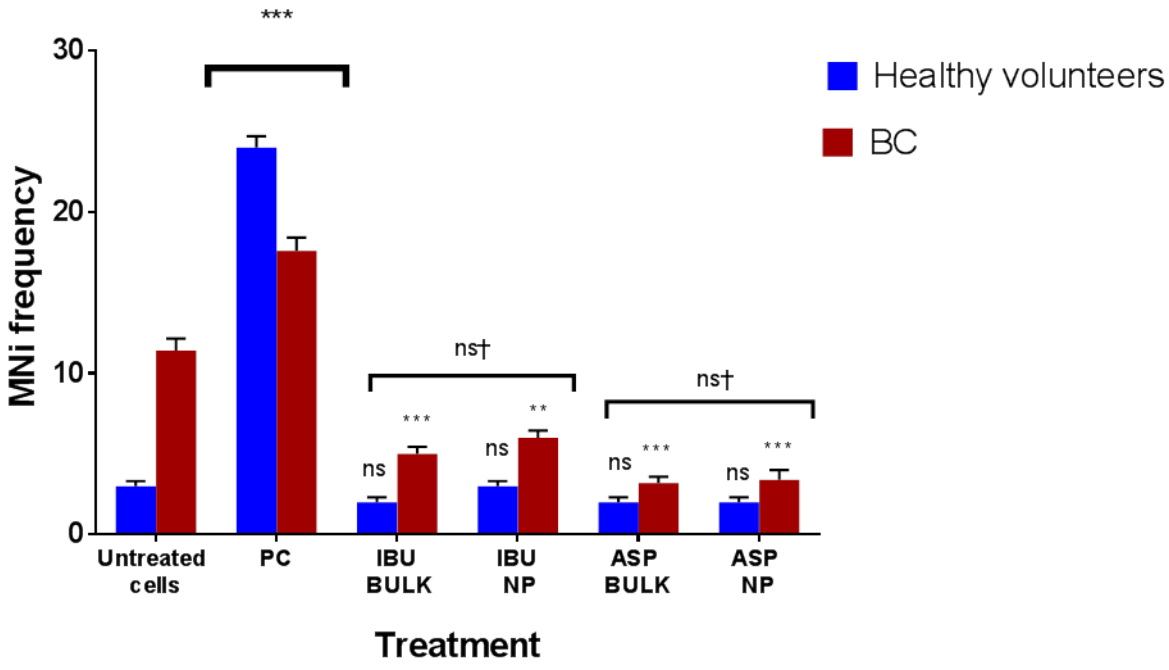

Figure 29 Comparison between MN frequencies in lymphocytes from healthy females and BC patients exposed to ibuprofen and aspirin including SE and significance. (N = 5)

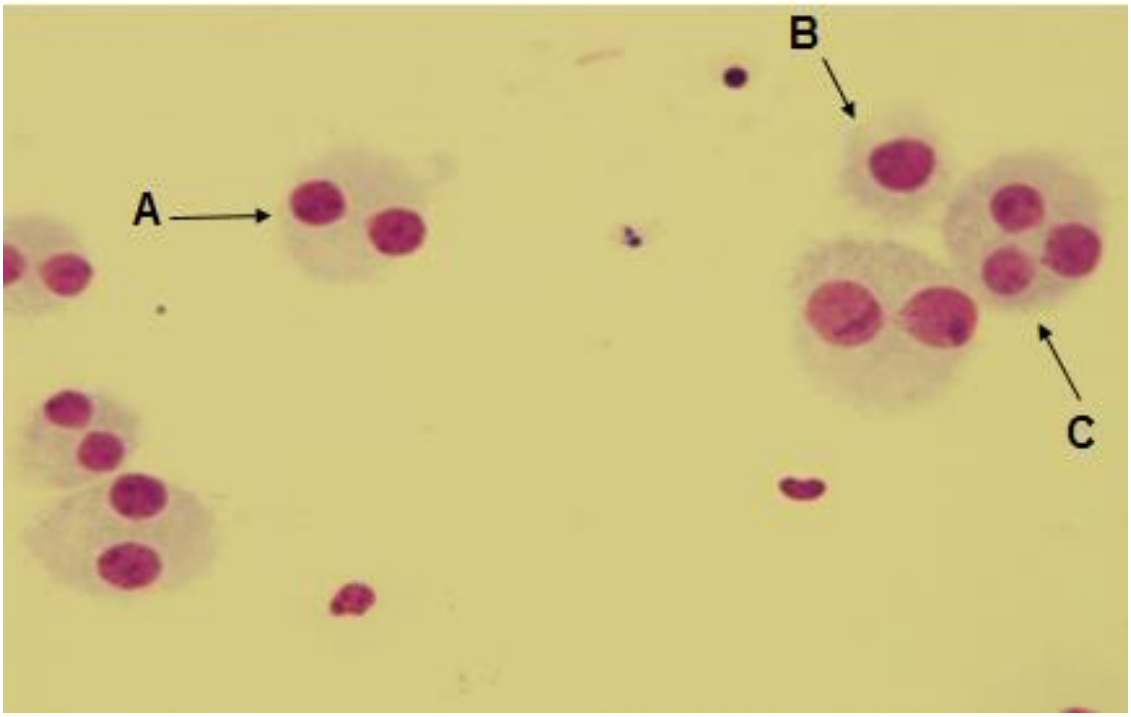

Figure 30 shows (A) Binucleated, (B) Mononucleated, and (C) Multinucleated cells.

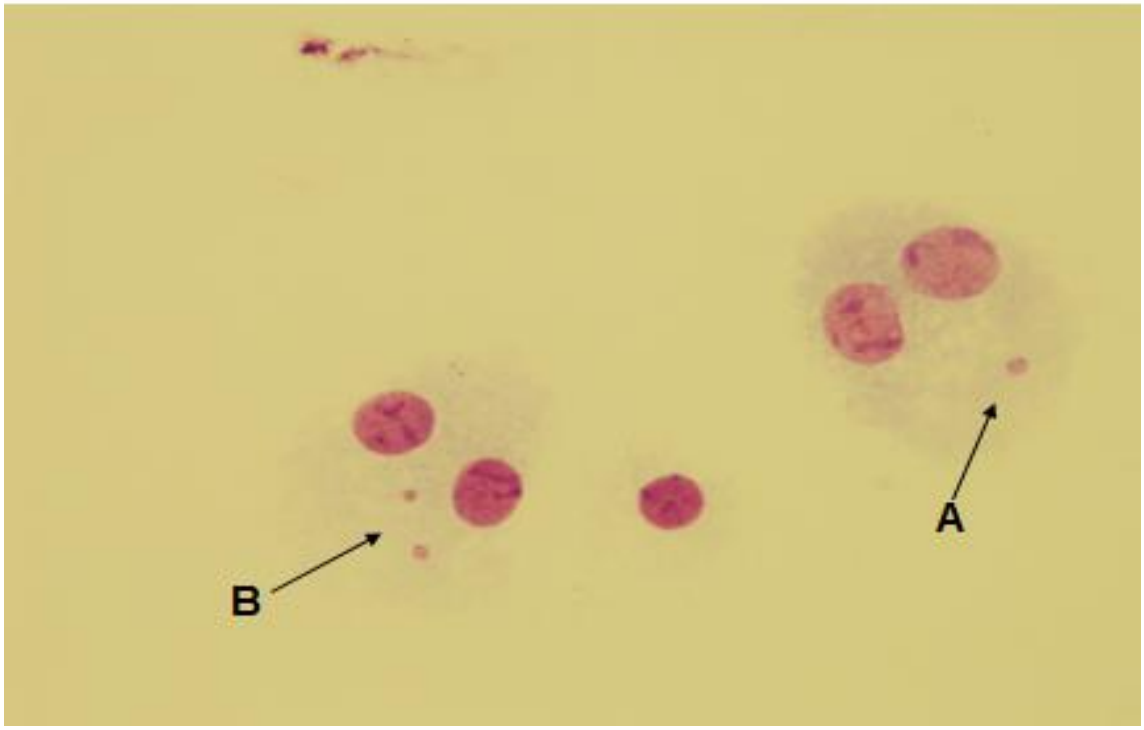

Figure 31 shows binucleated cells (A) with one MN and (B) with two MNi.

4.4 Discussion

Micronuclei are small extra-nuclear chromosomal fragments that can be observed in the cytosol after anaphase through nuclear division. MNi may originate from acentric chromosomal fragments or entire mis-segregated chromosomes which are usually not incorporated into either of the daughter nuclei (Fenech, 2007; Fenech et al., 2011; Torres-Bugarín et al., 2014). The roles of ibuprofen and aspirin in both sizes, NP and bulk, and their influence on MNi frequencies in binucleated lymphocytes was analysed in this study. The lymphocytes were obtained from both healthy females and breast cancer patients. Many studies recently suggested that regular intake of NSAIDs especially ibuprofen and aspirin have a role in cancer prevention (Johannesdottir et al., 2012). Concentrations of 250 ng/ml of ibuprofen NP, 250 ng/ml of ibuprofen bulk, 500 ng/ml of aspirin NPs, and 500 ng/ml of aspirin bulk were used in these experiments. As a PC 0.4 µM of MMC was used. Lymphocytes from both groups were exposed to both drugs in either form and cultured for 72 hours. Cyto-B was added to each culture after 44 hours to block cell cytokinesis by inhibiting the microfilament formation and cell division. At 72 hours, the cells were extracted, fixed, applied to glass slides, stained, and then scored (Fenech, 2007; Fenech et al., 2011). Our results showed (Tables 6 and 7) that the NDI values of all experiments were within the normal range (1.3 – 2.2) which indicates that the majority of viable cells had completed one cell division. Between 10% - 17% of healthy lymphocyte cells and 6% - 20% of lymphocyte cells from breast cancer patients were multinucleated showing a low proportion of the cell population had already have progressed to the second mitosis in the presence of Cyto-B. In experiments using lymphocytes from healthy individuals, ibuprofen and

aspirin showed no effect on MNi frequencies when compared to the untreated cells, i.e. the number of MNi within the untreated cells and the drug-treated cells were in the same range at about 2-3 per 1000 BiNC. Only the PC increased the MNi frequency sharply 2-fold compared to the untreated cells ***$p \leq 0.001$. Different results were obtained from experiments using lymphocytes from BC patients. It is clear that both drugs reduced cytogenic damage. Both drugs (see Table 7) showed an apparent reduction in MNi frequency compared to the untreated cells, in particular: ibuprofen NP ***$p \leq 0.001$, ibuprofen bulk **$P \leq 0.01$, aspirin NP ***$p \leq 0.001$ and aspirin bulk ***$p \leq 0.001$. This was due to the 2-folds higher baseline damage in lymphocytes from BC patients when compared to those from healthy individuals. By comparing the two drugs, it became clear that aspirin was more effective than ibuprofen (Figure 29) reducing the high cytogenetic baseline damage in lymphocytes of BC patients. Furthermore, the effects of NP of both ibuprofen and aspirin on MNi frequencies were stronger compared to bulk-sized drugs supporting the theory that NP have the ability to penetrate inside the nucleus faster than bulk sizes (Singh et al., 2009). From this work, it is clear that the outcome from BC patients corresponds to recent studies that suggested the ibuprofen and aspirin have the ability to reduce DNA damage which in turn may have a role in prevention of many cancer types (Ali, 2014)

Chapter 5 - Comet assay on BLM with and without recovery

5. Comet repair assay

5.1 Introduction

The Comet assay is a method used for direct assessment of DNA damage in mammalian cells (Azqueta and Collins, 2013). The Comet assay in recent years is a method of choice to measure genotoxic DNA damage in lymphocytes due to its time/cost efficiency, simplicity and high sensitivity (Kapka-Skrzypczak et al., 2011). The assay is using as a molecular biomarker for simultaneous monitoring to evaluate chemical genotoxicity in DNA damage and repair (Alvarez-Moya and Reynoso-Silva, 2015; Costa and Teixeira, 2014). Monitoring the repair of DNA is essential to assess cancer susceptibility (Gaivão and Sierra, 2015). All cells possess repair pathways for various types of DNA damage to avoid permanent changes being made to the DNA. The method used to assess DNA repair progress is also known as the challenge assay (Azqueta et al., 2014). The *In-vitro* cellular repair assay is a technique monitoring DNA repair process; this approach is also called the mutagen challenge assay. In this test, a genotoxic element such as MMC, doxorubicin, or BLM is utilised to challenge cells generating DNA damage. To provide the ability to monitor the DNA single strand breaks manner to re-join after repairing them (Azqueta and Collins, 2013; Azqueta et al., 2014). After incubation, cells are then transferred into a fresh buffer to eliminate the bleomycin genotoxic effect. A further incubation of cells is used with short time intervals (30 min) to allow the DNA to repair. In the next step, the DNA damage level is measured from samples at the end of the repair process then it is compared to the control samples (untreated cells). The Olive tail moment and % tail DNA

parameters are used for analysis and comparison with the DNA damage repair ability. The repair of single strand breaks can be monitored by observing other DNA lesions, such as oxidised bases which repair by using the base excision repair pathway. Additionally, altered bases can be evaluated, by using specific enzymes which have the capability to distinguish these lesions and consequently, alter these lesions to strand breaks (Azqueta and Collins, 2013; Azqueta et al., 2014).

The principle of the present study depends on the previous methodology described above. Cells from healthy female individuals and BC patients were challenged using the genotoxic agent bleomycin and then cells washed with RPMI and allowed to repair DNA damage in the absence of BLM. A group of samples were allowed to repair DNA damages in the presence of aspirin and ibuprofen (both nano and bulk forms) individually. This step was chosen due to previous work on the NSAIDs (aspirin and ibuprofen) using the Comet assay. This study is designed to examine whether these two compounds (both forms) increase the repair of DNA in the presence of the genotoxic agent (BLM).

BLM was chosen in this research as it was used in other repair studies. Various studies have examined DNA damage and repair mechanisms in lymphocyte cells obtained from patients with different cancer types after bleomycin exposure, such as lung cancer, mastocarcinoma patients, nasopharyngeal carcinoma, and oesophagus cancer (Laffon et al., 2010; Wei et al., 2005; Zhang and Gu, 2013). DNA damage resulting from BLM treatment is repaired through HRR, NHEJ and BER mechanisms (Wei et al., 2005). Bleomycin is a radiomimetic drug commonly used as part of chemotherapy for treatment of various solid tumours, especially for

cutaneous and subcutaneous tumours such as testicular carcinomas, lymphomas, and head and neck tumours (Chen and Stubbe, 2005; Gibot et al., 2013; Wang et al., 2013; Yu et al., 2013). Bleomycin induces DNA damage by binding to the transition metal iron (Fe(II)) or/ and oxygen to produce ROS which cause DNA damage (single and double-strand) in the presence of an electron reductant. Bleomycin can also cause lipid peroxidation and mitochondrial DNA damage (Chen and Stubbe, 2005; Wang et al., 2013). The most common cellular responses to BLM exposure are cell-cycle arrest, senescence, mitotic cell death, and apoptosis (Wang et al., 2013)

5.2 Materials and Methods

5.2.1 Materials

The materials required for this study have been described in section **2.1**

5.2.2 Methods

The treatment of whole blood cells treatment has been described in section **2.2**. The Comet assay was prepared under alkaline conditions. Treatment groups for all samples and treatment with NSAID compounds were described in sections **2.7.2, 2.7.4** and **2.7.5.** Embedding of cells in LMP agarose, DNA unwinding and electrophoresis, and neutralisation have been described in sections **2.7.5**, **2.7.6**, and **2.7.7** respectively. Scoring of cells and statistical analysis has been described in sections **2.7.8** and **2.11.**

This study was conducted to evaluate the DNA repair ability of lymphocyte cells, following BLM exposure for 30 min from healthy female control individuals and breast cancer patients. Cells were allowed to carry out DNA repair in the presence of aspirin and ibuprofen (NP and bulk forms), to determine the impact of these two compounds on cell DNA repair using the Comet assay technique. The results were compared to the treated and untreated cells and self-repaired cells without NSAIDs treatment (BLMR).

