CHAPTER ONE

INTRODUCTION

1.1. Background

Breast tumour is a benign or malignant growth that begins in the tissues of the breast. Most malignant breast tumours are derived from the epithelium lining the ducts or lobules, and are classified as mammary ductal carcinoma or mammary lobular carcinoma (Jeffrey et al., 2013, Amy *et al*., 2015). It is considered a heterogeneous disease differing by individual, age group and ethnic group. It can be Hormone receptor positive or negative; it can be growth factor receptor positive or negative. Breast tumours are caused by the mutation of the genetic material of normal cells, which upsets the normal balance between proliferation and cell death. The uncontrolled and often rapid proliferation of cells can lead to benign tumor which is self limiting or may turn into cancer when it begins to spread to nearby tissues or organs (Carlo and Croce, 2008). The development may go through a histological progression, from hyperplasia and *in situ* carcinoma to invasive cancer (Anita, 2013, Van *et al*., 2014).

Breast tumours are the most commonly diagnosed tumours in women worldwide (Lan *et al*., 2013). Research has shown a steady rise in breast cancer cases in Nigeria. This is an indication of inadequate or ineffective control measures to curtail the disease. Thus, there is need to control the upward trends and reduce the mortality rate of breast cancer, as the existing control measures on breast cancer are yet to yield any positive impact. In addition, the mortality caused by breast cancer has remained almost unchanged for the past 5 decades, becoming only second to lung cancer as a cause of cancer-related death. The failure of eradicating this disease is largely due to lack of identification of a specific etiologic agent, the precise time of initiation, and the molecular mechanisms responsible for cancer initiation and progression. Despite the numerous uncertainties surrounding the origin of cancer, there is substantial evidence that breast cancer risk relates to endocrinologic and reproductive factors (WHO, 2014). It is pertinent to check the interactive link between molecular expressions, female sex hormonal secretions and presence of microbial antigenic components of circulating immune complexes to access direct or indirect relationship between the reproductive female hormones, the expressed molecules and antigenic components of circulating immune complexes.

Breast tumours can be divided into those with identified genetic risk factors as inherited germline mutation from parents to offspring and those who are not genetically predisposed but encounter epimutational changes in their life time. While the former group accounts for only around 10% of total breast cancer cases, the later accounts for over 90% of total breast cancer cases (Anand *et al*., 2008). Inherited gene mutations such as BRCA1 and BRCA2 mutation lead to over 90% of inherited breast cancer. The majority of breast tumours are sporadic, caused by more complicated reasons such as the activation of mitogenic signaling pathways and loss of tumor suppressor gene expression due to epimutation (Wang and Li-Jun, 2014).

Epigenetics is the inheritance of information on the basis of gene expression rather then direct changes to sequence composition. Epigenetic alterations include methylation of CpG dinucleotides in promoters or alteration in the normal global DNA methylation and changes in chromatin structure that may lead to silencing of tumor suppressor genes. Activation of oncogenes may also be a result of epigenetic changes through post-translational modifications in histone acetylation or DNA conformation. DNA methylation is an epigenetic mark that has suspected regulatory roles in a broad range of biological processes and diseases. The technology is now available for studying DNA methylation genome-wide, at a high resolution and in a large number of samples (Bock, 2012). Evidence for the genetic predisposition to certain cancers exists, specifically because germ-line mutations of the DNA of an individual are inherited by future generations. These genetic predispositions to cancers are referred to as familial forms of cancer. There are instances in which non-familial cases of these cancers result (sporadic cancer) and the underlying cause has been suggested to involve epigenetic silencing. The epigenetic mark that has been most highly studied is DNA methylation, the presence of methyl groups at CpG dinucleotides. These dinucleotides are often located near gene promoters and associate with gene expression levels. Early studies indicated that global levels of DNA methylation increase over the first few years of life and then decrease beginning in late adulthood (Jones *et al*., 2015). These changes may involve chemical modifications of the DNA itself, such as DNA methylation or modifications of proteins that are closely associated with DNA, such as the histones that bind and compact DNA into chromatin packages. Research has shown that all recognized epigenetic marks (including DNA methylation, histone modification, and microRNA (miRNA) expression) are influenced by environmental exposures, including Infectious agents, diet, tobacco, alcohol, stress, genetic factors, which play important roles in the etiology of cancer (Zhang and Meaney, 2010). While the discovery that viruses can cause tumours in animals was traced back to 1st

century (De Flora and Bonanni, 2011), the implication of microbial and parasitic diseases in the causation of human cancers has been demonstrated.

Some of the epigenetic modifications change the expression of tumor suppressor genes and oncogenes and therefore, may be causal for tumorigenesis (Gómez-Díaz *et al*., 2012). There is now evidence that environmental events can directly modify the epigenetic state of the genome, thus studies with rodent models suggest that during both early development and in adult life, environment signals can activate intracellular pathways that directly remodel the epigenome leading to changes in gene expression (Zhang and Meaney, 2010). Both tumourigenesis and carcinogenesis are characterized by a progression of changes in cellular and genetic level that ultimately reprogram a cell to undergo uncontrolled cell division, thus forming a benign or malignant mass respectively (Carlo and Croce, 2008). The uncontrolled and often rapid proliferation of cells can lead to benign tumor which is self limiting or may turn into malignant tumor (cancer) (Carlo and Croce, 2008).

Tremendous progress have been made over the last decades at understanding the biology of breast tumours, however the mechanism for growth and progression of breast tumour with acquisition of invasive and metastatic phenotypes and therapeutic resistance are still not fully understood (Jamal *et al*., 2011, Xuan *et al*., 2014). Despite the $5.5 billion spent on breast cancer research over the past twenty years, the origins of a majority of breast cancer cases remain unknown (Xuan *et al*., 2014). It is crucial to understand the aetiology of these sporadic breast cancers in order to develop adequate therapeutic or preventive strategies against the devastating disease. The recent appreciation of the influence of microbiota on human health and disease begs the question of whether microbes play a role in sporadic breast tumours of unknown etiology or not. Moreover, so much importance has been attached to the role of immunological responses in tumour development. The interest in this study is focused on evaluation of immunologically inducible molecules expressed in the system, mediated and activated by the presence of certain antigenic components of circulating immune complexes and thus suspected to augment breast tumour development and progression. Research has shown that by circulation, circulating immune complexes can deposit in tissues and continue to induce secretion of pro-inflammatory molecules (Meyadas *et al*., 2009), thus mediating immune responses such as inflammatory processes capable of perturbing normal process of DNA methylation. This would be likely where enabling environmental factors seem to favour retention of immune complexes in circulation.

Insights into the molecular and cellular mechanisms underlying cancer development have revealed that immune cells functionally regulate epithelial cancer development and progression (DeNardo and Coussens 2007). Moreover, accumulated clinical and experimental data indicate that the outcome of an immune response towards an evolving breast neoplasm is largely determined by the type of immune response produced by the host. Acute tumor-directed immune responses involving cytotoxic T lymphocytes appear to protect against tumor development, whereas immune responses involving chronic activation of humoral immunity, infiltration by T helper lymphocyte type 2 (Th2) cells, and protumor-polarized innate inflammatory cells result in the promotion of tumor development and disease progression (DeNardo and Coussens 2007).

The binding of antigens to their corresponding antibodies tagged immune complexes have been shown to trigger a lot of immunological responses and have been associated with many pathological conditions such as autoimmune diseases, glomerulo-nephritis etc (Abhishek *et al*., 2015). Circulating immune complexes are physiologically removed by the Mononuclear Phagocytic System (MPS), however their relative persistence in known pathological subjects such as cancer patients in environmentally polluted areas, calls for need for their assessment. Under normal conditions, immune complexes (IC) are usually eliminated in the system and are not detectable in the system. Research has shown that detectable levels of IC are found in chronic or persistent exposure to foreign substances or ongoing infection (Brunner and Sigal 2000). The ability of CICs to classically and continuously activate immune responses, thus making it a potential immunological ligand that can progress tumourigenesis has been neglected overtime and as such deserves attention. Circulating immune complexes (CICs) are now viewed as regulators of both cellular and humoral immune responses by virtue of their capacities to interact with antigen receptors on T and B lymphocytes as well as with macrophages and neutrophils having FC receptors (Goins *et al*., 2010). Circulation of immune complexes, can induce complement activation, opsonization, phagocytosis, activation of immune cells (macrophages, neutrophils) and subsequent release of cytokines, chemokines and activation of protease pathways (Meyadas *et al*., 2009). Moreover immune complexes deposited intravascularly can directly engage circulating leukocytes thus releasing inflammatory mediators (cytokines and prostanoids) capable of activating the endothelium and their ability to recruit more cells (Meyadas *et al*., 2009). Based on these, it could be suggested that circulation of immune complexes is a potent source of acute and chronic inflammations which may continue to

stimulate the inflammatory pathways suspected to cause perturbation of epigenetic mechanism leading to epigenetic cell alteration such as DNA methylation shift.

De novo methylation, which involves the addition of a methyl group to unmodified DNA, is described as an epigenetic change resulting from environmental influence, because it is a chemical modification to DNA not a change brought about by a DNA mutation. Normal methylation of DNA would mark the DNA and enable such actions as turning genes on or off. Thus if epigenomic alteration persists in a tumour suppressor gene, expression of such gene could be altered and the cell may lose the normal pattern of interpretation of DNA instructions (altering the normal ON or OFF position). It has been well demonstrated that the decrease in global DNA methylation (demethylation) is one of the most important characteristics of cancer (Stefansson *et al*., 2013). Shift in normal DNA methylation could result to unmethylation, hypomethylation and hypermethylation. Thus, the quantification of 5-mC content or global methylation in tumour cells could provide very useful information for detection and analysis of breast tumours and other disease conditions. DNA methylation is important in the regulation of inflammatory genes. Hypermethylation of promoter regions of genes is typically associated with transcriptional silencing while hypomethylation obstructs gene expression (Matatiele *et al*., 2015). Consequently, epigenesis is an environmentally induced genetic condition other than the inherited gene mutation from the parents. This makes the study of epigenetics in our locality very important, sequel to positive correlative environmental influence on immune complex formation and clearance. This study thus, hypothesize that detectable level of immune complexes in Nigeria, may be a potential source of epigenetic cell alteration or modification, by inducing chronic inflammation, leading to formation of tumour microenvironment and subsequent tumorigenesis, cancer invasion or metastasis or exacerbation of poor prognosis in cancer patients.

The role of infection or inflammation in the initiation and progression of cancer has been an area of intense scientific interest and is usually considered from the perspective that persistent inflammation in the context of chronic infection or tissue injury might promote cell transformation through DNA damage or that tumor cells produce proinflammatory factors that encourages chronic inflammation and tumor growth (De Marzo *et al*., 2007, Elkahwaji, 2013).
This study is prompted to access the correlation of epigenetic cell alteration (DNA Methylation) with Nuclear Factor kappa B (NFkB) systemic expression and levels of microbial antigenic components of circulating immune complexes. Much more common are persisting microbes that

the host could not eliminate and which engage the host in an ongoing (long lasting) battle that damages tissues (Blaser, 2008, Wroblewski *et al*., 2010) and promotes malignancy. Some of the targeted microbial antigenic components in this study belong to: *Hepatitis C virus, Plasmodium falciparum, Hepatitis B virus, Salmonella spp, Helicobacter pylori, Treponema pallidum, Mcobacterium tuberculosis*. It should be noted that the immune complexes formed due to presence of these microbial antigens, may circulate freely in the blood thus could be deposited in any organ of the body including the breast. As aberrant DNA methylation is associated with cancer and other human diseases, there are growing interests in determining how environmental exposures may affect patterns of DNA methylation.

1.2. Statement of the Problem

Curiosity over the immunological and Immuno-molecular implications of circulating immune complexes, in patients with breast tumours and supposed healthy individual needs urgent and sustained attention due to increasing incidences of breast cancer cases and high motality due to the cancer amongst women as well as other environmental influences. Although the prevalence of breast cancer is higher in the western world, the success of control is also higher unlike in developing countries like in Nigeria, where the health care system is not efficient. Presently there has been increasing reports of breast cancer in Nigeria in the past decade (Jedy-agba *et al*., 2012). Additionally, Breast Cancer in developing countries like in Nigeria deteriorates faster, possing a poor prognostic status. Although incidence of cancer is higher in developed world than in developing countries, presently there is evidence of increasing incidence of cancer in developing countries (De Flora and Bonanni, 2011).

Apoptosis, necroptosis, and pyroptosis are different cellular death programs characterized in organs and tissues as consequence of microbial infection, cell stress, injury, and chemotherapeutics exposure (Garg *et al*., 2014). Dying and death cells release a variety of self-proteins and bioactive chemicals originated from cytosol, nucleus, endoplasmic reticulum, and mitochondria. These endogenous factors named cell death-associated molecular-pattern (CDAMP), damage-associated molecular-pattern (DAMP) molecules, and alarmins, cooperate or act as important initial or delayed inflammatory mediators upon binding to diverse membrane and cytosolic receptors coupled to signaling pathways for the activation of the inflammasome platforms and NF-κB multiprotein complexes (Sangiuliano *et al*., 2014). Based on these, circulating immune complexes continually form as a core adaptive immunological phenomenon. It is a pathway for elimination of such unwanted protein/antigens. Thus, where there is high

tendency of immune complex accumulation and its possible deposition, the apperent health conditions of individuals in such locality becomes questionable.

Though these immune complexes are continually removed from the system by mononuclear phagocytic system, many researchers have discovered high level of circulating immune complexes (CIC) in healthy individuals in Nigeria compared to low level of (CIC) in caucasians (Tanyigna *et al*., 2004). The reason for this development is yet to be properly ascertained. This can be understood against the background that in tropical environments such as in Nigeria, people are exposed to much environmental pollution including microbial agents capable of inducing immune response (Ezeani *et al*., 2011a). In this study the persistence of these immune complexes is suspected to be an enabling immunological risk factor capable of inducing persistent activation of proinflammatory molecules as well as perturbation of the normal global DNA methylation process which can result to epimutation and subsequent breast tumour development, progression and metastasis. This has raised the need for this study.

1.3. Justification of the Study

Research has shown a steady rise in breast cancer cases in Nigeria. This may be due to inadequate or ineffective control measures to curtail the disease or due to diversion of global attention to HIV/AIDS and tuberculosis in the country (Jedy-Agba *et al*., 2012). The involvement of antigen-antibody complexes in the pathogenesis of a large variety of human and animal diseases including cancer is well known. Immune complexes is before now viewed as regulators of both humoral and cellular immune responses by virture of presence of FcγRs present on most cell types of the innate and adaptive immune systems, including B cells, T cells, NK cells, macrophages, mast cells, dendritic cells and neutrophils (Rai and Mody 2012). Thus, persistence of such immunological activities in the presence of increased level of foreign proteins such microbial antigens, can sustain chronic inflammation capable of eliciting tumour microenvironment. Additionally, Immune complex is an evolving immunological product that can induce reactive oxygen species (ROS) production which would in turn attack DNA and cause breakage (Toong *et al*., 2011). This immunological phenomenon has not been properly worked on to determine its link with epigenetic cell alteration. The Fc-receptors for IgG (FcγR) comprise the largest family and mediate many biological functions such as activation of complement, phagocytosis, antibody-dependent cell-mediated cytotoxicity, induction of inflammatory cascades and modulation of immune responses (Bournazos *et al*., 2016). Thus, may continue to enhance chronic inflammation and subsequent tumour development.

1.3. Aim and Objectives

1.3.1 Aim

To evaluate the global (CpG) DNA methylation, the expression of pro-inflammatory molecules, oxidative stress marker, female sex hormones and presence of microbial antigenic components of circulating immune complexes in breast tumours in subjects attending clinics at Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra state, Nigeria.

1.3.2. Objectives of the Study

1. To determine the serum levels of pro-inflammatory molecules (NF-kB, TNF-alpha, IgG), Oxidative marker (OH2DG), circulating immune complexes and female sex hormones (oestrogen and progesterone) in subjects with breast tumours

2. To detemine the cut-off values of the pro-inflammatory molecules (NF-kB, TNF-alpha, IgG), Oxidative marker (OH2DG) and circulating immune complexes in apparently healthy female subjects.

3. To determine the pattern of global DNA methylation in subjects with breast tumours

4. To detect the serum level of CIC and presence of some specific microbial antigenic components of the circulating immune complexes in subjects with breast tumours.

5. To determine the possible association of the serum level of CIC, Global DNA methylation and pro-inflammatory molecules.

1.4. Research Questions

1. What is the serum level of pro-inflammatory molecules (NF-kB, TNF-alpha, IgG), Oxidative marker (OH2DG), circulating immune complexes and female sex hormones (oestrogen and progesterone) in subjects with breast tumours

2. What is the cut-off values of the pro-inflammatory molecules (NF-kB, TNF-alpha, IgG), Oxidative marker (OH2DG) and circulating immune complexes in apparently healthy female subjects in our environment.

3. What is the global DNA methylation status of subjects with breast tumours

4. What are the possible microbial agents detectable as components of circulating immune complexes in subjects with breast tumours.

5. Is there correlation of the pro-inflammatory molecules, global DNA methylation patterns with circulating immune complexes in subjects with breast tumours

1.5. Significance of the Study

High level of formation of immune complexes, their persistent circulation and deposition in breast tissues in pre-cancer and cancer subjects may be a fundamental immunological factor for cancer initiation, progression, invasion and metastasis. Following this line of argument can help us understand the contribution of circulating immune complexes in mediating breast tumour in Nigeria.

Analysis of DNA methylation would reveal the prevailing status of DNA methylation (hypomethylation, hypermethylation, unmethylation and normal methylation), in breast tumour subjects.

The use of serum sample containing cell free DNA (cfDNA-liquid biopsy) would reveal certain molecular expressions and DNA methylation status that could serve as pretumour markers enabling early clinical checkup for prevention of tumorigenesis.

CHAPTER TWO

LITERATURE REVIEW

2.1. History of Breast Cancer

The oldest description of cancer was discovered in Egypt and dates back to approximately 1600 BC. The Edwin Smith Papyrus describes 8 cases of tumours or ulcers of the breast that were treated by cauterization. The writing says about the disease, "There is no treatment (Olson, 2002). For centuries, physicians described similar cases in their practices, with the same conclusion. Ancient medicine, from the time of the Greeks through the 17th century, was based on humoralism, and thus believed that breast cancer was generally caused by imbalances in the fundamental fluids that controlled the body, especially an excess of black bile (Olson, 2002). Alternatively, patients often saw it as divine punishment. In the 18th century, a wide variety of medical explanations were proposed, including a lack of sexual activity, too much sexual activity, physical injuries to the breast, curdled breast milk, and various forms of lymphatic blockages, either internal or due to restrictive clothing (Olson, 2002). In the 19th century, the Scottish surgeon John Rodman said that fear of cancer caused cancer, and that this anxiety, learned by example from the mother, accounted for breast cancer's tendency to run in families (Aronowitz, 2007).

Although breast cancer was known in ancient times, it was uncommon until the 19th century, when improvements in sanitation and control of deadly infectious diseases resulted in dramatic increases in lifespan. Previously, most women had died too young to have developed breast cancer. Additionally, early and frequent childbearing and breastfeeding probably reduced the rate of breast cancer development in those women who did survive to middle age (Aronowitz, 2007). Because ancient medicine believed that the cause was systemic, rather than local, and because surgery carried a high mortality rate, the preferred treatments tended to be pharmacological rather than surgical. Herbal and mineral preparations, especially involving the poison arsenic, were relatively common.

Mastectomy for breast cancer was performed at least as early as AD 548, when it was proposed by the court physician Aetios of Amida to Theodora (Olson, 2002.) It was not until doctors achieved greater understanding of the circulatory system in the 17th century that they could link breast cancer's spread to the lymph nodes in the armpit. The French surgeon Jean Louis Petit

(1674–1750) and later the Scottish surgeon Benjamin Bell (1749–1806) were the first to remove the lymph nodes, breast tissue, and underlying chest muscle. Their successful work was carried on by William Stewart Halsted who started performing radical mastectomies in 1882, helped greatly by advances in general surgical technology, such as aseptic technique and anesthesia. The Halsted radical mastectomy often involved removing both breasts, associated lymph nodes, and the underlying chest muscles. This often led to long-term pain and disability, but was seen as necessary in order to prevent the cancer from recurring (Olson, 2002).

2.1.1. The Pink Ribbon

A pink ribbon is the most prominent symbol of breast cancer awareness (Sulik, 2010). Pink ribbons, which can be made inexpensively, are sometimes sold as fundraisers, much like poppies on Remembrance Day. They may be worn to honor those who have been diagnosed with breast cancer, or to identify products that the manufacturer would like to sell to consumers that are interested in breast cancer usually white, middle-aged, middle-class and upper-class, educated women (Sulik, 2010).

Figure 2.1: Pink ribbon symbol of breast cancer awareness (Sulik, 2010)

The pink ribbon is associated with individual generosity, faith in scientific progress, and a "can-do" attitude. It encourages consumers to focus on the emotionally appealing ultimate vision of a cure for breast cancer, rather than on the fraught path between current knowledge and any future cures (Sulik, 2010).

2.2. Definition of Cancer

There is no one definition that describes all cancers. They are a large family of diseases which form a subset of neoplasms, which show some features that suggest of malignancy. A neoplasm or tumor is a group of cells that have undergone unregulated growth, and will often form a mass or lump, but may be distributed diffusely (Ashworth *et al*., 2011, Narah *et al*., 2012). Cancer is a dreadful disease characterized by the irregular proliferation of the cells. As a cell progresses from normal to cancerous, the biological imperative to survive and perpetuate drives fundamental changes in cells behaviour. Hence, the actual cause of the disease in different sections is still to be explored clearly. Cancer is thus, a class of disease, classified by the type of cell that is initially affected. Today's global scenario indicates that breast cancer and colorectal cancer is the most prominent cancer in case of women and men (Pande and Tripathi 2014). Six characteristics of malignancies have been proposed: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. The progression from normal cells to cells that can form a discernible mass to outright cancer involves multiple steps (Ashworth *et al*., 2011).

Cancer is a cluster of diseases involving alterations in the status and expression of multiple genes that confer a survival advantage and undiminished proliferative potential to somatic or germinal cells (Ashworth *et al*., 2011). Alterations primarily in three main classes of genes viz., (proto) oncogenes such as *HRAS* genes, tumour suppressor genes such as *BRCA 1* and *BRCA2* and DNA repair genes collectively contribute to the development of cancer genotype and phenotype that resists the natural and inherent death mechanism(s) embedded in cells (apoptosis and like processes), coupled with dysregulation of cell proliferation events (American Cancer Society, 2016). Proto-oncogenes are genes that normally help cells grow. When a proto-oncogene mutates (changes) or there are too many copies of it, it becomes a "bad" gene that can become permanently turned on or activated when it is not supposed to be. When this happens, the cell grows out of control, which can lead to cancer. This bad gene is called an oncogene (American Cancer Society, 2016). Tumor suppressor genes are normal genes that slow down cell division, repair DNA mistakes, or tell cells when to die (a process known as *apoptosis* or

programmed cell death). When tumor suppressor genes don't work properly, cells can grow out of control, which can lead to cancer (American Cancer Society, 2016).

Cancers are classified by the type of cell that the tumour cells resemble and are therefore presumed to be the origin of the tumour as described by (American Cancer Society, 2016). These types include:

Carcinoma: Cancers derived from epithelial cells. This group includes many of the most common cancers, particularly in the aged, and include nearly all those developing in the breast, prostate, lung, pancreas, and colon.

Sarcoma: Cancers arising from connective tissue (i.e. bone, cartilage, fat, nerve), each of which develop from cells originating in mesenchymal cells outside the bone marrow.

Lymphoma and Leukemia: These two classes of cancer arise from hematopoietic (blood-forming) cells that leave the marrow and tend to mature in the lymph nodes and blood, respectively.

Germ cell tumor: Cancers derived from pluripotent cells, most often presenting in the testicle or the ovary (seminoma and dysgerminoma, respectively).

Blastoma: Cancers derived from immature "precursor" cells or embryonic tissue. Blastomas are more common in children than in older adults.

Breast cancer therefore is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk (Sariego, 2010). Cancers originating from ducts are known as ductal carcinomas, while those originating from lobules are known as lobular carcinomas. Breast cancer occurs in humans and other mammals. While the overwhelming majority of human cases are in women, breast cancer can also occur in men (Baselga *et al*., 2012).

2.3. Breast Development and Tumourigenesis

To understand breast cancer, it helps to have some basic knowledge about the normal structure of the breasts. The breast is made up mainly of lobules (glands that can produce milk if the right

hormones are present), ducts (tiny tubes that carry the milk from the lobules to the nipple), and stroma (fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels). Until puberty (usually around 13 or 14), young boys and girls have a small amount of breast tissue consisting of a few ducts located under the nipple and areola (area around the nipple) (Javed and Lteif 2013).

At puberty, a girl's ovaries make female hormones, causing breast ducts to grow, lobules to form at the ends of ducts, and the amount of stroma to increase. Men and boys normally have low levels of female hormones, and breast tissue doesn't grow much. Men's breast tissue has ducts, but only a few if any lobules. Like all cells of the body, a man's breast duct cells can undergo cancerous changes. But breast cancer is less common in men because their breast duct cells are less developed than those of women and because they normally have lower levels of female hormones that affect the growth of breast cells (Di Lauro *et al*., 2013). The development of the breast, which is rigorously controlled by the ovary, can be defined by several parameters, such as its external appearance, total area, volume, degree of branching, number of structures present in the mammary gland, and degree of differentiation of individual structures, i.e., lobules and alveoli (Javed and Lteif, 2013). The breast undergoes changes that are progressive from birth to early childhood, becoming striking at puberty. The adolescent period begins with the first signs of sexual change at puberty, which female's sets in between the ages of 10 and 12 years, and terminates with sexual maturity (Javed and Lteif, 2013).

The breast undergoes changes that are progressive from birth to early childhood, becoming striking at puberty. The adolescent period begins with the first signs of sexual change at puberty, which female's sets in between the ages of 10 and 12 years, and terminates with sexual maturity (Javed and Lteif, 2013, American Cancer Society, 2015). Although puberty is often considered to be the point of initiation of ovarian function, the development of the ovary is a gradual process that depends on pituitary gonadotropins. Normal ductal development, however, requires the presence of oestrogen and progesterone, the two ovarian steroid hormones that act on the mammary gland through their respective receptors. As puberty approaches, the rudimentary mammary ducts begin to show growth activity both in the glandular tissue and in the surrounding stroma. Glandular increase is due to the growth and division of small bundles of primary ducts originated during intrauterine life from invaginations of the superficial ectoderm (Choi and Johan, 2013).

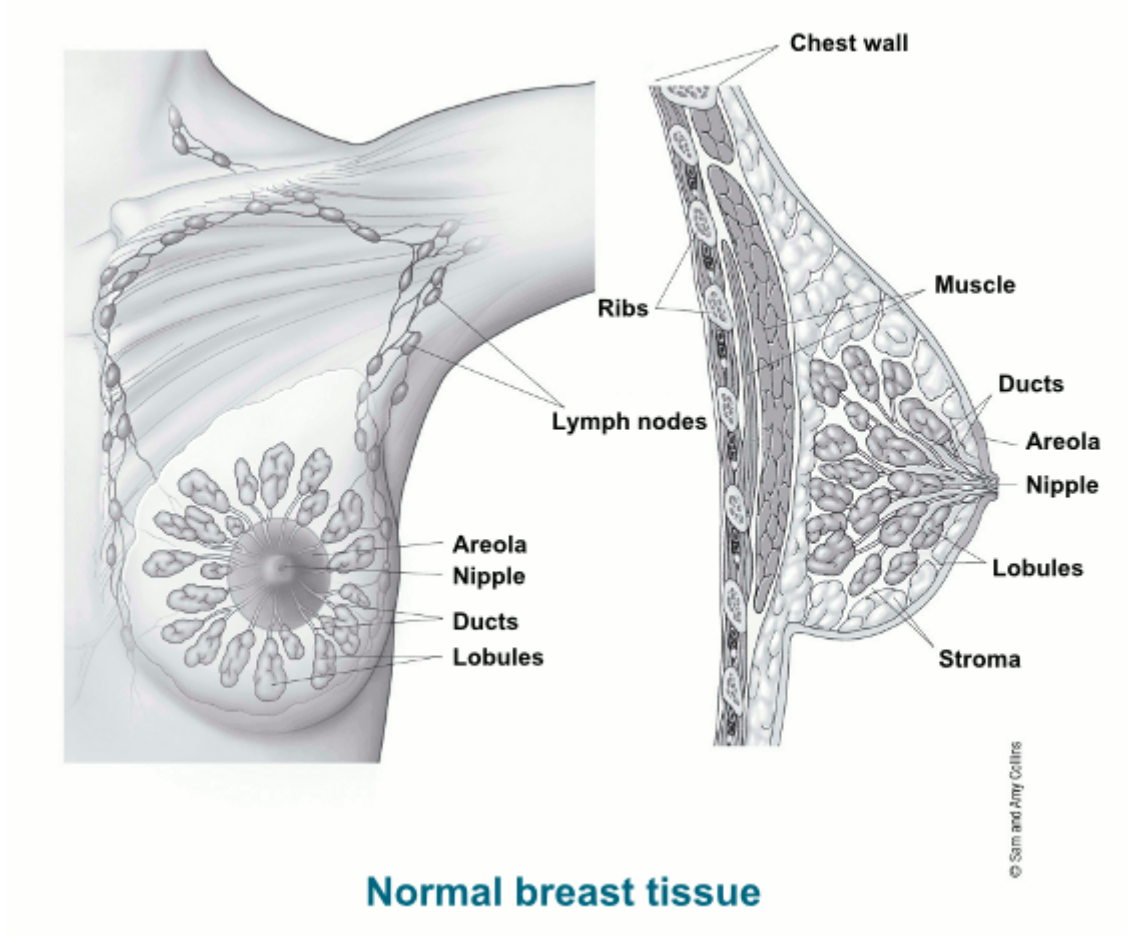

Figure 2.2 Normal breast tissue **(**American Cancer Society, 2015)

The ducts grow and divide through a combination of dichotomous and sympodial branching, forming at the distal epithelial–stromal boundary a club-shaped terminal end bud. Each terminal end bud bifurcates into two smaller ductules or alveolar buds (Gjorevski and Nelson, 2011). The term alveolar bud applies to those structures that appear morphologically more developed than the terminal end bud (Gjorevski and Nelson, 2011). With further branching, alveolar buds become smaller and more numerous, and then they are called ductules. When an average of 11 alveolar buds/ductules cluster around a terminal duct, they form the lobule type 1 (Lob 1) or virginal lobule. Terminal ducts and ductules are lined by a two-layered epithelium, whereas terminal end buds in the human fetus are lined by an epithelium composed of up to four layers of cells. Lobule formation in the female breast occurs within 1–2 years after onset of the first menstrual period. Afterward, the ulterior development of the gland varies greatly from woman to woman. Full differentiation of the mammary gland is a gradual process taking many years, and it can be assumed that in all women in whom pregnancy did not supervene, it was never attained (Gjorevski and Nelson, 2011).

The lymph (lymphatic) system of the breast is important to understand because it is one of the ways that breast cancers can spread. This system has several parts. Lymph nodes are small, bean-shaped collections of immune system cells (cells that are important in fighting infections) that are connected by lymphatic vessels. Lymphatic vessels are like small veins, except that they carry a clear fluid called lymph (instead of blood) away from the breast. Lymph contains tissue fluid and waste products, as well as immune system cells (American Joint Committee on Cancer, 2010, American Cancer Society, 2015). Most lymphatic vessels in the breast connect to lymph nodes under the arm (axillary nodes). Some lymphatic vessels connect to lymph nodes under the breast bone (internal mammary nodes) and either above or below the collarbone (supraclavicular or infraclavicular nodes) **(**American Cancer Society, 2015). If the cancer cells have spread to these lymph nodes, there is a higher chance that the cells could have also gotten into the bloodstream and spread (metastasized) to other sites in the body. The more lymph nodes with breast cancer cells, the more likely it is that the cancer may be found in other organs as well.

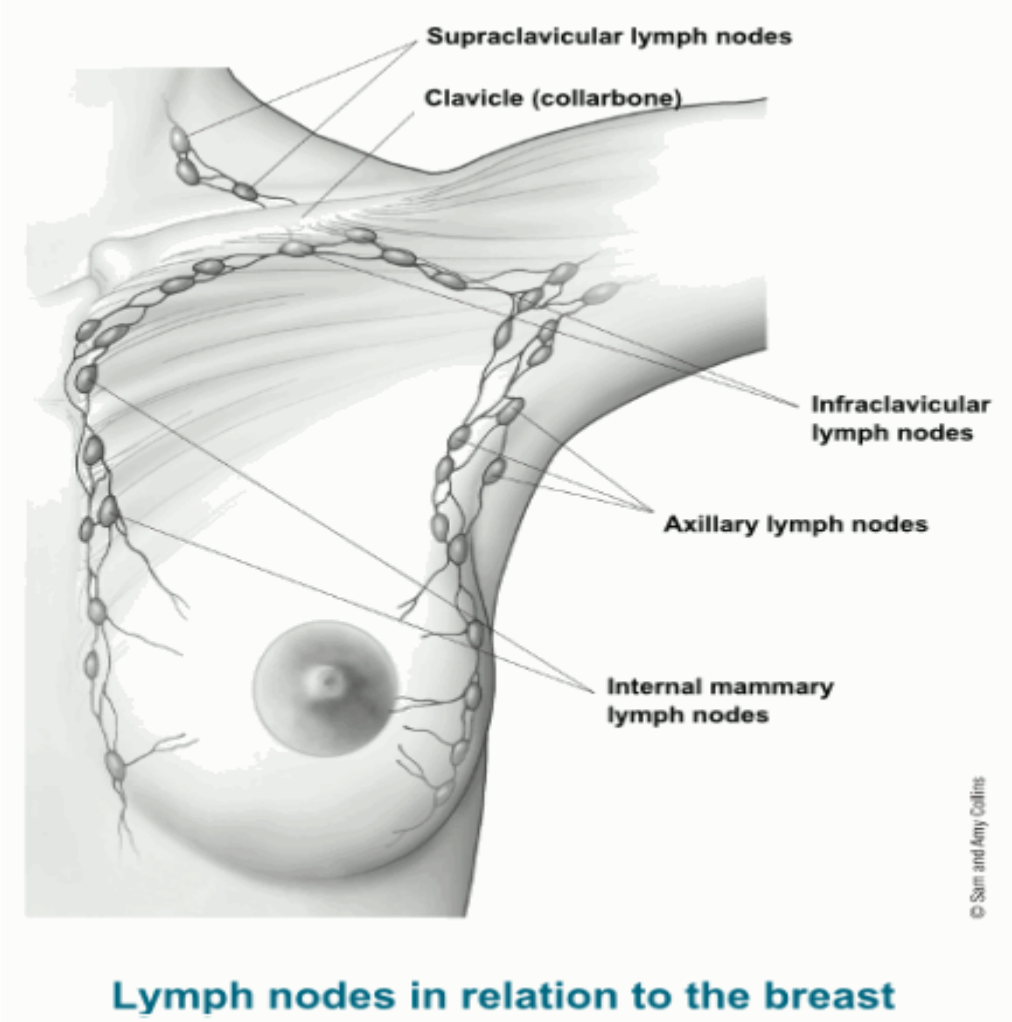

Figure 2.3. Lymph nodes in relation to the breast (American Cancer Society, 2015)

Because of this, finding cancer in one or more lymph nodes often affects the treatment plan. Still, not all men with cancer cells in their lymph nodes develop metastases to other areas, and some men can have no cancer cells in their lymph nodes and later develop metastases (American Cancer Society, 2015). Breast cancers are classified by several grading systems. Each of these influences the prognosis and can affect treatment response. Description of a breast cancer optimally includes all of these factors. (Viale, 2012). The breast is a hormone-responsive organ by excellence (American Cancer Society, 2015).

2.4. Classification of Breast Cancer

Breast cancer is a highly heterogeneous disease due to its diverse morphological features, the variable clinical outcome and the response to different therapeutic options. It is therefore necessary to devise a clinically meaningful classification of the disease, which has to be scientifically sound, clinically useful and widely reproducible. The established histopathological classification has a limited clinical utility, due to insufficient prognostic and predictive power. More recent classification schemes, based on the immunohistochemical characterization of breast cancer for the assessment of hormone receptor status, HER2 gene over-expression or amplification and the proliferative fraction or on gene expression profiles, correlate much better with the clinical outcome and may be used to inform the choice of the systemic therapy (Viale, 2012).

Research has divided breast cancer into categories according to different schemes, each based on different criteria and serving a different purpose. The major categories are the histopathological type, the grade of the tumor, the stage of the tumor, and the expression of proteins and genes. As knowledge of cancer cell biology develops these classifications are updated (American Cancer Society, 2015). The purpose of classification is to select the best treatment. The effectiveness of a specific treatment is demonstrated for a specific breast cancer (usually by randomized, controlled trials). That treatment may not be effective in a different breast cancer. Some breast cancers are aggressive and life-threatening, and must be treated with aggressive treatments that have major adverse effects. Other breast cancers are less aggressive and can be treated with less aggressive treatments, such as lumpectomy. U Treatment algorithms rely on breast cancer classification to define specific subgroups that are each treated according to the best evidence available. Classification aspects must be carefully tested and validated, such that confounding effects are minimized, making them either true prognostic factors, which estimate disease outcomes such as disease-free or overall survival in the absence of therapy, or true predictive

factors, which estimate the likelihood of response or lack of response to a specific treatment (Gonzalez-Angulo et al., 2007). Classification of breast cancer is usually, but not always, primarily based on the histological appearance of tissue in the tumor. A variant from this approach, defined on the basis of physical exam findings, is that inflammatory breast cancer (IBC), a form of ductal carcinoma or malignant cancer in the ducts is distinguished from other carcinomas by the inflamed appearance of the affected breast, which correlates with increased cancer aggressivity (Arunkumar, 2012).

Although breast cancer has much different histology, the considerable majorities of breast cancers are derived from the epithelium lining the ducts or lobules, and are classified as mammary ductal carcinoma. *Carcinoma in situ* is proliferation of cancer cells within the epithelial tissue without invasion of the surrounding tissue. In contrast, *invasive carcinoma* does not confine itself to the initial tissue compartment but invades the surrounding tissue (Allred, 2010; American Cancer Society, 2016). Perineural and/or lymphovascular space invasion is usually considered as part of the histological description of a breast cancer, and when present may be associated with more aggressive disease.

2.4.1. Grade

A pathologist also assigns a grade to the cancer, which is based on how closely the biopsy sample looks like normal breast tissue and how rapidly the cancer cells are dividing. The grade can help predict a woman's prognosis. In general, a lower grade number indicates a slower-growing cancer that is *less* likely to spread, while a higher number indicates a faster-growing cancer that is *more* likely to spread (American Cancer Society, 2016). The tumor grade is one factor in deciding if further treatment is needed after surgery. Histologic tumor grade (sometimes called the Bloom-Richardson grade, Nottingham grade, Scarff-Bloom Richardson grade, or Elston-Ellis grade) is based on the arrangement of the cells in relation to each other: whether they form tubules; how closely they resemble normal breast cells (nuclear grade); and how many of the cancer cells are in the process of dividing (mitotic count). This system of grading is used for invasive cancers but not for in situ cancers (American Cancer Society, 2015).

Grade 1 (well differentiated) low grade cancers have relatively normal-looking cells that do not appear to be growing rapidly and are arranged in small tubules. Grade 2 (moderately differentiated) intermediate grade cancers have features between grades 1 and 3. Grade 3 (poorly differentiated) high grade cancers, the highest grade, lack normal features and tend to grow and

spread more aggressively (American Joint Committee on Cancer, 2010, American Cancer Society, 2016). Grading compares the appearance of the breast cancer cells to the appearance of normal breast tissue. Normal cells in an organ like the breast become differentiated, meaning that they take on specific shapes and forms that reflect their function as part of that organ. Cancerous cells lose that differentiation. In cancer, the cells that would normally line up in an orderly way to make up the milk ducts become disorganized. Cell division becomes uncontrolled. Cell nuclei become less uniform.

2.4.2. Stages of Breast Cancer

The prognosis (forecast or outcome) of invasive breast cancer is strongly influenced by the stage of the disease – that is, the extent or spread of the cancer when it is first diagnosed. There are two main staging systems for cancer. The TNM classification of tumors uses information on tumor size and how far it has spread within the breast (T), the extent of spread to the nearby lymph nodes (N), and the presence or absence of distant metastases (spread to distant organs) (M) (Edge et al., 2010). Once the T, N, and M are determined, a stage of 0, I, II, III, or IV is assigned, with stage 0 being in situ, stage I being early stage invasive cancer, and stage IV being the most advanced disease. The TNM staging system is commonly used in clinical settings. Larger size, nodal spread, and metastasis have a larger stage number and a worse prognosis (Edge *et al*., 2010). The main stages are:

Stage 0 is a pre-cancerous or marker condition, either ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS). Stages 1–3 are within the breast or regional lymph nodes. Stage 4 is 'metastatic' cancer that has a less favorable prognosis (American cancer society 2015). Stage is usually expressed as a number on a scale of 0 through IV — with stage 0 describing non-invasive cancers that remain within their original location and stage IV describing invasive cancers that have spread outside the breast to other parts of the body (American Joint Committee on Cancer, 2010). Thus Cancer stage is based on four characteristics: the size of the cancer, whether the cancer is invasive or non-invasive, whether cancer is in the lymph nodes and whether the cancer has spread to other parts of the body beyond the breast.

Breast cancer stages may also be referred to as Local: The cancer is confined within the breast. Regional: The lymph nodes, primarily those in the armpit, are involved. Distant: The cancer is found in other parts of the body as well. Sometimes the term “locally advanced” or “regionally advanced”may be used to refer to large tumors that involve the breast skin, underlying chest

structures, changes to the breast's shape, and lymph node enlargement that is visible or that a doctor can feel during an examination (Voduc *et al*., 2010) . The cancer's stage is one of the most important factors in determining prognosis and treatment options. Staging is the process of finding out how widespread a cancer is when it is diagnosed. A staging system is a standardized way for the cancer care team to summarize information about how far a cancer has spread. The most common system used to describe the stages of breast cancer is as described by the American Joint Committee on Cancer (AJCC) (Edge *et al*., 2010).

2.4.3. Benign Breast Tumour

Some types of benign breast conditions are linked to breast cancer risk and this condition is often categorized into 3 general groups, reflecting the degree of risk: nonproliferative lesions, proliferative lesions without atypia (abnormal cells or patterns of cells), and proliferative lesions with atypia. Nonproliferative lesions are not associated with overgrowth of breast tissue and have little to no effect on breast cancer risk. Examples of nonpro- liferative lesions include fibrosis (also known as fibrocystic changes), simple cysts, and mild hyperplasia. Proliferative lesions without atypia are associated with a small increase in the risk of breast cancer (1.5 to 2 times the risk of those who do not have one of these lesions) and include non-atypical (or usual) ductal hyperplasia and fibroadenoma (Hartmann *et al*., 2005, Kabat *et al*., 2010). Proliferative lesions with atypia are associated with the greatest breast cancer risk – 4 to 5 times higher than average risk.55,56,58 These include atypical ductal hyperplasia (ADH) and atypical lobular hyperplasia (ALH). Women should keep detailed records of any benign breast biopsy results, as this information is valuable for risk assessment, screening, and counseling for chemoprevention and risk-reduction strategies (Hartmann *et al*., 2005, Kabat *et al*., 2010).

2.4.3.1 Fibrosis and cysts

Most lumps turn out to be caused by fibrosis and/or cysts, benign changes in the breast tissue that happen in many women at some time in their lives. (This is sometimes called fibrocystic changes and used to be called fibrocystic disease.) Fibrosis is the formation of scar-like (fibrous) tissue, and cysts are fluid-filled sacs. These conditions are most often diagnosed by a doctor based on symptoms, such as breast lumps, swelling, and tenderness or pain. These symptoms tend to be worse just before a woman's menstrual period is about to begin. Her breasts may feel lumpy and, sometimes, she may notice a clear or slightly cloudy nipple discharge.

2.4.3.2. Fibroadenomas and Intraductal Papillomas

Benign breast tumors such as fibroadenomas or intraductal papillomas are abnormal growths, but they are not cancerous and do not spread outside the breast to other organs. They are not life threatening. Still, some benign breast conditions are important because women with these conditions have a higher risk of developing breast cancer. For more information see the section "What are the risk factors for breast cancer?" and our information on Non-cancerous Breast Conditions (Hartmann *et al*., 2005 Kabat *et al*., 2010).

2.5. Types of Breast Cancer

There are several types of breast cancer, but some of them are quite rare. In some cases a single breast tumor can be a combination of these types or be a mixture of invasive and in situ cancer.

2.5.1. Ductal Carcinoma in situ

Ductal carcinoma in situ (DCIS); also known as intraductal carcinoma) is considered non-invasive or pre-invasive breast cancer. DCIS means that cells that lined the ducts have changed to look like cancer cells. The difference between DCIS and invasive cancer is that the cells have not spread (invaded) through the walls of the ducts into the surrounding breast tissue. DCIS is considered a pre-cancer because some cases can go on to become invasive cancers. Right now, though, there is no good way to know for certain which cases will go on to become invasive cancers and which ones won't. About 1 in 5 new breast cancer cases will be DCIS. Nearly all women diagnosed at this early stage of breast cancer can be cured (Solin *et al*., 2013, American Cancer Society, 2015). It is the most common type of in situ breast cancer, accounting for about 83% of in situ cases diagnosed during 2006-2010. DCIS may or may not progress to invasive cancer; in fact, some of these tumors grow so slowly that even without treatment they would not affect a woman's health. Studies suggest that about one-third, and possibly more, of DCIS cases will progress to invasive cancer if left untreated.1 Identifying subtypes of DCIS that are most likely to recur or progress to invasive cancer is an active area of research (Solin *et al*., 2013).

2.5.2. Lobular Carcinoma In situ

Lobular carcinoma in situ (LCIS, also known as lobular neoplasia) is not a true cancer or precancer, but an indicator of increased risk for developing invasive cancer. LCIS is much less common than DCIS, accounting for about 12% of female in situ breast cancers diagnosed during 2006-2010 (Solin *et al*., 2013). This uncommon condition is the result of abnormal cells forming in the lobules or milk-producing glands of the breast. Although LCIS seldom becomes invasive

cancer, women with LCIS are 7 to 12 times more likely to develop invasive cancer in either breast than women without LCIS. Lobular Carcinoma In-situ is not usually apparent on a mammogram and is typically discovered during a biopsy per- formed for another reason, such as an abnormal mammogram. Pure LCIS should be distinguished from DCIS and pleomorphic LCIS, as both of these conditions are considered precursor lesions for breast cancer and require cancer-directed therapy (Breen *et al*., 2008).

2.5.3. Invasive or Infiltrating Breast Cancer

Most breast cancers are invasive, or infiltrating. These cancers have broken through the ductal or glandular walls where they originated and grown into surrounding breast tissue. The prognosis (forecast or outcome) of invasive breast cancer is strongly influenced by the stage of the disease – that is, the extent or spread of the cancer when it is first diagnosed (Breen *et al*., 2008).

2.5.3.1 Invasive (or infiltrating) Ductal Carcinoma

This is the most common type of breast cancer. Invasive (or infiltrating) ductal carcinoma (IDC) starts in a milk duct of the breast, breaks through the wall of the duct, and grows into the fatty tissue of the breast. At this point, it may be able to spread (metastasize) to other parts of the body through the lymphatic system and bloodstream. About 8 of 10 invasive breast cancers are infiltrating ductal carcinomas (Breen *et al*., 2008).

2.5.3.2 Invasive (or infiltrating) Lobular Carcinoma

Invasive lobular carcinoma (ILC) starts in the milk-producing glands (lobules). Like IDC, it can spread (metastasize) to other parts of the body. About 1 invasive breast cancer in 10 is an ILC. Invasive lobular carcinoma may be harder to detect by a mammogram than invasive ductal carcinoma (Breen *et al*., 2008). This uncommon type of invasive breast cancer accounts for about 1% to 3% of all breast cancers. Usually there is no single lump or tumour. Instead, inflammatory breast cancer (IBC) makes the skin on the breast look red and feels warm, retracted nipple, ridges in skin, extensive discoloration of the skin. It also may give the breast skin a thick, pitted appearance that looks a lot like an orange peel texture around the nipple. Researchers now know that these changes are not caused by inflammation or infection, but by cancer cells blocking lymph vessels in the skin. The affected breast may become larger or firmer, tender, or itchy. In its early stages, inflammatory breast cancer is often mistaken for an infection in the breast (called mastitis) and treated as an infection with antibiotics. If the symptoms are caused by cancer, they will not improve, and a biopsy will find cancer cells

(Schlichting *et al*., 2012). Because there is no actual lump, it might not show up on a mammogram, which can make it even harder to find it early. This type of breast cancer tends to have a higher chance of spreading and a worse outlook (prognosis) than typical invasive ductal or lobular cancer. For more details about this condition, see our document, Inflammatory Breast Cancer (Dawood *et al*., 2011). Other types of breast cancers are:

2.5.4. Paget Disease of the Nipple

Paget disease of the breast (also known as Paget disease of the nipple and mammary Paget disease) is a rare type of cancer involving the skin of the nipple and, usually, the darker circle of skin around it, which is called the areola. Most people with Paget disease of the breast also have one or more tumors inside the same breast. These breast tumors are either ductal carcinoma in situ or invasive breast cancer (Caliskan *et al*., 2008). The symptoms of Paget disease of the breast are often mistaken for those of some benign skin conditions, such as dermatitis or eczema (Caliskan *et al*., 2008). These symptoms may include the following: Itching, tingling, or redness in the nipple and/or areola; flaking, crusty, or thickened skin on or around the nipple; A flattened nipple; Discharge from the nipple that may be yellowish or bloody (Kanitakis, 2007).

2.5.5. Phyllodes Tumour

Phyllodes tumor represents a broad range of fibroepithelial diseases and presence of an epithelial component with stromal components differentiates the phyllodes tumor from other stromal sarcomas (Liang *et al*., 2008, Mishra *et al*., 2013). They make up 0.3 to 0.5% of female breast tumors and have an incidence of about 2.1 per million, the peak of which occurs in women aged 45 to 49 years. The tumor is rarely found in adolescents and the elderly. They have been described as early as 1774, as a giant type of fibroadenoma (Mishra *et al*., 2013). It was believed to be benign until 1943, when Cooper and Ackerman reported on the malignant biological potential of this tumor in 1981 (Mishra *et al*., 2013). The World Health Organization adopted the term phyllodes tumor and as described by Rosen subclassified them histologically as benign, borderline, or malignant according to the features such as tumor margins, stromal overgrowth, tumor necrosis, cellular atypia, and number of mitosis per high power field.

The T stage of a breast cancer can range from T1 to T4, but all inflammatory breast cancers are T4. N stages range from N1 to N3. The clinical N stage is based on the presence of cancer in lymph nodes on the same side as the breast cancer under the arm (called axillary lymph nodes), around the collarbone (called supraclavicular or infraclavicular lymph nodes), or inside the chest

(called internal mammary lymph nodes). M stages are M0 and M1. If the cancer has not spread outside the breast and nearby lymph nodes it is M0. If the cancer has spread it is M1. (Schlichting *et al*., 2012).

2.5.6. Molecular Subtypes of Breast Cancer

Breast cancer is increasingly considered to be not one disease, but a group of diseases distinguished by different molecular subtypes, risk factors, clinical behaviors, and responses to treatment. Distinct molecular subtypes of breast cancer have been identified using gene expression profiles, a process that is both complex and costly. Molecular subtypes are increasingly being used for research purposes; however, questions remain about their usefulness to further tailor breast cancer treatments and predict breast cancer prognosis. Despite significant advances in diagnosing and treating breast cancer, several major unresolved clinical and scientific problems remain. These are related to (a) prevention (who needs it and when), (b) diagnosis (we need more specific and sensitive methods), (c) tumor progression and recurrence (what causes it and how to predict it), (d) treatment (who should be treated and how), and (e) therapeutic resistance (how to predict, prevent, and overcome it). Resolving all these problems is complicated by the fact that breast cancer is not a single disease but is highly heterogeneous at both the molecular and clinical level (Polyak, 2007; Lee 2016).

The St. Gallen International Expert Consensus of 2011 proposes a new classification system for breast cancer based on its division into five subgroups. The criteria to identify these subtypes were recently refined at the 2013 Conference (Inic *et al*., 2014). More convenient approximations of molecular subtypes have been identified using biological markers, including the presence or absence of estrogen receptors (ER+/ ER-), progesterone receptors (PR+/PR-), and human epidermal growth factor receptor 2 (HER2+/HER2-). The St Gallen International Expert Consensus proposed a new intrinsic biological classification system based on the expression of the estrogen receptor (ER), progesterone receptor (PgR), HER2 and Ki-67 (indicator of a large proportion of actively dividing cells) (Goldhirsch *et al*., 2011, Yanagawa *et al*., 2012). The classification system categorizes invasive breast carcinomas into the following five distinct molecular subtypes; as follows: luminal A (ER+, PgR+ or PgR-, HER2-, and low Ki-67 index); luminal B (HER2 -) (ER+, PgR+ or PgR-, HER2-, and high Ki-67 index); Luminal B (HER2+) (ER+, PgR+ or PgR-, and HER2+), HER2 (ER-, PgR- and HER2+), and basal-like (Triple Negative) (ER-, PgR-, and HER2-) (Inic et al., 2014). Determining the status of estrogen and progesterone receptors, HER2 amplification and Ki-67 antigen expression is practical and

valuable for estimating the patient prognosis and for determination of the treatment strategy. The classification can be performed in every pathological laboratory where Immuno histo-chemistry (IHC) with a simple panel of markers is possible, and this approach is designated as the IHC-based classification (Prat and Perou, 2011, Yanagawa *et al*., 2012). The clinical value of this classification system is still ongoing world wide.

The moderate to strong expression of Progesterone Receptor (PR) and Ki-67 level were both recognized as being important to the surrogate definition of a "Luminal A-like" disease (Inic *et al*., 2014). A higher Ki-67 index has been found to correlate significantly with young age, large tumors, positive lymph nodes, negative ER/PR, p53 overexpression, and positive HER2. A higher Ki-67 index has also been found to correlate with a poorer prognosis and early recurrence (<2 years). On the other hand, a lower Ki-67 index has correlated with a favorable prognosis and late recurrence (>10 years). Thus, proliferative activity as determined by Ki-67 may reflect the aggressive behavior of breast cancer and predict the time of recurrence and the appropriate therapy required in treatment. It is therefore important to take the Ki-67 index into consideration in the treatment and follow-up of breast cancer patients (Nishimura *et al*., 2010).

About 40% of breast cancers are luminal A, making it the most common breast cancer subtype. The Luminal A subtype was defined as ER and/or PR positive and HER2 negative. If the Ki-67 labeling index and/or nuclear grade was determined, the Luminal A subtype was also defined to a low Ki-67 labeling index (<14%) and/or nuclear grade 1 or 2. ER and PR positivity was confirmed by immunohistochemistry (IHC); greater than 1% of tumor cells staining positive was considered to be positive. A HER2-negative status was confirmed by the IHC (with 0, 1+, and 2+ scores indicating no cells stained, <10% of cells have membrane staining and >10% of cells with a low or medium membrane staining, respectively). Tumors that were 2+ by IHC were also examined by fluorescence in situ hybridization (with an amplification ratio <2.0, indicating a negative status). The Ki-67 labeling index determined by IHC shows the Ki-67 antigen staining in both its ER- and PR-positive sections (Inic *et al*., 2014). Luminal A tumors are associated with the most favorable short-term prognosis, in part because expression of hormone receptors are predictive of a favorable response to hormonal therapy, however, long-term survival is similar to or even lower than some other subtypes. Although the vast majority of ER-positive tumors show strong immunoreactivity, approximately 20% of tumors exhibit variable ER expression. At the 2013 Conference, the conclusion was made that Luminal A breast cancer treatment, in most cases, can be successfully treated with endocrine therapy alone, as well as in

cases of multiple positive nodes where chemotherapy may be supplemented to treatment. Moreover, while Luminal A patients are less responsive to chemotherapy, this treatment may be supplemented by endocrine therapy in cases of high bulk disease (eg, multiple positive nodes). In this respect, when patients with hormone receptor (HR)-positive tumors were treated with adjuvant tamoxifen, their risk for the composite outcome of recurrence or death was reduced by more than 30% (Berry *et al*., 2005, Inic et al., 2014). However, many patients with lymph node positive, ER-positive breast tumors, gain minimal benefit from adjuvant chemotherapy (Inic et al., 2014).

Like luminal A breast cancers, luminal B breast cancers are ER+ and/or PR+ and are further defined by being highly positive for Ki67 (indicator of a large proportion of actively dividing cells) or HER2. About 10% of breast cancers are ER+ and/or PR+ and HER2+. Luminal B breast cancers tend to be higher grade and more aggressive than luminal A breast cancers (Parise and Caggiano, 2014).

About 10% to 20% of breast cancers are basal-like, and the majority of basal-like breast cancers are referred to as "triple negative" because they are ER-, PR- and HER2-. Basal-like tumors are more common in African American women, premenopausal women, and those with a BRCA1 gene mutation (Reddy, 2011, Howlader *et al*., 2014). Women diagnosed with basal-like breast cancer have a poorer short-term prognosis than those diagnosed with other breast cancer types because there are no targeted therapies for these tumors. The basal-like subtype appears to overlap substantially with the population of breast cancers that are "triple negative" (ER/progesterone receptor (PR) and HER2 negative) and high grade but also are associated with characteristic histologic features such as solid-pushing borders, geographic areas of necrosis, and dense lymphocytic infiltrates (Foulkes *et al*., 2010). Cancers with a basal-like molecular profile can be identified with high specificity (but only about 75% sensitivity) by the following immunohistochemical profile: ER and PR negative, HER2 negative, and cytokeratin (CK) 5/6 or epidermal growth factor receptor (EGFR) positive (although other variations of this panel have also been used) (Badve *et al*., 2011, Kimberly, 2011).

The subtypes, originally defined by gene-expressing profiling, were also recently demonstrated as valid groupings using different platforms, including genomic DNA copy number arrays, DNA methylation, exome sequencing, microRNA sequencing, and reverse-phase protein assays (Kimberly, 2011). At the most basic level, the luminal subtypes share expression of estrogen

receptor (ER)–related genes and have better overall survival than the HER2-related and the basal-like subtypes, which are typically (but not uniformly) ER negative (Kimberly, 2012, Howlader *et al*., 2014). There is clinical interest in distinguishing the luminal B cancers from luminal A cancers because they may be a subset of ER-positive cancers that derive benefit from more aggressive therapy. However, it is also acknowledged that the differences between these 2 groups are largely based on differences in proliferation-related genes and, rather than representing distinct subtypes of breast cancer, are more likely 2 ends of a spectrum of ER-positive disease (Reis-Filho and Pusztai 2011).

2.6. Female Sex Hormones Receptor Status.

The importance of steroid hormone receptors to the biology of breast cancer was recognized over 40 years ago, when it was observed that radiolabeled estrogens concentrated preferentially in the estrogen-influenced target organs of both animal and human breast cancers. These findings gave rise to the concept of an estrogen receptor (ER). It has since become clear that human breast cancers are dependent upon estrogen and/or progesterone for growth and that this effect is mediated through ERs and progesterone receptors (PRs). Not surprisingly, ERs and PRs are both overexpressed in malignant breast tissue (Hammond *et al*., 2010). Three classes of sex hormones exist: androgens (mainly testosterone), estrogens (which is mainly 17beta-estradiol in the ovarian cycle), and progesterone. In males, plasma testosterone concentrations are relatively stable throughout life, although testosterone production declines with age. In females, the production of sex hormones 17beta-estradiol and progesterone fluctuates during the menstrual cycle. In response to pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH), fluctuations in sex hormone concentrations in the menstrual cycle include increasing 17beta-estradiol, but low progesterone plasma concentrations in the follicular phase and high plasma 17beta-estradiol and progesterone concentrations in the luteal phase (Bhatia *et al*., 2014). If pregnancy occurs, luteolysis is prevented and 17b-E2 and progesterone levels remain high. Later in life (menopause), with the depletion of follicles, sex hormone concentrations drop to very low levels. In oral contraceptive (OCC) users, the progestin component suppresses luteinizing hormone secretion, while the estrogenic component suppresses follicular stimulating hormone (FSH) secretion preventing selection and emergence of a dominant follicle and ovulation. Therefore, naturally 17b-E2 and progesterone plasma concentrations are low during oral contraceptive use, however, at the end of the pill free period the 17b-E2 concentration is comparable with the concentration, which characterizes the early follicular phase (Bhatia *et al*., 2014). New insights into hormone receptor biology and the increasing array of proteins that can

modify their function have already translated into better therapies for breast cancer. As an example, a number of drugs that interact with the receptor, including selective ER modulators (SERMs) and selective ER downregulators (SERDs), are approved for the treatment and prevention of breast cancer. At the same time, improved methods for assaying receptor proteins have led to less expensive and simpler measurements of ER and PR. Unfortunately, however, these methods have been fraught with variation that has resulted in inaccurate test results. Since these tests guide the use of endocrine therapy in patients with breast cancer, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) convened a panel to address causes of variation related to measurement of ER and PR by immunohistochemistry (IHC) in 2010 (Hammond *et al*., 2010).

Receptors are proteins in or on certain cells that can attach to certain substances, such as hormones, that circulate in the blood. Normal breast cells and some breast cancer cells contain receptors that attach to estrogen and progesterone. Breast cancer cells have these receptors on their surface and in their cytoplasm and nucleus. Chemical messengers such as hormones bind to receptors, and this causes changes in the cell. Breast cancer cells may or may not have three important receptors: estrogen receptor (ER), progesterone receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER-2). Estrogen Receptor+ (ER+) cancer cells (that is, cancer cells that have estrogen receptors) depend on estrogen for their growth, so they can be treated with drugs to block estrogen effects (e.g. tamoxifen), and generally have a better prognosis. Untreated, HER2+ breast cancers are generally more aggressive than HER2- breast cancers, (Sotiriou and Pusztai 2009, Kumar and Abbas, 2010).

But HER2+ cancer cells respond to drugs such as the monoclonal antibody trastuzumab (in combination with conventional chemotherapy), and this has improved the prognosis significantly (Romond *et al*., 2005). Cells that do not have any of these three receptor types (estrogen receptors, progesterone receptors, or HER2) are called triple-negative, although they frequently do express receptors for other hormones, such as androgen receptor and prolactin receptor (Foulkes *et al*., 2010, Hudis and Gianni 2011). Cancer cells may contain neither, one, or both of these receptors. Breast cancers that have estrogen receptors are often referred to as *ER-positive* (or ER+) cancers, while those containing progesterone receptors are called *PR-positive* (or PR+) cancers (American Cancer Society, 2015). If either type of receptor is present, the cancer is said to be *hormone receptor-positive*. Hormone receptor–positive breast cancers tend to grow more slowly and are much more likely to respond to hormone therapy than breast cancers without

these receptors. All breast cancers should be tested for these hormone receptors either on the biopsy sample or when they are removed with surgery. About 2 of 3 breast cancers have at least one of these receptors. This percentage is higher in older women than in younger women (American Cancer Society, 2015).

2.7. Female Sex Hormone and Breast Cancer Development

2.7.1. Oestrogen

Oestrogen is a female hormone that is mainly produced by the ovaries. It serves many purposes including helping to regulate the menstrual cycle, preserving bone density and increasing uterine growth. An imbalance in oestrogen levels can cause many problems and symptoms including abnormal bleeding, infertility menopause and cancer (Files *et al*., 2011). A simple blood, urine or saliva sample can be evaluated to determine estrogen levels. Oestrogen, a group of hormones, is produced in the pituitary gland and is mainly responsible for the development of female sex organs. It consists of three fractions: Oestrone (E_1), which mainly occurs after menopause; oestradiol (E_2), which is produced in the ovaries and is responsible for ovulation and affects conception and pregnancy; and estriol (E_3), which commonly occurs during pregnancy. These three factions are all measured during oestrogen testing (Files *et al*., 2011). Thus, estradiol is the most important oestrogen in non-pregnant females who are between the menarche and menopause stages of life. However, during pregnancy this role shifts to oestriol, and in postmenopausal women estrone becomes the primary form of estrogen in the body. Another type of oestrogen called oestetrol (E4) is produced only during pregnancy. All of the different forms of oestrogen are synthesized from androgens, specifically testosterone and androstenedione, by the enzyme aromatase. The steroid 17β-oestradiol is the most potent and prevalent endogenous oestrogen, but several metabolites of oestradiol also have oestrogenic hormonal activity. Synthetic oestrogens are used as part of some oral contraceptives, in oestrogen replacement therapy for postmenopausal women, and in hormone replacement therapy for women. Like all steroid hormones, oestrogens readily diffuse across the cell membrane. Once inside the cell, they bind to and activate oestrogen receptors (ERs) which in turn modulate the expression of many genes. Additionally, oestrogens bind to and activate rapid-signaling membrane oestrogen receptors (mERs) (Micevych and Kelly 2012; Soltysik and Czekaj, 2013), such as GPER (GPR30) *(*Prossnitz *et al*., 2007). The normal range of oestrogen varies depending upon the patient's age. Typically a women aged 20 to 29 will have an average level of 149 pg/ml (pictograms per milliliter). Females aged 30 to 39 will have an average level of 210 pg/ml. And those over 40 but not in menopause will have an average level of 152pg/ml. These average

levels can vary day to day depending on each female's menstrual cycle. Estrogen levels are considered low when the range is 10 to 20 pg/ml (Files *et al*., 2011). This can be caused by menopause, anorexia and Turner Syndrome. Extreme endurance exercise also can reduce estrogen levels. Some of the symptoms of low estrogen levels are fatigue, hot flashes, night sweats, vaginal dryness and difficulty concentrating. Low levels of estrogen can leave you feeling depleted and exhausted (Files *et al*., 2011). High Estrogen Levels above 200 pg/ml, are considered high. Oestrogen levels can be elevated because of obesity, cardiovascular disease and digestive problems. Stress also can contribute to a high oestrogen level. Symptoms of high oestrogen levels are anxiety, depression, mood swings and insomnia (Mahor *et al*., 2015). It's important to reduce levels of oestrogen to normal levels because high levels of oestrogen also are associated with breast and uterine cancer (Mahor *et al*., 2015). Multiple lines of evidence support a central role of hormones in the etiology of breast, endometrial and ovarian cancers. Evidence of an association between circulating hormones and these cancers varies by both hormone and cancer site, with the most consistent associations observed for sex steroid hormones and breast cancer risk among postmenopausal women (Eliassen and Hankinson, 2008). Various endogenous and exogenous sex hormones play a role in the etiology of breast cancer. Oestrogen has a proliferative effect on breast tissue. They are related to increased mitotic activity and believed to influence rather than an initiating effect. The proliferation of cells is essential for carcinogenesis because the risk of errors during DNA replication is increased during cell division. Excess endogenous or exogenous estrogens can enhance risk by stimulating proliferation of epithelial cells of breast that have undergone partial malignant transformation (Mahor *et al*., 2015; Roy and Vadlamudi 2012).

The genomic actions of oestrogens are mediated via oestrogen receptors (ERs), which are proteins that bind estrogens with high affinity and specificity. These receptors are members of a family of nuclear hormone receptors that include receptors that bind other steroids, thyroid hormone, and retinoids, and receptors such as peroxisome proliferator-activated receptor (PPAR), farnesoid X receptor (FXR), and liver X receptor (LXR) that mediate metabolic processes, as well as many "orphan" receptors for which no ligands have been identified. All these receptors function as ligand-modulated nuclear transcription factors (Brélivet et al., 2012). Two classes of estrogen receptor exist: ER, which is a member of the nuclear hormone family of intracellular receptors, and GPR30, which is a member of the rhodopsin-like family of G protein-coupled receptors (Menazza and Murphy, 2016).

Once activated by estrogen, the ER is able to translocate into the nucleus and bind to DNA to regulate the activity of different genes (i.e. it is a DNA-binding transcription factor). However, it also has additional functions independent of DNA binding (Gibson and Saunders, 2012, Menazza and Murphy, 2016). There are two different forms of the estrogen receptor, usually referred to as α and β, each encoded by a separate gene (*ESR1* and *ESR2*, respectively). Hormone-activated estrogen receptors form dimers, and, since the two forms are coexpressed in many cell types, the receptors may form ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers (Maggi, 2011, Menazza and Murphy, 2016). Oestrogen receptor alpha and beta show significant overall sequence homology, and both are composed of five domains. As signal transducer, estrogen receptors therefore do not need to be membrane-bound in order to bind with oestrogen, since oestrogen is a steroidal hormone, it can pass through the phospholipid membranes of the cell. On a genomic background, in the absence of hormone, estrogen receptors are largely located in the cytosol. Hormone binding to the receptor triggers a number of events starting with migration of the receptor from the cytosol into the nucleus, dimerization of the receptor, and subsequent binding of the receptor dimer to specific sequences of DNA known as hormone response elements. The DNA/receptor complex then recruits other proteins that are responsible for the transcription of downstream DNA into mRNA and finally protein that results in a change in cell function (Alluri *et al*., 2014). Oestrogen receptors can also act none genomically by associating with the cell surface membrane and can be rapidly activated by exposure of cells to estrogen (Alluri *et al*., 2014).

The impact of oestrogens on breast cancer etiology has been well established. A positive association between the breast cancer risk and the reproductive factors such as early menarche, late menopause, nulliparity, and postmenopausal obesity seems to indicate that oestrogens influence breast cancer risk. Higher serum oestradiol levels have also been observed in breast cancer cases than in controls. In addition, evidence linking the circulating oestrogen levels to breast cancer risk have also been reported both in the retrospective and in the prospective studies (Gloyeske *et al*., 2015) . Thus, the higher levels of estrogens as well as the longer time of estrogen exposure (high-estrogen milieu) are considered associated with the higher risk of breast cancer. It is speculated that a high-oestrogen milieu would stimulate the development of ER-positive breast cancer rather than ER-negative breast cancer because estrogens stimulate the carcinogenesis and development of breast cancer through ER. This speculation seems to be supported by the fact that women with hormone receptor therapy (HRT) are more likely to develop ER-positive breast cancer (Suba, 2013). Thus, women with high levels of serum

oestrogens are speculated to be at high risk for ER-positive breast cancer (Suba, 2013). Three main phases seem to be particularly dangerous for breast cancer initiation during the life of women (Suba 2013). Two of these are crucial periods inducing hormonal and metabolic storms, namely, adolescence (14–18 years) and the perimenopausal phase (45–55 years). Both periods present risks for overall breast cancer initiation if the biologic processes in the background become pathologic. The third, especially risky phase for breast cancer initiation is older age (over 60 years), when the hormonal and metabolic imbalance becomes stronger and the defense mechanisms against cancer initiation are debilitated (Suba 2013).

2.7.2. Progesterone

Progesterone is an ovarian steroid hormone that is essential for normal breast development during puberty and in preparation for lactation and breastfeeding. The actions of progesterone are primarily mediated by its high-affinity receptors, which include the classical progesterone receptor (PR)-A and -B isoforms, located in diverse tissues, including the brain, where progesterone controls reproductive behavior, and the breast and reproductive organs. Progestins are frequently prescribed for contraception or during postmenopausal hormone replacement therapy, in which progestins are combined with estrogen as a means to block estrogen-induced endometrial growth. The role of estrogen as a potent breast mitogen is undisputed, and inhibitors of the estrogen receptor and estrogen-producing enzymes (aromatases) are effective first-line cancer therapies. However, PR action in breast cancer is grossly understudied and remains controversial (Lange and Yee 2008). Early progesterone receptor (PR) antagonists, however, were dismissed because of severe side effects, but awareness is now increasing that progesterone is an important hormone in breast cancer. Oestrogen receptor-α (ERα) signalling and PR signalling have distinct roles in normal mammary gland biology in mice; both ERα and PR delegate many of their biological functions to distinct paracrine mediators (Brisken *et al*., 2015).

Progesterone levels increase after ovulation when the body anticipates pregnancy, and continue to rise when pregnancy is established (Brisken *et al*., 2015). Because PR is an ER target gene, it is co-expressed in the same cells, although evidence has emerged that, at least in the human breast, PR is also independently expressed (Hilton *et al*., 2014). A subset of mammary epithelial cells, in the breast that express both PR-A and PR-B also express ERs, and estrogen is usually required in order to induce the expression of PR in these ER^+ cells. For this reason, it has been difficult to separate the effects of progesterone alone from estrogen, itself a potent breast mitogen. Indeed, PR isoforms are grossly understudied relative to ER in both normal and

cancerous breast cells. Studies in ER and PR knockout mice have revealed that the concerted actions of estrogen and progesterone are required for normal mammary gland development (Feng *et al*., 2007, Lange and Yee 2008); oestrogen/ER promotes the early growth of milk ducts that invade the mammary fat pad emanating from the nipple, while oestrogen/ER and progesterone/PR isoforms are responsible for the development of the terminal end-buds. or acini located at the ends of ducts that will become the milk-producing structures in the lactating mammary gland. In contrast to the normal breast, where proliferating cells are clearly devoid of steroid hormone receptors, the majority of breast cancers (~70%) express ER and PR at the time of diagnosis. Although hormone receptor-positive tumors are often slower growing relative to receptor-negative tumors, (Lange and Yee 2008), ER^+/PR^+ breast epithelial cells may undergo an early switch to autocrine or paracrine signaling mechanisms, whereby negative controls on proliferation are somehow lifted. Tumor-promoting effects of progesterone are also observed in rodents, where chemically (7,12-Dimethylbenzanthracene)-induced carcinogenesis is enhanced/accelerated by progesterone/progestin administration. In support of this model, pharmacologically or genetically blocking RANKL delayed tumorigenesis (Schramek *et al*., 2010). Interestingly, RANKL inhibition was not effective anymore once the tumor was fully established, suggesting that the PR/RANKL axis is important specifically early in the pathogenesis of mammary carcinomas (Brisken *et al*., 2015).

2.8. Prevalence Of Breast Cancer

In 2015, an estimated 231,840 new cases of invasive breast cancer will be diagnosed among women, as well as an estimated 60,290 additional cases of in situ breast cancer in 2015, approximately 40,290 women are expected to die from breast cancer. Only lung cancer accounts for more cancer deaths in women. In 2015, about 2,350 men will be diagnosed with breast cancer and 440 men will die from the disease. More than 3.1 million US women with a history of breast cancer were alive on January 1, 2014. Some of these women were cancer-free, while others still had evidence of cancer and may have been undergoing treatment (DeSantis *et al*., 2014). To combat cancer, United States national cancer institute has undergone 2069 anticancer clinical trials, in which over 150 drug combinations have been successfully recorded against cancer (Youlden *et al*., 2014). Ductal breast cancer is the most common type of breast cancer. Worldwide, 70- 80 out of every 100 breast cancers diagnosed are of this type.

Breast cancer is considered to be the final outcome of multiple environmental and hereditary factors. Some of these factors include: i) lesions to DNA, such as genetic mutations (Cavalieri *et*

al., 2006) (ii) failure of immune surveillance (5); iii) abnormal growth factor signaling in the interaction between stromal and epithelial cells facilitating malignant cell growth; iv) inherited defects in DNA repair genes, such as BRCA1, BRCA2 and p53. Although numerous epidemiological risk factors have been identified, the cause of any individual's breast cancer is often unknown. In other words, epidemiological research provides information on the patterns of breast cancer incidence across certain populations, but not in a given individual. Oncogenes act cooperatively with other genetic or epigenetic changes (Tao *et al*., 2012).

Lyon/Geneva, 12th December 2013– The International Agency for Research on Cancer (IARC), the specialized cancer agency of the World Health Organization, released the latest data on cancer incidence, mortality, and prevalence worldwide. The new version of IARC's online database, GLOBOCAN 2012, provides the most recent estimates for 28 types of cancer in 184 countries worldwide and offers a comprehensive overview of the global cancer burden. The most commonly diagnosed cancers worldwide were those of the lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectum (1.4 million, 9.7%). The most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%), breast cancer 521,900 deaths in 2012). Projections based on the GLOBOCAN 2012 (Youlden *et al*., 2014) estimates predict a substantive increase to 19.3 million new cancer cases per year by 2025, due to growth and ageing of the global population. More than half of all cancers (56.8%) and cancer deaths (64.9%) in 2012 occurred in less developed regions of the world, and these proportions will increase further by 2025 (Jemal *et al*., 2010, Youlden *et al*., 2014, Siegel *et al*., 2015).

The incidence of breast cancer varies greatly around the world: it is lowest in less-developed countries and greatest in the more-developed countries. In the twelve world regions, the annual age-standardized incidence rates per 100,000 women are as follows: in Eastern Asia, 18; South Central Asia, 22; sub-Saharan Africa, 22; South-Eastern Asia, 26; North Africa and Western Asia, 28; South and Central America, 42; Eastern Europe, 49; Southern Europe, 56; Northern Europe, 73; Oceania, 74; Western Europe, 78; and in North America, 90 (Lindsey *et al*., 2015). Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012, and the most frequently diagnosed cancer among women in 140 of 184 countries worldwide Breast cancer alone accounts for 25% of all cancer cases and 15% of all cancer deaths among females (Ferlay *et al*., 2015). More developed countries account for about one-half of all breast cancer

cases and 38% of deaths. Rates are generally high in Northern America, Australia/New Zealand, and Northern and Western Europe; intermediate in Central and Eastern Europe, Latin America, and the Caribbean; and low in most of Africa and Asia (Lindsey *et al*., 2015). International variation in breast cancer incidence rates reflects differences in the availability of early detection as well as risk factors. There were 6.3 million women alive who had been diagnosed with breast cancer in the previous five years. Since the 2008 estimates, breast cancer incidence has increased by more than 20%, while mortality has increased by 14%. It now represents one in four of all cancers in women. "Breast cancer is also a leading cause of cancer death in the less developed countries of the world (Bray *et al*., 2013, Lindsey *et al*., 2015). This is partly because a shift in lifestyles is causing an increase in incidence, and partly because clinical advances to combat the disease are not reaching women living in these regions," says Dr David Forman, Head of the IARC Section of Cancer Information, the group that compiles the global cancer data. Generally, worldwide trends show that in developing countries going through rapid societal and economic changes, the shift towards lifestyles typical of industrialized countries leads to a rising burden of cancers associated with reproductive, dietary, and hormonal risk factors. Incidence has been increasing in most regions of the world, but there are huge inequalities between rich and poor countries. Incidence rates remain highest in more developed regions, but mortality is relatively much higher in less developed countries due to lack of early detection and access to treatment facilities. For example, in Western Europe, breast cancer incidence has reached more than 90 new cases per 100 000 women annually, compared with 30 per 100 000 in eastern Africa, (Bray *et al*., 2013). In contrast, breast cancer mortality rates in these two regions are almost identical, at about 15 per 100 000, which clearly points to a later diagnosis and much poorer survival in Eastern Africa. An urgent need in cancer control today is to develop effective and affordable approaches to the early detection, diagnosis, and treatment of breast cancer among women living in less developed countries. It is critical to bring morbidity and mortality in line with progress made in recent years in more developed parts of the world (Ferlay *et al*., 2015). Breast cancer in African continent was characterized by regional variation as the incidence was 27% of cancers in North African countries (Algeria and Egypt) compared with 15% in sub- Saharan Africa (Parkin *et al*, 2006) In the North-Western geopolitical zone of Nigeria, cancer of the breast was second to cancer of the cervix, while at University College Hospital (UCH), Ibadan (situated in the South-Western geopolitical zone of Nigeria) it was the leading malignancy among women (Afolayan, 2008, Ogunbiyi *et al*, 2010).

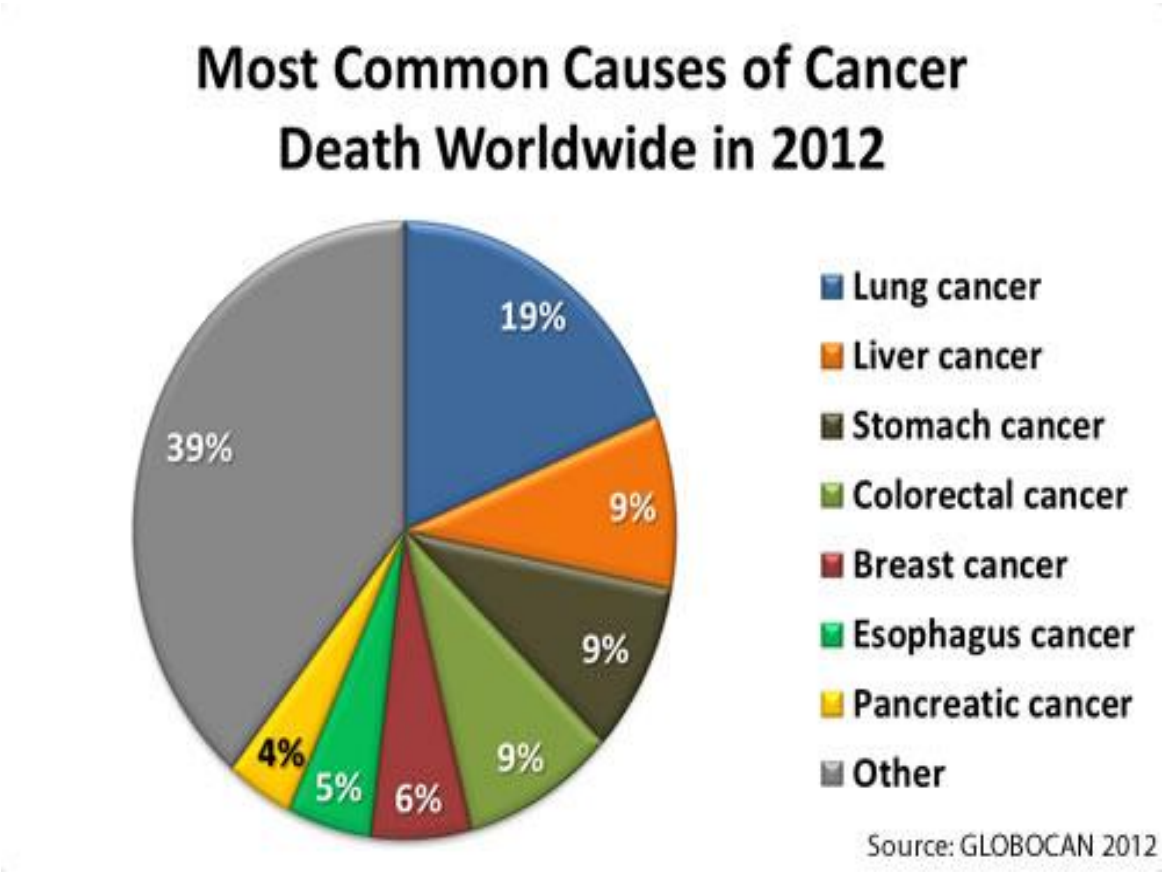

Figure 2.4. Most Common Causes of Cancer Death Worldwide (Ferlay *et al*., 2015).

In the North central geopolitical zone, breast cancer constituted 22.41% of new cancer cases registered in 5 years and accounted for 35.41% of all cancers in women (Jeddy-Agba *et al*, 2012). In developing or low income countries, breast cancer was characterized by late clinical presentation and in advance stage of the disease, when only chemotherapy and palliative care could be offered, and therefore associated with high mortality (Afolayan *et al*., 2012, Parkin *et al*, 2006).

Unfortunately there is paucity of data and sparse literature review on the trends of breast cancer in Nigeria due to few existing cancer registries most of which are either hospital-based or pathology based instead of the preferred population-based cancer registries. However, in low resource countries, hospital-based cancer registry has been serving as a fundamental source of information on cancer. Ilorin cancer registry, hospital-based, began active registration of cancer in 1997. In Nigeria, most cases presented during the late stage of the disease (Afolayan *et al*., 2012). In some previous studies, breast cancer is on top of the list of cancers among females and the commonest cancer when both sexes are combined (Jeddy-Agba *et al*, 2012). Up to 568 cases of breast cancer are registered over the period of ten-years representing approximately 57 cases per annum. Whereas in the published data on cancer from population-based Ibadan cancer registry at University College Hospital, Ogunbiyi et al (2010) reported 2225 new cases in breast cancer in 4 years giving annual incidence of 556 cases (Ogunbiyi *et al*, 2010). Thus, the disparity may be associated with improved diagnostic facilities (fine needle aspiration, USS, CT scan, and availability of manpower which hitherto that are not available before), apart from the use of hospital-based cancer registry data in this study which has a wider scope of sources of information (data) on cancer than the surgical pathology register. This also further demonstrates the justification for establishment of Population-Based Cancer Registry (PBCR) which covers a defined geopolitical area. From all indications, there is no existing data on the trend of breast cancer in South Eastern part of Nigeria. Jeddy-Agba *et al*., (2012) reported that the commonest cancers in Nigeria in 2009 to 2010 were breast and cervical cancer among women and prostate cancer among men. Breast cancer has been reported in male but it is very rare. Out of 1313 cases of breast cancer that presented over the period of a 12 year review, 26 were males giving male percentage to be 2% (Ezeome et al., 2010). They found significant increase in the incidence of breast cancer compared to historical records while the incidence of cervical cancer was relatively stable. There was very little disparity in the cancer incidence reported by registries in the northern and southern parts of the country regardless of differences in ethnicity and level of urbanization. Data suggests that the incidence of breast cancer in Nigeria has risen significantly.

The age standardized incidence rates for breast cancer in the period 1960–1969 was 13.7 per 100 000 and it rose to 24.7 per 100 000 by 1998–1999 – more or less a doubling of incidence over 4 decades or approximately 25% increase in incidence per decade (Jeddy-Agba *et al*., 2012). With incidence in 2009 to 2010 at 54.3 per 100 000, this represents a 100% increase in the last decade. The incidence rate of breast cancer in Nigeria in 2010 was higher than the GLOBOCAN 2008 estimate of 38.7 per 100 000 (Ferlay *et al*., 2015), although the GLOBOCAN estimate is for the whole country and for a different time period. The increase in cancer incidence in women may be both apparent and real. Some of the increase noted may result from improved diagnosis, better case finding and improved access to care. Despite this, some of the increase in incidence may be real due to increasing prevalence of risk factors for these cancers in populations that hitherto had low incidence (Forouzanfar *et al*., 2011, Jeddy-Agba *et al*., 2012).

2.9. Pathophysiology of Breast Tumour

Carcinoma arises from a single cell with a genome that either contains an inherited aberration (oncogene) or has acquired one as a consequence of spontaneous mutation or damage by a chemical toxin (carcinogen), radiation, viral infection, chronic inflammation or other external assaults (Kumar *et al*., 2015). The development of cancer can be divided into three distinct steps: initiation, promotion and progression (Kumar *et al*., 2015). The concept of the tumour microenvironment recognizes that the interplay between cancer cells and stromal cells is a crucial determinant of cancer growth. Research has shown that the tumour microenvironment is built through rate-limiting steps during multistage carcinogenesis (Barcellos-Hoff *et al*., 2013). Construction of a 'precancer niche' is a necessary and early step that is required for initiated cells to survive and evolve; subsequent niche expansion and maturation accompany tumour promotion and progression, respectively. As such, cancer niches represent an emergent property of a tumour that could be a robust target for cancer prevention and therapy (Barcellos-Hoff *et al*., 2013). During the initiation step, Extrinsic factors induce cells to acquire mutations by Epigenetic effects, and/or the activation of oncogenes, thereby providing mutant cells with a growth and survival advantage. However, these initial mutations are not sufficient for full neoplastic progression; tumour promotion and progression depend on signals that originate from non-mutant cells in the tumour microenvironment (Kumar *et al*., 2015). Oncogenes may also act cooperatively with other genetic or epigenetic changes. Breast cancer and other malignancies result from stepwise genetic alterations of normal host cells, and from other nongenetic (or epigenetic) changes in the behavior of not only malignant cells but also host cells that interact with the tumor, such as immune, vascular, and stromal cells.