All slides were coded before the scoring, and 100 cells were scored using a microscope equipped with a CCD camera and Computer system using Comet Kinetic Imaging Software© 6.0, Liverpool/Andor Technology, Belfast, UK. Each experiment used blood from BC patients or healthy individual were repeated fifteen times. Mean data were calculated with standard errors.

5.2.3 Statistics

Data from both Olive tail moment and % Tail DNA were used to accomplish SPSS Statistics (Version 18). Most of the data were normally distributed. The normality of distribution was checked using the Kolmogorov-Smirnoff and Shapiro Wilk's test. One-Way ANOVA test (>three variables) and t-test (two variables) were used determine the difference between healthy individuals' samples and BC samples.

Different kinds of significance were determined, all samples were compared to the untreated cells, including the BLM sample where DNA repair was not allowed to proceed (disallowed) ***p = ≤ 0.001, **p = ≤ 0.01, *p = ≤ 0.05 and ns (not significant).

The Second comparison was among treated with BLM and allowed to repair (BLMR) in the presence of NSAIDs (ASP B, ASP N, IBU B & IBU N) and the sample that was treated with BLM and disallowed DNA repair against the sample treated with BLM with allowing DNA self-repair without any of NSAIDs $^{\dagger\dagger\dagger}p$ = ≤ 0.001, $^{\dagger\dagger}p$ = ≤ 0.01, $^{\dagger}p$ = ≤ 0.05 and ns† (not significant).

Finally, the different forms of each NSAIDs were compared with each other. ASP N was compared to ASP B and IBU N was compared to IBU B $^{\wedge\wedge\wedge}p$ = ≤ 0.001, $^{\wedge\wedge}p$ = ≤ 0.01, $^{\wedge}p$ = ≤ 0.05, ns$^{\wedge}$ (not significant).

5.3 Results

5.3.1 Aspirin and ibuprofen concentration response on lymphocyte from healthy female volunteers treated with bleomycin

In figures 32 and 33 showed the concentration response of aspirin and ibuprofen both forms on lymphocytes DNA from healthy volunteers treated with BLM using Olive tail moment and % Tail DNA, standard errors SE and significance. Using the one-way ANOVA It was clear from the histogram in the Olive tail moment figure (32) that treatments showed significant increases in DNA damage compared to untreated cells, BLM, BLMR and ibuprofen-bulk $^{**}p \leq 0.001$ and ASP-bulk $^{**}p \leq 0.01$. However, both aspirin and ibuprofen NPs were not-significant (ns*) compared to untreated cells. Comparing to the BLMR treatment BLM, ibuprofen and aspirin NPs showed $^{\dagger\dagger\dagger}p \leq 0.001$, aspirin bulk $^{\dagger\dagger}p \leq 0.01$, whereas, ibuprofen bulk was ($ns^{\dagger}$). Another comparison was made between NPs and bulk forms of aspirin and ibuprofen; aspirin NP showed significance $^{\wedge}p \leq 0.05$ compared to the bulk form where there were no significance (ns^) between the two ibuprofen forms. In % Tail DNA figure (33) all treatments showed significance against untreated cells $^{***}p \leq 0.001$ except aspirin NP it was not-significant. In comparing with BLMR all treatments were significant (BLM, IBU bulk, IBU NP, ASP bulk and ASP NP) $^{\dagger\dagger\dagger}p \leq 0.001$. The effect of both ibuprofen and aspirin were significant when compared the NPs with bulk forms $^{\wedge\wedge\wedge}p \leq 0.001$.

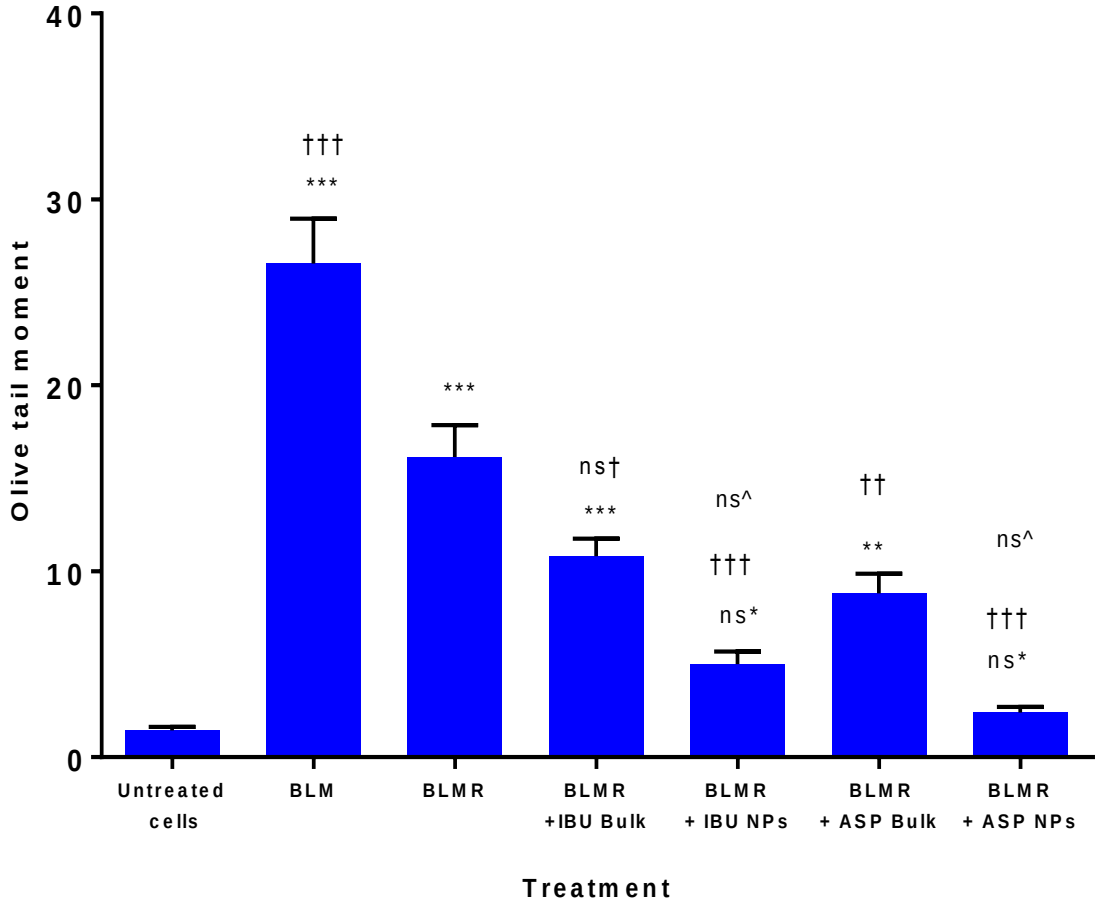

Figure 32 illustrates the comparison of the aspirin and ibuprofen (both forms) concentration-response, (±SE), on lymphocyte DNA from healthy female volunteers treated with bleomycin using Olive tail moment (n = 15).

BLM = without repair

BLMR = repair allowed

† = comparison with BLMR

^ = comparison between nano and bulk

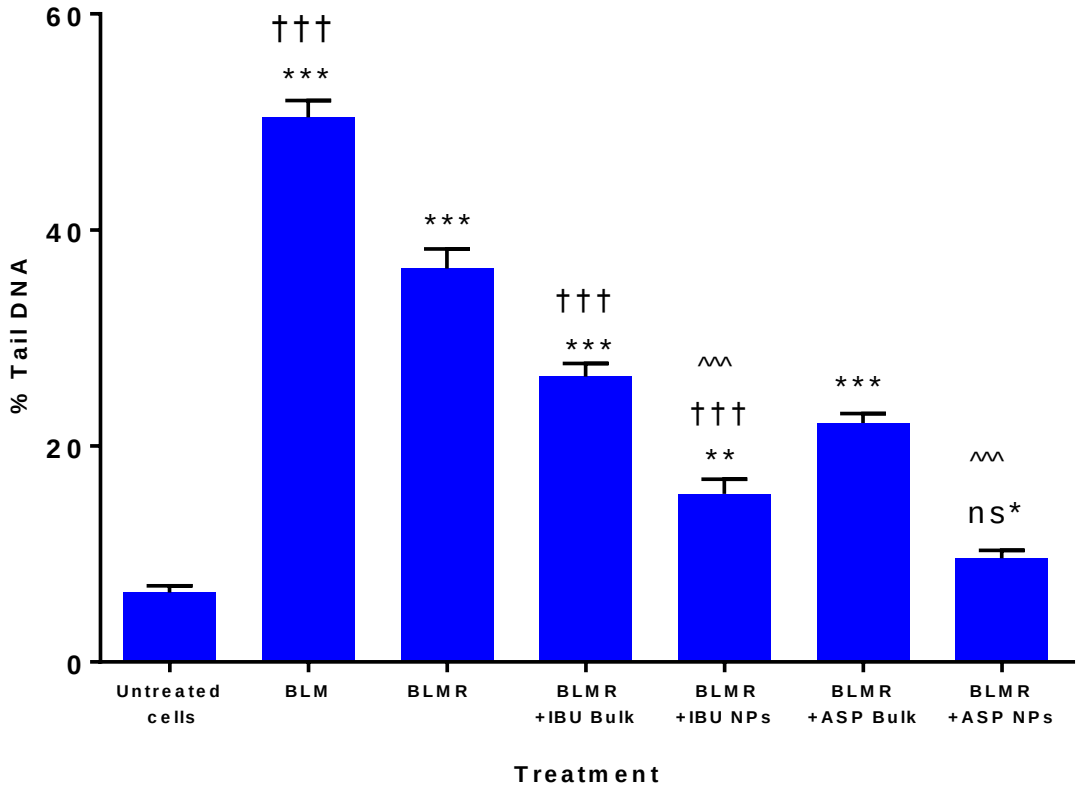

Figure 33 illustrates the comparison of the aspirin and ibuprofen (both forms) concentration-response, (±SE), on lymphocyte DNA from healthy female volunteers treated with bleomycin using % Tail DNA (n = 15).

BLM = without repair

BLMR = repair allowed

† = comparison with BLMR

^ = comparison between nano and bulk

5.3.2 Aspirin and ibuprofen concentration response on lymphocyte from BC patients treated with bleomycin

In figures 34 and 35 are displayed the concentration responses of aspirin and ibuprofen both forms on lymphocyte DNA from BC patient treated with BLM using Olive tail moment and % Tail DNA, standard errors SE and significance. Using the one-way ANOVA It was clear from the chart for Olive tail moment figure (34) that BLM, BLMR and BLMR + IBU bulk treatments showed a significant increase in DNA damage compared to untreated cells, BLM and BLMR ***$p \leq 0.001$ and BLMR + IBU bulk *$p \leq 0.05$. However, BLMR +ASP NPs, BLMR + IBU NPs and BLMR + ASP bulk were ns*. Furthermore, Comparing the treatments with BLMR BLM, BLMR + ASP bulk and BLMR + IBU bulk showed no-significance, whereas, BLMR + IBU NP $^{\dagger}p \leq 0.05$ and BLMR + ASP NP $^{\dagger\dagger}p \leq 0.01$. Another comparison was made between NPs and bulk forms of aspirin and ibuprofen, where there was no significance (ns^) between forms for both compounds. In % Tail DNA figure (35) BLMR + IBU NP, BLMR + ASP bulk and BLMR + ASP NP treatment showed (ns*) non-significance against untreated cells. By comparing treatments with BLMR, BLM and BLMR + IBU bulk were (ns$^{\dagger)}$ not-significant. However, there was significance with BLMR + ASP NP $^{\dagger\dagger}p \leq 0.01$ and both IBU NP and ASP bulk $^{\dagger}p \leq$ (0.05). Both ibuprofen and aspirin were not-significant when comparing the NPs with bulk forms (ns^).

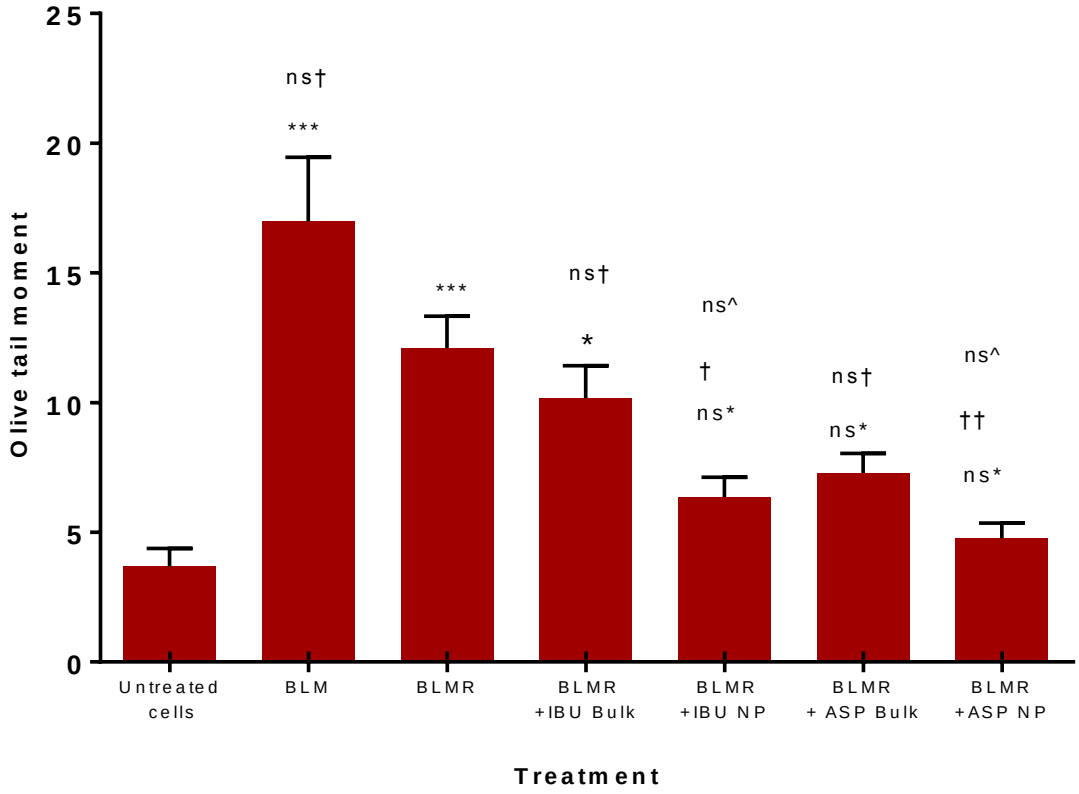

Figure 34 Comparison of the aspirin and ibuprofen (both forms) concentration-response, SE, and significance on lymphocyte DNA from BC patients treated with bleomycin using Olive tail moment (n=15)