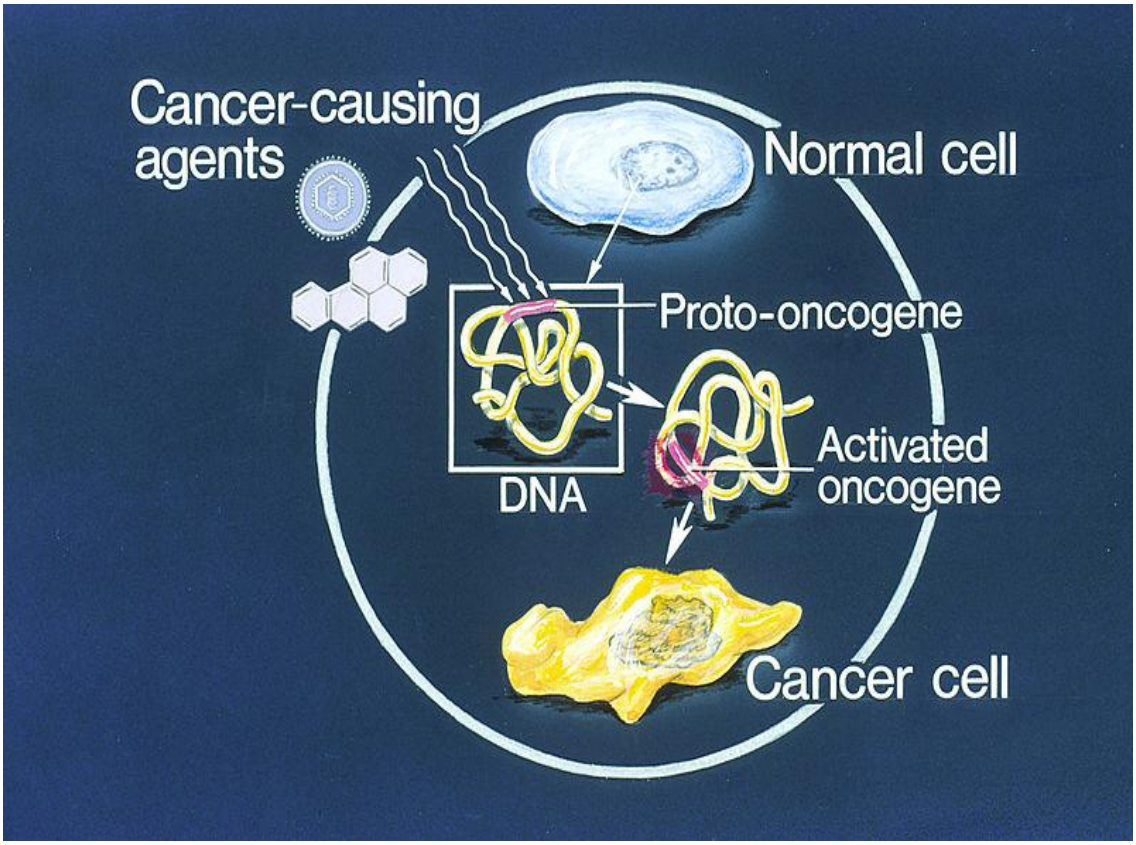

Figure 2.5: Normal cell damage and cancer development (Kumar *et al*., 2015).

One important factor to keep in mind is that breast (mammary) tissue has increased opportunity for DNA damage occurrence because of the extensive remodeling that occurs throughout a woman's life. Breasts are one of the few organs in the body that undergo precisely defined cell death and cellular proliferation on a moderate to large scale during *in utero* development, puberty, monthly pre-menopausal 28-day cycles, during pregnancy, lactation and involution (weaning-induced process of massive mammary remodeling) (Rijnkels *et al*., 2010, McCready *et al*., 2010). Thus, throughout a woman's lifetime her mammary tissue is undergoing proliferation, apoptosis and differentiation at a rate higher than most other tissues. Problems in DNA structure and function might accumulate in puberty due to a failure of cells to mature (differentiate). DNA damage can be detected even post-pubertal in animal models when female animals are exposed to certain foods either pre-pubertal or *in utero* (Hilakivi-Clarke *et al*., 2007, Olivo-Marston *et al*., 2008). Other common problems in the developing mammary gland might be a failure of fidelity in DNA replication or a failure of cells to die where appropriate during the rapid bodily growth and development that occurs during puberty. During pregnancy, the mammary environment changes at a very rapid pace (Radisky and Hartmann, 2009, McCready *et al*., 2010) and because of extensive proliferation, DNA replication is likely to produce mutations during this process. In adulthood, the mammary gland undergoes monthly hormonal fluctuations inducing cycles of proliferation, senescence and apoptosis. With the busy environment of the mammary gland it is no wonder that breast cancer is the most common female malignancy and many women (up to 1 in 5) will develop breast cancer some time in their life (McCready *et al*., 2010). However, it remains to be determined which dietary and environmental exposures may present the most DNA damage in early life or adulthood, and whether this accumulation of DNA damage is directly related to breast cancer risk. DNA assaults during these times of mammary remodeling may be endogenous (replication stress, oxidative species, replication errors), environmental (chemical exposures, food contaminants, naturally occurring endocrine disruptors in foods) or simply due to the process of aging which in itself can produce increased susceptibility to chromosomal abnormalities because of reduced expression of enzymes which protect the ends of chromosomes (telomerase). Because of the enormous activity of the mammary tissue, it is easy to understand that-of all the bodily tissues-it may be one of the most susceptible to DNA damage and one with the most need for intact, sensitive DNA damage response systems.

Cell division is a physiological process that occurs in almost all tissues and under many circumstances. Under normal circumstances, the balance between proliferation and programmed

cell death, usually in the form of apoptosis, is maintained by regulation of both processes to ensure the integrity of tissues and organs. Mutations and epimutations in DNA that lead to cancer (only certain mutations and epimutations can lead to cancer and the majority of potential mutations and epimutations will have no such effect) disrupt these orderly processes by disrupting the programming regulating the processes. Carcinogenesis is caused by mutation and epimutation of the genetic material of normal cells, which upsets the normal balance between proliferation and cell death. This results in uncontrolled cell division and the evolution of those cells by natural selection in the body. The uncontrolled and often rapid proliferation of cells can lead to benign tumors; some types of these may turn into malignant tumors (cancer). Benign tumors do not spread to other parts of the body or invade other tissues, and they are rarely a threat to life unless they compress vital structures or are physiologically active, for instance, producing a hormone. Malignant tumors can invade other organs, spread to distant locations (metastasis) and become life-threatening.

2.10. Genetic Alterations

Cancer critical genes are the genes that are responsible for causing cancer which contributes to the process of transformation where elevated cell proliferation, insensitivity to cell death and differentiation has been observed. Such genes drive the processes of cell cycle progression, cell differentiation, DNA repair and cell death (Garraway and Landeremail, 2013). These cancer critical genes can be classified into two broad categories:

Oncogenic: Genes that require gain of function for causing cancer that involve proto-oncogenes, metastatic genes and angiogenic genes while on the other hand, Anti-oncogenic: Genes that require loss of function for causing cancer that involve tumor suppressor genes and apoptotic genes. Moreover, mutations in cancer critical genes could be dominant or recessive, for instance proto-oncogene possess dominant mutation i.e. require mutation only in single copy for its effect while tumor suppressor genes exhibit recessive mutation i.e. they require mutation in both copies of genes for its inactivation (Kumar *et al*., 2015). Proto-oncogenes: These are the genes that control the normal process of cell growth and division. Proto-oncogenes encoded proteins are generally growth and transcriptional factors, growth factor receptors and signal transducers that govern the process of cell proliferation and suppressed cell death. Proto-oncogenes upon functional gain mutation become oncogenic in nature and thus cause cancers. E.g. EGFR, myc, src, Wnt etc. (Kumar *et al*., 2015). Metastatic genes: These are the genes that impart invasion and dissemination property to the cancerous cell. E.g. matrix metalloproteinases (MMP2/9),

cyclooxygenase (COX2) etc (Kumar *et al*., 2015). Angiogenic genes: These are the genes that aid and drive the formation of new blood vessels. In response to the growth factors secreted by cancerous cell, neovascularization take place around the tumor to provide oxygen and nutrients for tumor cell proliferation. E.g. vascular endothelial growth factor **(**VEGF), PDGF etc (Kumar *et al*., 2015). Tumor suppressor genes: In general tumor suppressor genes are known to restrain the cell division rate and thus have a role in preventing tumor progression. They often undergo mutational inactivation in cancerous cell. E.g. Protein 53 (p53), phosphatase and tensin homolog deleted on chromosome (PTEN), breast cancer type 1 and 2 (BRCA1/2) etc (Kumar *et al*., 2015). Apoptotic genes: These are the genes that govern programmed cell death of unwanted, damaged or dangerous cells in multicellular organisms. E.g. B cell lymphoma-2 associated protein X (Bax, anti-apoptotic), B cell lymphoma-2 homologous antagonist (Bak, pro-apoptotic) etc. There is an intricate interplay between these genes which signals for either cell proliferation or inhibition of cell proliferation and secrete chemical signal for tumour initiation and progression.

Breast cancer in humans is associated with genetic alterations of a number of oncogenes such as receptor tyrosine-protein kinase (ErbB2), myelocytomatosis Viral Oncogene Homolog (MYC) Phosphatidylinositol 3-Kinase, Catalytic, Alpha (PIK3CA) and tumor suppressors (TP53, BRCA1/2, PTEN *PTEN* (phosphatase and tensin homolog deleted on chromosome), (Perera and Bardeesy, 2012). Karyotypic and epidemiological analyses of mammary tumors at various stages suggest that breast carcinomas become increasingly aggressive through the stepwise accumulation of genetic changes. The majority of genetic changes found in human breast cancer fall into two categories: gain-of-function mutations in proto-oncogenes, which stimulate cell growth, division, and survival; and loss-of-function mutations in tumor suppressor genes that normally help prevent unrestrained cellular growth and promote DNA repair and cell cycle checkpoint activation (van Haaften *et al*., 2009, Dalgliesh *et al*., 2010;). In addition, the involvement of noncoding RNAs in tumorigenesis and tumor metastasis has been recently documented (Croce, 2008; Shimono *et al*., 2009).

Being the most common malignancy among females, 5%–10% of breast cancer cases are hereditary and are caused by pathogenic mutations in the considered reference BRCA1 and BRCA2 genes (Apostolou and Fostira, 2013). As sequencing technologies evolve, more susceptible genes have been discovered and BRCA1 and BRCA2 predisposition seems to be only a part of the story. These new findings include rare germline mutations in other high

penetrant genes, the most important of which include TP53 mutations in Li-Fraumeni syndrome, Serine/threonine kinase 11 (STK11) mutations in Peutz-Jeghers syndrome, and PTEN mutations in Cowden syndrome. Furthermore, more frequent, but less penetrant, mutations have been identified in families with breast cancer clustering, in moderate or low penetrant genes, such as Checkpoint kinase 2 tumour suppressor gene (CHEK2), Ataxia telangiectasia mutated (ATM), Partner and localizer of BRCA2 **(**PALB2), and BRCA1-interacting protein 1 (BRIP1) (Apostolou and Fostira, 2013). *BRCA1* and *BRCA2* mutations are associated with a significantly elevated risk for breast and ovarian cancers. Rare germ-line mutations in *TP53* (the gene encoding p53) and *PTEN* are associated with high risk for various cancers, including breast cancer. Germ-line mutations in *ATM, CHK2,* Nibrin *(NBS1), RAD50, PALB2,* and *BRIP* all moderately increase breast cancer risk. Among these tumor suppressors, ATM and CHK2 are kinases involved in the DNA damage response. RAD50 is a component of protein complex critical to DNA double-stranded-break end processing. *PALB2* encodes a BRCA2-interacting protein and *BRIP1* encodes a BRCA1-interacting protein with DNA helicase activity. These genes are also mutated in pancreatic, prostate, and other tumors. Tumors develop because of the loss-of-heterozygosity mutations in the remaining normal allele plus other somatic mutations. For example, *BRCA1*-driven breast cancers frequently harbor somatic mutations in *TP53* and *PTEN* (Holstege *et al*., 2009; Apostolou and Fostira, 2013).

2.11. Epigenetic Alteration

2.11.1. Epigenesis

Studies have begun to evaluate the role of epigenetics in tumor development. The epigenome is a multitude of chemical compounds that can tell the genome what to do (Gómez-Díaz *et al*., 2012). The human genome is the complete assembly of DNA (deoxyribonucleic acid)-about 3 billion base pairs - that makes each individual unique. DNA holds the instructions for building the proteins that carry out a variety of functions in a cell. The epigenome is made up of chemical compounds and proteins that can attach to DNA and direct such actions as turning genes on or off, controlling the production of proteins in particular cells (Gómez-Díaz *et al*., 2012). When epigenomic compounds attach to DNA and modify its function, they are said to have "marked" the genome. These marks do not change the sequence of the DNA. Rather, they change the way cells use the DNA's instructions. The marks are sometimes passed on from cell to cell as cells divide (Bannister and Kouzarides 2011). They also can be passed down from one generation to the next. Epigenetics is the inheritance of information on the basis of gene expression rather then direct changes to sequence composition. Epigenetic alterations include methylation of cytosine

and guanine (CpG) dinucleotides in promoters and changes in chromatin structure that may lead to silencing of tumor suppressor genes. Activation of oncogenes may also be a result of epigenetic changes through post-translational modifications in histone or DNA conformation (Blanca *et al*., 2011). Few areas in biology attract as much current attention and yet require as much presentation as the field of epigenetics. The term "epigenetics" was first used by Waddington to describe the process through which genotypes give rise to phenotypes during development (Weinhold, 2006). Since then, there has been a burgeoning interest in the field of epigenetics that has been coupled with a diversification in the use of the term: epigenetics means different things to the different fields of biology, and even within a given field, different authors may use it in somewhat different contexts, generating a great deal of confusion in the process. Epigenetics: "Epi" from Greek meaning "above or over genetics" Something that effects the Phenotype without changing the Genotype (Weinhold, 2006). A change in phenotype that is heritable but does not involve a change in nucleotide sequence in DNA; that is, a change in genotype. The term has evolved to include any process that alters gene activity without changing the DNA sequence, and leads to modifications that can be transmitted to daughter cells (Weinhold, 2006). Broadly speaking, epigenetics refers to stimuli-triggered changes in gene expression due to processes that arise independent of changes in the underlying DNA sequence. These changes may involve chemical modifications of the DNA itself, such as DNA methylation or modifications of proteins that are closely associated with DNA, such as the histones that bind and compact DNA into chromatin packages (Bannister and Kouzarides 2011; Gómez-Díaz *et al*., 2012). Research has shown that all recognized epigenetic marks (including DNA methylation, histone modification, and microRNA (miRNA) expression) are influenced by environmental exposures, including Infectious agents, diet, tobacco, alcohol, stress, genetic factors, which play important roles in the etiology of cancer. Some of these epigenetic modifications change the expression of tumor suppressor genes and oncogenes and, therefore, may be causal for tumorigenesis (Gómez-Díaz *et al*., 2012). Certain circumstances in life can cause genes to be silenced or expressed over time. In other words, they can be turned off (becoming dormant) or turned on (becoming active). What we eat, where we live, who we interact with, when we sleep, how we exercise, even aging – all of these can eventually cause chemical modifications around the genes that will turn those genes on or off. Tumourigenesis is a multistep process resulting from the accumulation of genetic alterations such as mutations, rearrangements and copy number variations, but also epigenetic alterations such as promoter methylation and histone modification (Dworkin *et al*., 2009, Holms *et al*., 2010).

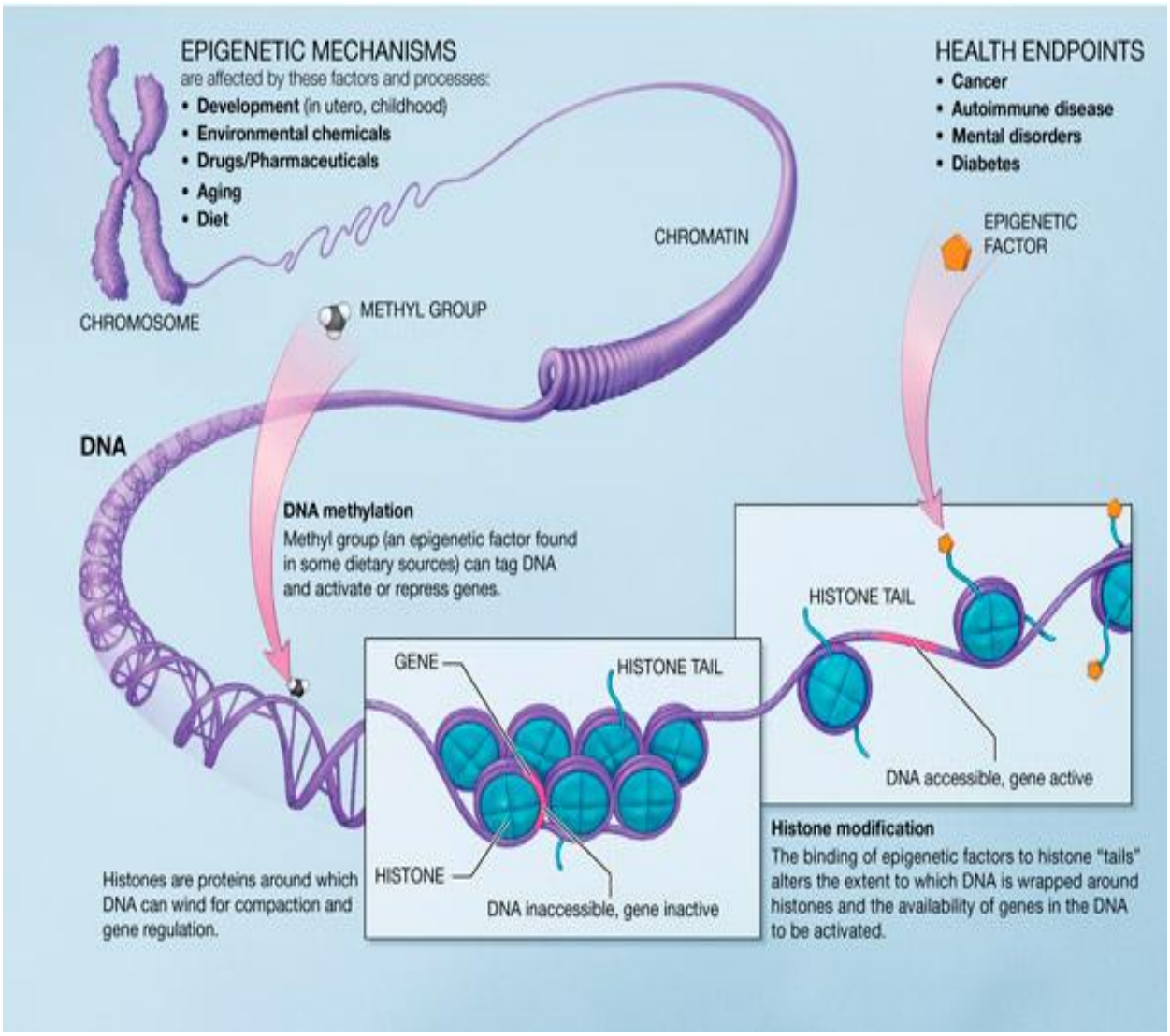

Figure 2.6: Epigenetic mechanism (DNA methylation) (Blanca *et al*., 2011).

2.11.2 DNA Methylation

DNA methylation is an epigenetic mark that has suspected regulatory roles in a broad range of biological processes and diseases. The technology is now available for studying DNA methylation genome-wide, at a high resolution and in a large number of samples (Bock, 2012). Evidence for the genetic predisposition to certain cancers exists, specifically because germ-line mutations of the DNA of an individual are inherited by future generations. These genetic predispositions to cancers are referred to as familial forms of cancer. There are instances in which non-familial cases of these cancers result (sporadic cancer) and the underlying cause has been suggested to involve epigenetic silencing. The epigenetic mark that has been most highly studied is DNA methylation, the presence of methyl groups at CpG dinucleotides. These dinucleotides are often located near gene promoters and associate with gene expression levels. Early studies indicated that global levels of DNA methylation increase over the first few years of life and then decrease beginning in late adulthood (Jones *et al*., 2015).

DNA methylation plays an essential role in development, chromosomal stability, and for maintaining gene expression states. DNA methylation occurs when methyl groups are added to cytosines in CpG dinucleotides, leading to a closed chromatin conformation and gene silencing. CpGs are often found at increased frequencies in promoter regions, forming CpG islands. Hypermethylation of CpG islands affects genes involved in cell cycle control, DNA repair, cell adhesion, signal transduction, apoptosis and cell differentiation (Holms et al., 2010). In tumour cells, local promoter hypermethylation is often accompanied by global hypomethylation. This results in more global patterns of methylation as compared with mutation spectra, which differ greatly in extent and patterns between tumours (Holms *et al*., 2010). In vertebrates, the cytosine/guanine (CG) dinucleotide sequence is a principal target of DNA methylation because it is preferentially recognized by vertebrate DNA methyltransferases (DNMT). Cytosine/guanine is the only dinucleotide sequence that contains a cytosine that is a palindrome and could be copied during cell division by a semiconservative DNMT from the parental strand onto the daughter strand (Szyf, 2012). Thus, changes introduced into the DNA methylation pattern either stoichastically or as an organized response to developmental or environmental signals could be maintained and memorized through DNA replication cycles. This is a mechanism through which a transient exposure to an environmental agent could result in lasting impact on DNA methylation and as a consequence on the phenotype (National Institute of Health, 2005). However, recent data including genomic sequencing suggest that DNA methylation occurs in other dinucleotide sequences in addition to CG in undifferentiated cells (Lister *et al*; 2011).

Different breast cancer subtypes are thought to arise through different “evolutionary paths” reflecting distinct patterns of mutated cancer genes (Stephens et al., 2012, Curtis et al., 2012). Less is known about the contribution of epigenetic changes to the development of biologically distinct breast cancer subtypes.

Breast Cancer is associated with different genetic and epigenetic events, such as DNA strand integrity, gene amplifications, gene mutations, DNA methylation, and microsatellite abnormalities. These alterations detected in the primary tumor may also be found in plasma/serum cfDNA of patients with breast cancer (Schwarzenbach and Pantel, 2015). DNA methylation is a biochemical process that is important for normal development in higher organisms. It involves the addition of a methyl group to the 5 position of the cytosine pyrimidine ring to form 5-methylcytosine. This modification can be inherited through cell division (Iqbal *et al*., 2011). DNA methylation occurs at cytosine bases located 5′ to a guanosine, so-called CpG dinucleotides. 5-Methylcytosine is an epigenetic modification formed by the action of DNA methyltransferases (Szyf, 2012).

CpG islands or CG islands are genomic regions that contain a high frequency of CpG sites. The "p" in CpG refers to the phosphodiester bond between the cytosine and the guanine, which indicates that the C and the G are next to each other in sequence, (Iqbal *et al*., 2011). The C-G nucleotide pairs are relatively sparse in the genome, and areas of comparatively high CpG density are referred to as CpG islands, identified as regions > 200 base pair (bp) with a > 50% G+C content and 0.6 observed/expected ratio of CpGs (Illingworth & Bird, 2009, Jones *et al*., 2015). These islands tend to be less methylated compared to non island CpGs and are often associated with gene promoters, while the regions immediately surrounding CpG islands are referred to as ‘shores’, followed by ‘shelves’. Approximately 60–70% of genes have a CpG island associated with their promoters, and promoters can be classified according to their CpG density (Weber *et al*., 2007, Jones *et al*., 2015). Levels of DNA methylation at a promoter-associated CpG island are generally negatively associated with gene expression, although some specific genes show the opposite effect (Lam *et al*., 2012; Gutierrez Arcelus *et al.,* 2013). Interestingly, this negative correlation is not upheld when comparing expression and DNA methylation for a specific gene across individuals (Gutierrez Arcelus *et al.,* 2013; Wagner *et al.,* 2014). Conversely, DNA methylation in the gene body is often positively associated with levels of gene expression (Gutierrez Arcelus *et al.,* 2013). Changes in DNA methylation occur throughout the lifetime, beginning at conception. Early studies assessing levels of DNA

methylation both globally and at specific regions observed age-associated changes. Kwabi-Addo *et al.,* (2007), examined DNA methylation through assessment of global methylcytosine/cytosine ratios by immune, colorimetric, and HPLC analyses, and occasionally by assaying DNA methylation at repetitive elements. Based on these early studies, it was hypothesized that DNA methylation was not accurately maintained over cell divisions, resulting in a gradual loss and increase in variability over the lifespan. This phenomenon has been referred to as 'epigenetic drift' (Jones *et al*., 2015). DNA methylation also functions to repress repetitive elements, such as *Alu* and long interspersed nucleotide element-1 *(LINE-1)*. They are non-coding genomic repetitive sequences and methylation of these elements can be used as a surrogate marker for genome-wide methylation status, which are generally highly methylated in the human genome. More works have shown that both Alu and LINE-1 repetitive elements exhibit decreased DNA methylation levels and increased variability with age (Bollati *et al.,* 2009; Talens *et al*., 2012). Unmethylated CpGs are often grouped in clusters called *CpG islands*, which are found in the proximal promoter region (regulatory regions) of over half of human genes (Jones *et al*., 2015). In many disease processes, such as cancer, gene promoter CpG islands acquire abnormal hypermethylation, which results in transcriptional silencing that can be inherited (Jones *et al*., 2015). In genetics, a promoter is a region of DNA that initiates transcription of a particular gene. Cytosine guanine (CpG) dinucleotides often are located in the regions of DNA that regulate DNA expression (promoter regions) (Daura *et al*., 2009). Unlike CpG sites in the coding region of a gene, in most instances the CpG sites in the CpG islands of promoters region are unmethylated (Feil and Berger, 2007). This observation led to the speculation that methylation of CpG sites in the promoter of a gene may inhibit gene expression (Jones *et al*., 2015). All of these factors and processes can have an effect on people's health and influence their health possibly resulting in cancer, autoimmune disease, mental disorders, or diabetes among other illnesses. Heritable and reversible mechanisms known as epigenetic alterations do not require direct alterations of DNA sequences, but they can be responsible for modifying gene expression and are related to cancer development.1-6Although the genetic information provides the sequence for protein synthesis, the epigenetic information provides instructions on how, where, and when the genetic information will be used. Szyf, (2012), came up with the following hypothesis: given that DNA methylation changes are plausibly critical components of the molecular mechanisms involved in breast cancer, breast cancers as a group and specific subtypes of breast cancer might be expected to show distinct DNA methylation states. These DNA methylation states could serve as diagnostic tools in breast cancer care. The important questions are: 1) Are the changes in DNA methylation in breast cancer limited to a narrow set of candidate

genes? 2) Could DNA methylation states serve as early predictors of breast cancer? 3) Could DNA methylation states provide information regarding the stage of breast cancer? 4) Could DNA methylation states provide tools for prognosis and stratification for different therapeutic approaches? (Szyf, 2012).

The location and distribution of 5mCs across the genome have important implications for understanding the roles of DNA methylation (Jones, 2012). In mammalian genomes CpGs are unevenly distributed: they are depleted on a global scale but enriched at short CpG-rich DNA stretches known as CpG islands (CGIs), which are preferentially located at transcription start sites of gene promoters (Deaton and Bird, 2011, Tania *et al*., 2014). In normal cells, cytosines within CGIs are generally protected from DNA methylation, in contrast to the vast majority of CpGs, which are usually methylated (that is, at non-coding regions and repetitive elements). Flanking regions of CGIs (±2 kilobases), referred to as CGI shores, show tissue-specific DNA methylation and are associated with gene silencing.

Global DNA methylation, also referred to as genomic DNA methylation, is the overall content of 5-methyl cytosine (5-mC) in the genome. Accumulating evidence suggests that global levels of DNA methylation are lower in tumor tissue when compared with adjacent tissue, indicating a significant role of DNA hypomethylation in cancer progression (Lissette *et al*., 2012). To investigate whether global DNA methylation in CpG sites can distinguish between normal and pancreatic cancer. It clearly shows that the normal and cancerous tissues can be separated by the global DNA methylation status of these CpG sites. This demonstrates that methylation profiles can be used to distinguish normal samples from cancers (Tan *et al*., 2009). Many studies have showed that methylation patterns are severely disrupted in human tumors. Aberrant hypermethylation of CpG islands in promoter regions represents one of the most frequent epigenetic events associated with gene silencing in cancer (Tan *et al*., 2009). Global DNA hypomethylation is likely caused by methyl-deficiency due to variety of environmental influences, and has been proposed as a molecular marker in multiple biological processes such as cancer. It is well demonstrated that the decrease in global DNA methylation is one of the most important characteristics of cancer. Thus, the quantification of global methylation in cancer cells could provide very useful information for detection and analysis of this disease. Epigenetic damage, such as aberrant DNA methylation, aberrant histone modification and miRNA expression is well recognized as a major driving force in cancer development and progression (Jeong *et al*., 2014). Through alterations in the accessibility of antigen receptor gene segment by

recombination activation gene (RAG) endonuclease, a misnomer may occur in the process of arranging for RAG endonuclease to have access to antigen receptor gene segments (Helmink and Sleckman, 2011). In this regard, *cis*-acting transcriptional regulatory elements such as promoters and enhancers that promote or enhance this accessibility, may bring about alterations in DNA methylation, chromatin structure, and nuclear positioning that affect the ability of RAG to access the appropriate regions of antigen receptor loci. This modulation of accessibility is likely important not only for the regulation of antigen receptor gene assembly but also for promoting genomic stability by limiting the generation of unnecessary RAG-mediated double strand breaks (DSBs) that could be resolved aberrantly (Helmink and Sleckman, 2011). Normal DNA methylation is required to enable gene expression in on and off position. To obtain a malignant phenotype, the cell needs to acquire genetic or epigenetic mutations to trigger transformation. This malignant phenotype must then be maintained. The inflammatory response in cancer tissues play an important role in maintaining the phenotype by inducing tumor tissue remodeling, angiogenesis, and metastasis; while suppressing the innate anticancer immune response (Queil and Joyce 2013).

There are two main mechanisms through which changes in DNA methylation relate to cancer initiation and progression (Kulis and Esteller., 2010). One is the silencing of expression of tumor suppressor genes by increased methylation of their promoter regions. The other one is general (global) hypomethylation of the genome that leads to genomic instability by activation of transposable elements, viral sequences and genes thus referred to as global DNA methylation. Genomic instability is an established hallmark of cancer progression and is characterized by increased mutation rates due to specific events such as the mutation of important gatekeeper genes and genome-wide events such as large chromosomal rearrangements (Lissette *et al*., 2012). Most epidemiologic studies of global methylation have focused on measuring overall 5-mC content or DNA methylation levels in retrotransposable. A transposable element (TE or transposon) is a DNA sequence that can change its position within the genome, sometimes creating or reversing mutations and altering the cell's genome size. Transposition often results in duplication of the TE) elements such as Long interspersed nucleotide element-1 **(**LINE-1) and Arthrobacter luteus (Alu) elements (Wilhelm *et al*., 2010, Cash *et al*., 2012). Some of these early studies considered LINE-1 and Alu as surrogate epigenetic measures of global DNA methylation levels because these elements are the most abundant retrotransposons in the human genome and their silencing is regulated by epigenetic mechanisms (Lissette *et al*., 2012). However, further research has indicated that DNA methylation levels at these sequences are not correlated with

global 5-mC content (Wu *et al*., 2011) and that these elements may be differentially associated with disease outcomes and lifestyle factors (Nelson *et al*., 2011). There are currently several different methods used to measure global DNA methylation in epidemiologic studies, and new assays for large samples are under development (Nelson *et al*., 2011).

In normal cells, repetitive genomic sequences reffered to as global (e.g., centromeric satellite α-DNA and juxtacentromeric satellite DNA) are heavily methylated (Lo and Sukumar, 2008). The maintenance of methylation in this repetitive DNA could be important for the protection of chromosomal integrity by preventing chromosomal rearrangements, translocations and gene disruption through the reactivation of transposable elements. Besides hypermethylation of gene-associated CpG islands, hypomethylation of repetitive genomic DNA has also been identified as a specific feature in human cancers (Lo and Sukumar, 2008). Although less well studied than DNA hypermethylation, several lines of investigation indicate that the global DNA hypomethylation identified in cancer cells might contribute to structural changes in chromosomes, loss of imprinting (LOI), micro satellite and chromosome instability through aberrant DNA recombination, aberrant activation of proto-oncogene expression and increased mutagenesis (Lo and Sukumar, 2008). Global genomic hypomethylation in breast cancer has been known to correlate with some clinical features such as disease stage, tumor size and histological grade. Some proto-oncogenes implicated in proliferation and metastasis (e.g., synuclein γ and urokinase genes) or drug resistance to endocrine therapy (e.g., *N-cadherin*, *ID4*, annexin *A4*, β-catenin and *WNT11* genes) have been found to be upregulated in breast cancer through the hypomethylation of their promoters (Lo and Sukumar, 2008)

CpG-island-containing gene promoters are usually unmethylated in normal cells to maintain euchromatic structure, which is the transcriptionally active conformation allowing gene expression. However, during cancer development, many of these genes are hypermethylated at their CpG-island-containing promoters to inactivate their expression by changing open euchromatic structure to compact heterochromatic structure (Jones and Baylin, 2007). These genes are selectively hypermethylated in tumorigenesis for inactivation owing to their functional involvement in various cellular pathways that prevent cancer formation (Lehmann *et al*., 2008).

DNA hypomethylation was the initial epigenetic abnormality recognized in human tumors. However, for several decades after its independent discovery by two laboratories in 1983, it was often ignored as an unwelcome complication, with almost all of the attention on the

hypermethylation of promoters of genes that are silenced in cancers (e.g., tumor-suppressor genes). Because it was subsequently shown that global hypomethylation of DNA in cancer was most closely associated with repeated DNA elements, cancer linked-DNA hypomethylation continued to receive rather little attention. DNA hypomethylation in cancer can no longer be considered an oddity, because recent high-resolution genome-wide studies confirm that DNA hypomethylation is the almost constant companion to hypermethylation of the genome in cancer, just usually (but not always) in different sequences. Methylation changes at individual CpG dyads in cancer can have a high degree of dependence not only on the regional context, but also on neighboring sites.

DNA methylation depends upon the availability of methyl groups from *S*-adenosylmethionine, which is derived from methionine (Niculescu and Zeisel, 2002). Transmethylation metabolic pathways closely interconnect choline, methionine and THF. The pathways intersect at the formation of methionine from homocysteine. Perturbing the metabolism of one of these pathways results in compensatory changes in the others. Tetrahydrofolate (THF), Choline, methionine and folate metabolism interact at the point that homocysteine is converted to methionine. Betaine: homocysteine *S*-methyltransferase catalyzes the methylation of homocysteine using betaine as the methyl donor (Niculescu and Zeisel, 2002). In an alternative pathway, 5-methyltetrahydrofolate: homocysteine *S*-methyltransferase regenerates methionine using a methyl group derived de novo from the one-carbon pool. Methionine adenosyltransferase converts methionine to SAM (the active methylating agent for DNA methylation) Niculescu and Zeisel, 2002).

Perturbing the metabolism of one of the methyl donors reveals the intermingling of these metabolic pathways. Total hepatic folate content decreased by 31–40% after 2 weeks on a choline-deficient diet in rats. This effect was reversible by refeeding choline. Tetrahydrofolate deficiency, induced by treatment with methotrexate or induced by dietary folate deficiency, resulted in diminished hepatic total choline, with the greatest decrease occurring in hepatic phosphocholine concentrations. During choline deficiency, hepatic SAM concentrations also decreased by as much as 50%. In rats, choline deficiency doubled plasma homocysteine levels (Niculescu and Zeisel, 2002).

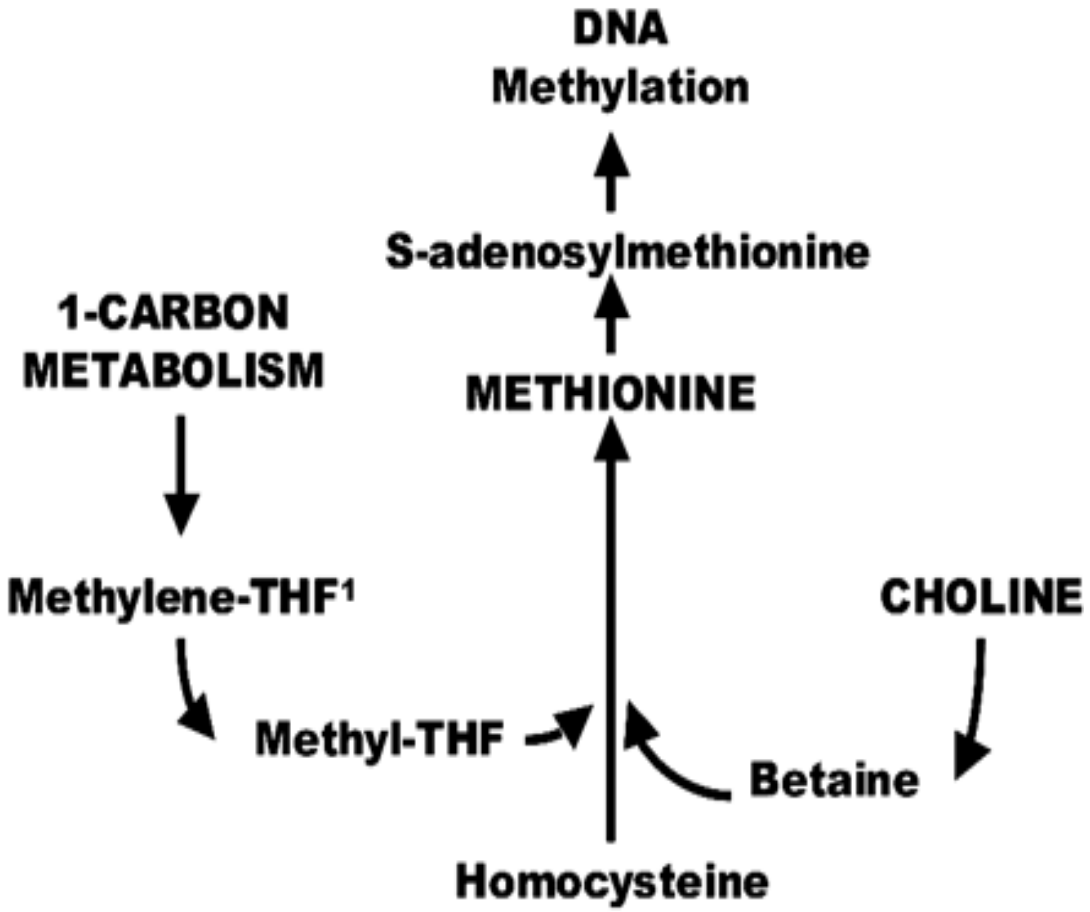

Figure 2.7. Sourcess of DNA methylation (Niculescu and Zeisel, 2002).

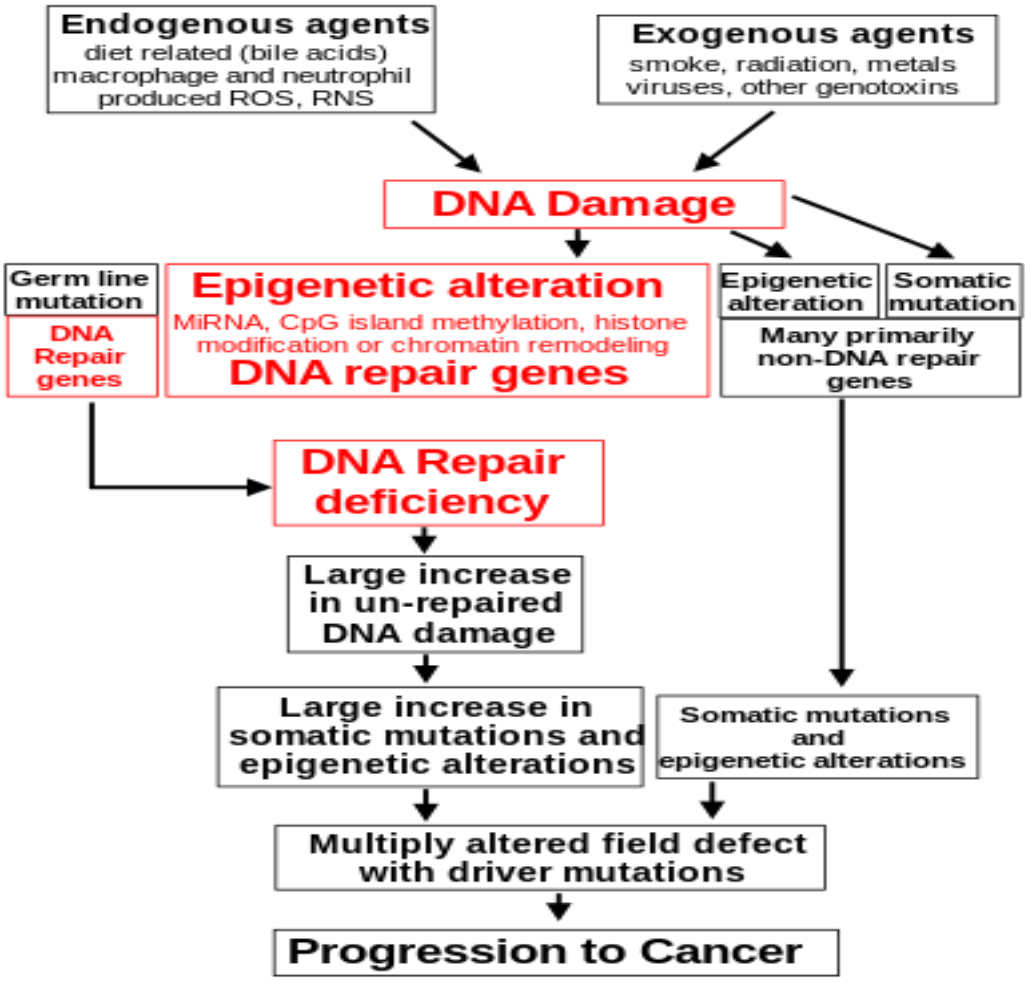

Figure 2.8: Endogenous and Exogenous sources of Epigenetic Alteratiion (National Institute of Health, 2005).

2.11.3. Methylation Enzyme

DNA methylation is mediated by DNA methyltransferases (DNMTs) that catalyze the transfer of the methyl group from *S*-adenosyl l-methionine (SAM) to the cytosine in CpG dinucleotide (Jin *et al*., 2011). To date, the known DNMTs are DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. DNMT1 shows preference for hemimethylated DNA *in vitro*, and is localized to replication foci during S phase. As such, it is the proposed maintenance methyltransferase responsible for copying DNA methylation patterns to the daughter strands during DNA replication (an enzyme that copies the DNA methylation pattern from the methylated parental strand to the unmethylated daughter strand during cell division). DNMT3a and DNMT3b are *de novo* DNMTs, as they methylate unmethylated and methylated DNA at an equal rate (Szyf, 2012). DNMT3L lacks the ability to bind to SAM, and is responsible for increasing the binding of DNMT3a to SAM DNMT2 - a small 391-amino-acid protein, is reported to possess weak DNA methyltransferase activity, but its biological function is not yet elucidated (Jin *et al*., 2011). Possessing homology to DNMT3A and DNMT3B, DNMT3L assists the *de novo* methyltransferases by increasing their ability to bind to the methyl group donor, S-adenosyl-L-methionine (SAM), and stimulating their activity *in vivo* (Jin *et al*., 2011). Regarding the role of DNMTs in breast tumorigenesis, it has been reported that DNMT3b mRNA is overexpressed in breast cancer, a finding that correlates well with the hypermethylator phenotype and poor prognosis in breast tumors (Roll *et al*., 2008). Cooperation among different DNMTs is also required in methylating some regions of the genome, particularly repetitive elements. As previously mentioned, it has been widely believed that DNMT1 acts mainly as a "maintenance" methyltransferase during DNA synthesis and that DNMT3A and DNMT3B act as "*de novo*" enzymes in development. However, mounting evidence indicates that DNMT1 may also be required for *de novo* methylation of genomic DNA and that DNMT3A and DNMT3B contribute to *maintenance* methylation during replication (Jin *et al*., 2011). In breast cancers and in many other disease states, hypermethylation of CpG islands results from overactivity of DNA methyltransferases (DNMTs).

2.11.4. Clinical Importance of DNA Methylation

DNA methylation may be classified as hyper- and hypo- methylation, according to increased and decreased levels of genomic modification, respectively. DNA methylation is one of the essential epigenetic mechanisms that are closely correlated with the mechanisms underlying cell growth, differentiation and transformation in eukaryotes. Global changes in the epigenetic landscape are considered to be a hallmark of cancer. Hypermethylation is an epigenetic alteration often leading

to gene-inactivating deletions and translocations. Hypermethylated cells may exhibit a phenotype of drug-resistance (Chang *et al*., 2010) or malignant proliferation. (Wu *et al*., 2010) demonstrated that 16 genes, including Bone morphogenetic protein 4 (*BMP4),* POU domain, class 4, transcription factor 3 **(***POU4F3*) and Glial cell line-derived neurotrophic factor (*GDNF),* are frequently hypermethylated in tumors. The other alteration is hypomethylation (reduced level or loss of methylation compared to that in normal cells). Thus, the DNA methylation status of such genes might show value as a predictive marker for therapy response (Neidhart, 2016).