BLM = without repair

BLMR = repair allowed

† = comparison with BLMR

^ = comparison between nano and bulk

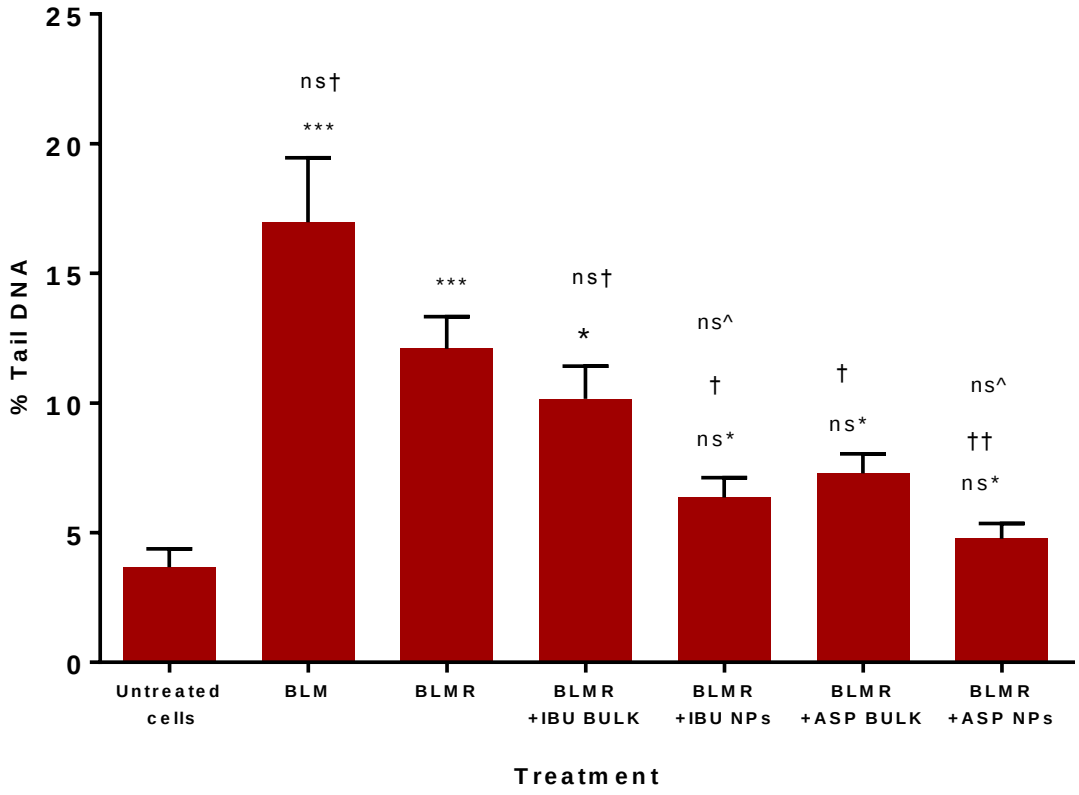

Figure 35 Comparison of the aspirin and ibuprofen (both forms) concentration-response, SE, and significance on lymphocyte DNA from BC patients treated with bleomycin using % Tail DNA (n=15)

BLM = without repair

BLMR = repair allowed

† = comparison with BLMR

^ = comparison between nano and bulk

5.4 Discussion

An interesting study was carried out by Kirkland and Speit (2008). They showed from studies, interalia Comet-assays, on 67 compounds that are classified carcinogens but showed negative or equivocal results in the micronucleus assay; 90 % were positive in the comet assay. Furthermore, 78 % of non-carcinogens were negative. The comet assay has been used since the very earliest days to monitor the repair of DNA damage. Thousands of DNA lesions per cell can be repaired in a matter of half an hour in conventional cultured mammalian cells (Azqueta and Collins, 2013). Milić and Kopjar (2004) confirmed the high sensitivity of the alkaline comet assay for evaluating bleomycin genotoxicity to human lymphocytes. It has also elucidated the advantages of the alkaline comet assay as one of the primary screening technique for *in vitro* investigations of drug-DNA interactions, particularly in assessing mechanisms of action of new drugs.

In this study, aspirin and ibuprofen have shown a chemo-protective influence against DNA damage induced by BLM in human lymphocytes obtained from BC patients and healthy female individuals using Comet assay. Aspirin and ibuprofen reduced the DNA damage in blood lymphocytes challenged with BLM. Both aspirin and ibuprofen exhibited a protective effect at concentrations of 500 µg/ml and 250 µg/ml respectively.

Data from all treatments illustrated that all samples challenged with BLM had high levels of DNA damage in both OTM and % tail DNA. This damage was, significant compared to the untreated cells $^{**}p \leq 0.01$ or $^{***}p \leq 0.001$. Aspirin and ibuprofen NPs showed ns compared to untreated cells in most samples especially in % tail

DNA after 30 minutes incubation with these two compounds. Non-significance implies that DNA was highly repaired and reduced to normal when compared to other treatments against untreated cell.

For healthy individuals, the decrease in DNA damage was obvious in the cells exposed to BLM and repaired in the absence of both NSAIDs (BLMR), compared to the cells treated with BLM and scored without repair incubation. Both aspirin and ibuprofen treatments showed higher reductions in DNA damage when compared to the BLM treatment with no repair. BC patients cells in general showed the same pattern as healthy individuals. Using OTM parameter in BC patients cells, the BLM treatment, BLMR + IBU bulk, and BLMR + ASP bulk were unable to show a significance reductions in DNA damage compared to the BLMR treatment, however, in % tail DNA parameter BLMR + ASP bulk treatment showed significance reduction in DNA damage $^{\dagger}p \leq 0.05$. When cells treated in the presence of NSAIDs, the ASP, and IBU NPs demonstrated greater reductions in DNA damage compared to the bulk forms. This result showing the importance of both compounds nano-forms in inducing DNA repair especially with the increased active surface area of nanoparticles, as a result of their large surface area to volume ratio.

It is interesting to combine NSAIDs with chemotherapy agent such as BLM; this combination suggested the possibility of enhancing the efficiency of the cancer chemotherapy and reducing toxicity on healthy cells. BLM is inducing the genotoxicity and pathogenesis by generating Oxidative stress and inflammation, which have a role in DNA damage, chromosome impairment, and mutagenesis (Arab et al., 2015; Zhou et al., 2014).

Chapter 6 - Investigation of XRCC3 and P53 proteins in Lymphocytes obtained from Healthy Female Individuals and BC Patients and Treated with Both forms of Aspirin and Ibuprofen Using Western Blot Technique

6. Western blot study

6.1 Introduction

The western blot technique is frequently used in research to isolate and identify proteins. In this method, a mixture of proteins is separated depending on molecular weight, during gel electrophoresis. The proteins are then transferred to a PVDF/nitrocellulose membrane producing a band for every protein. The membrane is then incubated with antibodies specific to the protein of interest (Liu et al., 2014; Mahmood and Yang, 2012). The unlinked antibody is rinsed off leaving only the linked antibody linked to the target protein. The bound antibodies are then identified by imaging the membrane. As the antibodies are only bound to the protein of interest, only specific bands should be visible. The thickness of the band correlates to the amount of protein present; thus a known standard can be used to indicate the quantity of protein present (Mahmood and Yang, 2012).

Use of non-steroidal anti-inflammatory drugs NSAIDs is correlated with a minor reduction in BC occurrence (de Pedro et al., 2015). However, it is not obvious whether NSAIDS can improve the outcome of patients already diagnosed with BC. Given that there are is an increase of 2.5 million BC survivors in the USA only, and that aspirin and other NSAIDs are frequently consumed medications, their influence on BC prognosis has significant clinical importance (Sutton et al., 2016). However, epidemiological studies on NSAIDs intake and BC prevalence have not yielded consistent results (Ou et al., 2013). Case–control studies globally show a small reduction in BC with NSAIDs use. Moreover, a protective effect of NSAIDs was verified in 13 studies (de Pedro et al., 2015), while only 7 papers demonstrated a higher risk of BC among NSAIDs medication users (de Pedro et

al., 2015; Vinogradova et al., 2011). A non-significant effect was determined in one case–control study (Kirsh et al., 2007). Furthermore, the vast majority of observational studies depended on the self-reported consumption of NSAIDs, generally obtained as over-the-counter medications; the few exemptions were studies based on prescriptions, which constitute a safer strategy to assess NSAIDs consumption. Finally, another prospective explanation for the contrast in results may lie on the theory that some NSAIDs inhibits COX-2 more intensely than others, which leads to different risk reductions (de Pedro et al., 2015).

XRCC3 is classified as one of the DNA repair genes that encodes for a protein that has an essential role in repairing the double-strand breaks, cross-link repair and the stability. XRCC3 has been associated with the emergence of Rad51 as a key component of the homologous DNA repair (HR) pathway (Bei et al., 2015; Han et al., 2006). Cells with XRCC3 disorders demonstrated defects in Rad51 genesis, which leads to genetic instability and increased sensitivity to DNA-damaging agents (Griffin, 2002; Zhao et al., 2013). People carrying the abnormal allele of XRCC3 Thr241Met had relatively high DNA adduct rate in lymphocyte DNA, demonstrating that this polymorphism was correlated with relatively low DNA repair capacity(Han et al., 2006; Matullo et al., 2001). Therefore, XRCC3 has been of substantial interest as a candidate susceptibility gene for cancer (Bei et al., 2015). A large number of molecular epidemiological studies have been conducted to assess the role of XRCC3 polymorphisms on several neoplasms, such as breast, lung, bladder, head and neck cancer (Figueiredo et al., 2004). Nevertheless, the outcomes remain apparently conflicting rather than decisive. As a result of this discrepancy among studies, it has been suggested that polymorphisms might play

a different role in different tumour sites. Also, even at the same cancer site, considering the prospective small effect size of these genetic polymorphisms to tumour and the relatively small specimen size in several studies. The contradiction will become evident since some single studies may have been underpowered to detect a small effect but real association (Bei et al., 2015; Peng et al., 2014).

The TP53 tumour suppressor gene encodes the p53 protein, which is an essential molecular decision maker of stress control in human cells (Horn and Vousden, 2007). Functioning within a complex signalling pathway, p53 senses excessive stress signals resulting from deregulated oncogenes expression, such as DNA damage, metabolic disorders or telomere erosion. The p53 signalling reaction is dependent on the cellular state and the type of oncogene. P53 triggers self-cell death (apoptosis), DNA repair, fugacious or permanent cell cycle arrest and metabolic homoeostasis maintenance (Li et al., 2012). P53 activation–inactivation depends on an accumulation of posttranslational modifications (PTMs) and interferes with proteins that induce p53 activation and subcellular relocation, allowing it to induce expression of appropriate sets of genes needed in the signalling pathway (Walerych et al., 2012). P53 alters cellular response to stress via either transcriptional or post-transcriptional pathway (Kelly et al., 2009).

A number of genomic studies in mouse models have shown that lack of p53 function leads to tumour formation and that restoration of p53 leads to a rapid and effective retraction of established, in situ tumours, providing vigorous evidence for designing chemotherapy that restores p53 function (Shangary and Wang, 2009).

In this study, we examined the expression of XRCC3 and P53 proteins in lymphocyte cells obtained from healthy females and BC patients, after treating clls using ibuprofen and aspirin, both forms individually. The Western blot technique was utilised in this study, to evaluate the impact of two NSAIDs on the expression of both proteins XRCC3 and P53.

6.2 Material and methods

Please refer to Chapter 2 Material and Methods section (**2.1**, **2.2** and **2.9**).

6.3 Results

6.3.1 XRCC3 expression in lymphocyte from healthy female individuals treated with both forms of aspirin and ibuprofen individually

XRCC3 protein expression in lymphocytes from healthy volunteers treated with both forms of aspirin (500µg) and ibuprofen (250 µg) was quantified using the Western blot assay. Two different incubation times were used 8 and 24 h individually. Untreated cells were used as a control to determine the value of XRCC3 expression for comparison. As depicted in Figure (36), the expression of XRCC3 was significantly increased after 8 h incubation for ASP bulk ***$p \leq 0.001$ and ASP nano ***$p \leq 0.01$, whereas the IBU nano was non-significant (ns). The IBU bulk showed a decrease in expression compared to the untreated cells. Following 24 h treatment, the XRCC3 expression decrease compared to 8h incubation in ASP nano and bulk treatment where it showed a non-significant increase compared to untreated cells. IBU bulk and nano showed no effect on XRCC3 expression after 24 h incubation.

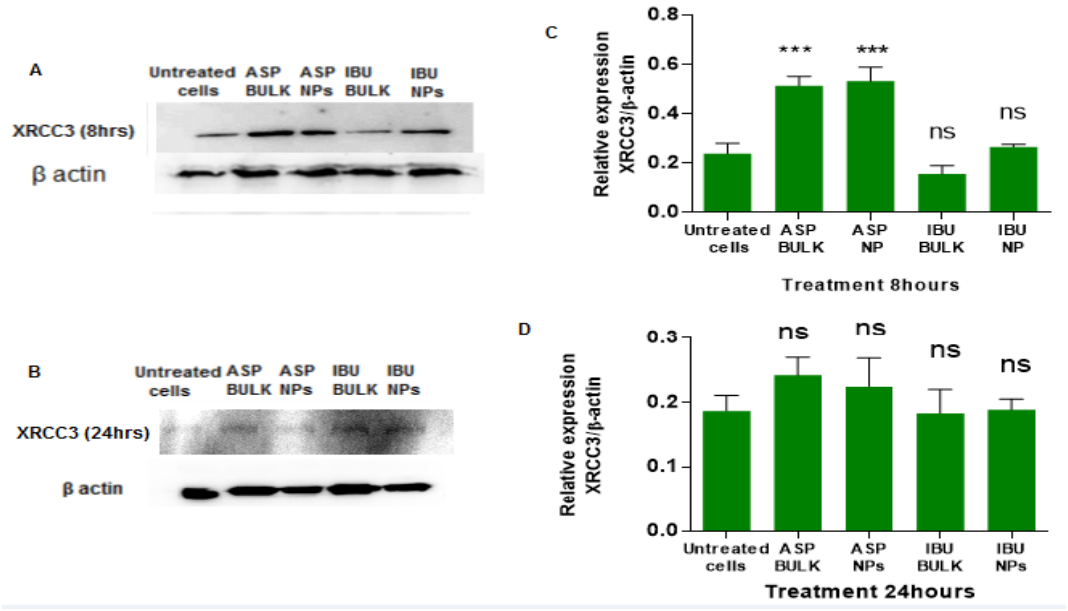

Figure 36 showing the effect of aspirin and ibuprofen both forms on XRCC3 protein expression in lymphocytes from healthy female individuals incubated for 8 and 24 h individually. (A, B) Developed bands of XRCC3, and β-actin by immunoblotting. β-actin was used as a control. (C, D) demonstrating relative expression of XRCC3 after treatment with aspirin and ibuprofen both forms individually, SE, and significance comparing to untreated cells. (N=3)

6.3.2 XRCC3 expression in lymphocyte from BC patients treated with aspirin and ibuprofen both forms

We were prompted to investigate the expression of XRCC3 in lymphocytes from breast cancer pateints treated with aspirin (500µg) and ibuprofen (250 µg) both forms (NPs, Bulk) for either 8 or 24 h. Untreated cells were used as a control to determine the basal level of XRCC3 expression for comparison. The results in Figure 37 showed that the expression of XRCC3 was decreased for all treatments ASP NPs *$p \leq 0.05$ ASP Bulk (ns), IBU NPs *$p \leq 0.05$ and IBU Bulk **$p \leq 0.01$. However, the expression remained unchanged (ns) for ASP NPs, ASP Bulk and IBU NPs following 24 h incubation, whereas, IBU Bulk showed a significant decrease compared to untreated cells.

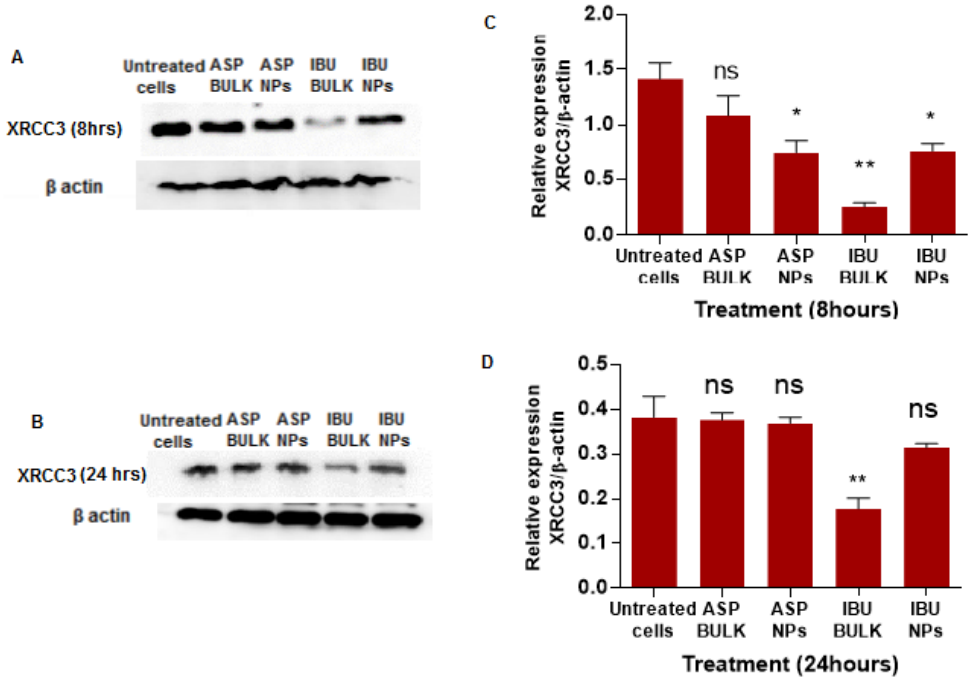

Figure 37 Western Blot validation results illustrating the effect of aspirin and ibuprofen both forms on XRCC3 protein expression in lymphocytes obtained from BC patients incubated for 8 and 24 h individually. (A, B) Images displaying Western blot results of XRCC3, β-actin was used as a control. (C, D) Graph displaying relative expression of XRCC3 after treatment with aspirin and ibuprofen both forms individually, SE, and significance comparing to untreated cells. (N=3)

6.3.3 P53 expression in lymphocyte from healthy female individuals treated with both forms of aspirin and ibuprofen individually

P53 protein expression in lymphocyte from healthy volunteers treated with both forms of aspirin (500µg) and ibuprofen (250 µg) were assessed using Western blot. As shown in Figure 38, the expression of p53 was not changed (ns) in either incubations 8 hrs or 24 h with ASP bulk, IBU nano and IBU bulk. Likewise, ASP NPs caused non significance change in p53 protein expression compared to the untreated cells.

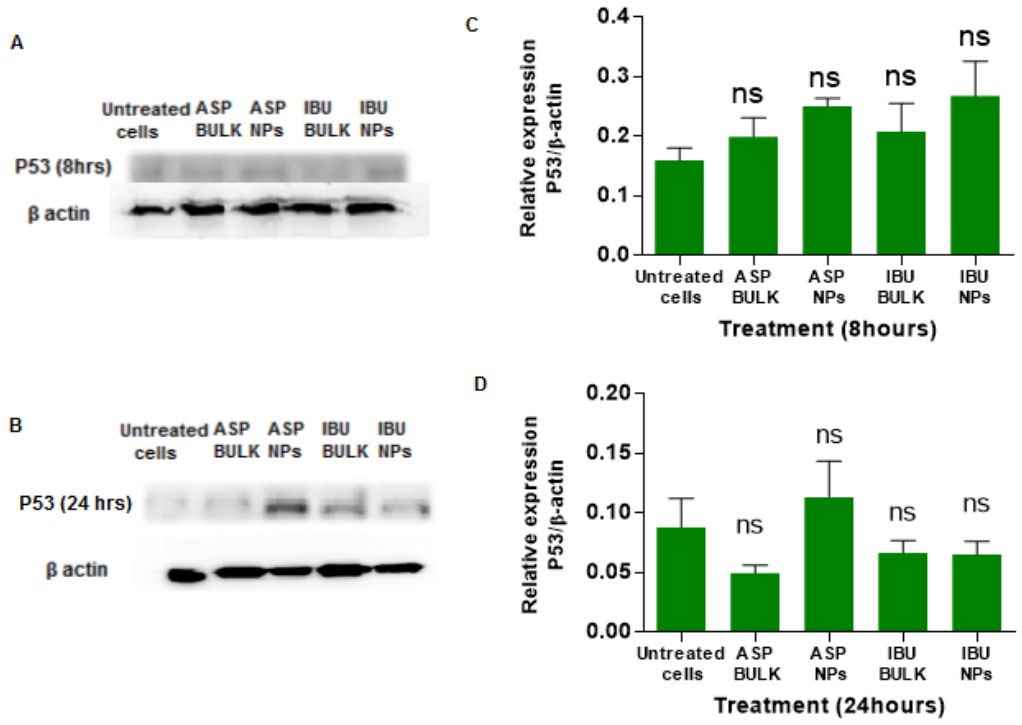

Figure 38 Western Blot verification results demonstrating the effect of aspirin and ibuprofen both forms on P53 protein expression in lymphocytes obtained from female healthy control incubated for 8 and 24 h individually. (A, B) Images displaying Western blot results of p53 and β-actin which was used as a control. (C, D) Graph displaying quantitative analysis of P53 relative expression after treatment with aspirin and ibuprofen both forms individually, SE, and significance comparing to untreated cells. (N=3)

6.3.4 P53 expression in lymphocyte from BC patients treated with aspirin and ibuprofen both forms

In studying the effect of aspirin and ibuprofen (bulk and NP) on p53 protein expression in lymphocyte from BC patients. The cells were treated individually with both forms of aspirin (500µg) and ibuprofen (250 µg) for 8 or 24 h, and p53 protein expression evaluated by western blot. Untreated cells were used as a control to quantify the value of the p53 expression for comparison. As depicted in Figure 39, the expression of p53 protein showed non-significant (ns) change when treated for 8 h with ASP NPs, ASP bulk and IBU NPs. By contrast, the IBU bulk showed significant downregulation $*p \leq 0.05$ when compared with untreated cells in 8 h incubation. In 24 h incubation both forms of aspirin (NPs and bulk) upregulated the expression of P53 significantly $**p \leq 0.01$ and $*p \leq 0.05$ respectivily. However, in ibuprofen nano and ibuprofen bulk treatment there was no difference in p53 expression compared to untreated cells.

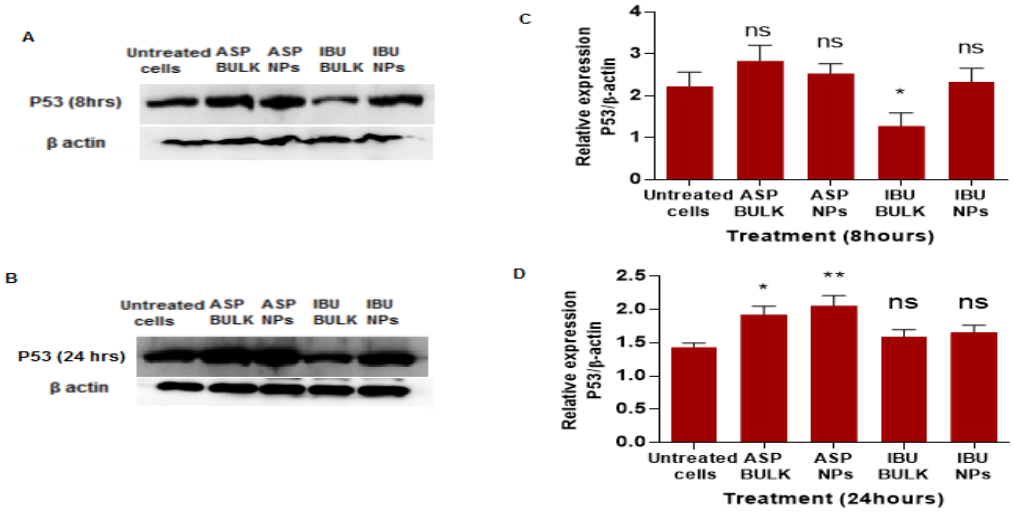

Figure 39 Western Blot validation results demonstrating the effect of aspirin and ibuprofen both forms on p53 protein expression in lymphocytes obtained from BC patients incubated for 8 and 24 h individually. (A, B) Western blot results show representative examples for expression of p53, as well as β-actin which served as an internal control. (C, D) Eexhibiting relative expression of p53 after treatment with aspirin and ibuprofen both forms individually, protein levels were expressed as means in bar graphs including SE and significance comparing to untreated cells. (N=3)

6.4 Discussion

Genomic DNA is exposed daily to a considerable number of damaging agents. In healthy cells, the cellular repair mechanisms are generally controlling the damage very efficiently, with only sporadic breaks or errors. Dysfunctional repair mechanisms may lead to genetic and/or multifactorial disorders. This effect depends on whether it occurs on germline or somatic cells, which allows for making an alteration in the genome that comprises one of the major motivations in evolution. Even amongst healthy individuals of similar species, however, the lack of protection against DNA damage including removal of lesions in DNA or the rate of unrepaired damage may vary (Chakarov et al., 2013). It is well known that genes and environmental elements are exceedingly engaged in the stages of the majority, common non-hereditary cancers. Regardless, the age at the first child's birth, nullparity and a family history are classified as most common risk factors but in the majority of BC cases, the cause of the disease remains unclear. The potential role for DNA erroneous repair in cancer development, namely in BC has been the subject of supplemental studies since it has been declared that BC patients might be deficient in one of the key DNA repair pathways (Ralhan et al., 2007).

The XRCC3 protein participates in homologous recombinational repair pathway (HRR) and is considered a member of a family of Rad-51-related proteins that probably participate in HRR to maintain chromosome stability. The XRCC3 (Ex8-5C > T, T241M, rs861539) polymorphism was studied in various case-control studies to clarify its role in susceptibility to breast cancer (Silva et al., 2010). And the results showed that there was an association between XRCC3 and BC risk (Fan Chai et al., 2015; Xu et al., 2016).

Responses to DNA damage are mediated through highly protected DNA damage checkpoint pathways that are essential for tumour suppression by arresting cell cycle development or triggering cellular senescence and programmed cell death. P53 is a vital participant in the tumour-suppressive pathway. p53 is mutated and inactivate in almost 50% of human cancers (Reinhardt and Schumacher, 2012). Recent studies propose that p53 not only antagonises oncogenic transformation but moreover regulates non-cell-independent responses to DNA damage by mediating liquidation of cells with damaged DNA through the native immune system. Additionally, p53 also dominates genes that are correlated to lifespan extension (Feng and Levine, 2010).

The present work studied the expression of XRCC3 and p53 protein in lymphocyte cells obtained from BC patients and healthy female individuals. Cells were treated with 250 ng/ml of ibuprofen (NPs and bulk) or 500 ng/ml of aspirin both forms for either 8 or 24 h. The results showed that, both aspirin and ibuprofen have effect on XRCC3 and p53 expression.

For XRCC3 protein expression, healthy cells treated for 8h had significant increase in its expression for both ASP bulk and ASP nano ***$p \leq 0.001$. However in both IBU treatments (NPs and Bulk) there was a non-significant change in XRCC3 protein expression compared to the untreated cells. Following 24 h treatment, all treatments displayed non-significant change compared to the untreated cells. In cells obtained from the BC patients, the expression exhibited a different trend, where ASP bulk increased the expression non-significantly in 8 h treatment. Both ASP nano and IBU nano showed significant decrease *$p \leq 0.05$ compared to untreated cells in 8 h treatment, however, ASP bulk remained unchanged in either

8 or 24 h incubation. IBU bulk followed the similar trend in both incubations, where it reduced the expression of XRCC3 significantly **p ≤ 0.01 compared to untreated cells. It is clear from the above that there is a difference in XRCC3 protein expression between the cells from healthy and BC patients.

In this study, it is clear that the treatment with NSAIDs produced a significant increase in XRCC3 protein expression in healthy lymphocytes. However, in BC patient's lymphocytes both ibuprofen and aspirin seemed to decrease the expression. Limited studies have been conducted to determine the relationship between NSADs intake and XRCC3 expression in different types of cells including cell lines from different tumours and healthy cells. Moreover, the results of two different studies in this area have been inconsistent or controversial (Dibra et al., 2010; Dibra et al., 2011).

The expression of p53 protein in healthy lymphocytes in either 8hrs or 24 h incubation was non-significantly (ns) changed for ASP bulk, ASP nano, IBU nano and IBU bulk compared to the untreated healthy lymphocytes.

Treatment with both forms of aspirin bulk and nano caused a significant increase in the expression of p53 protein *$p \leq 0.05$ and *$p \leq 0.01$ respectively in lymphocytes from BC patients incubated for 24 h. However, this increase was non-significant in 8 h treatment. for ibuprofen nano treatment the increase in protein expression was not significant at either incubation times. The ibuprofen bulk caused a significant decrease in protein expression *$p \leq 0.05$ after 8 h treatment, whereas, in 24 h incubation showed no change compared to untreated lymphocytes from BC

patients. The p53 protein expression was different between the two NSAIDs used in this study.

Our results are consistent with previous outcomes of studies displaying a protective effect on the part of NSAIDs in patients with High-grade dysplasia (HGD). However, their effect on the development of cancer cells is less clear (Vaughan et al., 2005). A number of studies suggested that the protective effect of NSAIDs initiates from the activating gene-1 (NAG-1), which acts as cox inhibitor. NAG-1 is a macrophage inhibitory cytokine-1 and a member of the transforming growth factor β family. Furthermore, increase in the expression of tumour suppressor protein p53 mediates the activation of NAG-1 (Baek et al., 2001; Kang et al., 2013).

Figueroa et al. (2009), studied the relation between several NSAIDs and p53 protein overexpression by examining 170 cases of esophageal adenocarcinomas, 147 gastric cardia adenocarcinomas, 220 non-cardia gastric adenocarcinomas, and 112 esophageal squamous cell carcinomas, using immunohistochemistry. They recorded the overexpression of p53 in 72% of esophageal adenocarcinoma, 69% of gastric cardia adenocarcinoma, 52% of non-cardia gastric adenocarcinoma, and 67% of esophageal squamous cell carcinoma. Bardia et al. (2016), suggested that only aspirin has an inverse correlation with breast cancer risk after multivariate analysis. However, there was no association for other (non-aspirin) NSAIDs with BC.

Further research is needed to identify the NSAIDs protective mechanisms of NSAIDs on BC.

Chapter 7 - Investigation of P53, COX1 and COX2 genes expression in lymphocytes from healthy females and BC patients treated with both forms of aspirin and ibuprofen using Real-Time PCR

7. QPCR study

7.1 Introduction

Real-Time PCR is a technique that allows for the logarithmic amplification and detection of a short sequence of specific nucleic acids in “real-time” by the measuring of fluorescent probes. In theory, a single copy of a particular DNA sequence can be amplified and identified by the use of proper primers and probes, with a direct relationship linking the starting target and the quantity of product at a given cycle (Zuo et al., 2010). The probe attaches to the target DNA sequence and cleaves, performing a fluorescence that is recognised by a qPCR machine after each thermal cycle is finished. This procedure is utilised for the detection and quantification of different viruses and parasites in various specimen mediums (Valasek and Repa, 2005).

Prostaglandin (PG) endoperoxide synthetase is also known as cyclooxygenase (COX) which plays an essential role in the inflammatory progression through altering the synthesis of prostaglandins from arachidonic acid (Gao et al., 2014). Higher COX expression in BC was first proposed on account of elevated PG production in BC cell lines (Brandão et al., 2013). To date there are two isoforms of cyclooxygenase: COX-1 and COX-2 (Gao et al., 2014). COX1 and COX2 are catalyse prostanoid biosynthesis in the same committed step with similar capabilities. However, the two COXs are encoded by different genes located on distinct chromosomes, and they differ considerably in their expression pattern (Nagao et al., 2013). COX1 is strongly expressed in most tissues and is accountable for the biosynthesis of PGs involved in different housekeeping functions, including the platelet function, gastrointestinal and regulation of renal

functions. However, COX-2 can be promptly inducible by inflammatory stimuli and a significant number of mitogenic and pro-inflammatory cytokines which lead to increased levels of prostaglandins which can impact on cell proliferation, apoptosis and angiogenesis, and finally contributing to neoplastic genesis and development (Kuroda and Yamashita, 2002).

Moreover, many studies have suggested that genetic polymorphisms in COX1 and COX2 might be associated with the liability to develop cancer or effect drug efficacy in humans. To date, various studies have investigated associations of the changes in expression of the COX1 and COX2 genes and NSAID consumption on cancer risk; Nevertheless, these studies have produced contradictory outcomes (Nagao et al., 2013).