The initiation and progression of cancer are mediated through epigenetic modifications along with genetic alterations. Aberrant methylation of promoter regions is an epigenetic abnormality of the human genome that is highly characteristic of cancer (Dong *et al*., 2014). Complex diseases, such as malignant tumors and diabetes, are a common occurrence and represent a major public health concern. Despite the significant advances in cancer treatment, the overall cancer-related mortality is ~90%, due to late-stage diagnosis and failure to optimize treatment. Therefore, effective biomarkers for cancer diagnosis are urgently needed. Cancers such as lung, colon and breast cancer are frequently diagnosed at a late stage. Despite intensified efforts focused on improving the survival of cancer patients, only a moderate improvement is generally achieved. Failure of early diagnosis often leads to low treatment efficiency and poor prognosis; thus, the identification of a signal characterizing the early stages of formation and progression of cancer may reduce the incidence of this disease (Heyn *et al*., 2013). Early findings of clinical studies indicated that early detection may offer a variety of novel efficient and cost-effective opportunities for cancer treatment (Smith *et al*., 2013). The mechanisms underlying cancer development are complicated and cancer was originally perceived as a genetic disease. However, it was previously demonstrated that the initiation and progression of cancer involve epigenetic abnormalities, such as DNA methylation, histone modifications, nucleosome positioning and aberrant expression of non-coding RNAs, specifically microRNAs (Aran and Hellman 2013). DNA methylation may be a biomarker of early cancer detection.

Besides the hypermethylated tumor suppressor genes, genes involved in DNA repair, apoptosis, metabolism, cell cycle regulation, cell adherence, metastasis, cellular homeostasis, and cell growth and genes encoding severalepigenetic enzymes are frequently hypermethylated inbreast cancer (Stefansson and Esteller 2013). Aberrant DNA hypermethylation of some key genes in breast cancer might be useful as prognostic or diagnostic markers. For instance, aberrant

hypermethylation of genes encoding estrogen receptor (ER)-α and progesterone receptor (PR) is correlated with silencing of these genes and with development of ER-and PR-negative breast cancer. Indeed, some hypermethylated genes, such as RASSF1A, are considered as potential diagnostic markers of breast cancer (Stefansson and Esteller 2013). Also, aberrant DNA hypermethylation of the gene PITX2 (paired like homeodomain transcription factor-2) in breast cancer was recently considered as a marker linked to tamoxifen resistance (Stefansson and Esteller 2013). Thus, the DNA methylation status of such genes might show value as a predictive marker for therapy response (Dong et al., 2014). A number of studies indicated that DNA methylation is crucial in the regulation of transcriptional silencing and transcription potential (Di Ruscio *et al*., 2013, Xie *et al*., 2013). Aberrant methylation of promoters in eukaryotic cells may lead to silencing of important genes, such as tumor suppressor genes, affecting their related transcriptional pathways and ultimately leading to the development of disease.

2.12. Cancer Associated Antigen 15-3 (CA 15-3)

The CA15-3 protein is a member of the family of proteins known as mucins, whose normal function is cell protection and lubrication. MUC1 is a large transmembrane glycoprotein which is frequently overexpressed and aberrantly glycosylated in cancer. It plays a role in reducing cell adhesion and is found throughout the body. Elevated levels of this antigen are found mainly in breast cancer where it appears to be involved in metastasis (Anant *et al*., 2010). CA15-3 level is elevated in nearly 11 percent of women with operable breast cancer, and 60 percent of women with metastatic disease. MUC1 functions in protection and lubrication of epithelial surfaces. It provides a protective layer on epithelial surfaces and is involved in cell-cell interactions, cell signaling and metastasis (Begum *et al*., 2012). MUC1 is expressed on the apical surfaces of most simple, secretory epithelia including the mammary gland, gastrointestinal, respiratory, urinary and reproductive tracts. Although MUC1 was thought to be an epithelial-specific protein (Tang *et al*., 2010), it is now known to be expressed on a variety of hematopoietic cells as well (Begum *et al*., 2012).

2.13. Breast Cancer Risk Factors

Risk factors for breast cancer may be divided into preventable and non-preventable. The studies of the risk factors belong to the field of epidemiology. Breast cancer, like other forms of cancer, can result from multiple environmental and hereditary risk factors. The term "environmental", as used by cancer researchers, means any risk factor that is not genetically inherited. For breast

cancer, the list of environmental risk factors includes the individual person's development, exposure to microbes, "medical interventions, dietary exposures to nutrients, energy and toxicants, ionizing radiation, and chemicals from industrial and agricultural processes and from consumer products...reproductive choices, energy balance, adult weight gain, body fatness, voluntary and involuntary physical activity, medical care, exposure to tobacco smoke and alcohol, and occupational exposures, including shift work" as well as "metabolic and physiologic processes that modify the body's internal environment (Institute of Medicine 2012). Some of these environmental factors are part of the physical environment, while others (such as diet and number of pregnancies) are primarily part of the social, cultural, or economic environment (Institute of Medicine 2012). Although many epidemiological risk factors have been identified, the cause of any individual breast cancer is most often unknowable. Epidemiological research informs the patterns of breast cancer incidence across certain populations, but not in a given individual. Approximately 5% of new breast cancers are attributable to hereditary syndromes, and well-established risk factors accounts for approximately 30% of cases.

2.13.1. Non Preventable Risk factors

2.13.1.1. Gender

Simply being a woman is the main risk factor for developing breast cancer. Men can develop breast cancer, but this disease is about 100 times more common among women than men. This is probably because men have less of the female hormones estrogen and progesterone, which can promote breast cancer cell growth (American Cancer Society, 2015).

Men have a much lower risk of developing breast cancer than women. In developed countries, about 99% of breast cancer cases are diagnosed in women; in a few African countries, which represent the highest incidence of male breast cancer, men account for 5–15% of breast cancer cases. The rate of breast cancer in men appears to be rising somewhat (Giordano *et al*., 2004). Men diagnosed with breast cancer tend to be older than women with breast cancer. They are more likely to be diagnosed with hormone-receptor positive tumors, with about six out of seven cases being estrogen-receptor positive. The overall prognosis is worse for men than for women.

2.13.1.2. Aging

Risks of developing breast cancer increase as we get older. About 1 out of 8 invasive breast cancers are found in women younger than 45, while about 2 of 3 invasive breast cancers are found in women age 55 or older (Abeloff *et al*., 2008). A woman is more than 100 times more

likely to develop breast cancer in her 60s than in her 20s. If all women lived to age 95, about one in eight would be diagnosed with breast cancer at some point during their lives. However, the actual lifetime risk is lower than that, because 90% of women die before age 95, most commonly from heart attacks, strokes, or other forms of cancer. The probability of breast cancer increases with age, but breast cancer tends to be more aggressive in younger people (Olsson, 2002).

2.13.1.3. Genetic risk factors

About 5% to 10% of breast cancer cases are thought to be hereditary, meaning that they result directly from gene defects (called *mutations*) inherited from a parent. (American Cancer Society, 2016). The most common cause of hereditary breast cancer is an inherited mutation in the *BRCA1* and *BRCA2* genes. In normal cells, these genes help prevent cancer by making proteins that keep the cells from growing abnormally. If you have inherited a mutated copy of either gene from a parent, you have a high risk of developing breast cancer during your lifetime. Although in some families with *BRCA1* mutations the lifetime risk of breast cancer is as high as 80%, on average this risk seems to be in the range of 55 to 65%. For *BRCA2* mutations the risk is lower, around 45% (American Cancer Society, 2015). Breast cancers linked to these mutations occur more often in younger women and more often affect both breasts than cancers not linked to these mutations. Women with these inherited mutations also have an increased risk for developing other cancers, particularly ovarian cancer (American Cancer Society, 2016)

2.13.1.4. Family history of breast cancer

Breast cancer risk is higher among women whose close blood relatives have this disease. Having one first-degree relative (mother, sister, or daughter) with breast cancer approximately doubles a woman's risk. Having 2 first-degree relatives increases her risk about 3-fold. The exact risk is not known, but women with a family history of breast cancer in a father or brother also have an increased risk of breast cancer. Altogether, less than 15% of women with breast cancer have a family member with this disease. This means that most (over 85%) women who get breast cancer *do not* have a family history of this disease (American Cancer Society, 2015).

2.13.1.5. Personal History of Breast Cancer

A woman with cancer in one breast has a 3- to 4-fold increased risk of developing a new cancer in the other breast or in another part of the same breast. This is different from a recurrence (return) of the first cancer (American Cancer Society, 2016).

2.13.1.6. Race and Ethnicity

Overall, white women are slightly more likely to develop breast cancer than are African-American women, but African-American women are more likely to die of this cancer. However, in women under 45 years of age, breast cancer is more common in African-American women. Asian, Hispanic, and Native-American women have a lower risk of developing and dying from breast cancer.

2.13.1.7. Dense Breast Tissue

Breasts are made up of fatty tissue, fibrous tissue, and glandular tissue. Someone is said to have dense breast tissue (as seen on a mammogram) when they have more glandular and fibrous tissue and less fatty tissue. Women with dense breasts have a higher risk of breast cancer than women with less dense breasts. Unfortunately, dense breast tissue can also make mammograms less accurate. A number of factors can affect breast density, such as age, menopausal status, the use of drugs (such as menopausal hormone therapy), pregnancy, and genetics.

2.13.1.8. Menstrual Periods

Women who have had more menstrual cycles because they started menstruating early (before age 12) and/or went through menopause later (after age 55) have a slightly higher risk of breast cancer. The increase in risk may be due to a longer lifetime exposure to the hormones estrogen and progesterone. Additional reproductive factors that influence hormonal exposure of the breast and breast cell proliferation include age at first pregnancy, number of children, and age at menarche. Evidence suggests that the first pregnancy, but not subsequent pregnancies, is related to a one-time increase in the risk of breast cancer that results in higher rates of breast cancer among parous as compared with nulliparous women through the early reproductive years (Britt *et al*., 2007). This cross-over of parous women experiencing higher rates of breast cancer than nulliparous women has been described in numerous settings and by use of a wide range of study and analytic approaches. Later age at first birth is associated with a greater adverse effect of the first pregnancy (Graham *et al*., 2012). Obesity is positively related to reproductive hormone levels among postmenopausal women.. This finding reflects the biologic function of fat cells, which metabolize androgens to estrogens among postmenopausal women. Lean, postmenopausal women have both lower estrogen levels and lower age-specific incidence of postmenopausal breast cancer (Suba 2013).

Obesity is also positively related to risk of breast cancer mortality (Giovanni and Silvestris, 2013). This relationship persists after control for stage at diagnosis and other modifiers of survival. This is consistent with a role for estrogen as a late promoter of breast cancer.

The duration from menarche to menopause summarizes the traditional lifetime exposure of women to significant levels of reproductive hormones. After menopause, hormone levels are substantially lower. During the premenopausal period, the timing of pregnancies conveys risk; late pregnancies are associated with increased risk of breast cancer, and early pregnancies are protective, presumably because first pregnancy is associated with terminal differentiation of breast cells and the cell cycle is longer after first pregnancy, allowing more time for DNA repair (Graham *et al*., 2012)

2.13.2. Preventable Risk Factor (Lifestyle-related factors)

Environmental assaults occur almost daily in adult tissue and can be due to exposure to radiation such as gamma irradiation or various chemicals known as carcinogens. Gamma irradiation can cause DNA lesions such as single and double-strand breaks by inducing breakage of phosphodiester bonds which form the back-bone of the DNA double-helical structure. This type of damage is normally repaired by homologous recombination (Davis and Lin, 2011). Chemical DNA damaging agents are present in the environment, water, air, and pollution. Individual job occupations can also increase the risk of exposure to these agents. Several categories of carcinogens exist, but we will only cover a few broad categories. DNA base-pair analogs that have a chemical structure almost identical to that of a DNA base can therefore change DNA base-pairing rules thus "tricking" DNA polymerase into substituting an incorrect base during replication. One common example is an analog of thymidine (5-bromouracil for example) causing a Thymidine: Adenine (T:A) base pairing to become a Cytocine:Guanine (C:G) substitution. Hydroxylating agents add a hydroxyl group, and for example hydroxylated cytosine pairs with adenosine instead of guanine causing a T:A substitution (Davis and Lin, 2011). Alkylating agents add an ethyl or methyl group to DNA and can cause either base-pair substitutions or epigenetic silencing of gene expression. Deaminating agents remove amine groups from base-pairs thereby causing instability in the DNA back-bone by interfering with normal formation of hydrogen bonds. A final broad category is insertion agents, often called intercalating agents, which cause DNA bulges that can be repaired to insert or delete a random base-pairing where the bulge was present. One example of a common intercalating agent is proflavin (Davis and Lin, 2011).

2.13.2.1. Previous chest Radiation

Women who, as children or young adults, had radiation therapy to the chest area as treatment for another cancer (such as Hodgkin disease or non-Hodgkin lymphoma) have a significantly increased risk for breast cancer. This varies with the patient's age when they had radiation. If chemotherapy was also given, it may have stopped ovarian hormone production for some time, lowering the risk. The risk of developing breast cancer from chest radiation is highest if the radiation was given during adolescence, when the breasts were still developing. Radiation treatment after age 40 does not seem to increase breast cancer risk (Houssami and Hayes 2009; Helvie, 2010).

2.13.2.2. Diethylstilbestrol Exposure

From the 1940s through the 1960s some pregnant women were given the drug diethylstilbestrol (DES) because it was thought to lower their chances of miscarriage (losing the baby). These women have a slightly increased risk of developing breast cancer. Women whose mothers took DES during pregnancy may also have a slightly higher risk of breast cancer (Briot *et al., 2010,* Hoover *et al*., 2011).

2.13.2.3. Having Children

Women who have had no children or who had their first child after age 30 have a slightly higher breast cancer risk. Having many pregnancies and becoming pregnant at a young age reduce breast cancer risk. Pregnancy reduces a woman's total number of lifetime menstrual cycles, which may be the reason for this effect (Nelson *et al*., 2016).

2.13.2.4. Birth control: Oral contraceptives

Studies have found that women using oral contraceptives (birthcontrol pills) have a slightly greater risk of breast cancer than women who have never used them. This risk seems to go back to normal over time once the pills are stopped. Women who stopped using oral contraceptives more than 10 years ago do not appear to have any increased breast cancer risk. When thinking about using oral contraceptives, women should discuss their other risk factors for breast cancer with their health care team (Li *et al*., 2012). Depot-medroxyprogesterone acetate (DMPA; Depo-Provera) is an injectable form of progesterone that is given once every 3 months as birth control. A few studies have looked at the effect of DMPA on breast cancer risk. Women currently using DMPA seem to have an increase in risk, but the risk doesn’t seem to be increased if this drug was used more than 5 years ago (Li *et al*., 2012).

2.13.2.5. Hormone Therapy after Menopause

Hormone therapy with estrogen (often combined with progesterone) has been used for many years to help relieve symptoms of menopause and to help prevent osteoporosis (thinning of the bones) (Goss *et al*., 2011). Earlier studies suggested it might have other health benefits as well, but these benefits have not been found in more recent, better designed studies. This treatment goes by many names, such as post-menopausal hormone therapy (PHT), hormone replacement therapy (HRT), and menopausal hormone therapy (MHT). There are 2 main types of hormone therapy. For women who still have a uterus (womb), doctors generally prescribe both estrogen and progesterone (known as combined hormone therapy or HT). Progesterone is needed because estrogen alone can increase the risk of cancer of the uterus. For women who no longer have a uterus (those who've had a hysterectomy), estrogen alone can be prescribed. This is commonly known as estrogen replacement therapy (ERT) or just estrogen therapy (ET) (Anderson *et al*., 2012). Using combined hormone therapy after menopause increases the risk of getting breast cancer. It may also increase the chances of dying from breast cancer. This increase in risk can be seen with as little as 2 years of use. Combined hormone therapy also increases the likelihood that the cancer may be found at a more advanced stage. (Usha *et al*., 2011).

The use of oestrogen alone after menopause does not appear to increase the risk of developing breast cancer. In fact, some research has suggested that women who have previously had their uterus removed and who take estrogen actually have a lower risk of breast cancer. Women taking estrogen seem to have more problems with strokes and other blood clots, though (Heiss *et al*., 2009). Also, when used long term (for more than 10years), ET has been found to increase the risk of ovarian cancer in some studies. At this time there appear to be few strong reasons to use post-menopausal hormone therapy (either combined HT or ET), other than possibly for the short-term relief of menopausal symptoms. (Heiss *et al*., 2009; Usha *et al*., 2011).

2.13.2.6. Breastfeeding

Some studies suggest that breastfeeding may slightly lower breast cancer risk, especially if it is continued for 1½ to 2 years. But this has been a difficult area to study, especially in countries such as the United States, where breastfeeding for this long is uncommon. One explanation for this possible effect may be that breastfeeding reduces a woman's total number of lifetime menstrual cycles (similar to starting menstrual periods at a later age or going through early menopause) (American cancer society 2016).

2.13.2.7. Drinking Alcohol

The use of alcohol is clearly linked to an increased risk of developing breast cancer. The risk increases with the amount of alcohol consumed. By the system of standard alcohol drinking units (SADU), which sums the number of glasses of wine (~ 10 gram), bottles of beer (~ 10 g) and units of spirits (~ 10 g) consumed regularly per week, compared with non-drinkers, individuals with habitual alcohol consumption of: 1–140 g/week, were considered light drinkers; 141–280 g/week, moderate drinkers and > 280 g/week, heavy drinkers (Gonzalez-Quintela *et al.*, 2008). Excessive alcohol consumption is also known to increase the risk of developing several other types of cancer (American Cancer Society, 2016). According to the International Agency for Research on Cancer, there is sufficient scientific evidence to classify alcoholic beverages as a Group 1 carcinogen that causes breast cancer in women. Group 1 carcinogens are the substances with the clearest scientific evidence that they cause cancer, such as smoking tobacco. The more alcohol a woman drinks, the more likely she is to get breast cancer. One or two drinks each day increases the relative risk to 150% of normal, and six drinks per day increases the risk to 330% of normal. Approximately 6% of breast cancers reported in the UK are due to women drinking alcohol. The primary mechanism through which alcohol causes breast cancer is increased estrogen levels (American Cancer Society, 2016).

Alcohol is readily distributed throughout the body in the blood stream and crosses biological membranes, which affect virtually all biological processes inside the cell. Excessive alcohol consumption induces numerous pathological stress responses, part of which is endoplasmic reticulum (ER) stress response. ER stress, a condition under which unfolded/misfolded protein accumulates in the ER, contributes to alcoholic disorders of major organs such as liver, pancreas, heart, and brain (Malhi and Kaufman 2011). Potential mechanisms that trigger the alcoholic ER stress response are directly or indirectly related to alcohol metabolism, which includes toxic acetaldehyde and homocysteine, oxidative stress, perturbations of calcium or iron homeostasis, alterations of S-adenosylmethionine to S-adenosylhomocysteine ratio, and abnormal epigenetic modifications. Interruption of the ER stress triggers is anticipated to have therapeutic benefits for alcoholic disorders. Most of the alcohol that enters the body is first oxidized to toxic acetaldehyde, which is catalyzed by the cytosolic alcohol dehydrogenase (ADH). Acetaldehyde is then converted by acetaldehyde dehydrogenase (ALDH) to acetic acid, which occurs primarily in the liver (Ji, 2012). Alcohol can also be oxidized to acetaldehyde by cytochrome P450IIE1 (CYP2E1) which generates hydrogen peroxide. The ER is an essential organelle for protein

synthesis and modifications, for storing and releasing Ca^{2+}, for the biosynthesis of lipids and sterols, and for detoxification of certain drugs. (Malhi and Kaufman 2011).

2.13.2.8. Being Overweight or Obese

In postmenopausal women, the risk of developing breast cancers that express the estrogen and progesterone receptors (ER and PR), is significantly elevated for those who are obese or overweight (Iyengar *et al*., 2013). Furthermore, obese and overweight patients, once diagnosed, suffer from worse disease-related outcomes than their leaner counterparts, regardless of breast cancer subtype. After menopause, estrogen is mostly derived peripherally from the non-cyclical conversion of androgen precursors within adipose tissue. The rate-limiting step in this conversion is catalyzed by the cytochrome P450 enzyme, aromatase, which is encoded by the *CYP*19 gene. Circulating estrogens, such as estradiol, are known to stimulate the proliferation of breast epithelial cells and potentially exert a mutagenic effect. Higher levels of circulating estradiol as a result of increased adiposity and aromatase expression are thought to contribute, in part, to the greater risk of ER/PR-positive breast cancer in obese postmenopausal women. However, compared to the premenopausal state, circulating estradiol levels are significantly lower as the ovaries no longer produce substantial amounts of estrogen. Nevertheless, the incidence of ER-positive disease rises with age, approaching nearly 85% of breast cancers diagnosed in women during their ninth decade of life (Iyengar *et al*., 2013).

Being overweight or obese after menopause, increase breast cancer risks. Before menopause, ovaries produce most of your estrogen, and fat tissue produces a small amount of estrogen. After menopause (when the ovaries stop making estrogen), most of a woman's estrogen comes from fat tissue. After menopause, having more fat tissues can increase chances of getting breast cancer by raising estrogen level. Also, women who are overweight tend to have higher blood insulin levels. Higher insulin levels have also been linked to some cancers, including breast cancer (Linda *et al*., 2012, American Cancer Society, 2016).

2.13.2.9. Physical Activity

Evidence is growing that physical activity in the form of exercise reduces breast cancer risk. The main question is how much exercise is needed. In one study from the Women's Health Initiative,

as little as 1.25 to 2.5 hours per week of brisk walking reduced a woman's risk by 18%. Walking 10 hours a week reduced the risk a little more (American cancer Society, 2016).

2.13.3. Unclear Factors:

2.13.3.1. Diet and Vitamin Intake

Many studies have looked for a link between what women eat and breast cancer risk, but so far the results have been conflicting. Some studies have indicated that diet may play a role, while others found no evidence that diet influences breast cancer risk. Studies have looked at the amount of fat in the diet, intake of fruits and vegetables, and intake of meat. No clear link to breast cancer risk was found (Kushi *et al*., 2012). Studies have also looked at vitamin levels, again with inconsistent results. Some studies actually found an increased risk of breast cancer in women with higher levels of certain nutrients. So far, no study has shown that taking vitamins reduces breast cancer risk. This is not to say that there is no point in eating a healthy diet. A diet low in fat, low in red meat and processed meat, and high in fruits and vegetables might have other health benefits (Kushi *et al*., 2012). Most studies have found that breast cancer is less common in countries where the typical diet is low in total fat, low in polyunsaturated fat, and low in saturated fat. But many studies of women in the United States have not linked breast cancer risk to dietary fat intake. Researchers are still not sure how to explain this apparent disagreement. It may be at least partly due to the effect of diet on body weight.

2.13.3.2. Chemicals in the Environment

A great deal of research has been reported and more is being done to understand possible environmental influences on breast cancer risk. Compounds in the environment that have estrogen-like properties are of special interest. For example, substances found in some plastics, certain cosmetics and personal care products, pesticides (such as DDE), and PCBs (polychlorinated biphenyls) seem to have such properties. These could in theory affect breast cancer risk. This issue understandably invokes a great deal of public concern, but at this time research does not show a clear link between breast cancer risk and exposure to these substances. Unfortunately, studying such effects in humans is difficult. More research is needed to better define the possible health effects of these and similar substances (American cancer Society, 2016).

2.13.3.3. Microbial Infection

Data covering infection-associated breast and lung cancers have been discussed and presented as possible involvements as pathogens in cancer. Because carcinogenesis is a multistep process with several contributing factors, we evaluated to what extent infection is significant, and concluded that members of the herpesvirus, polyomavirus, papillomavirus, and retrovirus families definitely associate with breast cancer (Alibek *et al*., 2013). Detailed studies of viral mechanisms support this conclusion, but have presented problems with experimental settings. It is apparent that more effort needs to be devoted to assessing the role of these viruses in carcinogenesis, by characterizing additional confounding and synergistic effects of carcinogenic factors. Alibek *et al* (2013) proposed that preventing and treating infections may possibly stop or even eliminate certain types of cancers. Nowadays, there is increasing evidence that some pathogenic bacteria can contribute to specific stages of cancer development. The concept that bacterial infection could be involved in carcinogenesis acquired a widespread interest with the discovery that *H. pylori* is able to establish chronic infections in the stomach and that this infection is associated with an increased risk of gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma. Chronic infections triggered by bacteria can facilitate tumor initiation or progression since, during the course of infection, normal cell functions can come under the control of pathogen factors that directly manipulate the host regulatory pathways and the inflammatory reactions.

2.13.3.4. Tobacco Smoke

For a long time, studies found no link between cigarette smoking and breast cancer. In recent years though, more studies have found that long-term heavy smoking is linked to a higher risk of breast cancer. Some studies have found that the risk is highest in certain groups, such as women who started smoking when they were young. In 2009, the International Agency for Research on Cancer concluded that there is limited evidence that tobacco smoking causes breast cancer (American Cancer Society, 2016). An active focus of research is whether secondhand smoke increases the risk of breast cancer. Both mainstream and secondhand smoke contain chemicals that, in high concentrations, cause breast cancer in rodents. Chemicals in tobacco smoke reach breast tissue and are found in breast milk. The evidence on second hand smoke and breast cancer risk in human studies is controversial, at least in part because the link between smoking and breast cancer hasn’t been clear. One possible explanation for this is that tobacco smoke may have different effects on breast cancer risk in smokers and in those who are just exposed to smoke (American Cancer Society, 2016).

2.14.1. Circulating Immune Complexes

Circulating Immune complex (CIC) is the binding of the antigen with its corresponding antibody by electrostatic van da Waal force, to form an interlock. The formation of immune complexes (IC), due to the interaction of foreign substances (endogenous and exogenous antigens) with specific antibodies, is a physiological process which constitutes an essential part of man's normal immune defence mechanisms against environmental factors and are usually eliminated by the mononuclear phagocytes system (MPS) without development of pathological changes (Mayadas *et al*., 2009). In any normal immune response, the half-life of CIC is transitory in nature. Continued presence of CIC over extended periods, however, is a cause of consequence of some pathological condition or infection (Stanilova and Slavov 2001, Maheswari *et al*., 2014). Elevated levels of CIC have been found in a variety of diseases including neoplasia. Serum CIC levels in cancer patients have been used for early diagnosis or measure of, metastatic spread, tumour burden, degree of aggressiveness, therapeutic response as well as prognosis (Maheswari *et al*., 2014). In most cancers, the antigenic part of the circulating immune complexes have been shown to originate from the disease affected tissues. As the disease progresses the shredding of the antigen by these tissues also increases and it is thought to increase the level of CICs (Jane et *al*., 2007). Pursuing the cause of this study therefore is a pursuit against environmental challenges accumulating through immunological responses as immunological risk factors suspected to induce epigenesis and subsequent carcinogenesis. This review looked at pure immunological risk factors that could serve as a pathway from normal immunological response to tumourigenesis.

These immune complexes are either destroyed in circulation, with no manifestation of symptoms, or fixed to a tissue where they are destroyed locally. If this becomes an ongoing process, due to regular intake of the antigenic food or regular exposure to fuelling factors, specific symptoms may occur and become chronic, depending on which organ has fixed the immune-complex. Thus persistence in the context of this work implies detectable level of immune complex in the system. Production of ICs requires ongoing antigen production, so persistence of IC might be a marker of ongoing or persisting infection (Brunner and Sigal 2001). In other wards if exposure to antigen is stopped, then immune complex would stop forming. In case of regular consumption of the foodstuffs against which IgG1-IgG3 antibodies are present, the deposition of immune complexes results in a chronic inflammation. This is true except for unspecific systemic reactions for which low-grade inflammations play a main role (high blood pressure, iron deficiency, metabolic syndrome, overweight). These antigens could be

exogenuous or endogenuous. Suffice it to say that detectable level of immune complexes indicates persistent or ongoing exposure to either exogenuous or endogenuous antigen. Immune complexes build up in 7 weeks in a rat that is being injected with antigen (Brunner and Sigal 2000). Presence of circulating immune complexes can trigger immune responses or immunological activities around a localized gene on the tissue where they deposited, and may turn those genes on or off. The same triggered immunological activities would release certain cytokines that would activate the translocation of NFkB pathway from cytosome to the nucleus. This would cause transcription of many factors top of which is pro-inflammatory molecules, leading to chronic inflammatory response. On the other hand, Immune Complexes may also persist in cancer patients because of persistence of Damage Associated Molecular Pattern, resulting from persistent immunological activities due to cancer antigens and mutational activities. The hypothesis is that continuous formation of these complexes may correlate with poor epigenetic status and fueling of NFkB translocation which may serve as a niche for cancer initiation, promotion and progression. Efforts would be made in this study to link or correlate detected levels of Immune complex with various molecular parameters that may serve as marker for tumorigenesis: such as DNA methylation, NFkB, 8-OH2DG, TNF –α, IgG, progesterone, estrogen, to ascertain the role of circulating immune complexes in cancer initiation promotion and progression. Then associate the microbial antigenic components of the immune complexes detected after characterization of the immune complexes to any of the pathological factors in breast cancer, as either contributing to initiation promotion or progression.

Both exogenous and endogenous sources can trigger pathogenic immune complexes, which are increasingly recognized as the cause of many diseases in animals and humans, including neoplasia (Rai and Mody, 2012). Deposition of circulating ICs in tissues, mainly in capillary beds, promoting inflammation and tissue damage, is the most relevant pathological mechanism underlying IC-mediated diseases (Ferrari *et al*., 2014). Anand *et al*., (2008) reported that cancers are primarily an environmental disease with 90–95% of cases attributed to environmental factors and 5–10% due to genetics. Environmental, as used by cancer researchers, means any cause that is not inherited genetically, not merely pollution. The term environment refers not only to air, water, and soil but also to substances and conditions at home and at the workplace, including diet, smoking, alcohol, drugs, exposure to chemicals, sunlight, ionizing radiation, electromagnetic fields, infectious agents, etc. Lifestyle, economic and behavioral factors are all aspects of our environment." (Parsa, 2012). Common environmental factors that contribute to cancer death include tobacco (25–30%), diet and obesity (30–35%), infections (15–20%),

radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity, and environmental pollutants (US Health in Perspective, 2013). As a result of these high environmental pollutions, individuals may continually be exposed to foreign substances (antigens) in the system and this would continually create the need for more and highly orchestrated immune responses, thus inducing proliferation of lymphocytes, couple with high rate of asembling antigen receptors gene in course of the lymphocyte development. Hence more antibodies are produced to mop up the invading antigens, thus forming immune complexes. Enormous challenges resulting from increased demand for diversity of antigen receptors in high proliferation of lymphocytes, may induce aberrations in the rearrangement process and induce chromosomal translocation (Tawn *et al*., 2016). DNA damage can result from exogenous stresses, such as ionizing radiation (IR), ultraviolet (UV) light and chemical compounds, or from endogenous insults such as reactive oxygen species (ROS) and DNA replication errors (Jackson and Jiri 2009). Researchers have shown that we are under serious environmental challenge (Omofonmwan and Osa-Edoh, 2008, Ezeani *et al*., 2011a). I hypothesize in this study that persistent circulation of immune complexes is an unconsidered endogenous DNA insult that may equally induce DNA damage.

In any normal immune response, the half-life of CIC is transitory in nature. Continued presence of CIC over extended periods, however, is a cause of consequence of some pathological condition or infection (Onyenekwe *et al*., 2000, Golda *et al*., 2004). Healthy humans (and, by inference, healthy nonhuman primates) form and clear approximately less than 1 to 30 µg/ml of ICs each day, with IgG-containing ICs slightly more frequent than IgM- or IgA-containing ICs. (Anderson and Stillman, 1980, Rojko *et al*., 2014). Elevated levels of CIC have been found in a variety of diseases including neoplasia. The suggestion that CIC might compromise in the host-tumor relationship on the immunological front, has led to extensive studies aiming at unraveling the correlation between CIC levels in serum and clinical course of neoplastic diseases (Golda et *al*., 2004). In most cancers, the antigenic part of the circulating immune complexes have been shown to originate from the disease affected tissues. As the disease progresses the shredding of the antigen by these tissues also increases and it is thought to increase the level of CICs (Jane *et al*., 2007). Pursuing the cause of this study therefore is a pursuit against environmental challenges accumulating through immunological responses as immunological risk factors suspected to enhance carcinogenesis. I am using this study to look at pure immunological risk factors that could serve as a pathway from normal immunological response to carcinogenesis.

Circulating Immune Complexes first localize within the vasculature and then translocate into extravascular tissue, attracting immune cells (Stokol *et al*., 2004). Circulation of immune complexes, can stir up different immune responses including complement activation, opsonization, phagocytosis, activation of immune cells (macrophages, neutrophils) and subsequent release of cytokines, chemokines and activation of protease pathways (Mayadas *et al*., 2009). Immune Complexes deposited intravascularly can directly engage circulating leukocytes. Both soluble and insoluble immune complexes can activate infiltrating immune cells such as T cells, neutrophils, mast cells and macrophages, that then release inflammatory mediators (cytokines and prostanoids) capable of activating the endothelium and their ability to recruit more cells (Elaine *et al*., 2014). Based on these, it is proposed that persistence of circulating immune complexes is a potent source of acute and chronic inflammations which continues to fuel the inflammatory pathways that leads may to tumour development.

The ability to classically and continuously activate immune responses is considered in this study as neglected immunological response that can induce cancer and as such deserves serious attention. Circulating immune complexes (CIC) are now viewed as regulators of both cellular and humoral immune responses by virtue of their capacities to interact with antigen receptor bearing lymphocytes and sub-population of T and B cells as well as with macrophages and neutrophils having FC receptors (Rai and Mody 2012). Both exogenous and endogenous antigens can trigger pathogenic immune complexes, which are increasingly recognized as the cause of many diseases in animals and humans, including neoplasia (National Institute of Health, 2005; Rai and Mody, 2012). Thus measurement of CIC in biological fluids has become increasingly important for diagnosis and assessment of prognosis in the management of patients of high risk group (Rai and Mody, 2012).

Deposition of circulating ICs in tissues, mainly in capillary beds, promoting inflammation and tissue damage, is the most relevant pathological mechanism underlying IC-mediated diseases (Ferrari *et al*., 2014). It is hypothesized in this study that persistent circulation of immune complexes could continue to exacerbate immune cell activities and subsequent production of cytokine such as Interleukin-1 Tumor necrosis factor-a (TNF-α), that has the potentials to induce translocation of Nuclear factor kappa-B (NF-kB). Immune complexes containing anti-Type II collagen (anti-CII) from arthritis patients induced the production of tumor necrosis factor alpha (TNF-alpha), interleukin-1beta (IL-1β), and Interleukin-8 (Mullazehi *et al*., 2006). The above mentioned cytokines are potent inducers of Nuclear factor kappa B activation. Immune

complexes (IC) induce a number of cellular functions, including the enhancement of cytokine production from monocytes, macrophages and plasmacytoid dendritic cells (Rönnelid *et al*., 2008).

Linking of reactive oxygen species (ROS) as an endogenous insult inducing DNA damage, is now an established fact (Jackson and Jiri, 2009). This may be a continuous process emanating from the immunological process induced by circulating immune complexes. Their capacities to interact with antigen receptor bearing lymphocytes and sub-population of T and B cells as well as with macrophages and neutrophils (Phagocytes) having FC receptors (Rai and Mody 2012), may emanate neutrophil activation and subsequent respiratory burst leading to release of reactive oxygen species (ROS). The high levels of circulating DNA-anti-DNA immune complexes may overwhelm the capacity of the reticuloendothelial system (RES) to clear them, and they are deposited in various tissues including glomeruli where local complement activation results in glomerular injury (Toong *et al*., 2011). Once DNA-anti-dsDNA immune complexes have been formed, they are normally cleared by the RES but defects of some of the clearance mechanisms have been described in Systemic Lupus Erythematosus (SLE), including aberrant interactions with Fcγ receptors (FcγRs), complement and complement receptors, and anti-C1q antibodies (Lee *et al*., 2009). Despite all the efforts to reduce these environmental pollutions, the problem persists due to ignorance and poor educational background. The status of our environment therefore may emanate the level of immune complex formation to be so high and overwhelm the capacity of the reticuloendothelial system (RES) to clear them; this would lead to their persistence in circulation and for a very long time.

It is worthwhile to look at the protein components of these immune complexes, characterize the antigenic components to detect possible microbial agents that may be pro-tumor or cancer metastatic specific in breast carcinogenesis. It is suggested that the high frequency of HBs-Ag in the precipitated CIC of healthy subjects could account for the occurrence of some post-transfusion hepatitis-B infections (Okerengwo and Atoba, 1992). Infection with *Leishmania donovani* had been associated with IL-10 as well as with GM-CSF (Elshafie *et al*., 2007). *Leishmania*-infected patients have been shown to have increased levels of circulating Complement lq-binding immune complex (CIC) which contain *Leishmania* Antigens. I propose that such immune complexes (IC) might affect cancer progression and disease outcome through induction of pro- or anti-inflammatory cytokines (Elshafie *et al*., 2007). It is worth given attention to the possibility of persistent circulation of immune complexes fuelling the connection between inflammation and cancer, as they would continue to attract macrophages and

neutrophils (phagocytes). At the very early stage of inflammation, neutrophils are the first cells to migrate to the inflammatory sites under the regulation of molecules produced by rapidly responding mast cells pre-stationed in tissues (Rashad *et al*., 2013). As the inflammation progresses, various types of leukocytes, lymphocytes, and other inflammatory cells are activated and attracted to the inflamed site by a signaling network involving a great number of growth factors, cytokines, and chemokines ((Rashad *et al*., 2013).). All cells recruited to the inflammatory site contribute to tissue breakdown and are beneficial by strengthening and maintaining the defense against infection. There are also mechanisms to prevent inflammatory response from lasting too long. A shift from antibacterial tissue damage to tissue repair occurs, involving both pro-inflammatory interleukin 1-alpha and tumour necrosis factor alpha (IL-1α, TNF- α) and anti-inflammatory molecules interleukin 10, 4 and transforming growth factor beta (IL-10, IL-4, TGF-β) (Piotr *et al*., 2014). The resolution of inflammation also requires a rapid programmed clearance of inflammatory cells (Efferocytosis) as mentioned above. Neighboring macrophages, dendritic cells, and backup phagocytes do this job by inducing apoptosis and conducting phagocytosis (Janssen and Henson 2012). However Toong *et al* (2011), revealed a positive feedback loop whereby inefficient clearance of apoptotic blebs by macrophages results in positive selection of germinal center B cells, which have self-reactivity against nuclear antigens exposed on these blebs. These self-reactive B cells undergo T cell-dependent affinity maturation and isotype switching and differentiate into long-lived plasma cells which reside in the bone marrow. The high affinity IgG anti-DNA antibodies secreted by these cells bind to the DNA to form immune complexes which activate plasmacytoid dendritic cells (pDCs) via toll-like receptor- (TLR-) to produce inflammatory cytokines such as interferon-alpha. These cytokines augment the humoral immune response and lead to further autoantibody production (Toong *et al* 2011). This systemic scenario would likely continue to induce more immune complex formation and by this standard, enabling its persistence with further immune cell activation and further inflammatory response. Constant production of immune complexes then may promote immune cell polarization to favour breast tumourigenesis. One plausible mechanism may have to do with the 'polarity' of the $CD4^+$ T-helper-cell response at primary tumour sites and/or their distant metastases. $CD4^+$ T-helper cells are activated in response to soluble factors and can be classified generally into different categories such as Th1 cells or Th2 cells (Piccinni *et al*., 2015). Following an activating stimulus, $CD4^+$ T-helper cells that are Th1-polarized secrete IFN-γ, transforming growth factor beta, TNF-α and IL-2. These cytokines enhance the cytotoxic/cell killing functions of $CD8^+$ T cells and can induce up regulation of antigen processing (in the proteasome), and induce expression of acute inflammatory responses

(left panel), Th1 $CD4^+$ and $CD8^+$ T cells directly regulate tumour cell cytotoxicity, while indirectly polarizing innate immune cells toward tumour suppression (such as M1 polarization of tumour-associated macrophages [TAMs] (DeNardo *et al*., 2010). In Th2-polarized $CD4^+$ T-helper cells express interleukin-4, interleukin-5, interleukin-6, interleukin-10, interleukin-13 (IL-4, IL-5, IL-6, IL-10 and IL-13), which induce T-cell anergy and loss of T-cell-mediated cytotoxicity while also enhancing humoral immunity (B-cell function) (DeNardo *et al*., 2010). During chronic inflammation, however (right panel), myeloid suppressor cells, Th 2 $CD4^+$ T cells and regulatory T (T-reg) cells function in combination to both repress $CD8^+$ cytotoxicity and to induce protumoral polarization of innate immune response (such as M2 polarization of TAMs) via cytokine secretion (IL-4, IL-13, IL-10, IL-6 and transforming growth factor beta (TGFβ)). Chronically activated B cells (typically in germinal centers) can produce granulocyte–macrophage-CSF, TNF-α, IL-6, and IL-10. These cytokines, in combination with Th2 cytokines such as IL-4, IL-13, and IL-10, are potent effectors of innate immune cell polarization (DeNardo et al., 2010). Circulating immune complexes is considered an enabling endogenous factor that can induce chronic inflammation by its persistence, by stimulation and activation of these cytokines through Th2 pathway. When polarized, as during chronic inflammation, these innate immune cells in turn provide a rich proangiogenic and pro-tumoural microenvironment (De Visser and Coussens, 2006, DeNardo *et al*., 2010). Example, protumor M2 polarization in macrophages is induced by the Th 2/humoral cytokines IL-4 and IL-10, while simultaneously repressing antitumor M1 macrophage polarization (De Visser and Coussens, 2006). Granulocyte–macrophage-CSF, IL-6 and IL-10 secreted by activated B cells suppress macrophage cytotoxic activity, while IL-10 inhibits both antigen presentation by macrophages as well as monocyte differentiation into DC lineages (Duque and Descoteaux 2014). Taken together, factors derived from chronically activated lymphocytes sculpt innate immune cell responses towards tumor tolerance and promotion of disease progression (Duque and Descoteaux, 2014). Persistent infection– The combined effects of a low-grade persistent infection and a weak antibody response lead to chronic immune-complex formation, and eventual deposition of complexes in the tissue. Diseases with this aetiology include: leprosy, malaria, dengue haemorrhagic fever, viral hepatitis and staphylococcal infective endocarditis.

Immune complexes are capable of triggering a wide variety of inflammatory processes: Complexes interact directly with basophils and platelets (via Fc receptors) to induce the release of vasoactive amines. Macrophages are stimulated to release cytokines, particularly TNF-α and IL-1, that are very important during inflammation. They interact with the complement system to

generate C3a and C5a (anaphylatoxins). These complement fragments stimulate the release of vasoactive amines (including histamine and 5 hydroxytryptamine) and chemotactic factors from mast cells and basophils. C5a is also chemotactic for basophils, cosinophils and neutrophils. Recent work with knockout mice indicates that complement has a less pro-inflammatory role than previously thought, wheras cell bearing Fc receptors for IgG and IgE appear to be critical for developing inflammation, with complement having a protective effect. The vasoactive amines released by platelets, basophils and mast cells cause endothelial cell retraction and thus increase vascular permeability, allowing the deposition of immune complexes on the blood vessel wall. The deposited complexes continue to generate C3a and C5a. Platelets also aggregate on the exposed collagen of the vessel basement membrane, assisted by interactions with the Fc regions of deposited immune complexes, to form microthrombi. The aggregate platelets continue to produce vasoactive amines and to stimulate the production of C3a and C5a. (Platelets are also a rich source of growth factors – these may be involved in the cellular proliferation seen in immune-complex diseases such as glomerulo-nephritis and rheumatoid arthritis).

Polymorphs are chemotactically attracted to the site by C5a. They attempt to engulf the deposited immune complexes, but are unable to do so because the complexes are bound to the vessel wall. Thus, they exocytose their lysosomal enzymes onto the site of deposition. If simply released into the blood or tissue fluids these lysosomal enzymes are unlikely to cause much inflammation, because they are rapidly neutralized by serum enzyme inhibitors. But if the phagocyte applies itself closely to the tissue-trapped complexes through Fc binding, then serum inhibitors are excluded and the enzymes may damage the underlying tissue.

2.14.2. Sources of Immune Complexes

Two types of antigens cause circulating immune complex (CICs): (1) individual can produce antibody against self-components (auto/endogenous antigens); antigen compounds of one's own cells/tissues. Mediated injury antigen may be exogenous (foreign protein, bacterium, or virus, chemical toxin and radiation).

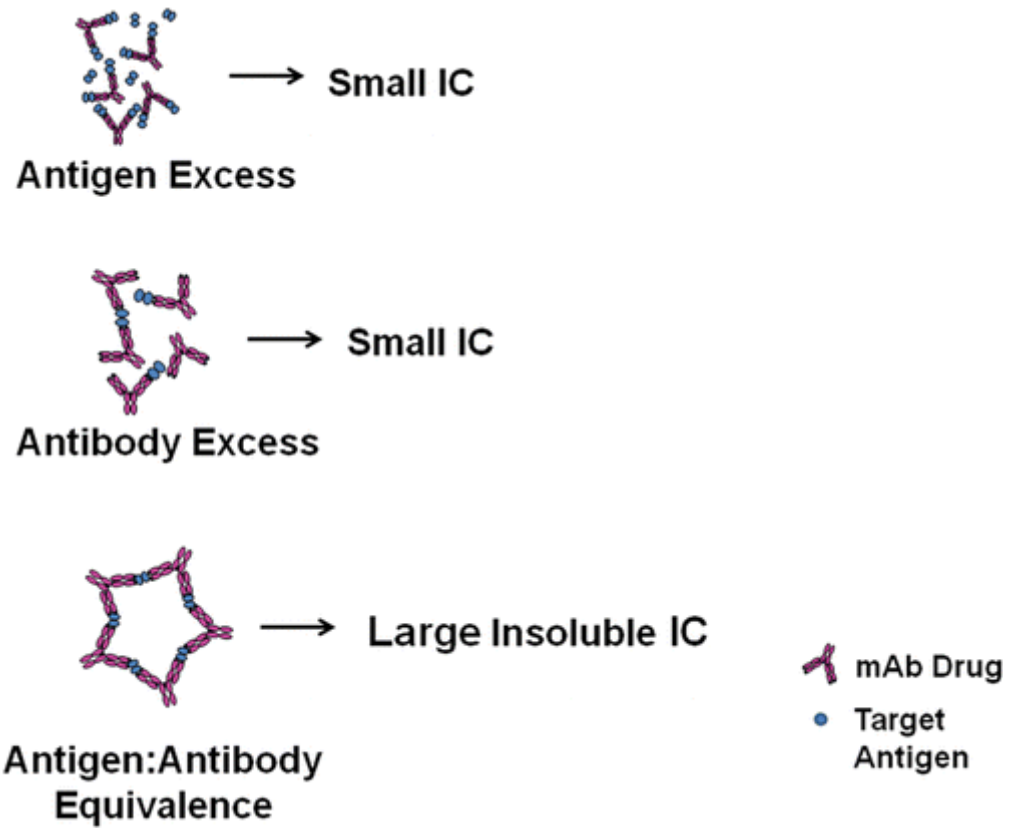

Figure 2.9

Immune Complex lattice formation at different molar ratios of antigen and antibody: When antigen or antibody is in great excess, small soluble complexes form. When antigen and antibody are in molar equivalence, large, insoluble complexes form. As antigen/antibody ratios approach molar equivalence, ICs are larger but remain soluble. IC = immune complex (Rojko *et al*., 2014)

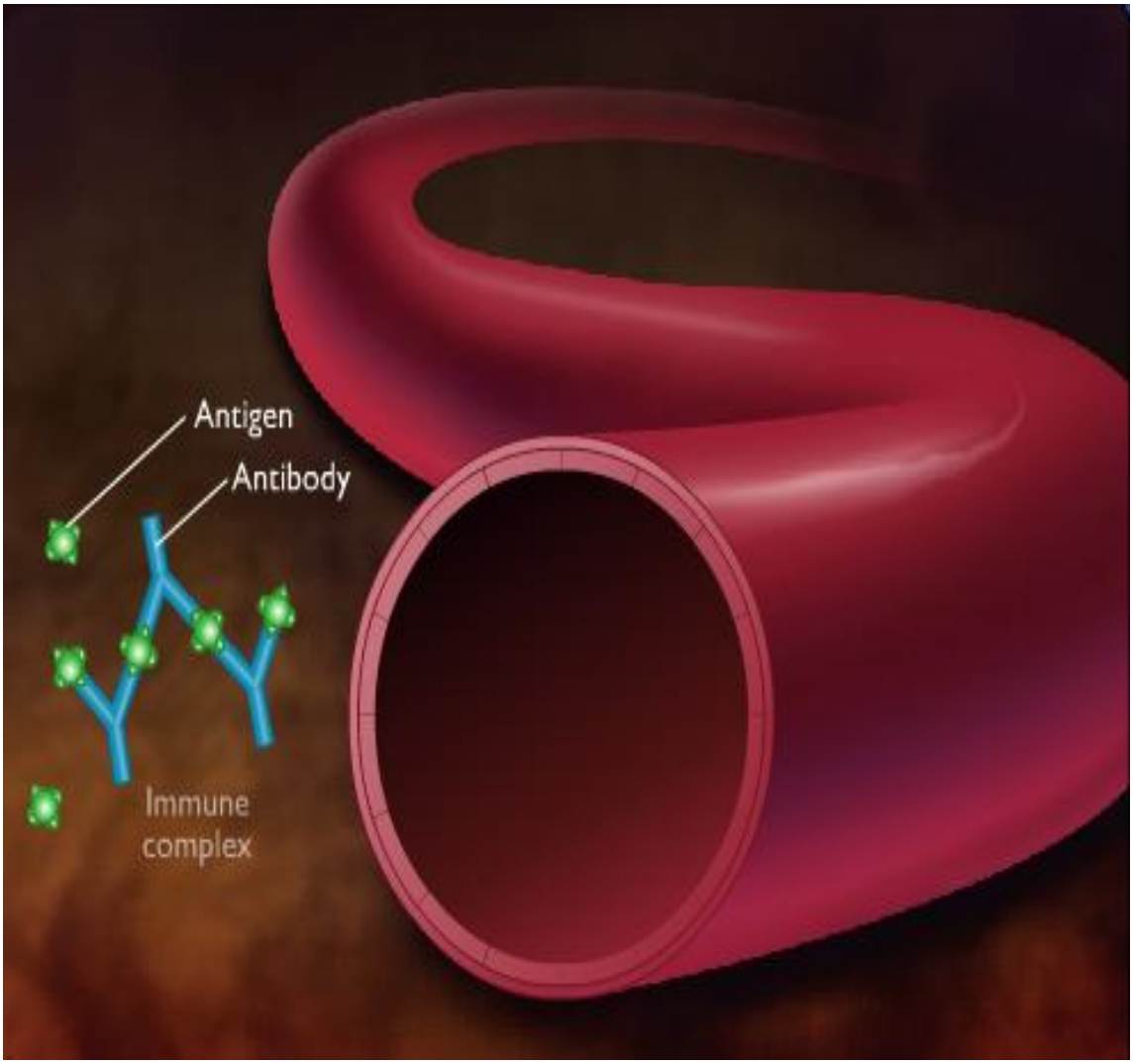

Figure 2.10: Immune Complexes and vascular system (Rojko *et al.*, 2014)

2.14.2.1. Endogenous (Assaults) Sources of Immune Complexes

Tissue damage caused by trauma, infection or inflammation is associated with the release of endogenous proteins that signal impending danger to the host. The terms "damage-associated molecular patterns (DAMPs)" or "alarmins" have been used to collectively describe endogenous proteins that signal tissue and cell damage which may be present in the absence of microbial pathogens. These molecules help to explain the initiation of an inflammatory response in the absence of infection such as in trauma or the classical example of acute pancreatitis (Coveney *et al*., 2015). Inflammatory mediators play important roles in the development and progression of cancer. Cellular stress, damage, inflammation, and necrotic cell death cause release of endogenous damage-associated molecular pattern (DAMP) molecules or alarmins, which alert the host of danger by triggering immune responses and activating repair mechanisms through their interaction with pattern recognition receptors. Recent studies show that abnormal persistence of these molecules in chronic inflammation and in tumour microenvironments underlies carcinogenesis and tumour progression, indicating that DAMP molecules and their receptors could provide novel targets for therapy (Srikrishna and Freeze, 2009). Multicellular animals detect pathogens via a set of receptors that recognize pathogen-associated molecular patterns (PAMPs). However, pathogens are not the only causative agents of tissue and cell damage: trauma is another one. Evidence is accumulating that trauma and its associated tissue damage are recognized at the cell level via receptor-mediated detection of intracellular proteins released by the dead cells. The term "alarmin" is proposed to categorize such endogenous molecules that signal tissue and cell damage. Intriguingly, effector cells of innate and adaptive immunity can secrete alarmins via nonclassical pathways and often do so when they are activated by PAMPs or other alarmins (Coveney *et al*., 2015). Endogenous alarmins and exogenous PAMPs therefore convey a similar message and elicit similar responses (forming circulating immune complexes); they can be considered subgroups of a larger set, the damage-associated molecular patterns (DAMPs) (Bianchi, 2006, Coveney *et al*., 2015). Multicellular animals must distinguish whether their cells are alive or dead and detect when microorganisms intrude, and have evolved surveillance/defense/repair mechanisms to this end (Bianchi, 2006, Coveney *et al*., 2015). Tissues can be ripped, squashed, or wounded by mechanical forces, like falling rocks or simply the impact of one's own body hitting the ground. Animals can be wounded by predators. In addition, tissues can be damaged by excessive heat (burns), cold, chemical insults (strong acids or bases, or a number of different cytotoxic poisons), radiation, or the withdrawal of oxygen and/or nutrients. Finally, humans can also be damaged by specially designed drugs, such as chemotherapeutics, that are meant to kill their tumor cells with

preference over their healthy cells. Very likely, we would not be here to discuss these issues if evolution had not incorporated in our genetic program ways to deal with these damages, which are not caused by pathogens but are nonetheless real and common enough. Inflammation is also activated by these types of insults. A frequently quoted reason for the similarity of the responses evoked by pathogens and trauma is that pathogens can easily breach wounds, and infection often follows trauma; thus, it is generally effective to respond to trauma as if pathogens were present. In my opinion, an additional reason is that pathogens and trauma both cause tissue and cell damage and thus trigger similar responses (Bianchi, 2007).

The best known DAMPs are high mobility group box-1 (HMGB1), S100A8 (MRP8, calgranulin A) HSP70, uric acid and S100A9 (MRP14, calgranulin B), and Serum amyloid A (SAA). Increased serum levels of these DAMPs have been associated with many inflammatory diseases, including sepsis, arthritis, atherosclerosis, lupus, Crohn's disease and cancer. Therapeutic strategies are being developed to modulate the expression of these DAMPs for the treatment of these diseases. These DAMDs have all been found to induce Toll-like receptor (TLR)-dependent inflammatory response (Cavassani *et al*., 2008) Significantly, some of these molecules, including HMGB1 and HSP70 are not released during apoptosis, which is in keeping with the idea that programmed cell death does not result in an inflammatory response. Some DAMPs can engage TLRs to induce and amplify the inflammatory response. TLR2 and TLR4 signaling have been shown to mediate NF-κB activation initiated by HMGB1, S100A8 and SAA. Different signaling pathways are involved that may cross-talk at several levels, but all culminate in the activation of NF-κB (Conevey *et al*., 2015). The sources of endogenous CICs formation involve physiological processes such as inadequate removal of apoptosed cells. Apoptosis produces cell fragments called apoptotic bodies that under go efferocytosis (phagocytic cells are able to engulf and quickly remove apoptotic bodies before the contents of the cell can spill out onto surrounding cells and cause damage) (Poon *et al*., 2014). The possible means of endogenous antigens that form immune complexes include the continual response of the body's immune system, which overloads the ability of the body to remove the immune complexes that formed, expression of mutated gene products (Poon *et al*., 2014) and proliferation of cancer cells, breakdown of tissue structure due to injuries, resulting from persistent inflammatory response. These have lead to release into circulation sequestered antigens (endogenous and/or auto-antigen) (Poon *et al*., 2014). It is important to note that continual progression of cancer cells and persistence of tissue injury, would continually induce the activation of the apoptotic pathway and activation of cytotoxic T cell activities, thereby generating more cell debris (apoptotic bodies

and/or cell fragments). The increase in these cell fragments may overwhelm the phagocytic cells thereby frustrating efferocytosis. Antibodies are formed against this debris leading to increase in immune complex formation, as well as fuelling the persistent circulation of immune complexes.

2.14.2.2. Exogenous Sources of Immune Complexes

Sources of exogenous CICs include microbial infections, Toxins and Chemicals Irritants. Chronic infections with persistent pathogens such as helminths, mycobacteria, *Plasmodium*, bacteria and hepatitis viruses affect more than a third of the human population and are associated with increased susceptibility to other pathogens as well as reduced vaccine efficacy. Although these observations suggest an impact of chronic infections in modulating immunity to unrelated antigens, little is known regarding the underlying mechanisms. Diseases from persistent infections impact a large portion of humanity (Stelekati and Wherry, 2012). In developing countries, in particular, infection with at least one persistent pathogen is common. Although major efforts are focused on the control of persistent pathogens, current vaccines and treatments for many of these infections are lacking, ineffective or unavailable (Stelekati and Wherry, 2012). Mounting evidence suggests that persistent infections can alter immunity to unrelated pathogens and vaccines. In some cases co-infections may provide a benefit to the host (Stelekati and Wherry, 2012). The high incidence of co-infection with multiple chronic pathogens suggests an increased susceptibility to secondary infections. Moreover, responses to many vaccines are reduced in chronically infected patients, rendering those individuals more susceptible to subsequent infections. Epidemiological studies suggest that chronic infections can pre-dispose patients to secondary infections (Stelekati and Wherry, 2012). Since many pathogens causing chronic pathology are co-endemic, one could argue that the high rate of co-infection is simply due to enhanced co-exposure. Although the geographical overlap of pathogen spread cannot be excluded as a potentially contributing factor, mathematical models suggest that chronic infections, such as malaria and human immunodeficiency virus (HIV), actively contribute to the increased rate of infection with unrelated pathogens (Abu-Raddad *et al*., 2006, Stelekati *et al*., 2014). The combined effects of a low-grade persistent infection (such as occur with a parasite such as *Plasmodium species* or in viral hepatitis) together with a weak antibody response, form chronic immune complexes (ICs) with the eventual deposition of the complexes in body tissues (Basile *et al*., 2012). Continuous exposure to these exogenous substances would be a source to retain pathological level of immune complexes in circulation. In this study, it is hypothesize that due to constant exposure to many infectious agents or foreign pathogens, with continuous infection and re-infection as may occur in some developing countries, IC accumulation may

reach a plateau, deposit on organs and constitute a great immunological risk factor to carcinogenesis. Hence it becomes imperative to measure the rate of formation. Supportively, Mart (1982), reported that concentration of immune complex at any giving time in circulation depends on the rate of immune complex formation and rate of removal (Mart 1982, Nydegger, 2007). The rate of immune complex formation in turn depends on the rate of antibody synthesis and rate of availability of specific antigen. The rate of immune complex removal in turn depends on the rate of removal by mononuclear phagocyte system (MPS), and on the deposition of immune complex on tissues. In cases with inefficient clearance by the mononuclear phagocytes system (MPS) only, pathological consequences will be expected, in particular by immune complexes formed with moderate excess of antigen (Nydegger, 2007).

2.14.3. Immune Complex Deposition

Immune complexes (ICs) are heterogeneous high-molecular-weight aggregates composed of antigens, immunoglobulins, and complement components (Stanilova and Slavov 2001 Senbagavalli *et al*., 2011). The fate of CIC has been examined by injection of preformed IC prepared with IgG class of antibodies (Mart 1982, Wang and Ravetch 2015). These investigations have shown that removal of CIC by MPS depends on the lattice of the IC, the status of the MPS, the nature of the antigen in IC and characteristic of the antibody in IC. The lattice of IC is defined as the number of antigen and number of antibody molecules in a given complex. When mixture of large latticed antibody defined as containing more than two antibody molecule and small lattice, defined as containing one or two ab molecule were injected into the mice, rabbit or monkeys, the large latticed complexes (above ag2-ab2) were removed relatively quickly while the small latticed complexes (either ag2-ab2, ag2-ab1, ag1-ab1) persisted longer in circulation (Mart, 1982, Wang and Ravetch 2015). The disappearance of large latticed complexes after the initial extravasations was best described by single exponential components; the disappearance of the small latticed complexes was best described by two exponential components, reflecting equilibrium between intravascular and extra vascular spaces and catabolism (Rojko *et al*., 2014). As increasing doses of IC were given, the clearance velocity of large latticed complexes and specific hepatic uptake of the materials reached a plateau, suggesting saturation of MPS with large latticed complexes. We hypothesize that this phenomenon could be the same when infection and re-infection continuously occur (Wang and Ravetch 2015). The physiologic role of complement in the biology of circulating immune complexes (CICs) includes solubilization of ICs, prevention of immune precipitation, and the clearance of ICs from circulation through erythrocyte complement receptor 1 (CR1). Changes in

vascular permeability induced by cytokines and/or lipid mediators secreted by hematopoietic cells and mast cells are likely the initial trigger for immune complex (IC) deposition (Mayadas *et al*., 2009). In addition, ICs themselves promote vascular leakage in "permeability"-susceptible tissues such as the joint tissue, which may explain why circulating ICs can promote tissue-specific disease such as RA (Binstadt *et al*., 2006, Mayadas *et al*., 2009*).* A change in the physicochemical parameters of ICs, reflected by a change in the properties of the antigen (eg, charge, valence, or size) or the antibody (eg, subclass, size, charge, or precipitability complement–fixing abilities), can modulate the extent and location of IC deposition (Mayadas *et al*., 2009). For example, ICs formed with an alteration in the ratio of antigen to antibody or composed of cation antigen have a higher potential to activate and bind complement C1q (Mayadas *et al*., 2009; Rojko *et al*., 2014). Once soluble ICs lodged in the capillaries reach the underlying tissue, activation of resident cells and subsequently the endothelium leads to the expression of leukocyte adhesion molecules and chemokines which enhance neutrophil recruitment. FcγRs also played an important role in neutrophil slow rolling and adhesion when neutrophils themselves were engaged by ANCAs (Nolan *et al*., 2008) Intravascular ICs trigger transient accumulation of neutrophils, which internalize the immune deposits and return to the circulation. Neutrophil FcγRIIIB present on microvilli tethers to ICs under physiological flow and clears them without damaging the vasculature. Right, when mechanism(s) of clearance are overwhelmed or defective during persistent IC generation, ICs translocate and accumulate in extravascular tissues. ICs, sensed by FcγRs and receptors for complement components (triangle) on resident mast cells, trigger the release of inflammatory mediators (eg, Leucotrene B_4 and tumor necrosis factor-α (TNFα). These directly or indirectly activate the endothelium, promote FcγRIIIB shedding, and increase the capacity of FcγRIIA to promote neutrophil recruitment and signal. Tissue-accumulated neutrophils release inflammatory mediators (eg, reactive oxygen species [ROS], proteases, eicosanoids, and cytokines) that activate resident cells. They also produce cytokines and chemokines that promote their own recruitment and the recruitment of other circulating leukocyte subsets. These actions together perpetuate inflammation and tissue damage.

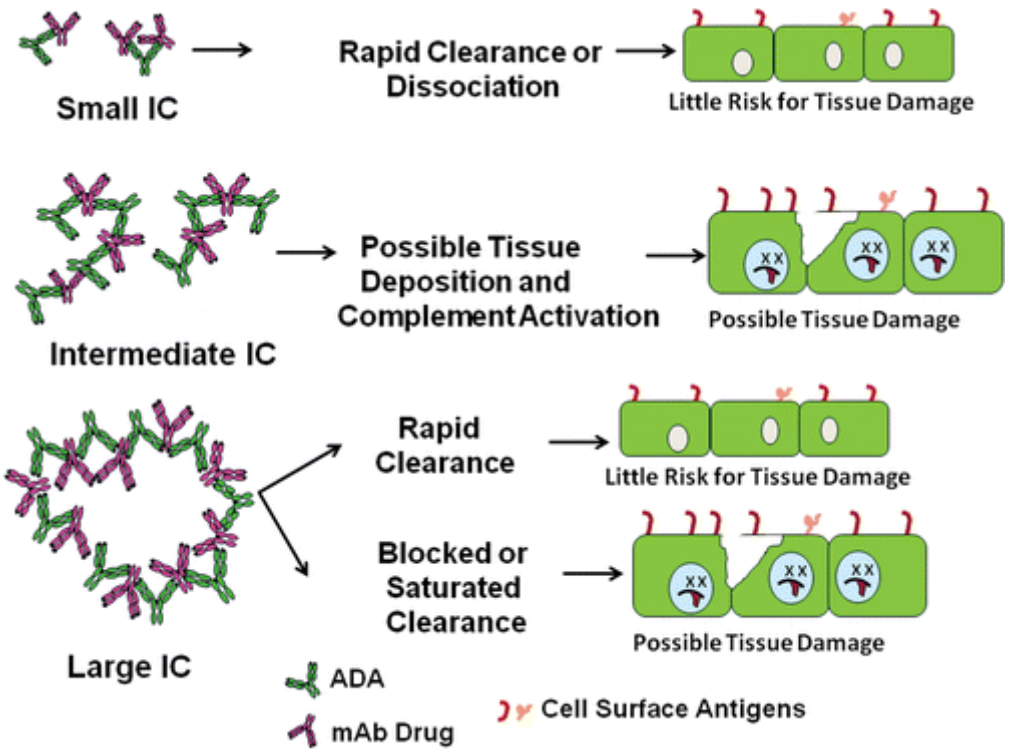

Figure 2.11 (Rojko *et al*., 2014)

Effect of IC lattice formation tissue deposition, and pathogenicity: Small ICs, which may form under great antigen or antibody excess, generally remain in circulation or are rapidly cleared or dissociated and pose little risk for tissue damage. Intermediate-sized IC can deposit in tissue and activate complement. Large ICs are generally rapidly cleared through the phagocytic system, but when clearance mechanisms are blocked or saturated, large ICs can deposit in tissue, activate complement, and cause tissue damage. IC = immune complex.

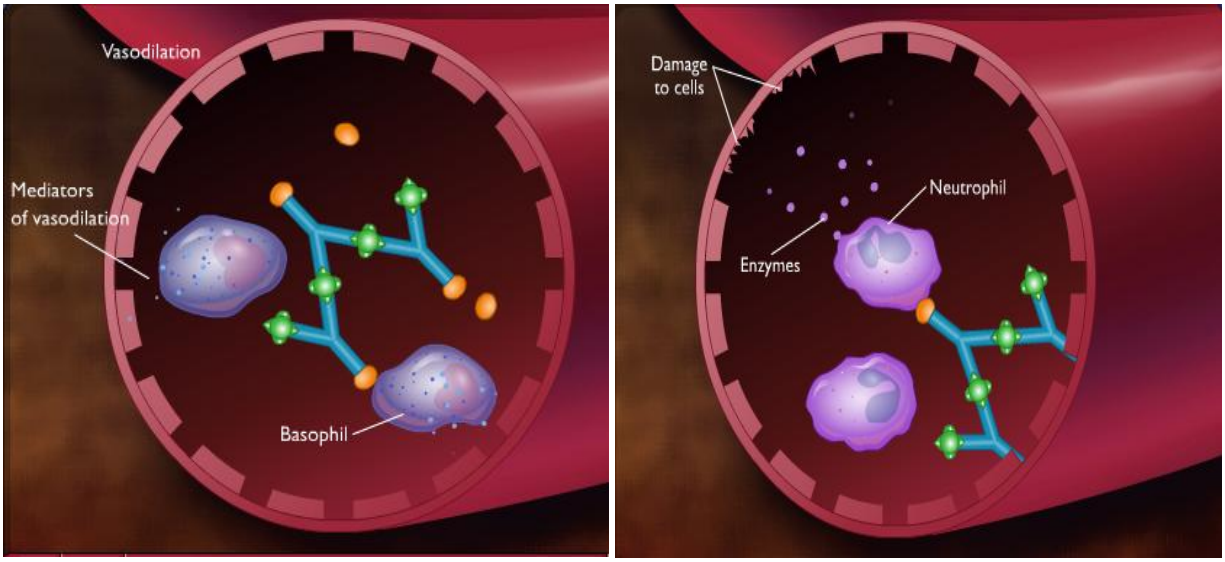

Figure 2.12 (Rojko *et al*., 2014)

Immune complexes in blood vessels bound by complement at the Fc portion inducing chemotaxis, thus mediating vasodilation and subsequent cell damage. Persistence of such delevelopment can increase the level of immune complexes and induce persistence chronic inflammation, formation and perturbation of the normal epigenetic flow in the cells.

2.15. Microbial Agents and Breast Cancer Development

Previous studies of microbial causes of breast cancer have focused on specific viruses and their potential contributions to breast cancer. While HPV infection has been reported by some groups to be associated with breast cancer (Heng *et al*., 2009), others have failed to find a correlation. Similarly, some groups have reported that up to 50% of breast tumors are EBV-positive, while others have been unable to detect the virus in breast tumors altogether. In contrast to viruses, bacteria in the breast have been studied to a far lesser extent. Several groups have investigated the bacteria responsible for infections stemming from breast implant procedures using culture-based methods (Xuan *et al*., 2014). Further, the breast milk of healthy women has been shown to harbour an abundance of bacterial species including commonly found skin bacteria (Cabrera-Rubio et al., 2012). Bacteria in the breast have been studied in the context of infections and in healthy individuals, but no comprehensive study of bacteria in breast cancer has been reported. Microbes inhabiting the human body outnumber human cells 10:1. Their influence on human health and disease is a new and rapidly expanding area of research. Microbes have been linked to diseases as varied as obesity (Turnbaugh et al., 2009), colon cancer (Castellarin *et al*., 2012) and colitis (Xuan *et al*., 2014).

The proportions of cancer deaths attributable to viral and bacterial infections and to parasitic diseases were tentatively estimated by Doll and Peto (1981) to be 10% in the USA in 1981 and by Doll (1998) to be 10–20% in UK in 1998 (Silvio and Puolo 2011). Pisani *et al* (1997) estimated that 15.6% (1 450 000 cases) of the worldwide incidence of cancers in 1990 could be attributed either to hepatitis B virus (HBV), hepatitis C virus (HCV), *Human papillomavirus* (HPV), *Epstein–Barr virus*, human T-cell lymphothropic (T-cell leukaemia/lymphoma) virus (HTLV)-1, human immunodeficiency virus (HIV), *Helicobacter pylori*, schistosomes or liver flukes. These data were updated in 2006 by Parkin who, based on the evidence of the strength of association and the prevalence of infection in different geographic areas, estimated that the total infection-attributable cancer in the year 2002 was 1.9 million cases, which accounted for 17.8% of all cancers in the world. By preventing cancer-associated infectious diseases, there would be 26.3% fewer cases in developing countries (<1.5 million cases/year) and 7.7% fewer cases in developed countries (<390 000 cases) (Parkin, 2006). zur Hausen (2009) estimated that slightly >20% of the global cancer burden can be linked to infectious agents and predicted that this fraction will increase in the future.

Upon a microbial attack, host cells undergo massive changes in their transcriptional program, mobilizing genes involved in key processes (e.g., immunity, cell death/survival, and adhesion/motility) to trigger an appropriate response (Jenner and Young 2005, Bierne *et al*., 2012). It is thus not surprising that successful pathogens have developed specific mechanisms to deregulate the expression levels and/or kinetics of these defence genes. Host transcription factors are first obvious targets to reprogram the genome and bacteria use diverse tricks to alter their function. For instance, bacterial factors can hijack cellular signaling pathways that activate or sequester transcription factors e.g. nuclear factor kappa B (NF-κB), signal transducer and activator of transcriptions **(**STATs), or activator protein 1 (AP-1) in the cytosol of targeted cells, or manipulate their half-lives via posttranslational modifications (Ribet and Cossart 2010; Perrett *et al*. 2011). Some bacteria, such as the phytopathogen *Xanthomonas*, even produce transcriptional activators that function as eukaryotic transcription factors. However, selective activation or silencing of specific genes not only depends on transcription factors, but also on their cross talk with epigenetic modulators, which regulate DNA accessibility by controlling the chromatin structure. Epigenetic modifications of chromatin during development and in response to distinct environmental factors contribute to adult phenotypic variability and susceptibility to a number of diseases, including cancers and metabolic and autoimmune disorders (Portela and Esteller 2010).

Several studies have shown that certain infectious agents (*Helicobacter pylori, Streptococcus bovis, Chlamydia pneumoniae, Campylobacter rectus, Epstein-Barr* virus, hepatitis viruses, Human papilloma virus, polyomaviruses, etc.) can contribute to the host epigenetic changes resulting in the onset and progression of some diseases, especially in malignancies (Gilbert *et al*., 2010). The importance of DNA methylation events associated with bacterial infections is also becoming increasingly appreciated. The best documented example is *H. pylori* infection that induces aberrant DNA methylation in the human gastric mucosa, strikingly at promoters of genes found methylated in gastric cancer cells (Ushijima and Hattori 2012). *H. pylori*-associated hypermethylation occurs, for instance, at the E-cadherin gene 1 (*CDH1)*, tumor-suppressor genes e.g., Upstream stimulatory factor 1*/2 USF1/2* (Yan *et al*. 2011), as well as to CpG islands of miRNA genes (Ando *et al*. 2009).

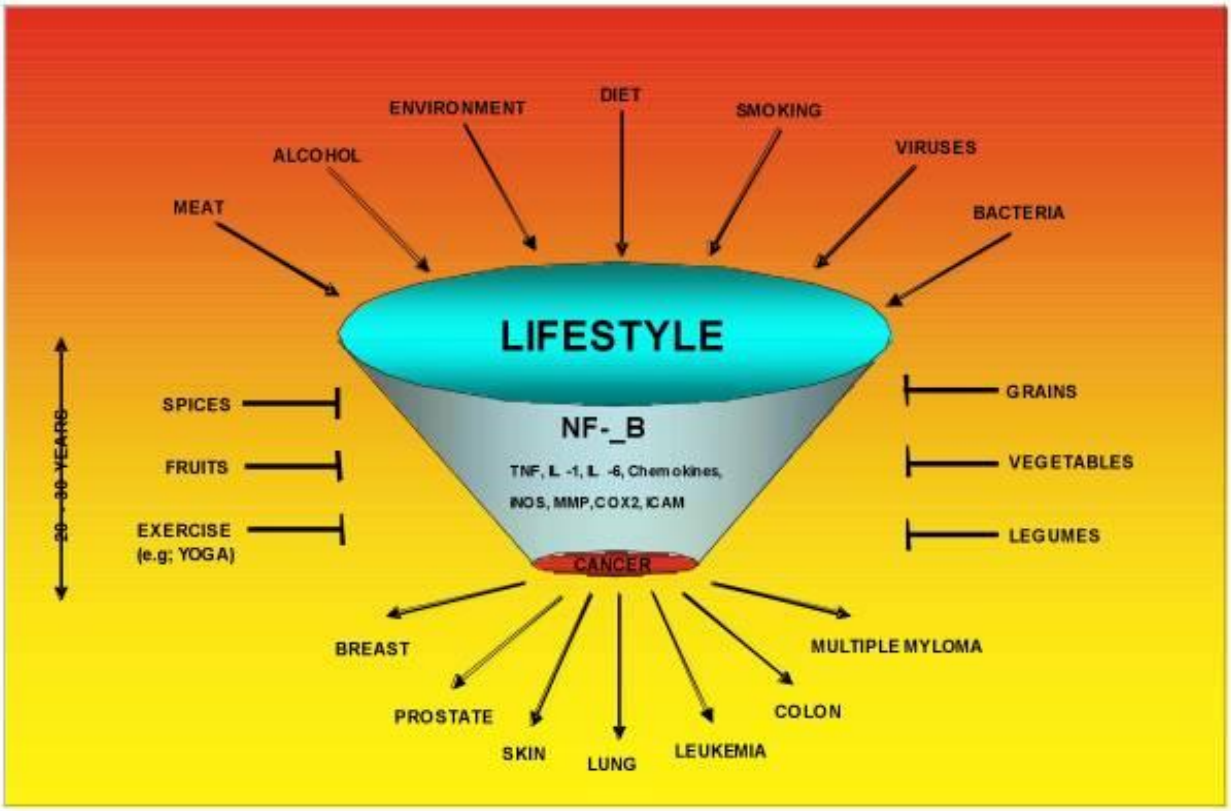

Figure 2.13. Environmental factors capable of inducing immune complexes and subsequent NF-kB activation and Translocation (Hoesel and Schmid, 2013)

The ability of *H. pylori* to induce DNA methylation in gastric mucosa was confirmed in the gerbil animal model and, interestingly, this effect was diminished on treatment with the immunosuppressor cyclosporin A (Niwa *et al*., 2010). Indeed, in contrast to ethanol or NaCl stimuli that induce neutrophil infiltration in the stomach, *H. pylori*-mediated inflammation triggered lymphocyte and macrophage infiltration, which appears to have a key role in induction of methylation (Hur *et al*., 2011). Thus, although the mechanisms by which *H. pylori* induces DNA hypermethylation are still unclear, the infection-associated inflammatory response is a tempting explanation (Ushijima and Hattori 2012). Among signals resulting from chronic inflammation, elevated levels of IL1β and nitric oxide (NO) are proposed to contribute to influence the recruitment of DNMTs at specific loci. A role of mammalian gut microbiota, as epigenetic modifying factor, in the pathogenesis of metabolic syndrome and associated diseases has been meant. Thus, the different biotic and abiotic signals can produce changes in gene expression that can persist after the effect has ceased (Oka *et al*., 2011).

The first step in any epigenetic study is global DNA methylation analyses which allow the detection and identification of DNA methylation. These approaches do not require previous knowledge of the genome of reference, and most rely on a prior enzymatic/chemical hydrolysis of DNA to obtain 2′-deoxymononucleosides, followed by the subsequent separation by chromatographic means such as High Performance Liquid Chromatography (HPLC) (Raid, 2013) or High Performance Capillary Electrophoresis (HPCE) (Raid, 2013), and a final detection step by UV spectroscopy or mass spectrometry. Alternatively, the global content of DNA methylation can also be quantified by enzymatic approaches such as the Luminometric Methylation Assay (LUMA) (Raid, 2013).

Interestingly, bacterial DNA load correlated inversely with advanced disease, a finding that could have broad implications in diagnosis and staging of breast cancer (Xuan et al., 2014). Previous studies of microbial causes of breast cancer have focused on specific viruses and their potential contributions to breast cancer. While HPV infection has been reported by some groups to be associated with breast cancer (Heng et al., 2009, Xuan et al., 2014), others have failed to find a correlation (Lindel et al., 2007). Similarly, some groups have reported that up to 50% of breast tumors are EBV-positive (Fina *et al*., 2001, Xuan et al., 2014), while others have been unable to detect the virus in breast tumors altogether (Glasser *et al*., 1998). In contrast to viruses, bacteria in the breast have been studied to a far lesser extent. Several groups have investigated the bacteria responsible for infections stemming from breast implant procedures using culture-

based methods (Pittet *et al*., 2005). Further, the breast milk of healthy women has been shown to harbour an abundance of bacterial species including commonly found skin bacteria (Cabrera-Rubio *et al*., 2012). Bacteria in the breast have been studied in the context of infections and in healthy individuals, but no comprehensive study of bacteria in breast cancer has been reported.

Microbes inhabiting the human body outnumber human cells 10:1. Their influence on human health and disease is a new and rapidly expanding area of research. Microbes have been linked to diseases as varied as obesity, colon cancer and colitis (Castellarin *et al*., 2012, Xuan *et al*., 2014). Examples of few microbe-induced epigenetic dysregulations include: *Campylobacterrectus* induces Methylation of Igf2 promoter region to cuase silencing of Igf2 P0 promoter by CpG methylation in the placenta; *Helicobacter pylori* induces Polycomb-repressive marks pinpoint the promoters to be silenced to enable silencing of selected promoters by CpG methylation; *Epstein-Barr* causes up-regulation of DNMT1, 3A, 3B via the JNK-AP-1 to induce silencing of E-cadherin promoter; *Human adenovirus* causes stimulation of E2F activity, up-regulation of DNMT1; association with DNMT1, stimulation of DNMT1 activity to induce dysregulation of DNMT1, 3A, 3B; *Human papillomavirus*, through association with DNMT1, cause stimulation of DNMT1 activity; increasing histone acetylation. Activation of E2F and CDC25A promoters; *Hepatitis B virus* induces Up-regulation of DNMT1 via the cyclin D1-CDK4/6-pRb-E2F1 and p38MAPK pathways; up-regulation of DNMT3A1 and DNMT3A2; Down-regulation of DNMT3B thereby causing silencing of tumor suppressor genes

Microbial manipulation of host epigenetic marks as obligate intracellular parasites has helped develop numerous ways of hijacking cell processes to facilitate the completion of their life cycle and sometimes to evade the immune responses of their host. Microbes that cause persistent infections are likely to benefit from heritable epigenetic changes in host transcription that produce an environment for their latent or persistent state without having to continuously express the initiating effectors (Virgin *et al*., 2009). Host genes involved in cell cycle progression, senescence, survival, inflammation and immunity are prime candidates as targets for such epigenetic control. Some chronic bacterial infections are also associated with malignancy, the most and widely studied being *Helicobacter pylori* infection of human gastric mucosa. Moreover, many microbes have evolved ways of eluding the immune response and, again, epigenetic changes in host cells have been implicated in these processes. Viral infection can deregulate patterns of repressive histone modifications that could then precipitate aberrant DNA methylation and the reprogramming of infected cells and their progeny.

Epigenetic mechanisms regulate expression of the genome to generate various cell types during development or orchestrate cellular responses to external stimuli. Recent studies highlight that bacteria can affect the chromatin structure and transcriptional program of host cells by influencing diverse epigenetic factors (i.e., histone modifications, DNA methylation, chromatin-associated complexes, noncoding RNAs, and RNA splicing factors), Bierne *et al*., (2012), revealed that the molecular bases of the epigenetic language and then describe the current state of research regarding how bacteria can alter epigenetic marks and machineries. Bacterial-induced epigenetic deregulations may affect host cell function either to promote host defence or to allow pathogen persistence. Thus, pathogenic bacteria can be considered as potential epimutagens able to reshape the epigenome. Their effects might generate specific, long-lasting imprints on host cells, leading to a memory of infection that influences immunity and might be at the origin of unexplained diseases (Bierne *et al*., 2012).

Various bacterial products can affect them in many ways, through activation of signaling cascades or directly in the nucleus. So far, most of the reported chromatin modifications induced by bacteria are histone acetylation/deacetylation and phosphorylation/dephosphorylation events generated through activation of host cell signalling cascades by bacterial components (e.g., microbe-associated molecular patterns, metabolites, and virulence factors). The effects are complex, because they differ according to the bacterial agonist, cell type, and kinetics parameters. Among the host signaling pathways that a number of bacteria activate, mitogen activation protein kinase (MAPKs) e.g. ERK and p38, NF-κB and Phosphatidylinositol 3-Kinase (PI3K) pathways are known to activate the kinases that phosphorylate H3S10 in the nucleus (i.e., Mitogen and stress-activated protein kinase 1/2 **(**MSK1/2), Inhibitor of KappaB Kinase alpha IKKα, and Protein kinase B (AKT/PKB), respectively) (Baek 2011). Any bacterial stimulus activating these pathways has therefore the potential to induce H3S10 phosphorylation and associated acetylated histones (Bierne *et al*., 2012).

The innate immune system senses the invasion of pathogenic microorganisms through the Toll-like receptors (TLR), which recognize specific molecular patterns present in microbial components. Microorganisms (prokaryotes) and tumor cells share a common feature: both contain unmethylated CpG motifs at a higher frequency than eukaryotes and normal cells, respectively. The innate immune system detects unmethylated CpG motifs using TLR-9 *(*Takeshita *et al*., 2004*).* The release of unmethylated CpG DNA during an infection provides a danger signal to the innate immune system, triggering a protective immune response that

improves the ability of the host to eliminate the pathogen (Klinman, 2004). Activation of the TLR-9 subsequently leads to the activation of a cascade including nuclear factor-κB (NFkB) that culminates in the activation and proliferation of immune cells.

2.16. Immune Complexes Mediated Immunological Pathways

During the primary response, naive B cell differentiation and antibody (Ab) production occur several days after antigen encounter. In contrast, following secondary antigenic exposure, B cells expand with a shortened lag phase and produce larger quantities of Abs. The difference between the primary and secondary exposures is the presence of memory B cells and pre-existing Ag-specific Abs. These antibodies (Abs) can form immune complexes (ICs) with the incoming antigen (Ag), and it is known that ICs can induce the production of higher Ab titers than Ag alone (Goins *et al*., 2010). One possible mechanism of IC-mediated enhancement is the activation of complement cascade. ICs, particularly those containing the Antibody isotypes IgG2a and IgG3, are able to activate the classical complement pathway (Goins *et al*., 2010). Because the complement receptor CD21 is part of the B cell coreceptor complex, this could lead to enhanced B cell activation. In addition, ICs are able to bind to a variety of cell types, particularly dendritic cells through FcγRs. For dendritic cells, engagement of FcγRs leads to cell activation, which results in enhanced antigen presentation and increased expression of costimulatory molecules (Goins *et al*., 2010). Because ICs are able to activate naive cells with enhanced kinetics, and because ICs are present after secondary Antigen encounter, it seems likely that ICs activate naive cells to participate in secondary responses. The formation of circulating immune complexes is the physiological consequence of antibody responses to different antigens, including microorganisms and intricate mechanisms for immune complex clearance have developed in mammals. Thus, immune complex formation is a physiological event, but their accumulation in the tissue or circulation, as seen in such disorders as rheumatoid arthritis or systemic lupus erythematosus, can be considered pathological. Immune complex accumulation leads to a broad spectrum of proinflammatory effects, including complement activation with release of phlogistic C3a and C5a peptides and cytokine secretion from FcγR-expressing cells (Senbagavalli *et al*., 2011). Immune Complex formation is generally followed by one or more secondary reactions, all of which enable the body to neutralize and clear microorganisms and non-self molecules (in the form of IC after antibody binding) that have penetrated the various body barriers (Schifferli and Taylor 1989). Inactivation and elimination of these "invaders" prevents their deposition (localization) where they might multiply (in the case of microorganisms) or induce specific damage (toxins or enzymes). IC formation followed by

these secondary reactions (such as complement fixation) enhances Mononuclear Phagocytic System (MPS) clearance mechanisms and prevents interaction with specific sites in the body that could be damaged by deposition. This entire dynamic process must be very efficient under normal circumstances, because although we are constantly exposed to and challenged by foreign pathogens, IC do not normally accumulate in blood or organs (Senbagavalli *et al*., 2011). Immune complexes have been found to be immunosuppressive in a variety of experimental systems and have been demonstrated in other parasitic diseases, such as malaria, trypanosomiasis, schistosomiasis, and onchocerciasis (Onyenekwe *et al*., 2000, Senbagavalli *et al*., 2011). Even though evidence suggests that, with few exceptions, circulating immune complex measurements do not correlate with disease activity, from the background of serum sickness animal models (Hong *et al*., 1991), it has been accepted that continuous formation of the immune complex is necessary to maintain chronicity.