In BC, different studies have indicated that moderate to high COX-2 expression is correlated with the formation of mammary tumour and its expression is related to parameters of aggressive BC with large tumour mass, positive axillary lymph node metastases, and positive human epidermal growth factor (HER2) (Gao et al., 2014). Inhibition of COX-2 expression could prevent the proliferation of BC cell lines in vitro (Dai et al., 2012). Genetic polymorphisms in COX-2 have shown alteration in its expression and influence the susceptibility to various carcinomas, including breast cancer (Yu et al., 2010).

The p53 tumour suppressor gene encodes the p53 protein, which is a fundamental molecule that is considered as the “decision maker of stress control” in human cells (Horn and Vousden, 2007). Functioning through a complicated signalling mechanism, p53, discriminates excessive stress signals emerging from regulating

oncogene expression, for instance, DNA damage, telomere erosion or metabolic disorders. The p53 signalling response relies on the variety of oncogenes and the cellular state. P53 induces apoptosis (self-cell death), DNA repair processes, perpetual or fugacious cell cycle arrest and metabolic equilibrium maintenance (Li et al., 2012). P53 activation–inactivation depends on an accumulation of posttranslational modifications (PTMs) and interacts with proteins that trigger p53 activation and relocates the subcellular, which in turn induces the expression of relevant sets of genes required in the signalling pathway (Walerych et al., 2012).

The aim of this study is to investigate the impact of aspirin and ibuprofen both forms on the expression of p53, COX1 and COX2 genes in lymphocytes from BC patients and compare them with those from healthy female individuals using the quantitative PCR technique.

7.2 Material and Methods

Please refer to Chapter 2 Material and Methods section (**2.1**, **2.2** and **2.8**).

7.3 Results

7.3.1 P53 mRNA abundance in lymphocytes from healthy female individuals treated with both forms of aspirin and ibuprofen individually.

P53 gene expression changes in lymphocyte from healthy volunteers treated with both forms of aspirin (500µg) and ibuprofen (250 µg) was quantified using quantitative PCR. Five different incubation times were used 0, 2, 4, 6 and 8 h individually. 0 h incubation was used as a control to determine the value of the p53 expression for comparison. All expressions were normalised against β actin using the ΔΔCT method. Preview results Figure 40 showed that there was no significant (ns) change in p53 expression in all four treatments aspirin bulk, aspirin nano, ibuprofen bulk and ibuprofen nano. This non-significant alteration was seen in all four incubation times 2, 4, 6 and 8 h compared to 0 hr incubation.

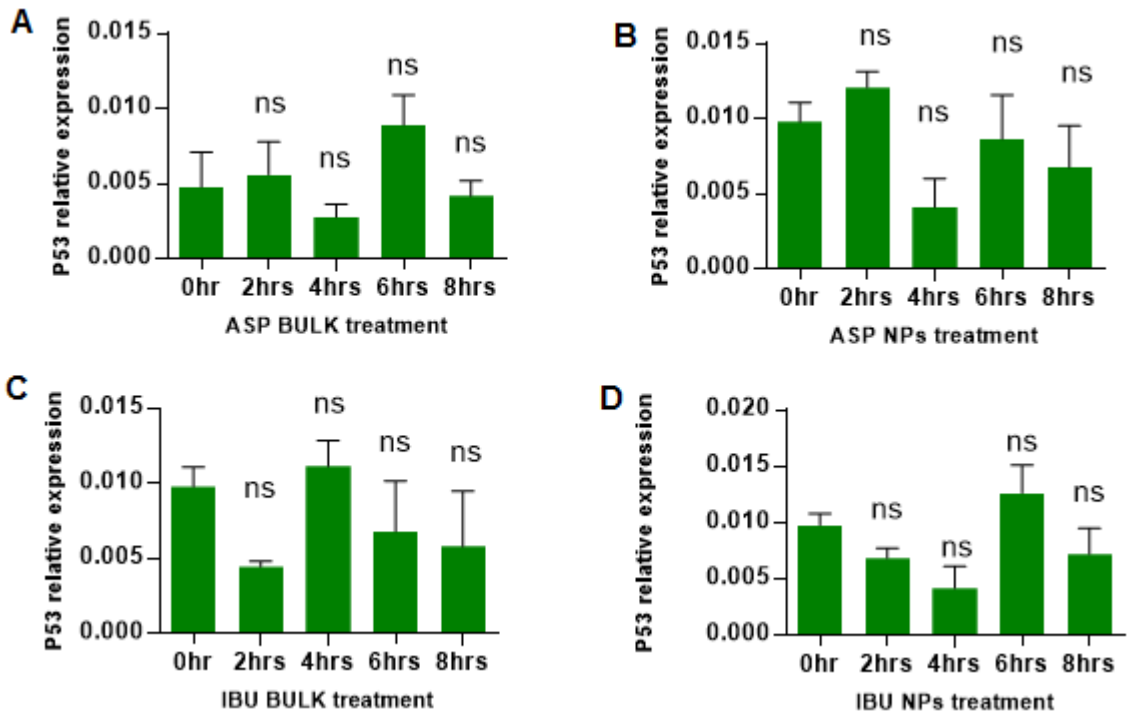

Figure 40 Real-time PCR (qPCR) results expressed in relative changes of p53 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes from healthy female volunteers. (A) Relative expression level of P53 mRNA in ASP BULK treatment. (B) Relative expression level of P53 mRNA in ASP NPs treatment. (C) Relative expression level of P53 mRNA in IBU BULK treatment. (D) Relative expression level of P53 mRNA in IBU NPs. Results are expressed as mean ± SEM and significance compared with the 0 h treatment. (N=3)

7.3.2 P53 mRNA abundance in lymphocytes from BC patients treated with both forms of aspirin and ibuprofen individually.

To assess whether both forms of aspirin (500µg) and ibuprofen (250 µg) have an influence on the expression of p53 gene, qPCR analysis on cDNA from RNA extracted from lymphocytes from BC patients was performed. Five different incubation times 0, 2, 4, 6 and 8 h were chosen for this experiment. 0 h incubation was used as a control to determine the value of the P53 expression for comparison. All expressions were normalised against β actin using the ΔΔCT method. Real-time PCR results Figure 41 showed that the increase of p53 expression in aspirin bulk was not- significant (ns) at 2, 4 and 6 h, however, the increase was significant **$p \leq 0.01$ at 8 h compared to the 0 h incubation. In cells treated with aspirin nano the p53 expression was non-significant (ns) at 2 and 4 h incubation, whereas, this expression was significant in 6 and 8 h *$p \leq 0.05$ and ***$p \leq 0.001$ respectively. In contrast, the ibuprofen bulk had no significant (ns) influence on the expression of p53 in all four treatments against 0 hr treatment, although, increase of expression can be seen in the graph 41C at 2 and 4 h. Ibuprofen nano showed significant increase *$p \leq 0.05$ at 6 and 8 h treatment compared to the 0 h treatment.

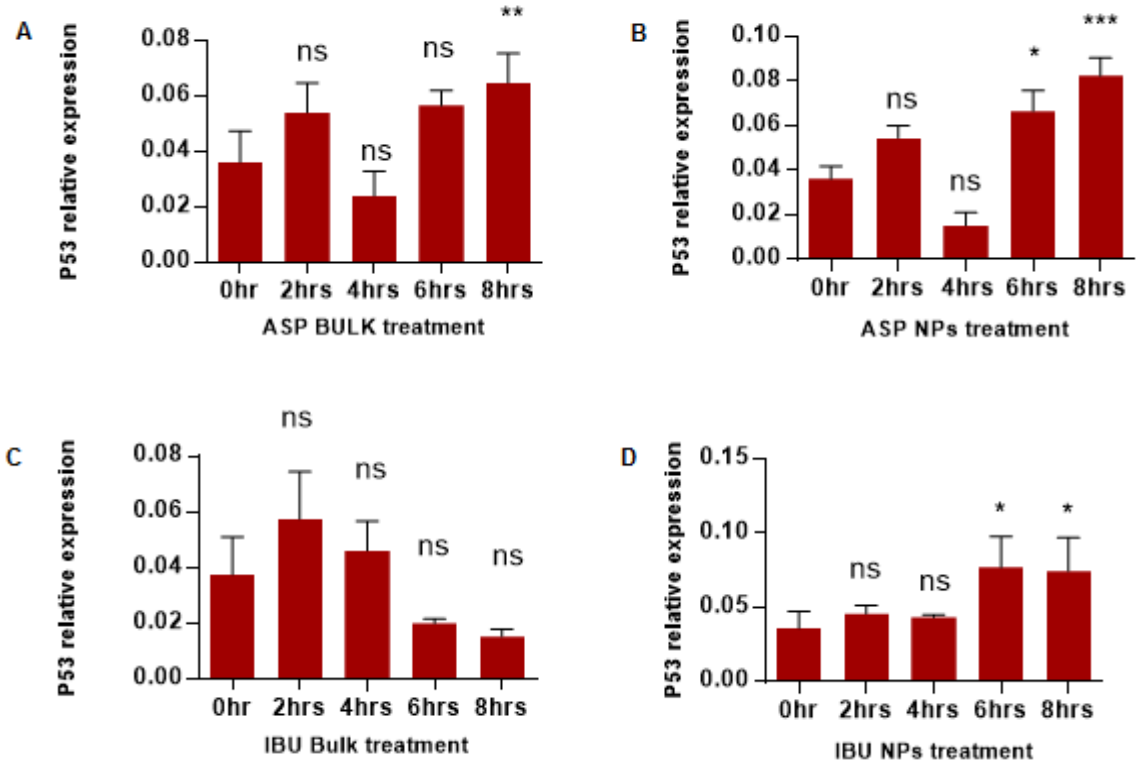

Figure 41 Real-time PCR (qPCR) results expressed in relative changes of P53 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes from BC patients. (A) Relative expression level of P53 mRNA in ASP BULK treatment. (B) Relative expression level of P53 mRNA in ASP NPs treatment. (C) Relative expression level of P53 mRNA in IBU BULK treatment. (D) Relative expression level of P53 mRNA in IBU NPs. Results are expressed as mean ± SEM and significance compared with the 0hr treatment. (N=3)

7.3.3 COX1 mRNA abundance in lymphocytes from healthy female volunteers treated with both forms of aspirin and ibuprofen individually

COX1 gene expression in lymphocytes from healthy female volunteers treated with both forms of aspirin (500µg), and ibuprofen (250 µg) was identified by quantitative PCR. Five different incubation times were namely 0, 2, 4, 6 and 8 h. Leave out individually. 0 h incubation was utilised as a control to determine the value of the COX1 expression for comparison. All expressions were normalised against β actin using the ΔΔCT method. It is evident from the Figure 42 that both forms of aspirin and both forms of ibuprofen down-regulated the expression of the COX1 gene. In aspirin bulk the decrease was significant *$p \leq 0.05$ at 2 h treatment and was ***$p \leq 0.001$ at 4, 6 and 8 h. for aspirin nano, ibuprofen bulk and ibuprofen nano the expression was decreased significantly ***$p \leq 0.001$ at all four incubations compared to the zero hour treatment.

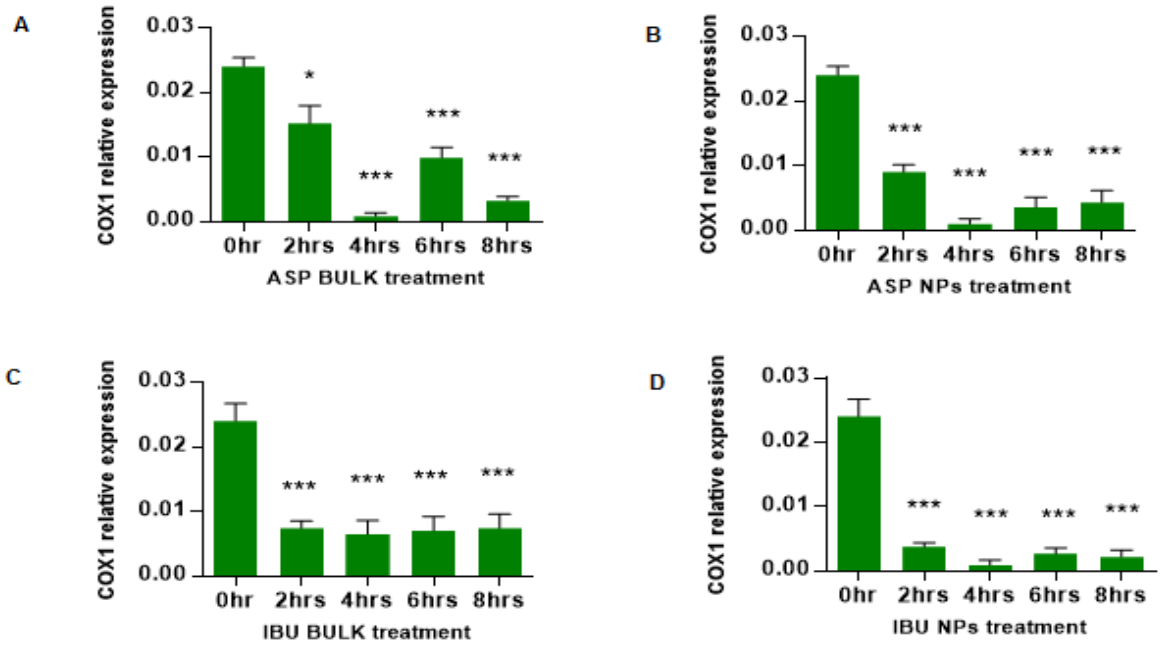

Figure 42 Real-time PCR (qPCR) results expressed in relative changes of COX1 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes from female healthy volunteers. (A) Relative expression level of COX1 mRNA in ASP BULK treatment. (B) Relative expression level of COX1 mRNA in ASP NPs treatment. (C) Relative expression level of COX1 mRNA in IBU BULK treatment. (D) Relative expression level of COX1 mRNA in IBU NPs. Results are expressed as mean ± SEM and significance compared with the 0hr treatment. (N=3)

7.3.4 COX1 mRNA abundance in lymphocytes from BC patient treated with both forms of aspirin and ibuprofen individually

The quantitative PCR technique determined expression patterns of COX1 in lymphocyte from BC patients treated with both forms of aspirin (500µg), and ibuprofen (250 µg). Five different incubation times were namely 0, 2, 4, 6 and 8 h. Leave out individually. 0 h incubation was used as a control to evaluate the value of the COX1 expression for comparison. All expressions were normalised against β actin with the ΔΔCT method. QPCR results showed Figure 43 the expression of COX1 was downregulated in the presence of aspirin or ibuprofen. In aspirin bulk treatment the expression was significantly decreased at 2 and 4 h incubation $*p \leq 0.05$ and $***p \leq 0.001$ respectively. However, there was no statistical difference at 6 h incubation and the expression increased in 8 h $**p \leq 0.01$. Aspirin nano downregulated the COX1 expression in all incubations where the significance was $*p \leq 0.05$ at 2 h incubation and $**p \leq 0.01$ at 4, 6, and 8 h. In ibuprofen bulk and nano the decrease was $*p \leq 0.05$ at 2 h and $***p \leq 0.001$ at 4, 6, 8 h.