Antigens, especially under inflammatory conditions, can be found already bound to antigen-specific antibodies, and these antigen–antibody complexes (referred to as immune complexes or immune-complexed antigen) can be recognized by Fc receptors through the Fc region of the antibodies. Binding of the immune complexes typically triggers crosslinking of the Fc receptors, their internalization together with the antigen, and shuttling of the immune complexes toward antigen presentation compartments (Baker *et al*., 2013, Guilliams *et al*., 2014). Immune Complexes interact with receptors for the Fc portion of Immunoglobulins the Fc Receptors (FcRs) which are expressed by many cells of the immune system (Platzer *et al*., 2014). Ligation of FcRs, specific for IgG, termed FcγRs, on myeloid cells induces cell activation which include phagocytosis of opsonized pathogens, Ab-dependent cell-mediated cytotoxicity (ADCC), release of proinflammatory mediators and reactive oxygen intermediates, and production of several cytokines and chemokines (Platzer *et al*., 2014). Circulating Immune Complexes (CICs) first localize within the vasculature and then translocate into extravascular tissue, attracting immune cells. Circulation of immune complexes, can stir up different immune responses including complement activation, opsonization, phagocytosis, activation of immune cells (macrophages, neutrophils) and subsequent release of cytokines, chemokines and activation of protease pathways (Meyadas *et al*., 2009). Immune Complexes deposited intravascularly can directly engage circulating leukocytes. Both soluble and insoluble immune complexes can activate infiltrating immune cells such as T cells, neutrophils, mast cells and macrophages that then release inflammatory mediators (cytokines and prostanoids) capable of activating the endothelium and their ability to recruit more cells (Meyadas *et al*., 2009).

The ability of CICs to classically and continuously activate immune responses is considered in this study as neglected immunological response that can induce carcinogenesis and as such deserves serious attention. Circulating immune complexes (CICs) are now viewed as regulators of both cellular and humoral immune responses by virtue of their capacities to interact with antigen receptor bearing lymphocytes and sub-population of T and B cells as well as with macrophages and neutrophils having FC receptors (Goins *et al*., 2010).

Furthermore, profound changes in neutrophil responsiveness to these complexes occur after cytokine priming. It has been established that under appropriate conditions these neutrophils can actively release large quantities of reactive oxidants and discharge the contents of their granules extracellularly. If such large scale release of these toxic molecules occurred in vivo, then it is likely that local antioxidants and antiproteinases would become saturated and tissue damage would ensue (Fossatti *et al*., 2002).

2.17. IgG and Breast Tumour Development

B-cell precursors mature within bone marrow, where somatic recombination of immunoglobulin genes results in expression of a diverse array of B-cell receptors. Mature antigen-committed B cells migrate to secondary lymphoid organs (lymph nodes or spleen, predominantly). Upon antigen recognition by B-cell receptors, the B lymphocytes become activated and undergo clonal expansion, resulting in their enhanced capacity to recognize foreign antigens (Zirakzadeh *et al*., 2013). Acute activation of B-lymphocyte responses (to foreign antigens or tissue damage) can also result in rapid induction of several soluble mediators, including diverse immunoglobulin subtypes, B-cell-derived cytokines such as IL-6 and activation of complement cascades, which together trigger recruitment of innate immune cells from the circulation. In this manner, acutely activated B cells orchestrate phagocytic or cytotoxic destruction of immunoglobulin-complexed antigens (pathogens or damaged cells) by innate immune cells. Such acute B-cell responses are critical for protecting tissues from pathogens and nonself-antigens.

Chronic activation of B cells can be deleterious, however, as evidenced by their association with several pathologic disease states (rheumatoid arthritis and other autoimmune diseases) and some cancer types (Mohamed *et al*., 2013). Lymphocytic infiltrates within solid tumors are well-recognized positive predictors of survival. Notably, B cells are a significant component of these infiltrates. For example, they have been reported to be present in ∼25% of breast cancers and constituted up to 40% of the tumor-infiltrating lymphocyte (TIL) population. Furthermore,

tumor-infiltrating B lymphocytes (TIL-Bs) have been correlated with survival in ovarian cancer. The survival was higher when tumors contained both CD20+ and CD8+ Cells than either of the TILs alone, which suggests an immunological ooperation between the two cell populations (Zirakzadeh *et al*., 2013).

During breast tumourigenesis, mature B cells (including naive cells and activated cells) can be found in secondary lymphoid tissues as well as in tumor-associated stroma. As compared with healthy patients without evidence of cancer, the sentinel (draining) lymph nodes of breast cancer patients contain enriched populations of proliferating and affinity matured (IgG^+) B lymphocytes. Moreover, data from retrospective studies examining the percentages of B cells present in sentinel and auxiliary lymph nodes of breast cancer patients reveal that their presence and/or maturation (IgG^+) correlates with increases in disease stage (stage I versus stage II) and in total tumor burden (Yang *et al*., 2013).

Contrasting roles of adaptive leukocytes during cancer development, show that during acute inflammatory responses (left panel), Th1 $CD4^+$ and $CD8^+$ T cells directly regulate tumor cell cytotoxicity, while indirectly polarizing innate immune cells toward tumor suppression (such as M1 polarization of tumor-associated macrophages [TAMs]) (Lazarevic and Glimcher, 2011). B-cell-derived factors (immunoglobulins and complement) facilitate recruitment of innate leukocytes and targeted destruction of neoplastic cells. During chronic inflammation, however (right panel), myeloid suppressor cells, Th2 $CD4^+$ T cells and regulatory T (T-reg) cells function in combination to both repress $CD8^+$ cytotoxicity and to induce protumoral polarization of innate immune response (such as M2 polarization of TAMs) via cytokine secretion (IL-4, IL-13, IL-10, IL-6 and transforming growth factor beta (TGFβ)) (Lazarevic and Glimcher, 2011). Chronically activated B cells promote accumulation of innate cells in the neoplastic stroma by immunoglobulin and cytokine production. When polarized, as during chronic inflammation, these innate immune cells in turn provide a rich proangiogenic and protumoral microenvironment.

Why are $CD8^+$ CTL-mediated responses not more effective in eradicating or minimizing cancer occurrence and how might $CD4^+$ T cells be involved in enhancing breast cancer progression? One plausible mechanism may have to do with the 'polarity' of the $CD4^+$ T-helper-cell response at primary tumor sites and/or their distant metastases. $CD4^+$ T-helper cells are activated in response to soluble factors and can be classified generally into two categories as either Th1 cells

or Th2 cells (Lazarevic and Glimcher, 2011). Following an activating stimulus, $CD4^+$ T-helper cells that are Th1-polarized, secrete IFNγ, transforming growth factor beta, TNFα and IL-2. These cytokines collaborate with the cytotoxic/cell killing functions of $CD8^+$ T cells (Lazarevic and Glimcher, 2011) and can induce upregulation of antigen processing (in the proteasome), can induce expression of MHC class I and II molecules, and can induce other antigen display cofactors in neoplastic cells. Th1 $CD4^+$ T-helper cells also enhance antitumor immune responses by secretion of INFγ, which in turn induces activation of macrophage cytotoxic activity. In contrast Th2-polarized $CD4^+$ T-helper cells express IL-4, IL-5, IL-6, IL-10 and IL-13, which induce T-cell anergy and loss of T-cell-mediated cytotoxicity while also enhancing humoral immunity (B-cell function) (Tanikawa *et al*., 2012). Taken together, Th1 responses are thought to be beneficial toward antitumor immunity whereas Th2 responses may downregulate cell-mediated antitumor immunity and enhance protumor humoral responses (Lazarevic and Glimcher, 2011). Model depicting the consequences of acute inflammation versus chronic inflammation. During acute antitumor inflammatory responses (left panel), Th1-polarized T cells secrete antitumor cytokines (IL-2 and INFγ, for example), which in combination with antitumor-directed B-cell-derived factors (such as immunoglobulins (Igs)) activate tumor inhibitory responses in recruited innate immune cells and cytotoxic T lymophocytes (CTLs) that together favor tumor rejection. In contrast, chronic activation of immune response (right panel) without resolution (of damage) often results in accumulation of regulatory T (Treg) cells, Th2 cells, and activated B cells, which in turn secrete progrowth factors (IL-4, IL-6, IL-10, IL-13, transforming growth factor beta (TGFβ) and immunoglobulins, for example) that enhance protumor responses in innate immune cells and inactivate CTL cytotoxicity, thus favoring tumor promotion. IgG expression in breast cancer cells is correlated with malignancy and AJCC stages of the cancers. This suggests that breast cancer derived IgG may be associated with genesis, development and prognosis of the cancer (Lazarevic and Glimcher, 2011, Zirakzadeh *et al*., 2013).

2.18. Immune Complexes Mediated Oxidative Stress

Immune complex is considered in this study as evolving immunological product that can induce ROS production which would in turn attack DNA and cause breakage. This immunological pathway through the persistence of circulating immune complexes remains an unattended pathway of DNA damage. The immunological activities of circulating immune complexes, may directly or indirectly induce DNA damage and its enabling repair in developing lymphocytes that have remained continuously challenged with reactive oxygen species. Our long, delicate DNA strands are easily broken. Ionizing radiation, such as x-rays and gamma rays, as well as

drugs like bleomycin (Blenoxane create reactive forms of oxygen, which in turn attack DNA and cause breakage (Azzam *et al*., 2012). DNA is continuously attacked by reactive species that can affect its structure and function severely. Structural modifications to DNA mainly arise from modifications in its bases that primarily occur due to their exposure to different reactive species. DNA damage plays a major role in mutagenesis, carcinogenesis and ageing. The vast majority of mutations in human tissues are certainly of endogenous origin (Bont and Larebeke 2004, Azzam *et al*., 2012). A thorough knowledge of the types and prevalence of endogenous DNA damage can be considered essential for an understanding of the interaction of exogenous agents and influences with endogenous processes in the induction of cancer and other diseases. In particular, this is important for risk analysis concerning low dose environmental factors (Azzam *et al*., 2012). Reactive oxygen species (ROS), despite being products of normal cellular metabolism, are considered to have a substantial influence on the development of cancer, in part, because of their ability to react with DNA. For example hydroxyl radical ($^{\bullet}OH$) can react with pyrimidines, purines and chromatin protein resulting in base modifications, genomic instability and alterations in gene expression. These reactions in connection with oncogenes or tumour suppressor genes may result in the initiation of cancer. Under normal conditions, ROS are maintained within narrow boundaries by scavenging systems, such as superoxide dismutases, peroxiredoxins (Prx) and glutathione-related antioxidant defences. Consequently, when the amount of ROS exceeds the capacity of the ROS scavenging systems, oxidative stress occurs and this imbalanced redox status leads to an increase in damage to DNA (Aprioku, 2013)..

8-Hydroxydeoxyguanosine (8-oxodG) is a specific marker of 2′-deoxyguanosine damage after ROS attack to DNA. 8-OxodG is one of the most widely used oxidative stress biomarkers. DNA damage may occur under the influence of factors external to the cell (factors of exogenous origin, e.g. environmental factors) or potentially aggressive agents produced by normal cell metabolism (factors of endogenous origin). The consequences for the cell's DNA damage caused by the action of endogenous factors may be more serious and/or more extensive than the effect of most of the exogenous DNA damaging factors (Chakarov *et al*., 2014). DNA damaging events caused by endogenous factors generally occur much more frequently than damage caused by exogenous factors. For example, several thousands of nitrogenous bases are lost daily from DNA in eukaryotic cells as a result of spontaneous base hydrolysis alone. DNA damaging factors of endogenous and of exogenous origin may exert their genotoxic action on DNA using the same mechanism. For example, both ionising radiation (an agent of environmental origin) and normal oxidative phosphorylation (obviously of endogenous origin) generate reactive

oxygen species which may damage the cell's DNA (Chakarov *et al*., 2014). In this work, immune complex is considered an exogenously fuelled endogenous genotoxic complex that can trigger DNA damage.

The highly, continuously generated reactive hydroxyl radical (˙OH) under the influence of persistence circulating immune complexes may react with DNA by addition to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and each of the C-H bonds of 2′-deoxyribose. Addition to double bonds of DNA bases occurs at or near diffusion-controlled rates with rate constants from 3 to10 × 10^9 M^{-1} s^{-1}; the rate constant of H abstraction amounts to 2 × 10^9 M^{-1} s^{-1} (Chakarov *et al*., 2014). Addition to the C5-C6 double bond of pyrimidines leads to C5-OH and C6-OH adduct radicals and H atom abstraction from thymine results in the allyl radical. Adduct radicals differ in terms of their redox properties, with C5-OH adduct radicals being reducing and C6-OH adduct radicals oxidizing. Were it not for cellular defences such as low molecular weight antioxidants, enzymic antioxidants, and DNA repair, levels of such oxidatively modified bases would rapidly represent the majority of bases in DNA. The antioxidant systems have been recognized for many years, and are relatively well defined. In contrast, although it has been some years since repair of oxidative DNA damage was first reported, the last decade has seen a notable increase in research effort directed toward unravelling DNA repair processes (Borut *et al*., 2013).

Immunological activities of CICs can generate TNF-α which has been reported to cause excessive free radical generation within cultured myocytes, endothelial cells, hepatocytes and cholangiocarcinoma cells (Aya *et al*., 2012). Proposed mechanisms involve up regulation or direct activation of several RONS producing enzymes including NADPH oxidase and inducible nitric oxide synthase (iNOS), altering levels of intracellular glutathione and damaging components of oxidative metabolism in the mitochondria resulting in excessive reactive oxygen species (ROS) production (Chen *et al*., 2008, Aya *et al*., 2012). Elevation of intracellular RONS and redox imbalance may therefore be responsible for TNF-α/TNFR signalling-induced DNA strand breaks, and as proposed in endothelial dysfunction (Chen *et al*., 2008). Circulating Immune Complexes Liganding of Fc receptors (on neutrophils, monocytes or macrophages) and mannose receptors (on macrophages) increases their O_2 uptake, called the respiratory burst. These receptors activate a membrane-bound NADPH oxidase that reduces O_2 to O_2^- (superoxide). Superoxide can be reduced to OH^- (hydroxyl radical) or dismutated to H_2O_2 (hydrogen peroxide) by superoxide dismutase. O_2^-, OH^-, and H_2O_2 are activated oxygen species

that are potent oxidizing agents in biological systems which adversely affect a number of cellular structures including membranes and nucleic acids (Kok *et al*., 2014). Furthermore, at least in the case of neutrophils, these reactive oxygen intermediates can act in concert with a lysosomal enzyme called myeloperoxidase to function as the myeloperoxidase system. During phagocytosis glucose is metabolized via the pentose monophosphate shunt and NADPH is formed. Cytochrome B which was part of the specific granule combines with the plasma membrane NADPH oxidase and activates it. The activated NADPH oxidase uses oxygen to oxidize the NADPH (Malka *et al*., 2012, Kok *et al*., 2014). The result is the production of superoxide anion. Some of the superoxide anion is converted to H_2O_2 and singlet oxygen by superoxide dismutase. In addition, superoxide anion can react with H_2O_2 resulting in the formation of hydroxyl radical and more singlet oxygen. The result of all of these reactions is the production of the toxic oxygen compounds superoxide anion (O_2-), H_2O_2, singlet oxygen ($^1O2$) and hydroxyl radical (OH) (Kok *et al*., 2014).

Oxidative stress has been defined as an elevation in the steady state concentration of various Reactive Oxygen State species (ROS) on a cellular level, such as the hydroxyl radical (OH), super oxide anion radical and the nitric acid radicals. Antioxidants defence mechanisms begin to work by preventing ROS formation and their induced damage through a number of enzymatic and non-enzymatic systems. Under normal physiological conditions, there is a balance maintained between endogenous oxidants and antioxidants, when imbalance occurs, through the excessive generation of oxidants or a decrease of antioxidants, this abnormal oxidant system then enters what is called oxidative stress (Di Renzo *et al*., 2010). Various markers of oxidative damage have been identified; the most popular markers designed for lipid peroxidation, were malondialdehyde (MDH) and oxidized low density lipoprotein (ox LDL). Recently 8-hydroxy-2-deoxy guanosine (8-oH-2-deoxy Guanosine) (8-OH-dG) emerged as a marker for oxidative stress and acts as a reliable biomarker for DNA oxidative damage (Taylor *et al*., 2010).

2.19. Immune Complexes Mediated Inflammatory Pathways

Of the ten leading causes of mortality in the United States, chronic, low-level inflammation contributes to the pathogenesis of at least seven. These include heart disease, cancer, chronic lower respiratory disease, stroke, Alzheimer's disease, diabetes, and nephritis (Cao 2011, Jha *et al*. 2009; Ferrucci *et al*. 2010, Singh and Newman 2011). Inflammation has classically been viewed as an *acute* (short term) response to tissue injury that produces characteristic symptoms and usually resolves spontaneously. More contemporary revelations show *chronic* inflammation

to be a major factor in the development of degenerative disease and loss of youthful functions. Chronic inflammation can be triggered by cellular stress and dysfunction, such as that caused by excessive calorie consumption, elevated blood sugar levels, and oxidative stress. It is now clear that the destructive capacity of chronic inflammation is unprecedented among physiologic processes (Karin and Hans 2016). The danger of chronic, low-level inflammation is that its silent nature belies its destructive power. In fact, stress-induced inflammation, once triggered, can persist undetected for years, or even decades, propagating cell death throughout the body. Due to the fact that it contributes so greatly to deterioration associated with the aging process, this silent state of chronic inflammation has been coined "inflammaging". Chronic inflammation in tissues arises from sustained activation of the innate immune system (neutrophils, macrophages, and fibroblasts) as well as the adaptive immune system (B and T cells) (de Visser *et al*., 2006).

This chronic inflammatory response to persistent infections or environmental insults increases cancer risk both directly, through DNA damage, and indirectly, through tissue remodeling and fibrosis (de Visser *et al*., 2006). One strategy to evaluate the relationship of cancer with chronic inflammation is to measure circulating levels of inflammatory markers. Most previous epidemiologic investigations of circulating inflammatory markers and cancer have included a narrow range of markers (e.g., CRP, IL-6, IL10, TNF-alpha etc.) (Chaturvedi *et al*., 2011). The process of inflammation is complex and involves multiple key mediators, including chemokines, pro-inflammatory cytokines, anti-inflammatory cytokines, growth factors. Research has shown that a key early event in chronic inflammation is the loss of epithelial barrier integrity leading to increased exposure of resident inflammatory cells (macrophages, mast cells, dendritic cells) to both pathogenic and non-pathogenic microbes (de Visser *et al*., 2006). Exogenous pathogen-associated molecular patterns (PAMPs) and endogenous ligands are recognized by the macrophages through pattern recognition receptors (PRRs). Engagement of the PRRs triggers signaling pathways that lead to the release of chemokines and cytokines, the recruitment and activation of lymphocytes, and the propagation of chronic inflammation. Genetic factors can influence the system at multiple checkpoints (de Visser *et al*., 2006). Cytokines and growth factors are secreted by inflammatory cells and stromal cells in the tumor microenvironment during chronic inflammation; they engage their cognate receptors to activate downstream kinases and transcription factors such as NF-κB, STAT, and HIFs.

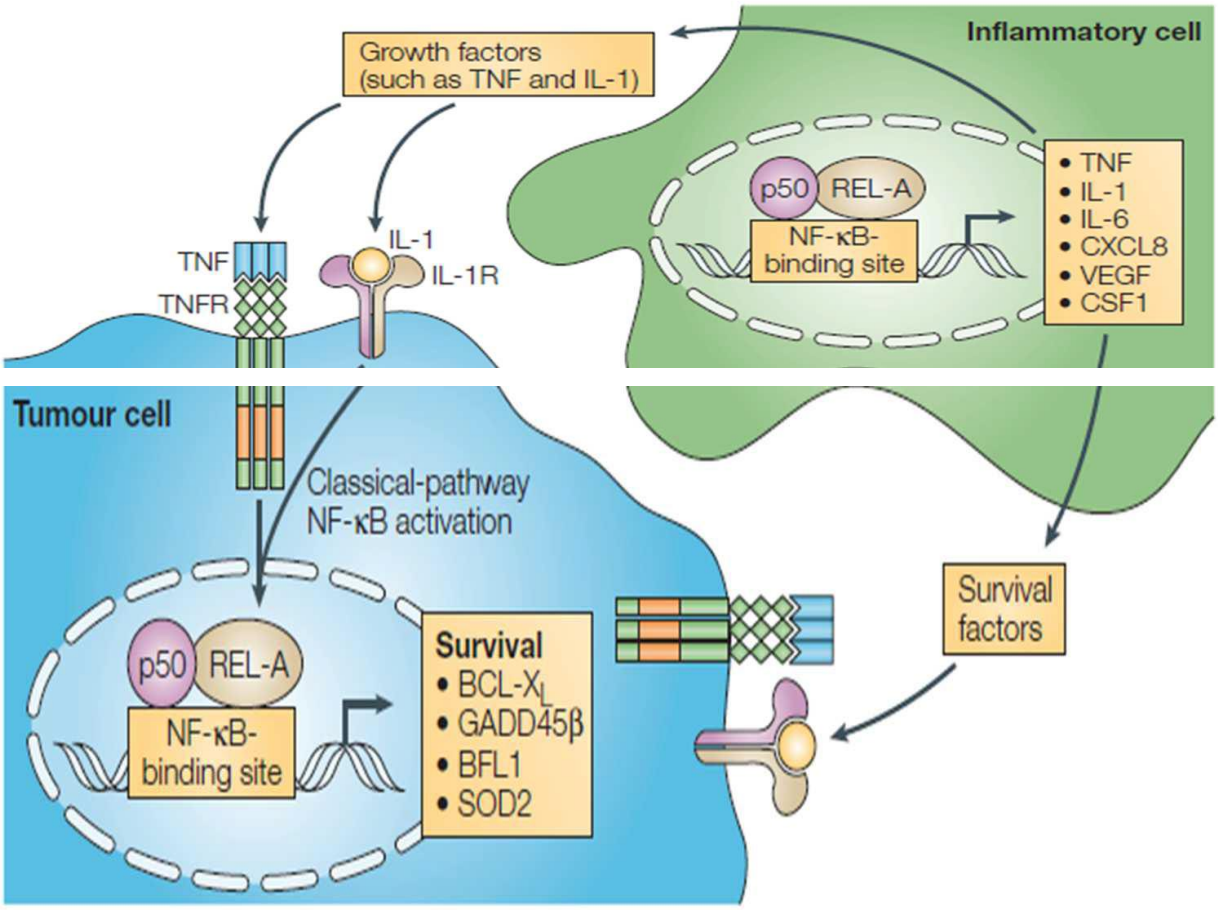

Figure 2.14: Progression of Inflammatory cell to Tumour cell Through NF-kB activation pathway (Chaturvedi et al., 2011).

These TNFs can up-regulate the expression, and enhance the activity of Noxs. They can also regulate target genes involved in cell cycle control, apoptosis, metabolism, angiogenesis, and metastasis. On the other hand, Nox-derived ROS can oxidize redox-sensitive cysteine residues in the catalytic domain of PTPs and inactivate their ability to limit the propagation of cytokine-derived signals. ROS can also directly oxidize the cysteine residues in some TFs and regulate their transcriptional activity (Wu *et al*., 2014).

2.19.1. Tumour Necrosis Factor-Alpha (TNF-Alpha)

Tumor necrosis factor alpha (TNF-α) is an intercellular signaling protein called a cytokine, which can be released by multiple types of immune cells in response to cellular damage, stress, or infection. Originally identified as an anti-tumor compound produced by macrophages (immune cells) (Dong *et al*. 2015), TNF-α is required for proper immune surveillance and function. Acting alone or with other inflammatory mediators, TNF-α slows the growth of many pathogens. It activates the bactericidal effects of neutrophils, and is required for the replication of several other immune cell types (Sethi *et al*. 2008). Excessive TNF-α, however, can lead to a chronic inflammatory state, can increase thrombosis (blood clotting) and decrease cardiac contractility, and may be implicated in tumor initiation and promotion (Kundu *et al*. 2008). Nuclear factor kappa-B (NF-kB) is important in the initiation of the inflammatory response. When cells are exposed to damage signals (such as TNF-α or oxidative stress), they activate NF-kB, which turns on the expression of over 400 genes involved in the inflammatory response (Sethi *et al*. 2008). These include other inflammatory cytokines, and pro-inflammatory enzymes including *cyclooxygenase-2* (COX-2) and *lipoxygenase*. COX-2 is the enzyme responsible for synthesizing pro-inflammatory prostaglandins, and is the target of non-steroidal anti-inflammatory drugs (ibuprofen, aspirin) and COX-2 inhibitors.

TNF-α is a major inflammatory cytokine shown to be highly expressed in breast carcinomas (Gustavo *et al*., 2016). TNF was thought to be produced primarily by macrophages. But it is produced also by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neurons *(*Swardfager *et al*., 2010*).* Large amounts of TNF are released in response to lipopolysaccharide, other bacterial products, Interleukin-1 (IL-1) and other environmental stresses. In the skin, mast cells appear to be the predominant source of pre-formed TNF, which can be released upon inflammatory stimulus (e.g., LPS) (Selwood and, Jaffe, 2011).

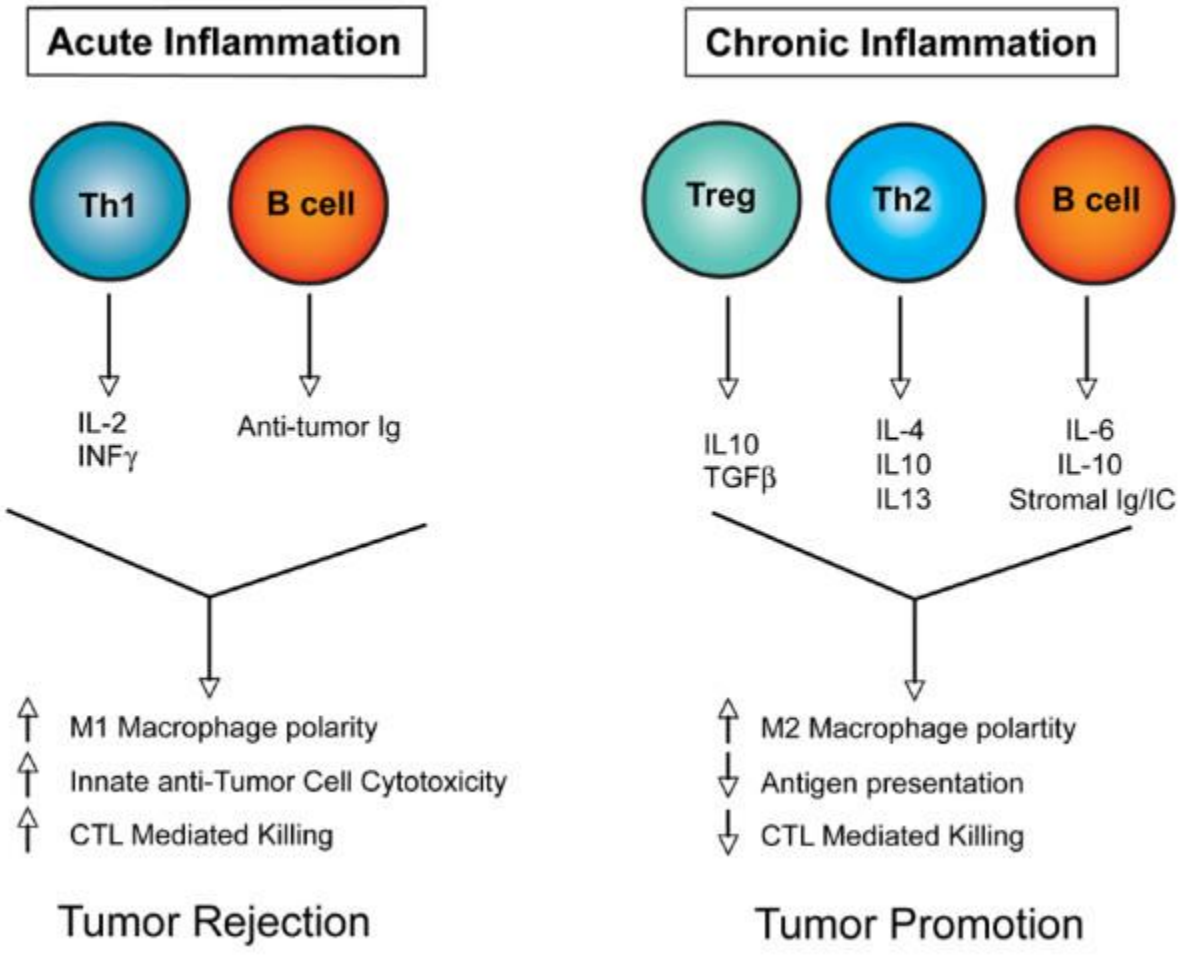

Figure 2.15. A Generalized Scheme Explaining the effects of Chronic Inflammation on tumour development (de Visser *et al*., 2006).

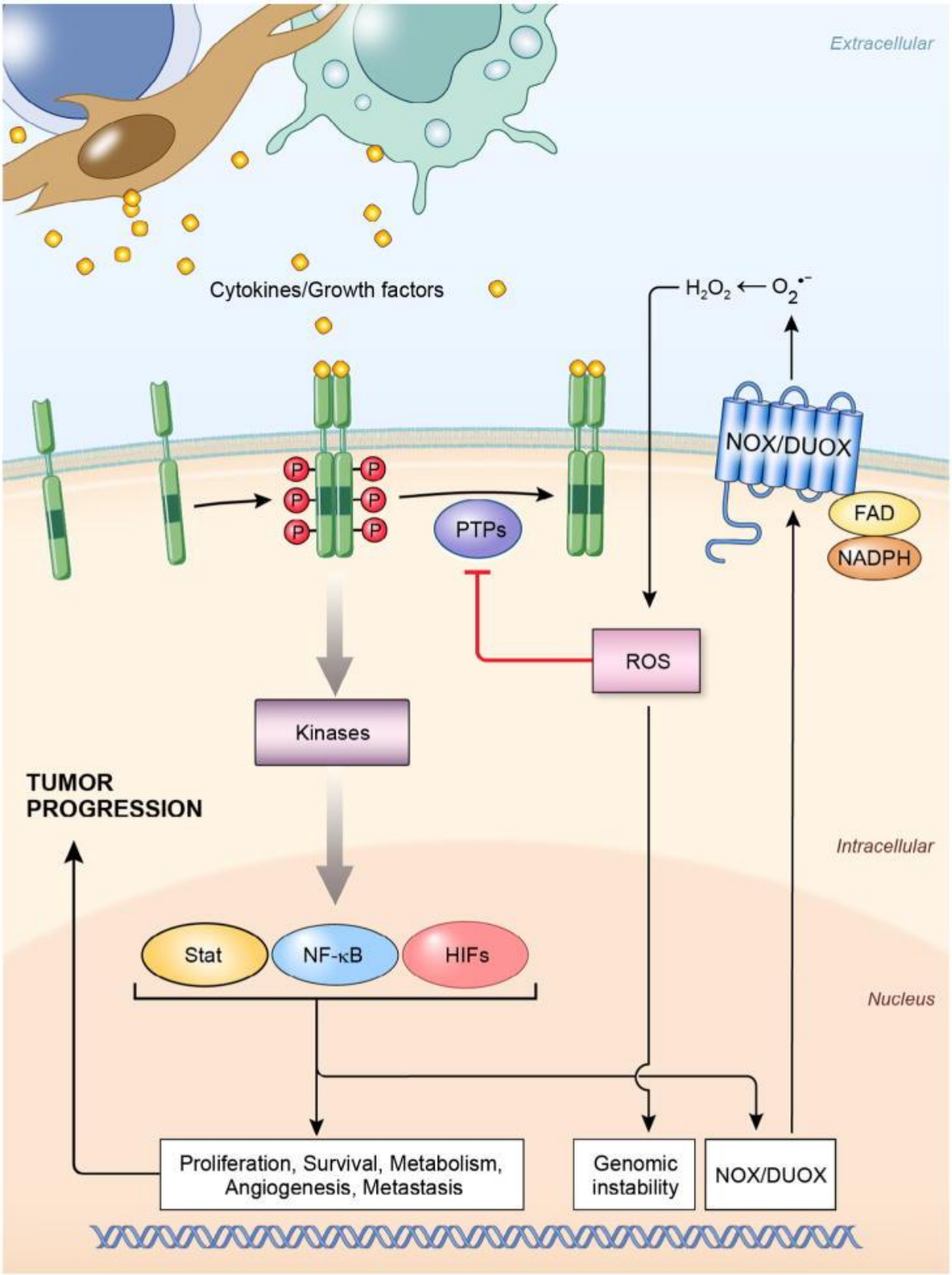

Figure 2.16: Role of cytokines, growth factors, and Noxs in tumor initiation, promotion, and progression (Wu *et al*., 2014).

Some studies showed evidence that chronic inflammation is linked to breast cancer recurrence and that elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients (Cole, 2009). In addition, experimental studies clearly indicate that inflammatory mediators promote tumor development in cancer prone animal strains. Moreover, inhibition of TNF-α and NF-kappaB (NF-κB) transcription factor is proved to be protective with respect to chemical induced mammary gland carcinogenesis (Connelly *et al*., 2011). Further, the in vitro activation of the TNF-α/NF-κB axis has induced an invasive and malignant behaviour in breast cancer cells. Indeed, investigations strongly suggest that the chronic expression of TNF-α in breast tumors actually supports tumor growth. The number of cells expressing TNF-α in inflammatory breast carcinoma was found to be correlated with increasing tumor grade and node involvement, and TNF-α expression was suggested to play a role in the metastatic behavior of breast carcinomas. Furthermore, patients with more progressed tumor phenotypes were shown to have significantly higher TNF-α serum concentration (Kamel *et al*., 2012). The tumor-promoting functions of TNF-α may be mediated by its ability to induce proangiogenic functions, to promote the expression of matrix metalloproteinases (MMP) and endothelial adhesion molecules, and to cause DNA damage via reactive oxygen, the overall effect of which is promotion of tumor related processes (Kamel *et al*., 2012).

Tumour necrosis factor (TNF) acts through two receptors, TNFR1 (TNF Receptor-1) and TNFR2 (TNF Receptor-2) (Petrus et al., 2011). TNFR1 is expressed by all human tissues and is the major signaling receptor for TNF-Alpha. TNFR2 is mostly expressed in immune cells and mediates limited biological responses. TNFR2 binds both TNF-Alpha and TNF-Beta. Binding of TNF-Alpha to its two receptors, TNFR1 and TNFR2, results in recruitment of signal transducers that activate at least three distinct effectors (Petrus *et al*., 2011). Through complex signalling cascades and networks, these effectors lead to the activation of Caspases and two transcription factors, Activation Protein-1 and NF-KappaB (Nuclear Factor-KappaB). The binding of the TNF trimer to TNFR1 causes trimerization of TNFR1, resulting in formation of the Complex I consisting of TNFR1, TNFR1-associated death domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2) and Receptor Interacting Protein (RIP) kinases (RIPK). Complex I mediates the NF-κB activation pathway through the Mitogen-activated protein kinase kinase kinase 3 (MEKK3-IKK-IκB- NF-κB cascade, leading to expression of a battery of genes including those encoding antiapoptotic factors such as inhibitors of apoptotic proteins (IAPs) and Cellular FLICE -inhibitory protein (c-FLIP) (Wang and Lin 2008, Bremer, 2013).

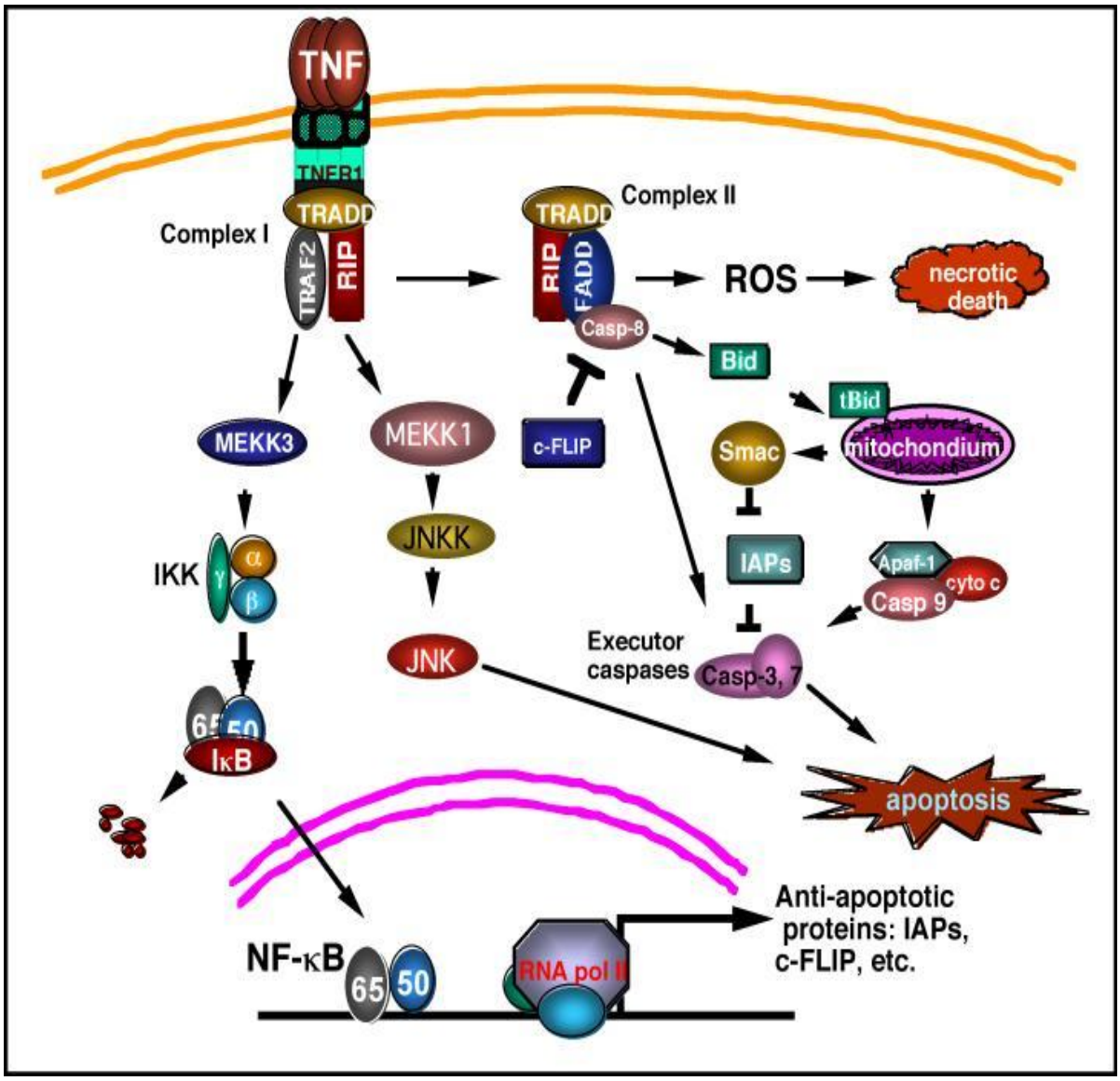

Figure 2.17. TNFR1 mediated signaling pathways (Wang and Lin, 2008).

The internalization of the TNFR1 complex enables formation of Complex II that contains RIP, TRADD and Caspase 8. Caspase 8 is autoactivated to trigger activation of the executor caspases −3, and −7, resulting in apoptosis. Cleavage of Bid by caspase 8 activates the mitochondrial apoptosis pathway that involves release of cytochrome c and Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis- binding protein (Smac/DIABLO) from mitochondria. Cytochrome c binds Apoptotic protease activating factor 1 (Apaf1) to activate caspase 9-mediated activation of executor caspases. The NF-κB activated factor c-FLIP suppresses caspase 8 activation while IAPs inhibit executor caspases. Smac released from the mitochndria suppresses IAPs to release the apoptosis brake. Complex II also mediates a necrotic cell death through ROS (Handa *et al*., 2011).

TNF-induced NF-κB activation is initiated by activation of inhibitor of κB (IκB) kinase (IKK). During TNFR-1 signaling IKK is recruited to the TNFR-1 signaling complex (Complex I), which consists of TRADD, TRAF2, and RIP. IKK is activated by a RIP-dependent mechanism that involves MEKK3 (Wang and Lin 2008). The activated IKK phosphorylates IκB, which retains NF-κB in the cytoplasm, to trigger its rapid polyubiquitination followed by degradation in the 26S proteasome. This process causes the NF-κB nuclear localization signal to be exposed, allowing its nuclear translocation to promote transcription of its target genes (Wang and Lin 2008). TNF-alpha has pleiotropic actions and has emerged as an especially important mediator in pro-inflammatory responses and activation of T cells. Various observations suggest that sex hormones may influence monocyte TNF-alpha production: in males, endotoxin-stimulated monocytes produce more TNF-alpha as compared to females (Aruna *et al*., 2014). Whether this is due to direct effects of high levels of testosterone in males remains uncertain since in vitro studies showed no effect of testosterone upon monocyte TNF-alpha production (Aruna *et al*.,

2014). Furthermore, endotoxin-stimulated monocytes of women in the luteal phase produce more TNF-alpha as compared to monocytes of women in the follicular phase. Kamel *et al*., (2012), explored a new mechanism by which inflammation may influence breast carcinogenesis through the estrogen metabolic pathway. The effect of the TNF-α, a hallmark of inflammation, on the estrogen metabolic pathway in MCF-7 estrogen dependent breast cancer cells has been investigated. The choice of MCF-7 as study model was built on several bases: First, studies have shown that regular use of nonsteroidal antiinflammatory drugs (NSAIDS), such as aspirin, significantly reduce the risk of ER-positive but not ER-negative breast cancers (Kamel *et al*., 2012). Second, it was recently suggested that inflammation may promote more aggressive ER-

positive tumors and that this may be one of the mechanisms by which a portion of ER-positive breast tumors fail to respond to endocrine therapy (Baumgarten and Frasor, 2012). In general, they sensed that further research is required to fully elucidate the mechanisms of action of TNF-α on breast carcinogenesis because TNF-α activity may vary under different physiological conditions and in a cell-type-dependent manner which contributes to a sense of ambiguity regarding its tumor effects. Thus proposed association of TNF-alpha molecule with other molecules under the influence of MAC or absence of MAC may throw more light in activities of TNF-alpha. Most of the reports examining the effects of TNF-α on MCF-7 breast cancer cells demonstrated its ability to induce apoptosis, inhibit proliferation and promote migration, invasion as well as resistance to chemotherapeutic drugs. These effects may however vary with other estrogen dependent (T47D) or independent (MDA-MB-231) cell lines (Goldberg and Schwertfeger, 2010). It is encouraged thus to explore this area of research because not many studies have examined the role of inflammation on the estrogen metabolic pathways in general inspite of this pathway being an important cause of carcinogenesis. In some of these studies, TNF-α has been found to have an important role in regulating estrogen synthesis in peripheral tissues, including normal and malignant breast tissues (Kamel *et al*., 2012).

2.19.2. Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB)

NF-κB is found in almost all animal cell types and it is an important transcription factor implicated in regulation of cytokines and chemokines. NF-*k*B regulates proinflammatory cytokines and these mediators act as stimulatory signals for NF-*k*B resulting in amplification that is often seen in inflammation. Such positive feed back mechanisms contribute to intensity of inflammation. In case of tumor growth these mechanisms may play a role in sustained tumorigenesis. NF-*k*B is a dimeric complex of Rel family proteins that functions as a transcription factor. It regulates more than 400 genes (Ledoux and Perkins, 2014). In resting state NF-*k*B is confined to the cytoplasm through its interaction with inhibitor of kappa B (IkB) proteins. Nucler translocation of NF-*k*B is a crucial step in its activation. NF-*k*B may be activated by various exogenous and endogenous factors including proinflammatory cytokines, T- and B-cell mitogens, biological, physical and chemical stressors such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low density lipoprotein, and bacterial or viral antigens (Perkins, 2007, Listwak *et al*., 2013, (Ledoux and Perkins, 2014). Constitutive activation of NF-*k*B has been reported for many cancers, including breast cancer. The activation of NF-κB, especially the constitutively activated NF-κB in chronic inflammatory patients, has been found to have critical linkage with a wide variety of human diseases, including asthma, atherosclerosis,

AIDS, Alzheimer's disease (AD), Parkinson's disease (PD), rheumatoid arthritis, cancer, diabetes, and osteoporosis which belong to autoimmune/inflammatory diseases (Shih *et al*., 2015). Besides constitutive expression in tumor cells, there are several lines of evidence that relate NF-*k*B to carcinogenesis: NF-*k*B regulates most of the genes linked to inflammation – some of those may have protumor effects; NF-*k*B regulates antiapoptotic genes, genes related to proliferation, invasion and angiogenesis; NF-*k*B has been linked to transformation; NF-*k*B is activated by numerous carcinogens while chemopreventive agents suppress its activation. In case of breast cancer NF-*k*B was related to progression of breast cancer to hormone-independent phenotype. Subsequent studies have shown thatNF-*k*B is activated in both hormone negative and hormone positive human breast cells. In several studies NF-*k*B was related to more aggressive phenotype of breast tumors.Increased NF-*k*B activity was related to breast canceroverexpressing HER-2/neu, to poorly differentiated tumours and tumours with high mitotic counts. NF-*k*B was reported to contribute to the unusual phenotype andaggressiveness of inflammatory breast cancer (Ledoux and Perkins, 2014). In study of ER-positive primary breast carcinoma, NF-*k*B was suggested to be a marker of high – risk subset of tumors.