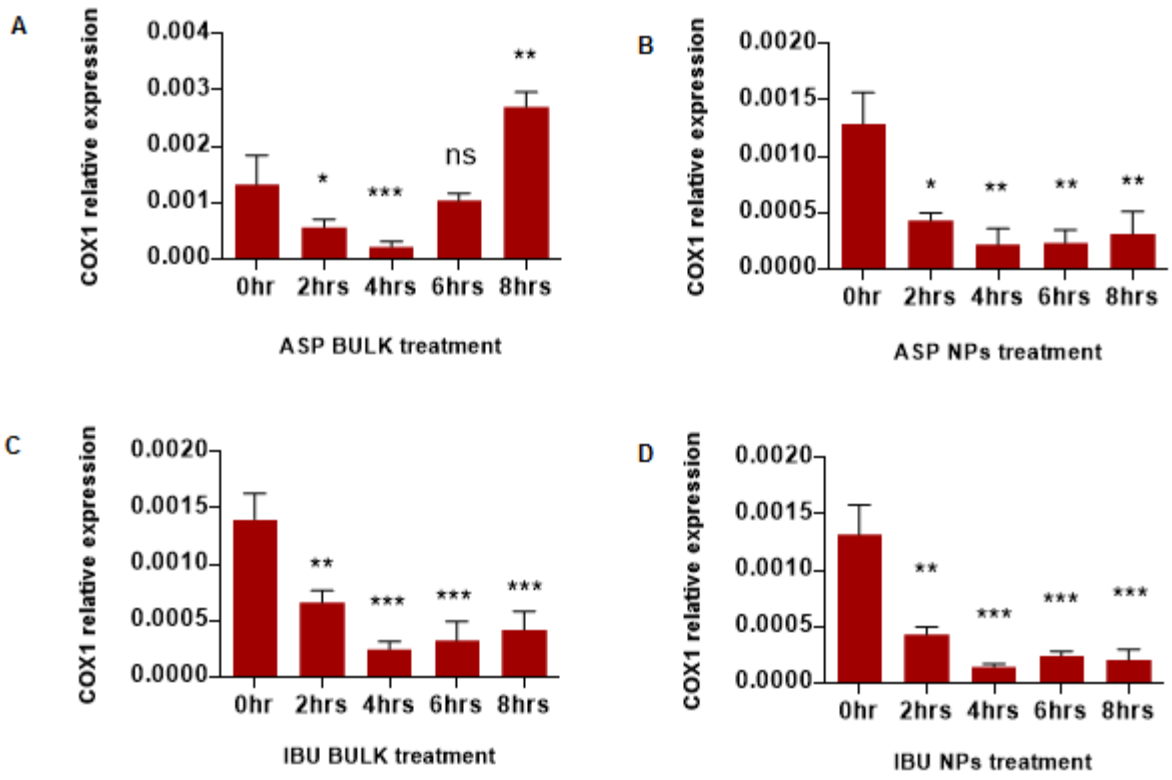

Figure 43 Real-time PCR (qPCR) results expressed in relative changes of COX1 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes from BC patients. (A) Relative expression level of COX1 mRNA in ASP BULK treatment. (B) Relative expression level of COX1 mRNA in ASP NPs treatment. (C) Relative expression level of COX1 mRNA in IBU BULK treatment. (D) Relative expression level of COX1 mRNA in IBU NPs. Results are expressed as mean ± SEM and significance compared with the 0hr treatment. (N=3)

7.3.5 COX2 mRNA abundance in lymphocytes from healthy female individuals treated with both forms of aspirin and ibuprofen individually.

COX2 gene expression changes in lymphocyte from healthy female volunteers treated with both forms of aspirin (500µg), and ibuprofen (250 µg) was specified using quantitative PCR. Five different incubation times were used 0, 2, 4, 6 and 8 h individually. 0 h incubation was used as a control to evaluate the value of the COX2 expression for comparison. All expressions were normalised against β actin using the ΔΔCT method. Preview results Figure 44 showed that there was no significant (ns) change in COX2 expression in all four treatments aspirin bulk, aspirin nano, ibuprofen bulk and ibuprofen nano. The non-significant (ns) changes were seen at all four incubation times 2, 4, 6 and 8 h compared to 0 h incubation.

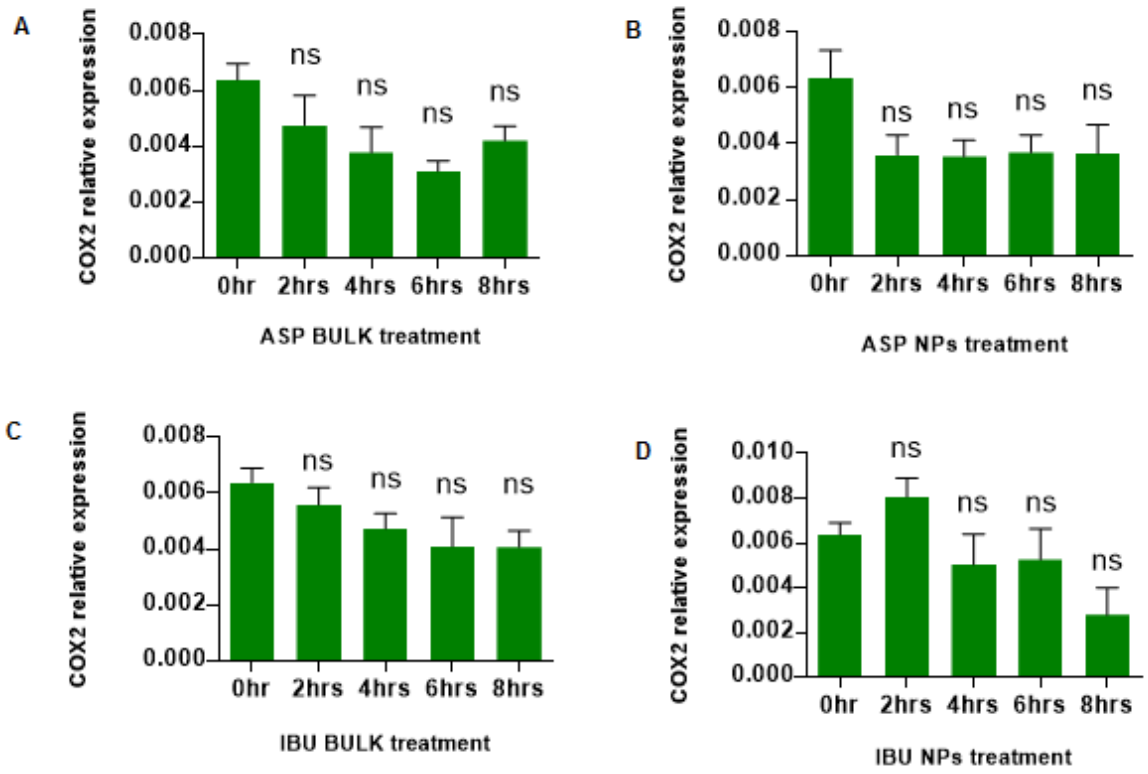

Figure 44 Real-time PCR (qPCR) results expressed in relative changes of COX2 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes from female healthy volunteers. (A) Relative expression level of COX2 mRNA in ASP BULK treatment. (B) Relative expression level of COX2 mRNA in ASP NPs treatment. (C) Relative expression level of COX2 mRNA in IBU BULK treatment. (D) Relative expression level of COX2 mRNA in IBU NPs. Results are expressed as mean ± SEM and significance compared with the 0hr treatment. (N=3)

7.3.6 The effects of aspirin and ibuprofen both forms, on the expression of COX2 in lymphocytes from BC patients.

The qPCR technique was used to assess whether the both forms of aspirin (500µg) and ibuprofen (250 µg) had an influence on the expression of the COX2 gene, by evaluating the cDNA from RNA extracted from lymphocytes obtained from BC patients. Five different treatment incubations were used 0, 2, 4, 6 and 8 h individually. The expression of COX2 was influenced statistically by the presence of the aspirin or ibuprofen whether bulk or nanoparticle. Figure 45 the aspirin nanoparticle showed a steady, significant decrease ***$p \leq 0.001$ at 2, 4, 6 and 8 h incubations compared with 0 h. Aspirin bulk illustrated significant change where it was **$p \leq 0.01$ at the first two hours and ***$p \leq 0.001$ at 4, 6, 8 h. Furthermore, ibuprofen bulk showed **$p \leq 0.01$ at 2 and 4 h then ***$p \leq 0.001$ for 6 and 8 h, however, ibuprofen nano demonstrated *$p \leq 0.05$ at 2 and 8 h then ***$p \leq 0.001$ at 4 and 6 h.

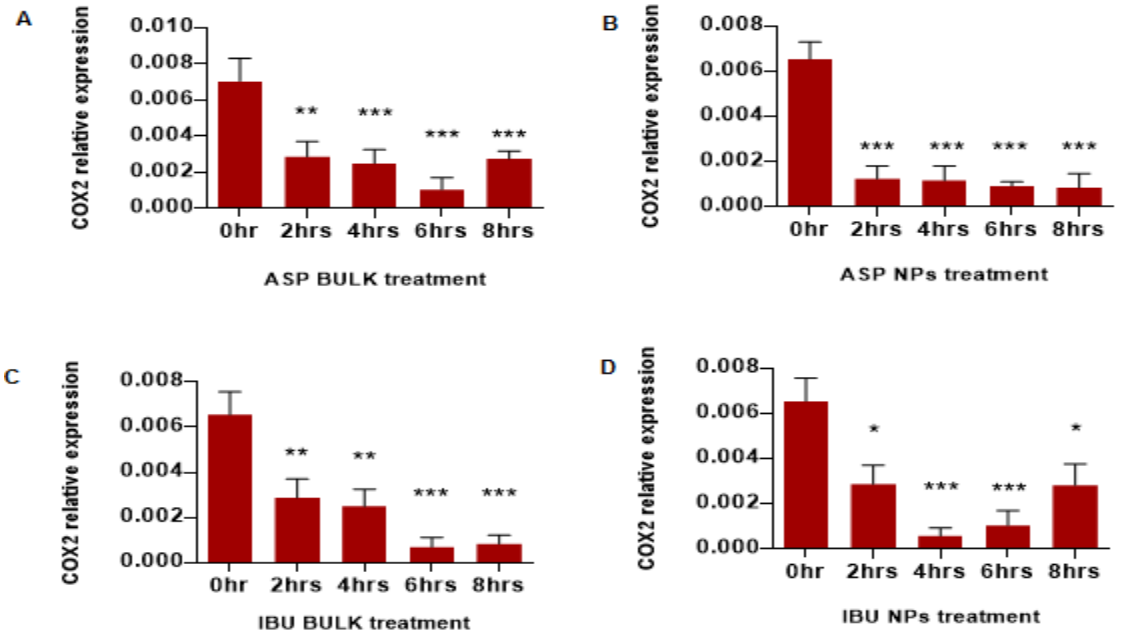

Figure 45 Real-time PCR (qPCR) results expressed in relative changes of COX2 mRNA over the control, using β actin as an endogenous housekeeping gene in lymphocytes from BC patients. (A) Relative expression level of COX2 mRNA in ASP BULK treatment. (B) Relative expression level of COX2 mRNA in ASP NPs treatment. (C) Relative expression level of COX2 mRNA in IBU BULK treatment. (D) Relative expression level of COX2 mRNA in IBU NPs. Results are expressed as mean ± SEM and significance compared with the 0hr treatment. (N=3)

7.4 Discussion

Various epidemiologic and observational studies have investigated the correlation between NSAIDs and BC, although the results remain controversial. A high proportion of case-control studies suggested that regular intake of NSAIDs is protective against BC. However, the majority of cohort studies did not detect this correlation. Several studies have dealt with NSAIDs as an entire group or with particular drugs, such as aspirin and ibuprofen, but not with NSAIDs subgroups. Moreover, limited attention has been given to the effect of NSAIDs on different tumour categories, for instance, ductal/non-ductal, stage at diagnosis or presence of hormonal receptors (Dierssen-Sotos et al., 2016; Pocock et al., 2004). In the predictable Women's Health Initiative Observational Study, regular use of NSAIDs was apparently associated with a reduction in the proportion of BC (van Nes et al., 2011).

In our study, we examined the prospective expression of the P53, COX1 and COX2 in lymphocytes from BC patients and healthy female volunteers, treated with aspirin and ibuprofen individually.

Our data demonstrated that aspirin and ibuprofen had no significant effect on p53 expression in lymphocytes from healthy female individuals either in the nano or bulk forms treatments including all incubations compared to 0 h incubation. In lymphocytes from BC cancer patients, both drugs impact was uneven depending on the compound and whether it was in the bulk or nano form. Aspirin bulk increased the expression of p53 significantly only after 8 h of incubation, whereas, in aspirin nano treatment, the statistical change was from 6 to 8 h. Interestingly, the ibuprofen bulk showed a non-significant change in gene expression. However, this

change was significant at 6 and 8 h for ibuprofen nano. Similar results were proposed by Tofiq (2015), that NSAID (Sulindac sulfone) increased the p53 activity in Hela cells.

The expression of the COX1 gene was significantly downregulated in lymphocyte from both healthy female and BC cancer patients when treated with either aspirin or ibuprofen. Furthermore, both forms were similar in decreasing the expression of COX1 gene. Aspirin and ibuprofen are nonselective COX inhibitors, which have illustrate a similar impact for both COX-1 and COX-2. Their inhibition of COX-1 leads to the decreased biosynthesis of homeostatic, cytoprotective, and hemostatic PGs, which further resulted in gastric ulcers and bleeding. Moreover, the inhibition of COX-1 in kidneys induced excessive water and sodium retention, which in turn reduced blood flow (Regulski et al., 2016).

In BC, the prognostic influence of COX2 expression varied widely among studies, and was emphasised by the fact that the majority of these studies did not stratify patients under study according to systemic therapy. Furthermore, in most of these studies, patients were given some forms of systemic therapy, so that the prognostic influence data of COX2 in the absence of systemic therapy was incomplete. This is especially the case for endocrine therapy, as COX2 stimulates the diversion of arachidonic acids into prostaglandins which catalyse aromatase and therefore the formation of oestrogens (van Nes et al., 2011).

In our study, we investigated and assessed the impact of both the NSAIDs aspirin and ibuprofen in nano and bulk forms on the expression of COX2 in lymphocytes from healthy females and BC patients. We found that both NSAIDs hade no

statistically significant effect on the change in COX2 expression in healthy female lymphocytes, and this applies to all treatments with aspirin nano, aspirin bulk, ibuprofen nano and bulk, regardless of the incubation times. However, this impact was completely different in the lymphocytes of BC patients , where both drugs downregulated the COX2 expression significantly. The expression decrease was comparatively small with aspirin bulk, ibuprofen bulk and nano, whereas, expression was sharply reduced with aspirin nano treatment.

Some studies suggested that there is an inverse correlation between p53 and COX2. Kim *et al*., (2010), found that inhibiting the expression of p53 by Pifithrin-α, led to upregulation of the COX-2 expression through a MAPK-Dependent Pathway in Breast and fibrosarcoma cells (Kim et al., 2010). By contrast, a previous study demonstrated that the anti-proliferative impact of NSAIDs was associated with an increased level of p53 acetylation and this effect appeared to be a COX-independent pathway (Tofiq, 2015).

Similar outcomes have been reported in some larger epidemiological studies examining NSAIDs, where it has a protective effect on BC. Furthermore, they have a role in inhibiting the expression of COX1 and COX2. The mechanisms behind these functions still need further investigation (Zhang et al., 2014).

Chapter 5 – General Discussion

8. General Discussion

Many studies recently suggested that regular intake of some NSAIDs have a protective effect against several types of tumours including BC. The protective effect of NSAIDs is thought to be mediated by the regulation of different pathways including inhibition of COX1, COX2 and expression of some tumour suppressor genes such as p53. Although, ibuprofen and aspirin are the most commonly used NSAIDs in these studies (Agrawal and Fentiman, 2008; Harris et al., 2005), none of the previous studies have investigated the effect of both drugs on DNA damage, specifically, damage to lymphocyte obtained from BC patients. Therefore, in this present study different sizes of ibuprofen and aspirin (NPs and bulk) were used to examine their protective effect on DNA damage of BC patients using lymphocytes as surrogate cells. Lymphocytes were selected for this study because they are exposed to different environments within the body by travelling in the bloodstream, and hence they can reflect DNA damage induced by endogenous and exogenous genotoxins from chemical and physical agents (Najafzadeh et al., 2012). NPs, on the other hand, have stable and known chemical and physical properties in their normal size. However, the smaller size of NPs allows them to penetrate the cell membrane by different mechanisms and finally they can diffuse through the nuclear membrane. In mitosis, NPs can become surrounded by the nucleus because the nuclear membrane disintegrates during mitosis. This can in turn lead to a direct interaction between the NPs and chromosomes that can subsequently promote contact with DNA (Singh et al., 2009). This fact was behind the selection of ibuprofen and aspirin NPs alongside the bulk size in this study.

Lymphocytes were treated with H_2O_2 and MMC which were used as a positive control to induce DNA damage in the Comet and micronucleus assays, respectively.

Suspending buffer was used as a negative control in both assays since it has no effect on DNA damage. BC patients lymphocytes and healthy volunteer lymphocytes were both treated with NPs and bulk forms of both drugs and the results were compared to the untreated controls.

8.1 Effect of aspirin and ibuprofen on DNA damage using the Comet assay

The Comet assay is a micro-electrophoretic method that facilitates the direct visualisation and measurement of DNA damage in several types of mammalian cells (Azqueta and Collins, 2013; Ostling and Johanson, 1984). The Comet assay has many advantages that make it the preferred technique for assessing DNA damage, because of, for example, simplicity, time efficiency, and low cost of the assay (Anderson et al., 1997; Anderson et al., 1998; Anderson et al., 1991). The Comet assay can recognise and follow the damage in single or double DNA strands and alkali labile sites. The principle of a Comet assay is based on investigation and measurment of the ratio between the head and tail of DNA where the head represents intact DNA while the tail corresponds to damaged DNA (Tice et al., 2000).

In this present study, lymphocytes were treated in vitro, with ibuprofen and aspirin (NP and bulk) individually for 30 min at 37°C followed by lymphocyte scoring. The lymphocyte nuclei displayed an increase in head DNA ratio after exposing the lymphocytes to the following doses: ibuprofen NP 250 ng/ml, ibuprofen bulk 250 ng/ml, aspirin NP 500 ng/ml, and aspirin bulk 500 ng/ml. In the case of lymphocytes from healthy volunteers, we observed minor and insignificant damage to DNA Figure 13, 14, 19 and 20. Lymphocytes obtained from BC patients, by

contrast, showed a decrease in DNA damage compared to the untreated cells. This decrease was significant for both aspirin forms (NP and bulk) ***p ≤ 0.001 and **p ≤ 0.01, respectively Figures 21 and 22. Despite the fact that there was a reduction in DNA damage observed with both forms of ibuprofen, this decrease was insignificant when data were analysed using ANOVA test Figures15 and 16. The insignificant effect of ibuprofen to protect against DNA damage perhaps may be attributed to the short incubation time (30 min) used with this assay. The short incubation might not be sufficient to cause significant DNA damage reduction. The result also showed that aspirin showed a greater reduction of the DNA damage compared to ibuprofen. Aspirin NP greater more significant DNA damage reduction compared to aspirin bulk form. This emphasises the theory that NPs have a better ability to penetrate into the nucleus compared to bulk size (Singh et al., 2009).

8.2 Ibuprofen and aspirin effect on MNi induction in the micronucleus assay

Detecting the effects of ibuprofen and aspirin on genomic DNA damage was established in lymphocyte cells using the Comet assay. Thereafter, micronucleus technique was used to study the influence of these drugs on lymphocytes at the cytogenetic level. During the cell cycle and growth many disorders at the stage of mitosis can lead to the appearance of MNi, which are smaller and separate extra nuclear fragment (Fenech, 2006, 2007). A MN is formed when the chromosomes fail to interact with spindle fibres in the mitosis phase. MN consists of acentric parts or whole chromosome, and MN presence results from chromosomal damage and cell damage. In this study, MNi were chosen over several types of cell abnormalities, to reflect and emphasise chromosomal breakage, rearrangement,

loss, and apoptosis (Fenech, 2007). In all experiments, the NDIs were within normal range Tables 6 and 7. The BiNC percentages after scoring 1000 cells in each experiment were within normal percentage range in lymphocyte cultures. The low % of multiNC in cell cultures indicated that the cyto B was efficient in blocking further cell division after one cell cycle (Fenech, 2007; Sgura et al., 1997).

In this assay ibuprofen and aspirin were also used in both NP and bulk forms, and lymphocytes from fresh blood were used. The cells were exposed to both ibuprofen and aspirin and cultured for 72 h. Cyto B was then added to the culture after 44 h to block cytokinesis during cell division. Lymphocytes were scored for the presence of MNi which indicates the chromosomal abnormality. Compared to the untreated cells, both ibuprofen and aspirin showed an insignificant effect on lymphocytes from healthy female volunteers Table 6 and Figure 27. By contrast, a significant reduction of MNi formation was observed on lymphocytes from BC patients with both ibuprofen and aspirin (NP and bulk) compared to untreated cells Table 7 and Figure 28. However, aspirin caused a greater reduction of MNi formation compared to ibuprofen. Furthermore, NPs of both drugs showed the superior reduction in MNi formation compared to their bulk forms Figure 29. Using the ANOVA test, aspirin NP, aspirin bulk and ibuprofen NP showed a significant ***$p \leq 0.001$ reduction in MNi formation compared to ibuprofen bulk **$p \leq 0.01$.

8.3 Comparing Comet and micronucleus assays results

Both aspirin and ibuprofen caused a reduction in DNA damage and micronucleus formation in lymphocytes from BC patients. Both aspirin bulk and NP forms showed a significant decrease in both the Comet and micronucleus assays. By contrast, ibuprofen showed a significant reduction with the micronucleus test but

not with the Comet assay. This discrepancy might be due to the difference in the exposure time of both assays since the Comet assay has an incubation time of only 30 minutes, whereas, the micronucleus assay has a longer incubation period of 72 hours. Therefore, long exposure makes the cells more exposed to ibuprofen, and this perhaps explains the significant reduction with the micronucleus but not with Comet assay.

The genotoxicity induced by ibuprofen has been debated in recent years. However, the controversy still exists as, for instance, the genotoxicity of ibuprofen on Salmonella strains did not result in conclusive and satisfactory results (Tripathi et al., 2012). Philipose et al. (1997), on the other hand demonstrated a genotoxic effect of ibuprofen in a mouse model where it caused a weak genotoxic effect observed on sister chromatid exchange. This is in line with our finding of minor DNA damage seen in healthy lymphocytes exposed to ibuprofen in the Comet assay. However, the dissimlar results were found when Ghosh *et al.* (2010) found that ibuprofen had no genotoxic effect over a two weeks' period. Conversely, Tripathi et al. (2012) conducted a similar study to investigate the genotoxic effect of ibuprofen on bone marrow cells of mice. Finally, they concluded that ibuprofen might induce a genotoxic effect in bone marrow cells of mice and this effect was more pronounced at 40 and 60 mg/kg bodyweight.

Although aspirin does not induce a genotoxic effect by itself, its protection against genotoxicity was observed when it was combined with the genotoxic anticancer agent MMC. Here mice treated with aspirin at 0.5, 5 and 50 mg/kg combined with 2mg/kg dose of MMC showed a reduced genotoxic effect. It was observed that aspirin led to a dose dependent reduction in the genotoxicity produced by MMC in various organs such as liver and spleen and the reduction was in a dose dependent fashion (Baron and Sandler, 2000; Niikawa et al., 2008). These findings correspond to this project's

outcomes that aspirin reduced the DNA damage in lymphocytes from BC patients. Niikawa et al. (2008) suggested that the mechanism behind the capacity of aspirin to reduce the DNA damage is its ability to scavenge or diminish or block different oxygen free radicals.

8.4 Comet assay on BLM with and without recovery

Our study using the Comet assay and micronucleus assay has demonstrated substantial reductions in DNA damage in lymphocytes when treated with the two forms of aspirin and ibuprofen, with NP forms causing more reduction. With these findings, the study aimed to examine the DNA repair influence in cells challenged with a genotoxic agent in the presence or absence of both aspirin and ibuprofen, to conclude whether these two NSAIDs induced DNA repair or not. Investigating DNA repair disorders has utmost importance in monitoring predisposition to the development of cancer (van Gent and Kanaar, 2016).

Lymphocytes from healthy females and female BC patients were challenged using BLM, and cells were allowed to carry out DNA repair without BLM. Some of the challenged cells were allowed to repair DNA in the presence of either aspirin or ibuprofen, as previously discussed.

Subsequent exposure to BLM, the greatest capacity for repairing of DNA was observed when cells were allowed to repair in the presence of NSAIDs. Furthermore, the treatment with nano forms showed higher repair in DNA compared to their bulk versions, where DNA damage was reduced to untreated cells levels.

In cells from BC patients, the DNA was still unrepaired in lymphocytes in BLMR, where DNA repair was allowed in the absence of either aspirin or ibuprofen

compared to untreated cells Figure 34 and 35. The reductions in DNA damage was present with the bulk forms of the NSAIDs, and this repair was higher in the presence of the NP form in both drugs. These findings illustrated the capacity of both compounds to induce the repair processes and reduce the DNA damage. The large surface of NPs could be the reason for the difference in DNA repair between nano and bulk forms. Where NPs have a large surface area to volume ratio, and maybe the nano size allowed for fast penetration to the nucleus compared to the bulk size (Huang et al., 2012).

Our findings were compatible with a study done on lymphocyte from lung cancer patients and challenged with BLM. Where the DNA damage was reduced in challenged cells in the presence of NSAIDs but not in the absence of aspirin and ibuprofen (Ali, 2014).

8.5 Investigation of XRCC3 and p53 proteins in Lymphocytes from Healthy Female and BC Patients Treated with Both forms of Aspirin and Ibuprofen using Western Blot

Mammalian cells have specific mechanisms for the repair of double-strand DNA breaks (DSBs): nonhomologous end joining (NHEJ) and homologous recombination repair (HRR). HRR is a vertebrate regulated pathway through which repair proteins accumulate sequentially at DSBs. In HRR, Rad51 and the five paralogues Xrcc3, Rad51B, Rad51C, Rad51D, and Xrcc2 accomplish distinct and collaborative functions to repair damage (Loignon et al., 2007).

Thus, we studied the effect of the two forms of aspirin and ibuprofen on the expression of XRCC3 and p53 proteins in lymphocytes from healthy female volunteers and BC patients using the western blot technique. Two different incubation times 8 and 24 h were chosen for each treatment. In the XRCC3 protein

expression experiment in cells from healthy individuals Figure 36, our results demonstrated that 8 h incubation with both aspirin nano and bulk significantly increased the protein expression. However, both forms of ibuprofen displayed no statistical change in XRCC3 expression. Both drugs showed no change in protein expression after 24 h incubation. The increase in protein expression after aspirin treatment may explain the minor increase in DNA damage that was observed in the Comet assay experiments in Chapter 3. Furthermore, aspirin may cause non-significant DNA damage in the first 8 h of the incubation in normal female lymphocytes. In samples from BC patients Figure 37, aspirin bulk showed a non-significant change in XRCC3 expression after 8 or 24 h incubations. However, ibuprofen bulk decreased the expression approximately 2- fold at both 8 and 24 h. Both aspirin nano and ibuprofen nano decreased the expression nearly 1-fold in 8 h incubation, whereas, both drugs in nano forms showed no change in XRCC3 protein expression after 24 h of incubation. Both NSAIDs showed a non-significant change in p53 expression in lymphocytes from healthy control samples after 8 or 24 h incubation Figure 38. The p53 expression results in lymphocytes from BC patients was varied among treatments Figure 39, where aspirin bulk showed no change in 8 h and a significant increase $*p \leq 0.05$ at 24 h incubation. However, aspirin nano was not significant at 8 h while the statistical significance $**p \leq 0.01$ at 24 h. Ibuprofen nano had no effect on p53 expression after incubation for 8 or 24 h. Ibuprofen bulk significantly reduced p53 expression $*p \leq 0.05$ at 8 hrs, whereas, this change disappeared after 24 h.

Dibra et al. (2010) proposed that exposure to aspirin (1 mM) for 48 h, caused a significant increase in the transcription of XRCC3 in the SW480 colorectal cancer

cell line. They suggested that the toxic influence of aspirin on the transcriptional machinery could explain this effect. By contrast, our results showed no XRCC3 overexpression in BC patients lymphocytes when treated with aspirin or ibuprofen. Furthermore, both aspirin and ibuprofen nano decreased the protein expression significantly $*p \leq 0.05$, where, ibuprofen bulk reduced it $**p \leq 0.01$, whilst considering the difference between BC and colorectal cancer and the type of cells used in both studies.

A study by Loignon et al. (2007) indicated that the absence of exogenous DNA damage, reduced the expression of XRCC3 which then decreased cell proliferation by slowing replication. Also, decreasing the XRCC3 expression in lymphocytes from BC patients after treatment with aspirin and ibuprofen perhaps means that both NSAIDs stimulated DNA repair process.

8.6 The effect of both aspirin and ibuprofen on COX1, COX2 and P53 genes expression in lymphocytes from healthy female and BC Patients using Real Time PCR

Many studies have been done recently to assess the ways that aspirin acts against BC and many mechanisms have been suggested for the phenomenon. Inhibition of cyclooxygenase is the most common known pathway. COX enzymes are enzymes that have an important function in the synthesis of some mediators particularly prostaglandin-endoperoxide. The prostanoids including prostaglandins are essential biological mediators, which serve different biological roles. So far three isoenzymes of COX are known COX1, COX2 and COX3. Aspirin and ibuprofen can block both COX1 and COX2 (Simmons et al., 2004).

The main function of COX enzymes Figure 5 to convert the arachidonic acid to prostaglandin H2 and finally synthesise the biological form of prostaglandin which

has roles in cell division, migration, angiogenesis, and apoptosis (Ulrich et al., 2006). In addition, many preclinical studies found that pro-inflammatory prostaglandin E2 (PGE2) catalyses the production of oestrogen by increasing the expression of the aromatase gene. The latter belongs to the P450 family of enzymes and stimulates the production of oestrogen from androgen. Furthermore, various studies have found that COX enzymes stimulate PGE2 to catalyse CYP19 transcription and lead to increases in the aromatase production and activity. These findings support the observation of a positive correlation between COX enzymes level and CYP19 expression in human breast carcinoma (Elvin et al., 2000). Therefore, the preventative effect of aspirin and ibuprofen observed in BC can be linked to inhibition of prostaglandin by these agents and this ultimately stops the production of oestrogen from androgen as this conversion needs prostaglandin to proceed successfully. This in turn inhibits breast cell proliferation as it needs oestrogen to function (Lazzeroni et al., 2013).

The COX2 signalling pathway has been marked as a fundamental mediator in the pathogenesis of a range of malignancies. Consistent with this concept, it has been evidenced that the COX2 pathway inhibition can be a key approach to the treatment of a variety of cancer models, including BC (Gharghabi et al., 2016). Previous studies have described a functional interaction between COX2 and some molecular targets, especially p53. Regarding this theory, the role of COX2 inhibitors as a potential candidate to induce apoptosis through cell death machinery has been examined in various reports so far (Corcoran et al., 2005; Swamy et al., 2003).

In this respect, we examined the effect of NSAIDs aspirin and ibuprofen (nano and bulk forms) on the expression of p53, COX1 and COX2 in lymphocytes from BC patients and compared responses with those from healthy female volunteers.

Our results showed a non-significant change in the expression of both p53 and COX2 genes in lymphocyte from healthy females after treatment with either aspirin or ibuprofen Figure 40 and 44. However, COX1 was downregulated significantly Figure 42. In lymphocytes from BC patients, the effect of both drugs was different, where; both forms of aspirin and ibuprofen NPs significantly increased the expression of p53 Figure 41. In the case of ibuprofen bulk, there was an increase in p53 expression but the effect was not statistically significant. However, the expression of COX1 and COX2 in patients cells was significantly reduced after treatment with both compounds either bulk or nano form Figure 43 and 45.

It is evident that the effect of both NSAIDs on the expression of p53 was different compared to their impact on COX1 and 2. Moreover, p53 and COX responded to this effect differently. This outcome may support the studies suggesting that NSAIDs inhibit cyclooxygenase via increasing the expression of tumour suppressor gene p53 (Wilson et al., 2003).