NF-κB has been recognized as a member of Rel family of transcription factors. In mammals, there are five different members to compose the NF-κB family: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) which have the similar amino acid sequence, the RHD (Rel homology domain, over approximate 300 amino acids) of these proteins (Shih *et al*., 2015). The activated NF-κB subunits will assemble to form the homo-or hetero-dimerized transcription factor complexes displaying the DNA-binding ability and transactivation potentials. The most widely studied form of NF-κB is a heterodimer of the p50 and p65 subunits and is a potent activator of gene transcription. NF-κB is activated by a wide variety of agents including viruses, bacterial toxins such as lipopolysaccharide (LPS), UV light, oxidative stresses such as free radicals and cigarette smoke, inflammatory stimuli, cytokines, carcinogens, tumor promoters, and various mitogens (Shih *et al*., 2015). NF-κB regulates the expression of almost 500 different genes, including enzymes [e.g., cyclooxygenase (COX)-2, 5-lipoxygenase (LOX), and inducible NO synthase (iNOS)], cytokines [such as interleukin (IL)-1, IL-6, IL-8, chemokines, and tumor necrosis factor (TNF)], adhesion molecules, cell cycle regulatory molecules, and angiogenic factors (Gupta *et al*., 2010).

This is a nuclear factor protein complex that controls transcription of DNA, cytokine production and cell survival. NF-κB plays a key role in regulating the immune response to infection.

Incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development (Listwak *et al*., 2013). Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis. Defects in NF-κB result in increased susceptibility to apoptosis leading to increased cell death. This is because NF-κB regulates anti-apoptotic genes especially the TRAF1 and TRAF2 and, therefore, checks the activities of the caspase family of enzymes, which are central to most apoptotic processes (Rahman and McFadden, 2011)

The NF-κB family of transcription factors has an essential role in inflammation and innate immunity. Furthermore, NF-κB is increasingly recognized as a crucial player in many steps of cancer initiation and progression. During these latter processes NF-κB cooperates with multiple other signaling molecules and pathways. When cells are exposed to damage signals such as (TNF-α, oxidative stress or lipopolysaccharide), Stimulation through these receptors leads to activation of the IκB kinase (IKK) complex, which in turn phosphorylates IκBα primarily by IKK2. They activate NF-kB, which turns on the expression of over 400 genes involved in the inflammatory response (Hoesel and Schmid, 2013). These include other inflammatory cytokines, chemokines and pro-inflammatory enzymes including *cyclooxygenase-2* (COX-2) and *lipoxygenase*. COX-2 is the enzyme responsible for synthesizing pro-inflammatory prostaglandins, and is the target of non-steroidal anti-inflammatory drugs (ibuprofen, aspirin) and COX-2 inhibitors (Celebrex) (Katharina *et al*., 2012). The transcription factor NFkB is widely studied due to its implication in the regulation of genes that control inflammation, cell proliferation and cell survival. NFkB is comprised of homo- or heterodimers of different subunits. These subunits are members of the structurally related Rel family of transcription factors. Five different Rel proteins (also called Rel/NFkB proteins) have been identified: p50 (NF-kB1), p52 (NF-kB2), p65 (RelA), RelB and c-Rel (Hoesel and Schmid, 2013). p65 (RelA), RelB and c-Rel contain a transactivation domain (TD) in their C-termini, which is required for the transport of active NFkB complexes into the nucleus. In contrast, subunits p50 and p52 do not contain transactivation domains; they are unable to transactivate on their own and must form heterodimers with RelA, RelB or c-Rel. The p50/p65 heterodimers and the p50 homodimers are the most common dimers found in the NFκB signaling pathway (Hoesel and Schmid, 2013).

NFkB is activated in response to several endogenous and exogenous ligands, including pathogen-associated molecular patterns (PAMPs), Damage Associated Molecular Pattern

(DAMP), reactive oxygen species (ROS), tumor necrosis factor alpha (TNFα), interleukin 1-beta (IL-1β), bacterial lipopolysaccharides (LPS), isoproterenol, cocaine, and ionizing radiation (Vallabhapurapu and Karin, 2009, Tolle and Standiford 2013). In tumor cells, NF-κB is active either due to mutations in genes encoding the NF-κB transcription factors themselves or in genes that control NF-κB activity (such as IκB genes); in addition, some tumor cells secrete factors that cause NF-κB to become active. Blocking NF-κB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents. Thus, NF-κB is the subject of much active research among pharmaceutical companies as a target for anti-cancer therapy (Escárcega et al., 2007). While in an inactivated state, NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IκB kinase (IKK) (Rahman and McFadden, 2011). IKK, in turn, phosphorylates the IκBα protein, which results in ubiquitination, dissociation of IκBα from NF-κB, and eventual degradation of IκBα by the proteosome. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE) (Perkins, 2007). The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein, which results in a change of cell function (Rahman and McFadden, 2011).

In the nucleus, NF-κB binds to target DNA elements and positively regulates the transcription of genes involved in immune and inflammatory responses, cell growth control, and apoptosis. Genes encoding cytokines, cytokine receptors, cell adhesion molecules, chemoattractant proteins, and growth regulators are positively regulated by NF-κB (Rahman and McFadden, 2011)). For example, NF-κB-mediated IL-6 promotes cancer cell proliferation and invasion by secreting IL-6, TGF-β, VEGF and MMP9 which are required for cancer cellular invasion and metastasis. VEGF is known to sustain tumor growth through the inhibition of dendritic cell maturation, thus enhancing tumor survival and growth. NF-κB activation is essential for STAT3 activation in breast cancer cells (Xie *et al*., 2010).

Immunological activities of Circulating Immune Complex activities could be implicated in the activation and the translocation of NF-kB into the nucleus and initiate transcription of many gene factors and expression of many molecules which can promote cancer development, invasion and metastasis. This could be achieved by inducing immunological cell responses and subsequent activation of proinflammatory cytokines and molecules (Elinav *et al*., 2013).

Owing to the importance of the NF-κB signalling pathway in many crucial cellular processes, cells have developed multiple mechanisms to regulate the function of this pathway. One negative regulatory mechanism is deubiquitylation of signalling molecules that activate the IKK complex, by deubiquitylase enzymes such as CYLD and zinc finger protein A20 (also known as TNFAIP3) (Rahman and McFadden, 2011). Several bacteria have co-opted this mechanism using virulence factors that deubiquitylate NF-κB signalling molecules. The type III effector protein AvrA of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. PhoPc, a non-pathogenic strain, is a deubiquitylase that is closely related to YopJ and that inhibits the NF-κB pathway by removing ubiquitin from IκBα and from β-catenin, a negative regulator of the pro-inflammatory NF-κB pathway in epithelial cells (Ye *et al*., 2007). Another *S.* Typhimurium type III effector protein, SseL (encoded in *Salmonella* pathogenicity island 2), also possesses a deubiquitylase activity that inhibits degradation of IκBα. *Salmonella* typhimurium lacking SseL causes increased NF-κB activation in macrophages as a result of ubiquitin-mediated degradation of IκBα (Le Negrate *et al*., 2008).

2.20. Diagnosis of Breast Tumour and Epigenetic Cell Alteration

Breast cancer typically is detected either during a screening examination, before symptoms have developed, or after symp- toms have developed, when a woman feels a lump. Most masses seen on a mammogram and most breast lumps turn out to be benign; that is, they are not cancerous, do not grow uncontrol- lably or spread, and are not life-threatening. When cancer is suspected based on clinical breast exam or breast imaging, microscopic analysis of breast tissue is necessary for a definitive diagnosis and to determine the extent of spread (in situ or inva- sive) and characterize the pattern of the disease. The tissue for microscopic analysis can be obtained via a needle or surgical biopsy. Selection of the type of biopsy is based on individual patient clinical factors, availability of particular biopsy devices, and resources (American cancer Society 2015).

DNA methylation represents a new opportunity in cancer genetics to provide biomarkers for diagnostic and prognostic use in clinical environment (Di Ruscio et al., 2013). Many different techniques for DNA methylation determination exist, and choosing the most appropriate one largely depends on the nature and number of the samples, information required, and costs. The three main approaches are: methylation-specific restriction enzyme digestion, affinity purification of methylated DNA and bisulfite conversion of DNA (Chang *et al*., 2010). The DNA obtained is further subjected to molecular-genetic approaches, which are for single locus

analysis based on PCR, whereas in the case of genome-wide interrogation they are based either on microarray technology, mass spectroscopy or next generation sequencing analysis. Investigating the potential and practical methods of DNA methylation is essential for determining whether there is an association between aberrant DNA methylation within CpG-rich sequences and cancer. Several different methods have been proposed for DNA methylation analysis and some of the advantages and disadvantages of the different approaches, particularly concentrating on genome scale DNA methylation, were outlined based on enzymes combined with PCR methods. Conducting a DNA methylation analysis requires a highly precise and accurate determination of the methylation status.

Several methods based on PCR have been developed to evaluate the methylation level of genes. Bisulphite treatment and PCR amplification are used for locus-specific detection. For example, a quantitative methylation-specific PCR assay was developed for high-throughput analysis and a real-time assay for individual methylated targets. Assays of other useful techniques may be applied to genes with 5-methylcytosine (m5C). The distribution of m5C within DNA is unique and may be used for genome-scale methylation analysis (Laird, 2010). For example, restriction landmark genome scanning was the first DNA methylation profiling technique that was widely used in identifying methylated loci in species or in a tissue-specific manner. Chromatin immunoprecipitation, based on microarray or next-generation sequencing, used antibodies or methyl-binding proteins for massive methylated DNA profiling. These powerful approaches provide accurate, reproducible and sensitive data in comprehensive methylation epigenomic and genomic typing (Dong *et al*., 2014). Thus Enzyme-linked immunosorbent assay (ELISA) based, that enable the quick assessment of DNA methylation status has been applied. ELISA-based assays are quick and easy to perform methods that serve well for the identification of large changes in global DNA methylation (Kurdyukov and Bullock 2016). Apart from detection of methylated gene (gene with 5-methylcytosine) and expression of enzyme methyl transferase, determination of the methylation levels of LINE-1 (long interspersed nuclear elements-1) retrotransposons, of which ~17% of the human genome is composed has been applied using ELISA. These are well established as a surrogate for global DNA methylation (Ohka *et al*., 2011)

In the laboratory setting one of the mostly used methods for locus specific methylation biomarkers is pyrosequencing, which is very appropriate for degraded formalin-fixed, paraffin-embedded (FFPE) samples that are an important part of tissue bio-banks. The technique enables

quantitative analysis of each CpG position (Hama *et al*., 2012). Another method, which is fast and also robust, is high resolution melting (HRM) curve analysis. Two typical methods in wider use are methylation-sensitive HRM (MS-HRM) curve analysis and quantitative real time PCR, such as SMART-MSP (Dong *et al*., 2014). Both techniques are relatively cheap and sensitive, allow relatively good throughput and quantification, and are closed tubes techniques. The latter minimizes the risk for sample confusion and cross-contamination which is of vital importance in clinical laboratory (Di Ruscio *et al*., 2013). The MS-HRM method has already been tested on samples with small amounts of DNA, like stool, and has proven to be sensitive and reliable enough to be used for screening purposes (Laird, 2010). Both techniques have demonstrated to be successful also on old FFPE tissues. However, these techniques require well designed primer pairs and stringent annealing temperatures in order to overcome the problem with false positive results. They are also not completely reliable when analysing heterogeneous DNA methylation patterns.

Another useful technique, still considered as a single locus analysis, is the matrix-assisted laser desorption ionization - time of flight (MALDI-TOF). The Sequenom Inc. has developed a sensitive and high throughput assay MassARRAY EpiTYPER, which enables quantitative screening and differential methylation analysis in cancer samples. Roche 454 Genome Sequencer and Illumina Genome analyser are the most used next generation sequencing platforms in research (Dong *et al*., 2014) and therefore likely to be validated for clinical use. They are becoming the key players in cancer genome-wide methylome determination, which could result in determination of an array of biomarkers. These arrays should subsequently be subjected to thorough testing on larger sample cohorts using more-cost effective methods. The costs of next-generation sequencing are currently still too high to allow larger sample testing. So far research performed on these platforms proves to be useful to provide fingerprints of cancer methylomes, which could help in cancer subtypes classification. However, due to the limited knowledge of functional consequences of methylation aberrations, enormous number of discovered changes and overlapping changes between different cancers, establishment of cancer specific methylation signatures is still far away.

The methods for DNA methylation analysis are abundant, however, when considering their application for use in clinical diagnostics the main drawbacks are the standardization of methods between laboratories, and costs, associated with training of personnel and obtaining new equipment (Di Ruscio *et al*., 2013). DNA methylation techniques that could be used in clinical

setting should be easy to use, high throughput, preferably automatable, applicable on degraded DNA, cost-effective, and should provide quantitative methylation data (Tan *et al*., 2010). On the other hand, DNA methylation is a stable covalent modification, present at single or multiple CpG sites, and as such may be easily translated into robust and high performance laboratory tests. Furthermore, biomarker evaluation should be performed in readily accessible diagnostic specimens, such as blood, urine, faeces or saliva in order to detect early stages of the disease.

DNA methylation biomarkers could be used in two different ways: as confirmatory diagnostic, prognostic and predictive markers in an already diagnosed tumour or as markers for early disease and/or residual disease screening (Tan *et al*., 2010). An important feature of cancers is global hypomethylation across the genome, whereas hypermethylation is mostly observed locally. It is assumed that the consequence of hypomethylation of single-copy genes is activation of expression, leading to enhanced oncogenic potential. The role of hypomethylation of repeated sequences, which comprise 45% of human genome, is less understood, although it has been hypothesized to facilitate genomic instability (Hartmann *et al*., 2009). Hypermethylation, on the other hand is associated with inactivation of tumour suppressor genes and genes implicated in normal homeostasis of tissues (Hrasovec *et al*., 2013).

When the methylation status of whole genome is determined it is referred to as a cancer methylome fingerprint, while designation of methylation biomarker applies to methylation status of particular sites in the genome. Both are important in the development of clinical protocol that could enable the clinician to better diagnose and stage the disease, and to predict the prognosis and monitor the response to therapy (Carone *et al*., 2010).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Subjects

Ninety nine (99) female subjects were recruited for this study and were grouped into three. They included 24 female subjects with benign breast tumour (benign subjects), 25 female subjects with malignant breast tumour (Cancer subjects) and 50 female subjects without breast tumour (Control Subjects). The subjects with benign and malignant breast tumours were attending clinic at the surgical unit of Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra State. The cutoff values were drawn from 100 healthy female volunteers. The 100 healthy female volumteers and the 50 healthy female control subjects were confirmed free from breast tumours by clinical examination using physical breast examination by Surgeon (Ravi and Rodrigues, 2012). None of the subjects had received any form of treatment (chemotherapy, surgery, radiotherapy or immunotherapy) for breast tumour prior to the study. All the subjects were screened clinically and biochemically to exclude any autoimmune diseases and Human Immunodeficiency Virus. The diagnosis of breast tumour was established by histo-pathological examination of biopsy and detection of cancer associated antigen 15-3 (CA 15-3). Staging of cancer was done according to American Joint Committee for Cancer (Tumour Node Metastasis (TNM) classification). Approval for the study was obtained from the ethics committee of Nnamdi Azikiwe University Teaching Hospital. Informed consent was obtained from the subjects before participation. All the subjects were administered questionnaire to obtain medical history and demographic information.

3.1.1. Inclusion Criteria

Female subjects with benign or malignant breast tumour. Female subjects without tumour and are not under cytotoxic drug or any anti-immune therapy such as in auto-immune disease.

3.1.1.2. Exclusion Criteria

Immuno-deficient patients or patients with HIV infection, Female subjects without cancer but are under cytotoxic drug or any anti-immune therapy such as in auto-immune disease. Female subjects with breast tumour and had received any form of treatment (chemotherapy, surgery, radiotherapy or immunotherapy) for breast tumours.

3.1.2. Sample Size

This was calculated based on the Cohen's sample size formula stated below (Ogunbode et al., 2015).

$$n = \frac{z^2 \times p(1-p)}{d^2}$$

Description:

n = required sample size

Z = confidence level at 95% (standard value of 1.96)

p = estimated prevalence of breast cancer in the project area = 0.0053% (Nnamdi Azikiwe University Teaching Hospital Cancer Registry)

D = Precision (margin of error at 5%) (Standard value of 0.05).

Based on this calculation, the minimum sample size was 8.

3.2. Specimen Collection

Ten (10) ml of fasting blood sample was drawn by veni-puncture from all the participants. Serum was obtained from the fasting blood samples. Criteria for blood sample collection were made to suit the various parameters required to be tested in this study. Five (5) drops of blood was put in Ethylene Di amine Tetraacetic Acid (EDTA) container for *Plasmodium falciparum* detection, while the remaining blood sample was put in a plain vacutainer tube. The blood samples were allowed to clot at room temperature, for 30 minutes. The retracted clot was removed by centrifugation (Sorvall RC5C HS-4 rotor at 1500 x g for 15 min) at room temperature and the formed serum was carefully pipetted into another tube. Immune complex precipitation and dissociation, DNA extraction was carried out from the serum immediately. The sera were stored at -20^0C in aliquots until used for the analysis of Nuclear Factor kappa B (NF-kB), Immunoglobulin G (IgG), Tumour Necrosis Factor-alpha (TNF-α), 8-hydroxy-2'-deoxyguanosine (8-OH2DG), Estrogen (Estradiol 2), Progesterone and CA15-3 antigen.

3.3. Site of Study

The laboratory analysis was carried out at the Chemical Pathology hormonal Assay unit and HIV Laboratory of Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra State Nigeria.

3.4. Methods

3.4.1. Immune Complex Precipitation and Estimation

Approximately 0.2ml (1 part) of the freshly obtained serum was mixed with 0.4ml (2 parts) of 0.01M-borate buffer, pH 8.4. To this mixture, 27 parts of 4.166% PEG was added (final 1:30 serum dilution and 3.75% PEG concentration was obtained) (Brunner and Sigal, 2000). The mixture was incubated at room temperature for 60 minutes; the turbidity developed was measured spectrophotometrically at 450nm against control containing 1:30 diluted serum in borate buffer without PEG. The level of CIC in serum was expressed in terms of OD450 measured at the end of 60 minutes. The result was expressed as Peg Index derived by the formula: Peg Index= OD450 with Peg – OD450 with Borate Buffer Saline (BBS) without PEG x 1000 (Brunner and Sigal, 2000). Mean +2SD (at 95% confidence interval) method, was used to determine the reference values of Circulating immune complex in 100 healthy subjects (Singh, 2006). Value of 56.7ugEq/ml was regarded as cut off values. Values above 56.7ugEq/ml were regarded as high.

3.4.2. Immune Complex Dissociation

The polyethylene glycol (PEG) method was used to precipitate immune complexes (Schutzer et al., 1994). 1ml of 4% PEG (average molecular weight, 6,000; Sigma) and 0.44% NaCl in 0.1M borate buffer (pH 8.4) was added drop wise with constant stirring to 1ml of serum in a micro-centrifuge tube (Eppendorf). Tubes were vortexed, left at 4°C for at least 3 hours and centrifuged at 10,000 rpm ((Sorvall RC5C HS-4 rotor 8,320 × g) for 15 min in the cold. Supernatants were carefully removed with a pipette. The pellet was re-suspended and washed twice with 2ml of 2% PEG solution in the same buffer, removing the supernatant carefully each time by means of aspiration using Pasture pipette. After the second spin, samples were re-suspended in 2ml of 0.1 M sodium borate buffer, pH 10.2 (Brunner and Sigal, 2000). Dissociated ICs were kept in buffer at 4°C until use. The dissociated immune complex solution was assayed serologically for *Salmonella typhi* antigens, Malaria parasites (*Plasmodium falciparum)*, *Hepatitis B surface antigen* (HbsAg), *Hepatitis C virus* (HCV), *Mycobacterium tuberculosis* antibodies, *Helicobacter pylori* antibodies and *Treponema pallidum* antibodies using Immuno-chromatographic assay technique (Brunner and Sigal, 2000). See pages 131 to 133.

3.4.3. Extraction of Serum DNA (Epigentek, USA)**.**

Principle

After treatment with the DNA digestion buffer, strong ionic strength is formed which enhances DNA precipitation. If adequate ethanol is added, the electrical attraction between the DNA phosphates would increase ionic bond and DNA precipitation.

Procedure

The procedure was as described by the manufacturer (Epigentek, USA).

Volume of 1 ml of digestion solution was added to digestion powder. The tube was vortexed until solution is clear. Upto 500µl of DNA isolation buffer then 20µl of the mixed (digestion solution/digestion powder) solutions were added to 500µl of serum sample, and mixed very well. The mixture was incubated at 65ºC for 10 minutes. Meanwhile, a spin column was placed into a 2 ml collection tube. Maximum of 500µl of mixture was transferred to the column and Centrifuged at 12,000 rotor per minute (rpm) for 30 seconds. The flow through was discarded and the column replaced to the collection tube and the remaining volume of mixture was transferred to the column, centrifuged again at 12,000 rpm for 30 seconds. The flow through was again discarded and the column replaced to the collection tube.

Three hundred micro-litre (300µl) of 70% ethanol was added to the spin column and centrifuge at 12,000 rpm for 20 seconds. The flow through was discarded and the column replaced to the collection tube. Two hundred micro-litre (200µl) of 90% ethanol was added to the column and centrifuge at 12,000 rpm for 20 seconds. The flow through was discarded and the column replaced to the collection tube. Another 200µl of 90% ethanol was added to the column and centrifuged at 12,000 rpm for 40 seconds. The column was placed in a new 1.5ml vial and 100µl of DNA Elution Solution was added directly to the column filter and centrifuged at 12,000 rpm for 20 seconds to elute DNA. The eluted DNA was stored at –20°C for one week before use

3.4.4 Methylated DNA Quantification (Colorimetric) (Epigentek, USA)**.**

3.4.4.1 Principle

The principle of indirect ELISA was applied. The ability of DNA to bind to strip wells that are specifically treated to have a high DNA affinity enables the methylated fraction of DNA to be detected using capture and detection antibodies and quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of methylated DNA is proportional to the optical density (OD) intensity measured.

3.4.4.2. Assay Procedure

The procedures were stated below as directed by the manufacturer (Epigentek, USA)

3.4.4.2.1. DNA Binding

Eighty micro-litres (80µl) of binding solution was added to each well for DNA binding. Then 1µl of negative control, 1µl of diluted positive control were added, followed by 8µl of the isolated DNA sample. The solutions were mixed by shaking the plate on the bench several times, ensuring that the solutions coat the bottom of the well evenly. The strip plate was covered with plate seal and incubated at 37°C for 90 minutes. The binding reaction solution was removed from each well and the wells were washed three times with 150µl of diluted wash buffer.

3.4.4.2.2 Methylated DNA Capture

Capture antibody was diluted (at 1:1000 dilution) with diluted wash buffer. 50µl of the diluted capture antibody was added to each well, then covered and incubated at room temperature for 60 min. After 60 minutes, the diluted capture antibody solution was removed from each well. The wells were washed three times with 150µl of diluted wash buffer. Detection antibody was diluted at 1:2000 dilution) with the diluted wash buffer, then 50µl of the diluted detection antibody was added to each well, then covered and incubated at room temperature for 30 minutes. The diluted detection antibody solution was removed from wells, and the wells were washed four times with 150µl of diluted wash buffer). Enhancer solution was diluted at 1:5000 dilutions with the diluted wash buffer. Afterwards, 50µl of the diluted enhancer solution was added to each well. The wells were covered and incubate at room temperature for 30 minutes. The diluted enhancer solution was removed from each well and the wells were washed five times with 150µl of diluted Wash Buffer).

3.4.4.2.3. Optical Density

Total of 100µl of developer solution was added to each well and incubated at room temperature for 10 minutes away from light. Colour change was monitored in the sample wells and control wells. Then 100µl of stop solution was added to each well to stop enzyme reaction when colour in the positive control wells turned medium blue. The solution was mixed by gently shaking the frame on the bench and waited for 2 minutes to allow the colour reaction to be completely stopped. The colour change to yellow after adding stop solution and the absorbance was read on a microplate reader at 450 nm within 2 to 15 min.

3.4.4.2.4. 5-mC (5-carbon of cytosine ring where DNA methylation occurs) Calculation and Relative Quantification.

To determine the relative methylation status of two different DNA samples, calculation of percentage of 5-mC in total DNA was carried out using the following formula as described by the manufacturer (Epigentek, USA).

$$5\text{-mC\%} = \frac{(\text{Sample OD} - \text{ME3 OD}) \div S(100)}{\text{ME4OD} - \text{ME3OD} \times 2^{*} \div P(5)} \times 100$$

Sample OD= optical density of the sample, ME3 OD= optical density of negative control, ME4 OD= optical density of positive control. The 2* is a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC; S= the amount of input sample DNA in ng = 100ng; P= the amount of input positive control in ng = 5ng; Positive control is a methylathed polynucleotide containing 50% of 5-methylcytosine; Negative control is an unmethylathed polynucleotide containing 50% of cytosine.

3.4.5. Immunoglobulin G Assay

3.4.5.1 Principle of the Assay

The principle of sandwich ELISA was applied. Plate coated with a capture antibody (anti-IgG) can capture antigen (IgG) in the test sample, if any, in other to immobilize the antigen on the walls of the plate. Subsequently, detecting antibody directed against IgG can specifically bind to the immobilized antigen. The detecting antibody is probed with enzyme-linked secondary antibody (horseradish peroxidase conjugated antibody). On addition of the chromogenic substrate (3,3',5,5'-tetramethylbenzidine (TMB) the enzyme converts the substrate to a detectable form inducing a colour change. The intensity of the colour is directly propotional to the quantity of IgG present in the serum sample.

3.4.5.2 Procedure

The procedure was done as directed by the manufacturer (Immunology Consultants Laboratory, Inc. USA). All the reagents were brought to room temperature before use. Then 100µL of Standards was pipetted. Standard 0 (0.0 ng/ml) in duplicate; Standard 1 (15.6 ng/ml) in duplicate; Standard 2 (31.25 ng/ml) in duplicate; Standard 3 (62.5 ng/ml) in duplicate; Standard 4 (125 ng/ml) in duplicate; Standard 5 (250 ng/ml) in duplicate; Standard 6 (500ng/ml) in

duplicate. One hundred microliter (100µL) of sample (in duplicate) was pipetted into pre designated wells. The microtiter plate was incubated at room temperature for sixty (60) minutes. The plate was covered and kept at level surface during incubation. Following incubation, the contents of the wells were aspirated. The wells were completely filled with appropriately diluted Wash Solution and the plate was inverted after shaking on the bench surface to pour out the content in a waste container. This was followed by (blotting) sharply striking the wells on absorbent paper to remove residual buffer, this was repeated 3 times for a total of four washes. One hundred microlitre (100µL) of appropriately diluted Enzyme-Antibody Conjugate was pipetted into each well and incubated at room temperature for twenty (20) minutes. The plate was kept covered in the dark and on level surface during incubation. The plate was washed and blotted as described above. One hundred micrlitre (100µL) of TMB substrate solution was pipetted into each well and incubated in the dark at room temperature for precisely ten (10) minutes. After ten minutes, 100µL of stop solution was added to each well. The absorbance of the contents of each well was determined (450 nm). The quantity of IgG in the test sample was interpolated from the standard curve constructed from the stand.

3.4.6. CA15-3 Assay (Accubind Inc. United States of America).

3.4.6.1 Principle

The principle of sandwich enzyme linked immuno sorbent assay (ELISA) was applied. Plate coated with a capture antibody can capture native antigen (CA15-3) in the test sample in other to immobilize the antigen on the walls of the plate. Subsequently, detecting antibody directed against Ca15-3 specifically bind to the immobilized antigen. The detecting antibody is probed with enzyme-linked secondary antibody (horseradish peroxidase conjugated antibody). On addition of the chromogenic substrate (3,3',5,5'-tetramethylbenzidine (TMB) the enzyme converts the substrate to a detectable form inducing a colour change. The intensity of the colour is directly propotional to the quantity of CA15-3 present in the serum sample.

3.4.6.2. Test Procedure

The procedure is described by the manufacturer Accubind Inc. United States of America (USA). All reagents, and samples were brought to room temperature (20 - 27°C), then 0.1 ml (100µl) of the biotinylated labeled antibody was added to coat each well, thereafter, 0.025 ml (25µl) of the appropriate diluted serum reference control and specimen were separately added into their assigned wells. The microplate was swirled gently for 20-30 seconds to mix; the plate was covered after mixing and Incubated for 60 minutes at room temperature. Content of the

microplate was discarded by decantation, tapped and blotted dry with absorbent paper. 350µl of wash buffer was added and decanted (tap and blot) after shaking. This was repeated two (2) additional times for a total of three (3) washes. One hundred microliter (100µl) of the anti-Ca15-3 Enzyme conjugated reagent was added to each well. At this time shaking was avoided. The plate was covered and incubated for 60 minutes at room temperature. Content of the microplate was discarded by decantation, tapped and blotted dry with absorbent paper. 0.1 ml (100µl) of substrate reagent was added to all wells. The addition of the reagent was in the same order to minimize reaction time, avoiding shaking of the plate. The plate was incubated at room temperature for twenty (20) minutes. Fifty microliter (50µl) of stop solution was added to each well and gently mixed for 15-20 seconds. The absorbance in each well was read at 450nm (using a reference wavelength of 620-630nm) in a microplate reader. The results was read within thirty (30) minutes of adding the stop solution

3.4.6.3 Calculation of Results

A standard curve was used to ascertain the concentration of CA15-3 in unknown specimens by locating the average absorbance of the duplicates for each unknown on the vertical axis of the graph, to find the intersecting point on the curve, and read the concentration (in U/ml) from the horizontal axis of the graph.

3.4.7. Oestradiol (E2) Estimation: (Accubind Inc. USA)

3.4.7.1 Principle

The principle of competitive ELISA was employed. Upon mixing anti-estradiol biotinylated, enzyme-antigen conjugate and a serum containing the native antigen, labelled antigen, competes for a limited number of antibody binding sites with the sample antigen in a solid microtitre well ($^{Enzyme}Ag + {}^{Native}Ag + Ab_{Biotinylated} \leftrightarrow {}^{Native}AgAb_{Biotinylated} + {}^{Enzyme}AgAb_{Biotinylated}$). A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation. $\{{}^{Native}AgAb_{Biotinylated} + {}^{Enzyme}AgAb_{Biotinylated}$ + Streptavidin → Immobilized complex (antigen antibody complex on the solid surface)}. The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. The less the native antigen in the sample, the more enzyme labelled antigen is retained in the well to react with the substrate and the stronger the signal. On the other hand the higher the concentration of native antigen in the sample, the less the enzyme antigen to react with the substrate and the lower the absorbance. By

utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

3.4.7.2. Test Procedure

The procedure is described by Accubind Inc. USA. All reagents, including the serum samples were brought to room temperature (20 - 27°C). Volume of 0.025m (25 µL) of test serum and serum reference was pipetted into the assigned wells. Volume of 0.05ml (50µl) of the anti-Estradiol Biotin Reagent was added to all wells. The microplate was swirled gently for 20-30 seconds to mix the content. The plate was covered and incubated for 30 minutes at room temperature. Volume of 0.05ml (50µl) of Estradiol Enzyme Reagent was added to all wells. The microplate was swirled gently for 20-30 seconds to mix the content. The plate was covered and incubated for 90 minutes at room temperature. Content of the micro plate was discarded by decantation, and blotted dry with absorbent paper. 350µl of wash buffer was added, decant (tap and blot), and repeated two (2) additional times for a total of three (3) washes. Decant the wash and repeat two (2) additional times. Volume of 0.1ml (100µl) of substrate solution (Tetramethylbenzidine-Hydrogen peroxide) was added to all wells, shaking was avoided at this stage. The plate was incubated at room temperature for twenty (20) minutes and 0.05ml (50µl) of stop solution was added to each well and gently mixed for 15-20 seconds. The absorbance was read in each well at 450nm. The results were read within thirty (30) minutes of adding the stop solution.

3.4.7.3. Calculation of Results

The absorbance for each duplicate serum reference was plotted against the corresponding estradiol concentration in pg/ml. The standard curve was used to ascertain the concentration of estradiol in unknown specimens using the absorbance of the unknown.

3.4.8. Serum Progesterone Estimation (Accubind Inc. USA)

3.4.8.1 Principle

The principle of competitive ELISA was applied. Upon mixing anti-progesterone biotinylated, enzyme-antigen conjugate and a serum containing the native antigen, labelled antigen, competes for a limited number of antibody binding sites with the sample antigen in a solid microtitre well ($^{Enzyme}Ag + {}^{Native}Ag + Ab_{Biotinylated} \leftrightarrow {}^{Native}AgAb_{Biotinylated} + {}^{Enzyme}AgAb_{Biotinylated}$). A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation.

{$^{Native}AgAb_{Biotinylated}$ + $^{Enzyme}AgAb_{Biotinylated}$ + Streptavidin → Immobilized complex (antigen antibody complex on the solid surface)}. The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. The less the native antigen in the sample, the more enzyme labelled antigen is retained in the well to react with the substrate and the stronger the signal. On the other hand the higher the concentration of native antigen in the sample, the less the enzyme antigen to react with the substrate and the lower the absorbance. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

3.4.7.2. Test Procedure

The procedure is directed by Accubind Inc. USA. All reagents, including the serum samples were brought to room temperature (20 - 27°C). Volume of 0.025m (25 µL) of test serum and serum reference was pipetted into the assigned wells. Volume of 0.05ml (50µl) of the anti-Estradiol Biotin Reagent was added to all wells. The microplate was swirled gently for 20-30 seconds to mix the content. The plate was covered and incubated for 30 minutes at room temperature. Volume of 0.05ml (50µl) of progesterone enzyme reagent was added to all wells. The microplate was swirled gently for 20-30 seconds to mix the content. The plate was covered and incubated for 90 minutes at room temperature. Content of the micro plate was discarded by decantation, and blotted dry with absorbent paper. 350µl of wash buffer was added, decant (tap and blot), and repeated two (2) additional times for a total of three (3) washes. Decant the wash and repeat two (2) additional times. Volume of 0.1ml (100µl) of substrate solution (Tetramethylbenzidine-Hydrogen peroxide) was added to all wells, shaking was avoided at this stage. The plate was incubated at room temperature for twenty (20) minutes and 0.05ml (50µl) of stop solution was added to each well and gently mixed for 15-20 seconds. The absorbance was read in each well at 450nm. The results were read within thirty (30) minutes of adding the stop solution.

3.4.8.3 Calculation of Results

The absorbance for each duplicate serum reference was plotted against the corresponding progesterone concentration in ng/ml. The standard curve was used to ascertain the concentration of progesterone in unknown specimens using the absorbance of the unknown.

3.4.9. Nuclear Factor kappa B Detection (Rockland Immunochemicals (Gilbertsville, Pennsylvania, USA**)**

3.4.9.1. Principle

Principle guiding indirect ELISA was employed. A specific double stranded DNA (dsDNA) sequence containing the NF-kB response element immobilized onto the bottom of wells of a 96 well plate. NF-kB contained in a nuclear extract specifically binds to the NF-kB response element. NF-kB (p65) is then detected by addition of a specific primary antibody (detecting antibody) directed against NF-kB (p65). A secondary antibody conjugated to emzyme (Horse Radishperoxidase (HRP) to bind the detecting antibody. On addition of the chromogenic substrate (peroxide) the enzyme converts the substrate to a detectable form inducing a colour change. The intensity of the colour is directly propotional to the quantity of NF-kB present in the nuclear extract.

3.4.9.2. Test Procedure

Indirect ELISA method was employed. The procedure is as described by the manufacturer (Rockland Immunochemicals (Gilbertsville, Pennsylvania, USA). The plates and buffers were equilibrated to room temperature prior to opening. One hundred microliter (100µL) complete Transcription Factor Binding Assay Buffer (CTFB) was added to the Blank wells (Blk); 100 µL of CTFB was added to Non-specific Binding wells (NSB). Eighty microliter (80µL) of CTFB was added to Competitor wells (CI) prior to adding 10µL of competitor double stranded DNA (dsDNA) to the wells. Ninety microliter (90µL) of CTFB was added to sample wells (U1-U44) - prior to adding 10µL of nuclear extract to the wells. Ninety microliter (90µL) of CTFB was added to positive Control wells prior to adding 10µL of positive control to appropriate wells. The plate was covered with a seal and incubated for 1 hour at room temperature without agitation. The plates were emptied and washed 5 times with 200µL of 1X wash buffer. After each wash, the plate was emptied in the sink. After the final wash (i.e. 5th wash), the plate was tapped on a paper towel to remove any residual wash buffer.

Addition of Anti-NF-kB (p65) Primary Antibody: Anti-NF-kB (p65) antibody was diluted 1:100 in 1X antibody binding buffer (ABB). Total volume of 100µL of diluted Anti-NF-kB (p65) antibody was added to each well except the Blank (Blk) wells. The adhesive cover was used to seal the plate. The plate was incubated for 1 h at room temperature without agitation. The plate was emptied and the wells washed 5 times with 200µL of 1X wash buffer. After each wash, the

contents of the plate were emptied into the sink. After the final wash (i.e. 5th wash), the plate was tapped 5 times on a paper towel to remove any residual wash buffer.

Addition of the HRP Goat anti-Rabbit conjugated Secondary Antibody: The HRP-conjugated secondary antibody was diluted 1:100 in 1X Antibody binding buffer (ABB) and 100 µL antibody was added to each well except the Blank (Blk) wells. The adhesive cover was used to seal the plate, incubated for 1 hour at room temperature without agitation. The plate was emptied and washed 5 times with 200µL of 1X wash buffer. After each wash, the contents of the plate were empty into the sink. After the final wash, the plate was tapped 5 times on a paper towel to remove any residual wash buffer. The plate was developed by adding to each well 100µL of developing solution which has been equilibrated to room temperature. The plate was incubated for 45 minutes at room temperature with gentle agitation protected from light. The plate was allowed to turn medium to dark blue prior to adding stop solution. Absorbance was read at 450nm within 5 minutes of adding the stop solution. The plate reader was blanked according to the manufacturer's requirements using the blank wells.

3.4.10. 8-hydroxy-2-deoxy Guanosine (8-OH-dG) Assay (StressMarq Biosciences Inc. USA)

3.4.10.1 Principle

This assay is based on the competition between 8-hydroxy-2-deoxy guanosine (8-OH-dG) and 8-OH-dG-acetylcholinesterase (AChE) conjugate (8-OH-dG Tracer) for a limited amount of 8-OH-dG Monoclonal Antibody. Because the concentration of the 8-OH-dG Tracer is held constant while the concentration of 8-OH-dG varies, the amount of 8-OH-dG Tracer that is able to bind to the 8-OH-dG Monoclonal Antibody will be inversely proportional to the concentration of 8-OH-dG in the well. This antibody-8-OH-dG complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. Ellman's Reagent (which contains the substrate to 8-OH-dG-acetylcholinesterase AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 8-OH-dG Tracer bound to the well, which is inversely proportional to the amount of free 8-OH-dG present in the well during the incubation.

3.4.10.2. Preparation of the standard for use in EIA

One hundred microliter (100µl) of the 8-OH-dG standard (Catalog# SKC-120C) was transferred into a clean test tube, and diluted with 900µl distilled water. The concentration of this solution (the bulk standard) will be 30ng/ml. This was stored at 4°C. Clean test tubes were numbered 1 - 8. Then 900µl EIA Buffer was aliquoted to tube 1 and 500µl EIA Buffer to tubes 2-8. 100µl of the bulk standard (30ng/ml) was transferred to tube 1 and mixed thoroughly. The standard was serially diluted by removing 400µl from tube 1 and placing in tube 2; this was mixed thoroughly, then 400µl was removed from tube 2 and place it into tube 3 and mixed thoroughly. This process was repeated till tube 8.

3.4.10.3. Test Procedure

The procedure is as described by the manufacturer StressMarq Biosciences Inc. USA. One hundred microliter (100µl) Enzyme Immunoassay (EIA) Buffer was added to Non-Specific Binding (NSB) wells, while 50µl EIA Buffer was added to Maximum Binding wells. Upto 50µl of 8-hydroxy-2-deoxy Guanosine Standard from tube 8 was added to both of the lowest standard wells (S8), while 50µl from tube 7 was added to each of the next two standard wells (S7). This was continued until all the standards were aliquoted. Then 50µl of sample was added per well. Each dilution was assayed in duplicate. Fifty microliter (50µl) of 8-hydroxy-2-deoxy Guanosine AChE Tracer was added to each well except the Total Activity (TA) and the Blank (Blk) wells, then 50µl 8-hydroxy-2-deoxy Guanosine Monoclonal Antibody was added to each well as well, except in the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells. The plate was covered with plastic film and incubated for 18 hours at 4°C. Ellman's Reagent was reconstituted immediately before use (20 ml of reagent is sufficient to develop 100 wells) distilled water. The well was emptied and rinsed five times with Wash Buffer. Two hundred microliter (200µl) of Ellman's Reagent was added to each well and 5µl of tracer to the Total Activity wells. The plate was covered with plastic film. The plate was developed with a flat cover in the dark. The bottom of the plate was wiped with a clean tissue to remove fingerprints and dirt. The plate cover was removed being careful to keep Ellman's Reagent from splashing on the cover. Any loss of Ellman's Reagent will affect the absorbance readings. The plate was read at 420nm wavelength and the concentration of each sample was determined using the standard curve plot.

3.4.11. TNF-alpha Assay (ABCAM USA)

3.4.11.1 Principle

The principle of sandwich ELISA was applied. Plate coated with a capture antibody can capture native antigen (TNF-α) in the test sample if any in other to immobilize the antigen on the walls of the plate. Subsequently, detecting antibody directed against (TNF-α) can specifically bind to the immobilized antigen. The detecting antibody is probed with enzyme-linked secondary antibody (horseradish peroxidase conjugated antibody). On addition of the chromogenic substrate (3,3',5,5'-tetramethylbenzidine (TMB) the enzyme converts to the substrate to a detectable form inducing a colour change. The intensity of the colour is directly propotional to the quantity of (TNF-α) present in the serum sample.

3.4.11.2. Preparation of TNF-alpha Standard

Tumour Necrosis Factor-alpha TNF-alpha standard sample was reconstitute by adding 100µL of standared diluents buffer to stock vial to get 800pg/ml stock Standard 1 using pipette. This was mix thoroughly and gently and left at room temperature for 10 minutes. Other tubes were labeled from 2 to 8. 100µL of diluents buffer was added into each tube. Standard 2 was prepared by adding 100µL of Standard 1 to tube 2 and mix thoroughly. Standard 3 was prepared by adding 100µL of Standard 2 to tube 3 and mix thoroughly. Standard 4 was prepared by adding 100µL of Standard 3 to tube 4 and mix thoroughly. Standard 5 was prepared by adding 100µL of Standard 4 to tube 5 and mix thoroughly. Standard 6 was prepared by adding 100µL of Standard 5 to tube 6 and mix thoroughly to get concentrations of 800pg/ml, 400pg/ml, 200pg/ml, 100pg/ml, 50pg/ml, 25pg/ml, 12.5pg/ml and 6.25pg/ml.

3.4.11.3 Assay Procedure

The procedure is as directed by ABCAM USA. All reagents were brought to room temperature (18-25°C) prior to use. One hundred micro-litre (100µL) of each standard and sample was added into appropriate wells. The wells were covered and incubated for 2 hours 30 minutes at room temperature, enabling antigen (TNF-alpha)-capture antibody complex. The solutions were discarded and washed 4 times, by filling each well with 1X Wash Solution (300µL) using a multi-channel Pipette. After the last wash, any remaining wash buffer was removed by decanting and the plate blotted against clean paper towels. One hundred microliterr (100µL) of 1X Biotinylated TNF alpha Detection Antibody was added to each well and incubated for 1hour at room temperature with gentle shaking. The solution was decanted and the wash repeated. One hundred microliter (100µL) of 1X HRP-Streptavidin solution was added to each well. The plate

was incubated for 45 Minutes at room temperature with gentle shaking. The solution was decanted and the wells washed. Then 100µL of TMB One-Step Substrate Reagent was added to each well and incubated for 30 Minutes at room temperature in the dark with gentle shaking, and 50µL of Stop Solution added to each well. The wells were read at 450Nm immediately. Standard curve was used to determine the amount of TNF-a in the samples.