The nuclear factor of kappa light polypeptide gene enhancer in B cells (NFkB) is another mediator that is also inhibited by aspirin and this inhibition has been observed in both *in vivo* and *in vitro* studies (Lazzeroni et al., 2013). Surprisingly, the prevention of NFKB transcription results in an increase in the apoptosis ratio and this effect was found to be limited to neoplastic epithelial cells. Among others some *in vitro* studies suggested that aspirin may have a direct impact on elevating the levels of some important apoptotic genes namely BAX, BCL2 and caspase-3

genes and this eventually results in stimulation of breast tumour cell apoptosis (Yan et al., 2013). Aspirin also blocks angiogenesis (formation of new blood vessels from the pre-existing blood vessels) by inhibiting the secretion of some angiogenic factors and this in turn leads to promote tumour growth and this effect was assessed using the 3-dimensional collagen angiogenesis assay, utilising (0.5 mM) therapeutic aspirin, which showed a reduction in tubule formation (Lazzeroni et al., 2013).

Sun et al. (2012) suggested that the phosphate-ibuprofen has a considerable influence on two protein members of thioredoxin (Trx) system, Trx-1 and TrxR. phosphate-ibuprofen has the ability to change the Trx-1 to its oxidised form and finally reduced the expression of Trx-1. Phosphate-ibuprofen also suppressed the activity of TrxR. These findings demonstrated the major inhibition effect of Phosphate-ibuprofen on the Trx system through effects on Trx-1 and TrxR. The effect of Phosphate-ibuprofen on the Trx system had indirect impacts on signalling cascades dependent on the Trx system, for instance NFKB and MAPK. The NFkB is predominantly inhibited by oxidation of the Trx system through its Cys62 of p50. This oxidation makes NFkB unable to bind the DNA. NFKB regulates the two anti-apoptotic proteins Bcl-2 and Mcl-1 and the inactivation of NFkB by phosphate-ibuprofen leads indirectly to suppression of the expression of Bcl-2 and Mcl-1 proteins. This finally increases the apoptotic process (Mitsiades et al., 2002). The phosphate-ibuprofen also has a role in the MAPK pathway regulation, by down-streaming the Trx-1. This pathway remains inactive as a result of ASK1 bound to Trx-1. Once the Trx-1 is oxidised, this activates the pathway by releasing the ASK1. Furthermore, phosphate-ibuprofen has the ability to induce apoptosis and

suppress proliferation, that is mainly mediated by NF-_B and MAPK (Sun et al., 2012).

8.7 Conclusion

Data from the Comet assay showed that ibuprofen and aspirin (both bulk and NP sizes) caused a reduction in DNA damage in lymphocytes from BC patients but not in healthy volunteers. However, the reduction of DNA damage was higher for aspirin compared to ibuprofen. The results were the same for both parameters Olive tail moment and % tail DNA. The micronucleus assay data showed a decrease in MNi frequency, which followed a similar pattern the results of the Comet assay. In both assays, aspirin was more effective than ibuprofen in reducing DNA damage and MNi formation seen in the Comet and micronucleus assays, respectively. NPs of both agents were more effective than bulk sizes. In the recovery study, the DNA repair process was more effective in the presence of both NSAIDs, where the aspirin nano form had the greatest effect compared to aspirin bulk, ibuprofen bulk and ibuprofen nano. Also, both forms of aspirin increased the expression of p53 protein, and aspirin nano increased the expression of the tumour suppressor gene p53. Both aspirin and ibuprofen reduced the expression of the COX2 gene. The results of this work are consistent with the view that NSAIDs, particularly aspirin and ibuprofen have a promising role in cancer treatment including BC.

8.8 Future work

From the above discussion, it can be concluded that these findings have to be studied extensively and using additional techniques to confirm and prove these results. Micronucleus FISH (Fluorescence in situ hybridisation) is one of these

techniques which allow the evaluation of the nature of chromosome damage lesions. Since in this project ibuprofen and aspirin were used with lymphocytes from BC patients, it would be interesting if this work could be repeated with lymphocytes from different types of cancer patients to determine whether the results will be similar or different compared to BC. Aspirin and ibuprofen downregulated the expression of some genes, and it could be possible to study if both NSAIDs have any effect in the formation of micro-RNA (miRNA) perhaps this could be an additional protective effect mechanism against cancer. Finally, regarding the studies suggesting that aspirin and ibuprofen could have an effect on the expression of BAX, BCL2 and caspase-3 genes, it is possible to study this effect on these genes in lymphocytes from BC patients and compare them with healthy female volunteers.

Chapter 9 - References

9. References

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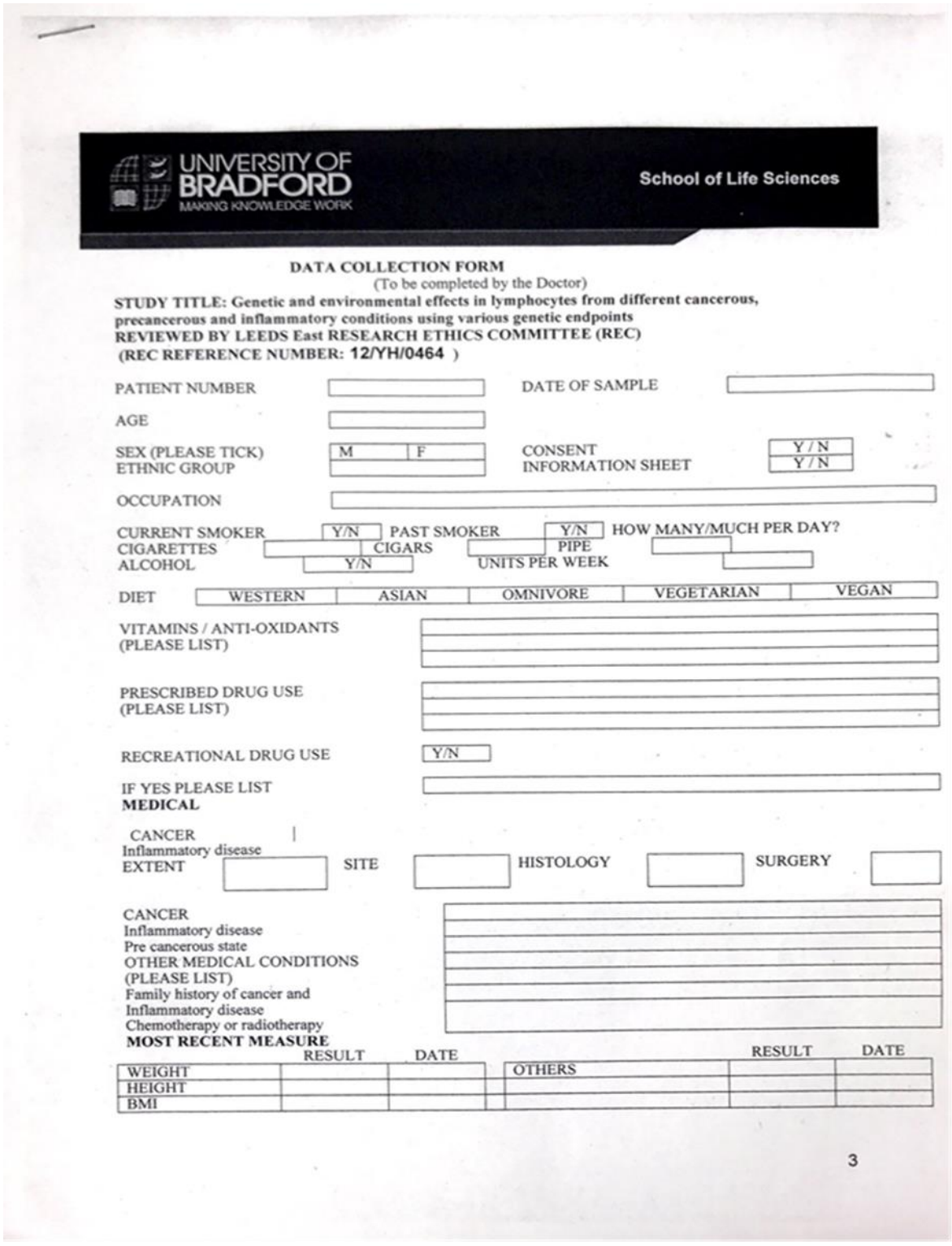
Appendix

Appendix 1

Sample of Survey



UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

School of Life Sciences

DATA COLLECTION FORM

(To be completed by the Doctor)

STUDY TITLE: Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints

REVIEWED BY LEEDS East RESEARCH ETHICS COMMITTEE (REC)

(REC REFERENCE NUMBER: 12/YH/0464)

PATIENT NUMBER | DATE OF SAMPLE

AGE

SEX (PLEASE TICK) M F | CONSENT Y / N

ETHNIC GROUP | INFORMATION SHEET Y / N

OCCUPATION

CURRENT SMOKER Y/N PAST SMOKER Y/N HOW MANY/MUCH PER DAY?

CIGARETTES CIGARS PIPE

ALCOHOL Y/N UNITS PER WEEK

DIET WESTERN ASIAN OMNIVORE VEGETARIAN VEGAN

VITAMINS / ANTI-OXIDANTS (PLEASE LIST)

PRESCRIBED DRUG USE (PLEASE LIST)

RECREATIONAL DRUG USE Y/N

IF YES PLEASE LIST

MEDICAL

CANCER

Inflammatory disease

EXTENT SITE HISTOLOGY SURGERY

CANCER

Inflammatory disease

Pre cancerous state

OTHER MEDICAL CONDITIONS (PLEASE LIST)

Family history of cancer and Inflammatory disease

Chemotherapy or radiotherapy

MOST RECENT MEASURE

	RESULT	DATE		RESULT	DATE
WEIGHT			OTHERS		
HEIGHT					
BMI					

3

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

School of Life Sciences

Centre Number:

CONSENT FORM FOR HEALTHY VOLUNTEERS

Title of Project: **Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints**
Reviewed by Leeds East Research Ethics Committee (REC)
(REC reference number: 12/YH/0464)

Names of Researchers: Prof. D Anderson, Dr. Mojgan Najafzadeh, Mr M Salhab.

	Please **initial** box
1. I confirm that I have read and understand the information sheet (version 2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
3. I agree that the sample I have given and the information gathered about me can be stored at the University of Bradford, as described in the attached information sheet.	☐
4. I agree to take part in the above study.	☐

Name of Volunteer	Date	**Signature**
Name of Person taking consent	Date	Signature

When completed, 1 for patient; 1 for researcher site file;

5

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

Reviewed by Leeds East Research Ethics Committee (REC)
(REC reference number: 12/YH/0464)

Invitation to the research study

We should like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take the time to read the following information carefully. Talk to others about the study if you wish and you will be allowed around 24 hours to consider this.
(Part 1 tells you the purpose of this study and what will happen to you if you take part.
Part 2 gives you more detailed information about the conduct of the study).
Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you want to take part.

Part 1

What is the purpose of the study?

In this study white blood cells will be treated in a test tube with very small chemical particles or UVA (Ultra Violet A light) to determine if patients with different diseases are more at risk after exposure compared to healthy individuals. For example, chemicals and UV (Ultra Violet) can break and damage the DNA of white blood cells. Further examination of this resulting damage may improve our knowledge of the cancers and other inflammatory diseases. The tests are not predictive for any kind of diseases and the test results will not impact on you or the patients with whom you are compared.
A blood sample of around 2-4 teaspoons (20 ml) will be taken. Samples will be stored only until the end of the study (after 8 years) and used for studies of a similar nature or to check original responses. The research is also used for some PhD programmes.

Why have I been invited

You have been invited because you are healthy and do not have the disease of the patients we are comparing you with. We should like to determine if these small chemical particles or UVA could be more harmful to people with diseases than those without diseases.

Do I have to take part?

No, it is up to you to decide. We shall outline the study and go through this information sheet, which we shall then give to you. We shall ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason.

Part 2

What will happen to me if I take part?

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

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

The study tests are not predictive for you

The data obtained will only be available to the research team and will **not** be returned to you. Responses will be compared only on group basis i.e. collective responses from patients with that individual disease compared to collective responses from people without that disease. Results could be published in the form of scientific papers. The work may benefit the medical and scientific community at large, but will not be of direct benefit to you as an individual. If, however, you would like more information, the appropriate consultant will be prepared to talk to you individually about study results.
The data will be stored until the study is completed at the end of 8 years.

People who cannot take part in the study.
People who are not well enough to take part will be excluded (e.g those with anaemia).

If you have any further questions, you could contact the research team:
Mr Salhab, Bradford Teaching Hospitals NHS Foundation Trust, St Lukes' Hospital Bradford Teaching Hospitals NHS Foundation Trust, BD5 0NA .

Telephone: 01274 36 5734

Professor Diana Anderson, Established Chair in Biomedical Sciences, BSc, MSc, PhD, DipEd, FIBiol, FATS, FRCPath, FIFST, FBTS, FHEA, University of Bradford, Richmond Road, Bradford, BD7 1DP and Honorary Research Consultant to Bradford NHS Trust.
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Appendix 2

Healthy female individuals and Breast cancer patients database details.

Control sample No	Age	Ethnicity	Smoking pack year	Smoking history	Family history	Past Medical history
A1	34	Asian	----	No	No	No
M1	40	Asian	----	No	No	No
R1	34	Asian	----	No	No	No
S1	30	Asian	----	No	No	No
K1	44	Asian	----	No	No	No
CH1	29	Asian	5	10/d	BC	No
SH1	33	Asian	----	No	No	No
A18	41	Asian	----	No	No	No
O1	36	Asian	----	No	No	No
1723	45	Caucasian	----	No	No	No
4107	56	Caucasian	----	No	No	No
4113	80	Caucasian	----	Ex-smoker	Heart disease	Osteoprosis
4114	45	Caucasian	----	No	No	No
4115	45	Caucasian	----	No	No	Arthritis
4122	50	Caucasian	----	No	No	No
4522	52	Caucasian	----	No	No	No
4523	53	Caucasian	21	10/d-42y	No	Lumbar disc
4524	60	Caucasian	----	No	No	No
4526	61	Caucasian	----	No	No	No
4922	53	Caucasian	16	10/d-32y	No	No

BC sample No	Age	Ethnicity	Smoking pack year	Smoking history	Family history	Past Medical history
4796	80	Asian	----	No	No	No
4802	51	Caucasian	7.5	15/d	No	No
4805	85	Caucasian	----	No	No	No
5076	71	Caucasian	----	No	No	kidney transplant
5189	41	Asian	----	No	No	No
5351	70	Caucasian	10	10/d	No	IHD, Chol
5357	81	Caucasian	----	No	No	HTN, Chol
5363	47	Caucasian	6	20/d	No	No
5364	47	Caucasian	----	No	No	No
5372	32	Caucasian	2.1	7/d	No	N
5375	65	Asian	----	No	No	HRT
5554	36	Asian	----	No	No	No
5558	46	Caucasian	----	No	No	No
5572	57	Caucasian	4.5	15/d	No	No
5604	58	Asian	----	No	No	No
5608	50	Caucasian	6	10/d	No	No
5723	60	Caucasian	----	No	No	No
6003	58	Caucasian	13	20/d-13y	BC	No
6010	53	Caucasian	1	10/d-2y	Ovarian cancer	No
6011	55	Caucasian	----	No	BC	No

Fifteen percent of control individuals and 40% of BC cancer patients were smokers. It would appear that smoking and ethnicity did not confound the responses due to uniformity of the data collected. The t-test showed no change in significance between the two groups (p = 0.337) and (p = 0.489) respectively. In both, gender was not an issue since only females were examined.

With regard to age:

Age	20	30	40	50	60	70	80
Control	1	5	6	5	2	0	1
BC	0	2	4	7	2	2	3

The difference in the age might contribute some enhancement of response in the cancer group, since damage can increase with age. However both control and patients fall within the same age range. Therefore, it is unlikely that only age contributes to the response. Furthermore, some older patients had lower value than those in the ages between forty and fifty.