3.4.12. Detection of Hepatitis C Virus (HCV) (Global Sources-Bio Focus South Korea).

3.4.12.1 Principle

The test is based on the principle of indirect enzyme immunoassay. The mixture of HCV antigen in serum/immune complex solution if any reacts with the protein A coated particles (colloidal gold conjugate) on the sample pad and migrates on the membrane chromatographically by capillary action to react with recombinant HCV antibody on the test region, to generate red coloured line, indicating a positive result, absence of the line indicates a negative result. For procedural control, the antibody in the solution migrates further to react with the anti-IgG antibody in the control region to generate another coloured band in the control region.

3.4.12.2 Procedure

The test procedure is as directed by the manufacturer (Global Sources-Bio Focus South Korea).

3.4.13. Detection of *Plasmodium falciparum* (Global Sources-Bio Focus South Korea)

3.4.13.1. Principle

The test is based on the principle of sandwich immunoassay. Qualitative, membrane based immunoassay for the detection of circulating *Plasmodium falciparum* (P.f) Histidine rich protein (HRP-2) antigen in immune solution was applied. The membrane is pre-coated with *Plasmodium falciparum* antibody. During testing, the *Plasmodium falciparum* antigen HRP-2 in the immune solution specimen reacts with the dye conjugate, which has been pre-coated in the application point of the test strip. The mixture migrates upward on the membrane chromatographically by capillary action and reacts with *Plasmodium falciparum* antibody on the test region. If the blood/immune solution contains *Plasmodium falciparum* antigen, a red coloured line will appear on the test region indicating positive result. The absence of the red coloured line indicates negative result. To serve as a procedural control, the antibody migrates further to react with the anti-IgG antibody in the control region to generate another coloured band in the control region.

3.4.13.2. Procedure

The test procedure is as directed by the manufacturer (Global Sources-Bio Focus South Korea).

3.4.14. Detection of Hepatitis B Surface Antigen (HBsAg).

3.4.14.1. Principle

The test is based on the principle of sandwich immunoassay. The mixture of HBsAg in serum/immune complex solution if any reacts with the antibody coated nanoparticles (colloidal gold conjugate) on the sample pad and migrates on the membrane chromatographically by capillary action to react with anti-HBsAg antibody on the test region, to generate red coloured line, indicating a positive result; absence of the line indicates a negative result. For procedural control, the antibody in the solution migrates further to react with the anti-IgG antibody in the control region to generate another coloured band in the control region.

3.4.14.2 Procedure

The test procedure is as directed by the manufacturer (Global Sources-Bio Focus South Korea).

3.4.15. Detection of *Helicobacter pylori*

3.4.15.1 Principle of the Test

The test is based on the principle of indirect enzyme immunoassay. In the test procedure, recombinant *H. pylori* antigen is coated on the test band region. The antibody in serum/immune solution, when placed on the specimen pad, reacts with the antigen -colloidal gold conjugate. Running buffer facilitates the movement of the sample and antigen -colloidal gold conjugate along the length of the membrane to the test region. If antibody to *H. pylori* is present in the sample, an antigen-antibody-antigen complex will form. This is shown by a red colored line in the test area. To serve as a procedural control, further migration of the antigen will react with the anti human *H. pylori* IgG in the control region to give a red colored line at the control region if the test has been performed correctly

3.4.15.2 Procedure

The test procedure is as directed by the manufacturer (Global Sources-Bio Focus South Korea).

3.4.16. Detection of *Treponema pallidium*.

3.4.16.1 Principle

The test is based on the principle of indirect enzyme immunoassay. In the test procedure, recombinant *Treponema pallidium* antigen is coated on the test band region. The antibody in serum/immune solution, when placed on the specimen pad, reacts with the antigen -colloidal gold conjugate. Running buffer facilitates the movement of the sample and antigen -colloidal gold conjugate along the length of the membrane to the test region. If antibody to *Treponema pallidium* is present in the sample, an antigen-antibody-antigen complex will form. This is shown by a red colored line in the test area. To serve as a procedural control, further migration of the antigen will react with the anti human *Treponema pallidium* IgG in the control region to give a red colored line at the control region if the test has been performed correctly.

3.4.16.2. Procedure

The test procedure is as directed by the manufacturer (Global Sources-Bio Focus South Korea).

3.4.17. Detection of *Salmonella typhi.*

3.4.17.1. Principle

The test is based on the principle of sandwich immunoassay by the specific binding of antigens from S. typhi on a nitrocellulose membrane. The mixture of *S. typhi* antigen in serum/immune complex solution if any reacts with the antibody cold nanoparticles (colloidal gold conjugate) on the sample pad and migrates on the membrane chromatographically by capillary action to react with antibody of *S.typhi* on the test region, to generate red coloured line, indicating a positive result; absence of the line indicates a negative result. For procedural control, the antibody in the solution migrates further to react with the anti-IgG antibody in the control region to generate another coloured band in the control region.

3.4.17.2. Procedure

The test procedure is as directed by the manufacturer (Cortez Diagnostic Inc USA).

3.4.18. Detection of *Mycobacterium tuberculosis*.

3.4.18.1. Principle

A combination of recombinant antigens was applied to detect elevated levels of TB antibodies in serum specimen, using a qualitative solid phase, applying indirect enzyme immunoassay method. The strip membrane is pre-coated with TB recombinant antigen on the test line region

of the device. The TB antibodies if present in the serum specimen react with the particles coated with TB recombinant antigen. The mixture migrates upwards chromatographically by capillary action to react with TB recombinant on the test region and generate a coloured line indicating a positive result, while its absence indicates a negative result. To serve as a procedural control, further migration of the antigen will react with the anti-TB antibodies in the control region to give a red colored line at the control region if the test has been performed correctly

3.4.18.2. Procedure

The test procedure is as directed by the manufacturer (Global Sources-Bio Focus South Korea).

3.5. Statistical Methods

SPSS version 23 (IBM Inc., USA) was used to analyze the data. Descriptive statistics (mean ± SD) were performed for distribution patterns. Mean +2SD (at 95% confidence interval) method was used to determine the reference values in healthy subjects (Singh, 2006). The P values lower than 0.05 were considered statistically significant. Chi-square was used to determine the significant association of Norminal variables. Welch's analysis of varience (W-ANOVA) was used (where homogeneity or equal varience were not met (Levene's test) for analysis of more than 2 groups. Games Howell package was used for post hoc test (Tabachnick & Fidell, 2007). Pearson product-moment correlation coefficient was used to determine association between the continous variables.

3.6. Quality Control

The validity of the assay results was determined by coefficient of variation, analysis on three different levels of pool (low, normal and high) control sera (CV= Std/Mean x100).

CHAPTER FOUR

4.0 RESULTS

4.1: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with Combined Antigenic Components (Mean ±SD).

Circulating Immune Complexes (ugeq/ml)

The mean level of circulating immune complexes (CIC) in subjects with malignant breast tumour (142±57), those with benign tumour (92±51) and control subjects (49±28) differed significantly (F=39.2, P=0.000).

Post Hoc Analysis

The mean level of circulating immune complexes (CIC) in 25 subjects with malignant breast tumour (142±57) was found to be significantly raised, compared with the mean level of CIC in 24 benign breast tumour subjects (92±51; P=0.006). The mean level of circulating immune complexes (142±57) in 25 subjects with malignant breast tumour was also found to be significantly raised compared with the mean level in 50 control subjects (49±28; P= 0.000). The mean level of circulating immune complexes (CIC) (92±51) in 24 subjects with benign breast tumour was found to be significantly raised compared to the mean level of CIC in 50 control subjects (48±28; P=0.002) (Table 4.1).

Nuclear factor kappa B (ng/ml)

The mean level of Nuclear factor kappa B (NF-kB) in subjects with malignant breast tumour subjects (0.363±0.055), those with benign tumour (0.330±0.044) and control subjects (0.317±0.019) diferred significantly (F=12.7; P=0.000).

Post Hoc Analysis

The mean level of Nuclear factor kappa B (NFkB) (0.363±0.055) in 25 subjects with malignant breast tumour was found to be significantly raised compared to the level of NFkB in 50 control (0.317±0.019; P= 0.001). The mean level of Nuclear factor kappa B (NFkB) (0.363±0.055) in 25 subjects with malignant breast tumour was not significantly raised compared to the mean level of NFkB (0.330±0.044) in 24 benign breast tumour subjects, (P=0.067). The mean level of Nuclear

factor kappa B (NFkB) (0.330±0.044) in 24 benign breast tumour subjects was not significantly raised compared to the level of NFkB in 50 control (0.317±0.019; P=0.334) (Table 4.1)

Immunoglobulin G (mg/ml)

The mean level of Immunoglobulin G (IgG) in subjects with malignant breast tumour (26.9±5.1); those with benign breast tumour (22.0±6.1) and control subjects (19.8±5.2), diferred significantly (F=14.8; P=0.000).

Post Hoc Analysis

The mean level of serum Immunoglobulin G (IgG) (26.9±5.1) in 25 subjects with malignant breast tumour was found to be significantly raised compared to the mean level of serum IgG (22.8±6.1) in 24 subjects with benign tumour (P=0.009). The mean level of serum Immunoglobulin G (IgG) (26.9±5.1) in 25 subjects with malignant breast tumour, was also found to be significantly raised compared to the level of IgG (19.8±5.1) in 50 control subjects without breast tumour P=0.000. The mean level of serum Immunoglobulin G (IgG) (22.8±6.1) in 24 benign breast tumour subjects was not significantly raised compared to the level of IgG (19.8±5.2) in 50 control subjects without breast tumour (P=0.292) (Table 4.1).

Tumour Necrosis Factor-Alpha (pg/ml)

The mean levels of Tumour Necrosis Factor-Alpha (TNF-α) in subjects with malignant breast tumour (20.3±2.6); those with benign tumour (15.5±6.2); control subjects (9.5±2.2) diferred significantly (F=76.4, P=0.000).

Post Hoc Analysis

The mean level of Tumour Necrosis Factor-Alpha (TNF-α) (20.3±2.6) in 25 subjects with malignant breast tumour was significantly raised compared to the mean level of TNF-α (15.5±6.2) in 24 subjects with benign breast tumour, P=0.004. The mean level of Tumour Necrosis Factor-Alpha (20.3±2.6) in 25 subjects with malignant breast tumour was significantly raised compared to the mean level of TNF-α (9.5±2.2) in 50 control subjects without tumour, P=0.000. The mean level of Tumour necrosis factor-alpha (TNF-α) (15.5±6.2) in 24 benign breast tumour subjects was significantly raised compared to the mean level of TNF- α (9.5±2.2) in 50 control subjects without tumour, (P=0.000) (Table 4.1).

8-Hydroxy-2-Deoxy Guanosine (ng/ml)

The mean levels of 8-Hydroxy-2-Deoxy Guanosine (8-H2DG) in subjects with malignant breast tumour (26.2±8.6); those with benign tumour (15.9±5.8) and control subjects (9.7±4.2) diferred significantly (F=62.9, P=0.000).

Post Hoc Analysis

The mean level of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) (26.2±8.6) in 25 subjects with malignant breast tumour was significantly raised compared to the mean level of 8-OH-dG (15.9±5.8) in 24 subjects with benign breast tumour, P=0.000. The mean level of 8-OH-dG (26.2±8.6) in 25 subjects with malignant breast tumour was significantly raised compared to the mean level of 8-OH-dG (9.7±4.2) in 50 control subjects P=0.000, as well as (11.8±5.4) without tumour. The mean level of 8-OH-dG (15.9±5.8) in 24 subjects with benign breast tumour was significantly raised compared to the mean level of 8-OH-dG (9.7±4.2) in 50 control subjects without tumour (P=0.000) (Table 4.1).

Estrogen (pg/ml)

The mean levels of estrogen in subjects with malignant breast tumour (173±78); those with benign tumour (212±97) and control subjects (137±62) diferred significantly (F=8.2; P=0.001).

Post Hoc Analysis

The mean level of Estrogen (173±78) in 25 subjects with malignant breast tumour was lower than the mean level of estrogen (212±97) in 24 subjects with benign breast tumour, P=0.283. The mean level of Estrogen (173±78) in 25 subjects with malignant breast tumour was raised higher than the mean level of estrogen (137±62) in 50 control subjects P=0.121. Meanwhile the mean level of estrogen (212±97) in 24 subjects with benign breast tumour was significantly higher than the mean levels (137±62) in control subjects (P=0.004) (Table 4.1).

Progesterone (ng/ml)

The mean levels of progesterone in subjects with malignant breast tumour (0.6±0.4); those with benign tumour (1.1±0.5) and control subjects (2.0±1.3) diferred significantly (F=18.8; P=0.000).

Post Hoc Analysis

The mean level of progesterone (0.6±0.4) in 25 subjects malignant breast tumour was significantly low compared to the mean level of progestereone (1.1±0.5) in 24 subjects with benign breast tumour, (P=0.001). The mean level of progesterone (0.6±0.4) in 25 subjects with malignant breast tumour was significantly low compared to the mean level of progesterone (2.0±1.3) in 50 control subjects (P=0.000). The mean level of progesterone (1.1±0.5) in 24 benign breast tumour subjects was significantly low compared to the mean level of (2.0±1.3) in 50 control subjects without tumour, (P=0.000) (Table 4.1).

Table 4.1: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with Combined Antigenic Components (Mean ±SD).

	CIC/ mgeq/ml	**NFkB /ng/ml**	**IgG /mg/ml**	**TNFα /pg/ml**	**8OH2DG /ng/ml**	**Estrogen pg/ml**	**Progest ng/ml**
Benign[1] N=24	92±51	0.330±0.044	22.0±6.1	15.5±6.2	15.9±5.8	212±97	1.1±0.5
Malignant[2] N=25	142±57	0.363±0.055	26.9±5.1	20.3±2.6	26.2±8.6	173±78	0.6.±0.4
Control[3] N=50	49±28	0.317±0.019	19.8±5.2	9.5±2.2	9.7±4.2	137±62	2.0.±1.3
F(P-value)	39.2(0.000)	12.7(0.000)	14.8(0.000)	76.4(0.000)	62.9(0.000)	8.2(0.000)	18.8(0.000)
	Post Hoc						
1vs2	**0.006**	0.067	**0.009**	**0.004**	**0.000**	0.283	**0.001**
1 vs 3	**0.002**	0.334	0.292	**0.000**	**0.000**	**0.004**	**0.000**
2 vs 3	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**	0.121	**0.000**

KEY:

CIC= Circulating Immune Complexes; NFkB= Nuclear Factor Kappa B; IgG= Immunoglobulin G; TNF-α= Tumour Necrosis Factor-Alpha; 8-OH2DG=8-hydroxy-2-deoxy Guanosine; Progest= Progesterone.

4.2: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with Evidence of Microbial Antigenic Components (Mean ±SD).

Circulating Immune Complexes (ugeq/ml)

The mean level of circulating immune complexes (CIC) in subjects with malignant breast tumour subjects (139±51), those with benign tumour (97±53) and control subjects (71±36) were significantly different (F=7.5; P=0.001).

Post Hoc Analysis

The mean levels of circulating immune complexes (CIC) in 20 subjects with malignant breast tumour (139±51), was significantly raised compared to the mean level of CIC in 21 subjects with benign breast tumour (97±53; P=0.036). The mean levels of circulating immune complexes (CIC) in 20 subjects with malignant breast tumour (139±51) was significantly raised compared to the mean level of CIC in 11 control subjects without tumour (71±34; P=0.001). No significant difference was seen between the mean CIC of 21 subjects with benign breast tumour (97±53) and 11 control subjects without tumour (71±36) (P=0.250) (Table 4.2).

Nuclear Factor kappa B (NF-kB) (ng/ml)

The mean level of Nuclear Factor kappa B (NF-kB)) in subjects with malignant breast tumour (0.374±0.051), those with benign tumour (0.330±0.046) and control subjects (0.312±0.013) were significantly different (F=8.9, P=0.000).

Post Hoc Analysis

The mean level of Nuclear Factor kappa B (NF-kB) in 20 subjects with malignant breast tumour (0.374±0.051), was significantly raised compared to the mean level of (NF-kB) in 21 subjects with benign breast tumour (0.330±0.046), P=0.014. The mean levels of NF-kB in 20 subjects with malignant breast tumour (0.374±0.051); was significantly raised compared to the mean level of NF-kB in 11 control subjects without breast tumour (312±0.013), P=0.000. No significant difference was seen between the mean NF-kB expression in 21 subjects with benign breast tumour (0.330±0.046) and 11 control subjects without tumour (312±0.013), (P=0.261) (Table 4.2).

Immunoglobulin G (IgG) (mg/ml)

The mean level of Immunoglobulin G (IgG) in subjects with malignant breast tumour subjects (28.2±5.1), those with benign tumour (21.9±6.5) and control subjects (19.9±4.0) diferred significantly (F=12.0, P=0.000).

Post Hoc Analysis

The mean level of Immunoglobulin G (IgG) in 20 subjects with malignant breast tumour (28.2±5.1), was significantly raised compared to the mean level of IgG in 21 subjects with benign breast tumour (21.9±6.5), P=0.002. The mean level of IgG in 20 subjects with malignant breast tumour (28.2±5.1); was significantly raised compared to the mean level of IgG in 11 control subjects without breast tumour (19.9±4.0), P=0.000. No significant difference was seen between the mean level of IgG in 21 subjects with benign breast tumour (21.9±6.5) and 11 control subjects without tumour (19.9±4.0) (P=0.556).

Tumour Necrosis Factor Alpha (pg/ml)

The mean level of Tumour Necrosis Factor alpha (TNF-α) in subjects with malignant breast tumour subjects (20.6±2.5), subjects with benign tumour (15.7±6.4) and control subjects (9.2±2.8) diferred significantly (F=22.4; P=0.000).

Post Hoc Analysis

The mean level of Tumour Necrosis Factor alpha (TNF-α) in 20 subjects with malignant breast tumour (20.6±2.5) was significantly raised compared to the mean TNF-α in 21 subjects with benign breast tumour (15.7±6.4) P=0.009. The mean level of TNF-α in 20 subjects with malignant breast tumour (20.6±2.5) was significantly raised compared to the mean level of TNF-α (9.2±2.8) in 11 control subjects, P=0.000. The mean level of TNF-α in 21 subjects with benign breast tumour (15.9±5.9) was significantly raised compared to the mean level of TNF-α in 11 control subjects (9.2±2.8) (P=0.001) (Table 4. 2).

8-Hydroxy-2-Deoxy Guanosine (ng/ml)

The mean level of 8-Hydroxy-2-Deoxy Guanosine (8-OH2DG) in subjects with malignant breast tumour subjects (27.5±8.2), subjects with benign tumour (15.8±6.2) and control subjects (9.2±4.1) diferred significantly (F=30.3, P=0.000).

Post Hoc Analysis

The mean level of serum 8-OH2DG in 20 subjects with malignant breast tumour (27.5±8.2) was significantly raised compared to the mean level of serum 8-OH2DG in 21 subjects with benign breast tumour (15.8±6.2) (P=0.000). The mean level of 8-OH2DG in 20 subjects with malignant breast tumour (27.5±8.2) was significantly raised compared to the mean level of 8-OH2DG in 11 control subjects (9.1±4.0) (P=0.000). The mean level of 8-OH2DG in 21 subjects with benign breast tumour was significantly raised compared to the mean level of 8-OH2DG in 11 control subjects (9.1±4.0), (P=0.003) (Table 4.2).

Estrogen (pg/ml)

The mean level of estrogen in subjects with malignant breast tumour subjects (175±81), those with benign tumour (222±90) and control subjects (142±50) were significantly different (F=4.0; P=0.024).

Post Hoc Analysis

The mean serum level of estrogen in 20 subjects with malignant breast tumour (175±81) was decreased compared to the mean serum level of estrogen in 21 subjects with benign breast tumour (222±90), (P=0.195). Also the mean level of estrogen in 20 subjects with malignant breast tumour (175±81) was not significantly raised compared to the mean level of estrogen in 11 control subjects (142±50), (P=0.351). Conversely, the mean level of estrogen in 21 subjects with benign breast tumour (222±90), was significantly raised compared to the mean level of estrogen (142±50) in 11 control subjects tumour subjects, (P=0.008) (Table 4.2).

Progesterone (ng/ml)

The mean level of progesterone in subjects with malignant breast tumour subjects (0.6±0.4), those with benign tumour (1.2±0.5) and control subjects (2.2±1.2) were significantly different (F=20.1, P=0.000).

Post Hoc Analysis

The mean level of progesterone in 20 subjects with malignant breast tumour (0.6±0.4) was significantly low compared to the mean level of progesterone in 21 subjects with benign breast tumour (1.2±0.5) (P=0.000). The mean level of progesterone in 20 subjects with malignant breast tumour (0.6±0.4) was significantly low compared to the mean level of progesterone in 11 control subjects without tumour (2.2±1.2) (P=0.003). On the other hand, the mean level of

progesterone in 21 subjects with benign breast tumour (1.2±0.5) was not significantly low compared to the mean level of progesterone in 11 control subjects (P= 0.053) (Table 4.2).

Table 4.2: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with Evidence of Microbial Antigenic Components (Mean ±SD).

	CIC/ mgeq/ml	**NFkB /ng/ml**	**IgG /mg/ml**	**TNFα /pg/ml**	**8OH2DG /ng/ml**	**Estrogen pg/ml**	**Progest ng/ml**
Benign[1] N=21	97±53	0.330±0.046	21.9±6.5	15.7±6.4	15.8±6.2	222±90	1.2±0.5
Malignant[2] N=20	139±51	0.374±0.051	28.2±3.9	20.6±2.5	27.6±8.2	175±81	0.6.±0.4
Control[3] N=11	71±36	0.312±0.013	19.9±4.0	9.2±2.8	9.2±4.0	141±50	2.2.±1.2
F(P-value)	7.5(0.001)	8.9(0.000)	12.0(0.000)	22.4(0.000)	30.3(0.000)	4.0(0.024)	20.1(0.000)
	Post Hoc						
1vs2	**0.036**	**0.014**	**0.002**	**0.009**	**0.000**	0.195	**0.000**
1 vs 3	0.250	0.261	0.556	**0.001**	**0.003**	**0.008**	0.053
2 vs 3	**0.001**	**0.000**	**0.000**	**0.000**	**0.000**	0.351	**0.003**

KEY:

CIC= Circulating Immune Complexes; NFkB= Nuclear Factor Kappa B; IgG= Immunoglobulin G; TNF-α= Tumour Necrosis Factor-Alpha; 8-OH2DG=8-hydroxy-2-deoxy Guanosine; Progest= Progesterone.

4.3: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with DNA Methylation Shift in the Presence of Mixed Antigenic Components (Mean ±SD).

Circulating immune complexes (CIC)

The mean levels of Circulating immune complexes (CIC) in subjects with: benign breast tumour and DNA methylation shift (96±54); benign breast tumour and normal DNA methylation (79±45); malignant breast tumour and DNA methylation shift (146±52); control subjects with DNA methylation shift (49±24) and control subjects with normal DNA methylation (49±30), were significantly different (F=22.8, P= 0.000).

Post Hoc Analysis

Circulating immune complexes (96±54), in 18 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean levels of CIC (49±30) in 40 control subjects with normal DNA methylation P=0.017. The mean level of Circulating immune complexes (96±54), in 18 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean levels of CIC (49±24) in 10 control subjects with DNA methylation shift (P=0.028). The mean level of The mean level of Circulating immune complexes (96±54), in 18 subjects with benign breast tumour and DNA methylation shift, was significantly different compared to the mean levels of CIC (157±35) in 24 subjects with malignant breast tumour and DNA methylation shift (P=0.030). The mean level of Circulating immune complexes (157±35), in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of CIC (49±24) in 10 control subjects with DNA methylation shift (P=0.000). The mean level of Circulating immune complexes (157±35), in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of CIC (49±30) in 40 control subjects with normal DNA methylation (P=0.000). The mean level of Circulating immune complexes (96±54), in 18 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of CIC (79±45) in 6 subjects with benign breast tumour and normal DNA methylation (P=0.941) (Table 4.3).

Nuclear Factor Kappa B (NFkB) (ng/ml)

The mean levels of Nuclear Factor Kappa B (NFkB) in subjects with: benign breast tumour and DNA methylation shift (0338±0.039); benign breast tumour and normal DNA methylation (0.306±0.056); malignant breast tumour and DNA methylation shift (0.364±55); control subjects with DNA methylation shift (0.327±20) and control subjects with normal DNA methylation (0.314±0.017), were significantly different (F=7.9.8, P= 0.000).

Post Hoc Analysis

The mean level of Nuclear Factor Kappa B (NFkB) (0338±0.039), in 18 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of NFkB (0.306±0.056) in 6 subjects with benign breast tumour and normal DNA methylation, the mean levels of NFkB (0.327±20) in 10 control subjects with DNA methylation shift, the mean levels of NFkB (0.314±0.017) in 40 control subjects with normal DNA methylation and the mean levels of NFkB (0.364±55); in 24 subjects with malignant breast tumour and DNA methylation shift (Table 4.3).

The mean level of NFkB (0.364±55) in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of NFkB (0.314±0.017) in 40 control subjects with DNA methylation shift (P=0.000). But the mean level of NFkB (0.364±55) in 24 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of NFkB (0.327±20) in 10 control subjects with DNA methylation shift, (Table 4.3).

Immunoglobulin G (mg/ml)

The mean levels of Immunoglobulin G (IgG) in subjects with: benign breast tumour and DNA methylation shift (24.3±4.5); benign breast tumour and normal DNA methylation (15±4.6); malignant breast tumour and DNA methylation shift (26.9±5.2); control subjects with DNA methylation shift (20.9±5.1) and control subjects with normal DNA methylation (19.5±5.2), were significantly different (F=12.2, P= 0.000).

Post Hoc Analysis

The mean level of Immunoglobulin G (24.3±4.5), in 18 subjects with benign breast tumour and DNA methylation shift, was significantly different compared to the mean levels of IgG (15.0±4.6) in 6 subjects with benign breast tumour and normal DNA methylation P=0.015. The

mean level of IgG (24.3±4.5), in 18 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean level of IgG (19.5±5.2) in 40 control subjects with normal DNA methylation (P=0.009. The mean level of IgG (24.3±4.5), in 18 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of IgG (20.9±5.1) in 10 control subjects with DNA methylation shift, the mean level of IgG (26.9±5.2) in 24 subjects with malignant breast tumour and DNA methylation shift (P=0.417).

The mean level of IgG (15.0±4.6), in 6 subjects with benign breast tumour and normal DNA methylation, was significantly different compared to the mean levels of IgG (26.9±5.2) in 24 subjects with malignant breast tumour and DNA methylation shift (P=0.003).

The mean level of IgG (26.9±5.2), in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of IgG (20.9±5.1) in 10 control subjects with DNA methylation shift (P=0.044).

The mean level of IgG (26.9±5.2), in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of IgG (19.5±5.2) in 40 control subjects with normal DNA methylation (P=0.000) (Table 4.3).

Tumour Necrosis Factor-alpha (pg/ml)

The mean levels of Tumour Necrosis Factor-alpha (TNF-α) in subjects with: benign breast tumour and DNA methylation shift (16.9±5.4); benign breast tumour and normal DNA methylation (11.6±7.1); malignant breast tumour and DNA methylation shift (20.5±2.5); control subjects with DNA methylation shift (11.1±1.5) and control subjects with normal DNA methylation (9.1±2.2), were significantly different (F=47.1, P= 0.000) (Table 4.3).

Post Hoc Analysis

The mean level of TNF-α (16.9±5.4), in 18 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean level of TNF-α (11.1±1.5) in 10 control subjects with DNA methylation shift (P=0.003).

The mean level of TNF-α (16±5.4), in 18 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean levels of TNF-α (9.1±2.2) in

40 control subjects with normal DNA methylation (P=0.000). However, the mean level of Tumour Necrosis Factor-alpha (TNF-α) (16.9±5.4), in 18 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of TNF-α (11.6±7.1) in 6 subjects with benign breast tumour and normal DNA methylation, the mean level of TNF-α (20.5±2.5) in 24 subjects with malignant breast tumour and DNA methylation shift P=0.098. The mean level of TNF-α (20.5±2.5), in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of TNF-α (11.1±1.5) in 10 control subjects with DNA methylation shift (P=0.000).

The mean level of TNF-α (20.5±2.5), in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of TNF-α (9.1±2.2) in 40 control subjects with normal DNA methylation (P=0.000).

The mean level of TNF-α (11.1±1.5), in 10 control subjects with DNA methylation shift, was significantly different compared to the mean levels of TNF-α (9.1±2.2) in 40 control subjects with normal DNA methylation (P=0.024).

8-hydroxy-2-deoxy Guanosine (ng/ml)

The mean levels of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) in subjects with: benign breast tumour and DNA methylation shift (16.7±5.3); benign breast tumour and normal DNA methylation (13.5±7.2); malignant breast tumour and DNA methylation shift (26.6±8.6); control subjects with DNA methylation shift (11.5±4.2) and control subjects with normal DNA methylation (9.3±4.2), were significantly different (F=33.5, P= 0.000) (Table 4.3).

Post Hoc Analysis

The mean level of 8-OH-dG (16.7±5.3), in 18 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean levels of 8-OH-dG (9.3±4.2) in 40 control subjects with normal DNA methylation (P=0.000). The mean level of 8-OH-dG (16.7±5.4), in 18 subjects with benign breast tumour and DNA methylation shift, was significantly different compared to the mean levels of 8-OH-dG (26.6±8.6) in 24 subjects with malignant breast tumour and DNA methylation shift (P=0.000) (Table 4.3).

The mean level of 8-hydroxy-2-deoxy Guanosine (16.7±5.3), in 18 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels

of TNF-α (13.5±7.1) in 6 subjects with benign breast tumour and normal DNA methylation, the mean level of 8-OH-dG (11.5±4.2) in 10 control subjects with DNA methylation shift.

The mean level of 8-OH-dG (13.5±7.1), in 6 subjects with benign breast tumour and normal DNA methylation, was significantly different compared to the mean level of 8-OH-dG (26.6±8.6) in 24 subjects with malignant breast tumour and DNA methylation shift (P=0.025) (Table 4.3).

The mean level of 8-OH-dG (26.6±8.6), in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of 8-OH-dG (11.5±4.2) in 10 control subjects with DNA methylation shift (P=0.000). The mean level of 8-OH-dG (26.6±8.6), in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of 8-OH-dG (9.3±4.2) in 40 control subjects with normal DNA methylation (P=0.000) (Table 4.3).

Oestrogen (pg/ml)

The mean levels of estrogen in subjects with: benign breast tumour and DNA methylation shift (197±98); benign breast tumour and normal DNA methylation (258±84); malignant breast tumour and DNA methylation shift (176±79); control subjects with DNA methylation shift (154±52) and control subjects with normal DNA methylation (133±64), were significantly different (F=5.1, P= 0.001) (Table 4.3).

Post Hoc Analysis

The mean level of estrogen (197±98), in 18 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of estrogen (157±84) in 6 subjects with benign breast tumour and normal DNA methylation, the mean level of estrogen (154±52) in 10 control subjects with DNA methylation shift, the mean level of estrogen (133±64) in 40 control subjects with normal DNA methylation, the mean levels of estrogen (176±79) in 24 subjects with malignant breast tumour and DNA methylation shift (Table 4.3).

The mean level of estrogen (157±84), in 6 subjects with benign breast tumour and normal DNA methylation, was not significantly different compared to the mean level of estrogen (176±79) in 24 subjects with malignant breast tumour and DNA methylation shift (P=0.289). The mean level

of estrogen (176±79), in 24 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of estrogen (154±52) in 10 control subjects with DNA methylation shift and the mean level of estrogen (133±64) in 40 control subjects with normal DNA methylation (Table 4.3).

Progesterone (pg/ml)

The mean levels of progesterone in subjects with: benign breast tumour and DNA methylation shift (1.0±0.5); benign breast tumour and normal DNA methylation (1.4±0.3); malignant breast tumour and DNA methylation shift (0.5±0.4); control subjects with DNA methylation shift (2.1±1.2) and control subjects with normal DNA methylation (2.0±1.3), were significantly different (F=10.3; P= 0.000) (Table 4.3).

Post Hoc Analysis

The mean level of progesterone (1.0±0.5), in 18 subjects with benign breast tumour and DNA methylation shift, was significantly different compared to the mean levels of progesterone (0.5±0.4) in 24 subjects with malignant breast tumour and DNA methylation shift (P=0.014). The mean level of progesterone (1.0±0.5), in 18 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean level of progesterone (2.0±1.3) in 40 control subjects with normal DNA methylation (P=0.001).

The mean level of progesterone (1.0±0.5), in 18 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of progesterone (1.4±0.3) in 6 subjects with benign breast tumour and normal DNA methylation and the mean level of progesterone (2.1±1.2) in 10 control subjects with DNA methylation shift (Table 4.3). The mean level of progesterone (1.4±0.3), in 6 subjects with benign breast tumour and normal DNA methylation, was significantly different compared to the mean level of progesterone (0.5±0.4) in 24 subjects with malignant breast tumour and DNA methylation shift P=0.004.The mean level of progesterone (0.5±0.4), in 24 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of progesterone (2.0±1.3) in 40 control subjects with normal DNA methylation (P=0.000), but the mean level of progesterone (0.5±0.4), in 24 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of progesterone (2.1±1.2) in 10 control subjects with DNA methylation shift (P=0.057) (Table 4.3).

Table 4.3: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with DNA Methylation Shift in the Presence of Mixed Antigenic Components (Mean ±SD).

	CIC/ mgeq/ml	**NFkB /ng/ml**	**IgG /mg/ml**	**TNFα /pg/ml**	**8OH2DG /ng/ml**	**Estrogen pg/ml**	**Progest ng/ml**
Benign Ab[1] N=18	96±54	0.338±0.039	24.3±4.5	16.9±5.4	16.7±5.3	197±98	1.0±0.5
Benign nr[2] N=6	79±45	0.306±0.056	15.0±4.6	11.6±7.1	13.4±7.2	258±84	1.4±0.3
Malign Ab[3] N=24	146±52	0.364±0.055	26.9±5.2	20.5±2.5	26.6±8.6	176±79	0.5.±0.4
Control Ab[4] N=10	49±24	0.327±0.020	20.9±5.1	11.1±1.5	11.5±4.2	154±52	2.1.±1.2
Control nr[5] N=40	49±30	0.314±0.017	19.5±5.2	9.1±2.2	9.3±4.2	133±64	2.0±1.3
F(P-value)	22.8(0.001)	7.9(0.000)	12.2(0.000)	47.1(0.000)	33.5(0.000)	5.1(0.001)	10.3(0.000)
	Post Hoc						
1vs2	0.941	1.638	**0.015**	0.496	0.834	0.602	0.325
1 vs 5	**0.017**	0.120	**0.009**	**0.000**	**0.000**	1.118	**0.001**
1 vs 3	**0.030**	0.409	0.417	0.098	**0.000**	0.944	**0.014**
3 vs 5	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**	0.178	**0.000**
4 vs 5	1.000	0.369	0.922	**0.024**	0.588	0.813	0.999

Key

Benign Ab= Benign tumour with abnormal methylation; Benign nr = Benign tumour with normal methylation; Malign Ab= Malignant tumour with abnormal methylation; Control Ab= Control subjects with abnormal methylation; Control nr= Control subjects with normal methylation

4.4: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with DNA Methylation Shift and Evidence of Microbial Antigenic Components (Mean ±SD).

Circulating immune complexes (CIC)

The mean levels of Circulating immune complexes (CIC) in subjects with: benign breast tumour and DNA methylation shift (104±55); benign breast tumour and normal DNA methylation (79±45); malignant breast tumour and DNA methylation shift (145±44); control subjects with normal DNA methylation (68±39) and control subjects with normal DNA methylation (44±25), were significantly different (F=20.4, P= 0.000).

Post Hoc Analysis

The mean level of Circulating immune complexes (104±55), in 15 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean level of CIC (44±25) in 31 control subjects with normal DNA methylation (P=0.007), but the mean level of Circulating immune complexes (104±55), in 15 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean level of CIC (79±45) in 6 subjects with benign breast tumour and normal DNA methylation, the mean level of CIC (68±39) in 9 control subjects with normal DNA methylation and the mean levels of CIC (145±44) in 19 subjects with malignant breast tumour and DNA methylation shift (P=0.163).

The mean level of Circulating immune complexes (145±44), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of CIC (68±39) in 9 control subjects with normal DNA methylation (P=0.002). The mean level of Circulating immune complexes (145±44), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of CIC (44±25) in 31 control subjects with normal DNA methylation (P=0.000).

Nuclear Factor Kappa B (NFkB) (ng/ml)

The mean levels of Nuclear Factor Kappa B (NFkB) in subjects with: benign breast tumour and DNA methylation shift (0339±0.042); benign breast tumour and normal DNA methylation (0.306±0.052); malignant breast tumour and DNA methylation shift (0.377±0.051); control

subjects with normal DNA methylation (0.310±0.013) and control subjects with normal DNA methylation (0.315±0.018), were significantly different (F=11.0, P= 0.000).

Post Hoc Analysis

The mean level of Nuclear Factor Kappa B (NFkB) (0.339±0.042), in 15 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of NFkB (0.306±0.056) in 6 subjects with benign breast tumour and normal DNA methylation, the mean level of NFkB (0.310±0.013) in 9 control subjects with normal DNA methylation, the mean level of NFkB (0.315±0.018) in 31 control subjects with normal DNA methylation and the mean level of NFkB (0.377±0.051); in 19 subjects with malignant breast tumour and DNA methylation shift (Table 4.4).

The mean level of NFkB (0.377±51) in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of NFkB (0.310±0.013) in 9 control subjects with normal DNA methylation (P=0.000). The mean level of NFkB (0.377±51) in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of NFkB (0.315±0.018) in 31 control subjects with normal DNA methylation (P=0.000) (Table 4.4).

Immunoglobulin G (mg/ml)

The mean levels of Immunoglobulin G (IgG) in subjects with: benign breast tumour and DNA methylation shift (24.6±4.9); benign breast tumour and normal DNA methylation (15.0±4.6); malignant breast tumour and DNA methylation shift (28.3±4.0); control subjects with normal DNA methylation (19.3±4.0) and control subjects with normal DNA methylation (19.5±5.5), were significantly different (F=14.8, P= 0.000).

Post Hoc Analysis

The mean level of Immunoglobulin G (24.6±4.9), in 15 subjects with benign breast tumour and DNA methylation shift, was significantly different compared to the mean levels of IgG (15.0±4.6) in 6 subjects with benign breast tumour and normal DNA methylation (P=0.012). The mean level of IgG (24.6±4.9), in 15 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean levels of IgG (19.5±5.5) in 31 control subjects with normal DNA methylation (P=0.024), but the mean level of IgG (24.6±4.9), in 15 subjects with benign breast tumour and DNA methylation shift was not significantly different

compared to the mean levels of IgG (19.3±4.0) in 9 control subjects with normal DNA methylation and the mean levels of IgG (28.3±4.0) in 19 subjects with malignant breast tumour and DNA methylation shift (Table 4.4).

The mean level of IgG (15.0±4.6), in 6 subjects with benign breast tumour and normal DNA methylation, was significantly different compared to the mean levels of IgG (28.3±4.0) in 19 subjects with malignant breast tumour and DNA methylation shift (P=0.002).

The mean level of IgG (28.3±4.0), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of IgG (19.3±4.0) in 9 control subjects with normal DNA methylation (P=0.000). The mean level of IgG (28.3±4.0), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of IgG (19.5±5.5) in 31 control subjects with normal DNA methylation (P=0.000) (Table 4.4).

Tumour Necrosis Factor-alpha (pg/ml)

The mean levels of Tumour Necrosis Factor-alpha (TNF-α) in subjects with: benign breast tumour and DNA methylation shift (17.3±5.5); benign breast tumour and normal DNA methylation (11.6±7.1); malignant breast tumour and DNA methylation shift (20.9±2.2); control subjects with normal DNA methylation (8.7±2.6) and control subjects with normal DNA methylation (9.2±2.0), were significantly different (F=40.5, P= 0.000) (Table 4.4).

Post Hoc Analysis

The mean level of TNF-α (17.3±5.5), in 15 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean level of TNF-α (8.7±2.6) in 9 control subjects with normal DNA methylation (P=0.000). The mean level of TNF-α (17.3±5.5), in 15 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean levels of TNF-α (9.2±2.0) in 31 control subjects with normal DNA methylation (P=0.000).

The mean level of Tumour Necrosis Factor-alpha (TNF-α) (17.3±5.5), in 15 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean level of TNF-α (11.6±7.1) in 6 subjects with benign breast tumour and normal DNA

methylation and the mean level of TNF-α (20.9±2.2) in 19 subjects with malignant breast tumour and DNA methylation shift (P=0.183).

The mean level of TNF-α (20.9±2.2), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of TNF-α (8.7±2.6) in 9 control subjects with normal DNA methylation (P=0.000). The mean level of TNF-α (20.9±2.2), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of TNF-α (9.2±2.0) in 31 control subjects with normal DNA methylation (P=0.000) (Table 4.4).

8-hydroxy-2-deoxy Guanosine (ng/ml)

The mean levels of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) in subjects with: benign breast tumour and DNA methylation shift (16.8±5.8); benign breast tumour and normal DNA methylation (13.5±7.2); malignant breast tumour and DNA methylation shift (28.1±8.0); control subjects with normal DNA methylation (9.3±4.2) and control subjects with normal DNA methylation (9.4±4.2), were significantly different (F=33.8; P= 0.000) (Table 4.4).

Post Hoc Analysis

The mean level of 8-OH-dG (16.8±5.8), in 15 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean level of 8-OH-dG 9.3±4.2) in 9 control subjects with normal DNA methylation (P=0.010). The mean level of 8-OH-dG (16.8±5.8), in 15 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean levels of 8-OH-dG (9.4±4.2) in 31 control subjects with normal DNA methylation (P=0.002). The mean level of 8-OH-dG (16.8±5.8), in 15 subjects with benign breast tumour and DNA methylation shift, was significantly different compared to the mean levels of 8-OH-dG (29.1±8.0) in 19 subjects with malignant breast tumour and DNA methylation shift (P=0.000), but the mean level of 8-hydroxy-2-deoxy Guanosine (16.8±5.8), in 15 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of TNF-α (13.5±7.1) in 6 subjects with benign breast tumour and normal DNA methylation (Table 4.4).

The mean level of 8-OH-dG (13.5±7.2), in 6 subjects with benign breast tumour and normal DNA methylation, was significantly different compared to the mean levels of 8-OH-dG (28.1±8.0) in 19 subjects with malignant breast tumour and DNA methylation shift (P=0.013).

The mean level of 8-OH-dG (28.1±8.0), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of 8-OH-dG (9.3±4.2) in 9 control subjects with normal DNA methylation (P=0.000) The mean level of 8-OH-dG (28.1±8.0), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean levels of 8-OH-dG (9.4±4.2) in 31 control subjects with normal DNA methylation (P=0.000) (Table 4.4).

Oestrogen (pg/ml)

The mean levels of estrogen in subjects with: benign breast tumour and DNA methylation shift (208±91); benign breast tumour and normal DNA methylation (258±84); malignant breast tumour and DNA methylation shift (179±82); control subjects with normal DNA methylation (137±54) and control subjects with normal DNA methylation (132±67), were significantly different (F=5.4, P= 0.004) (Table 4.4).

Post Hoc Analysis

The mean level of estrogen (208±91), in 15 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of estrogen (258±84) in 6 subjects with benign breast tumour and normal DNA methylation, the mean level of estrogen (137±54) in 9 control subjects with normal DNA methylation (P=0.148), the mean level of estrogen (132±67) in 31 control subjects with normal DNA methylation (P=0.058) and the mean level of estrogen (179±82) in 19 subjects with malignant breast tumour and DNA methylation shift (P=0.860).

The mean level of estrogen (258±84), in 6 subjects with benign breast tumour and normal DNA methylation, was not significantly different compared to the mean level of estrogen (179±82) in 19 subjects with malignant breast tumour and DNA methylation shift (P=0.333).

The mean level of estrogen (179±82), in 19 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of estrogen (137±54) in 9 control subjects with normal DNA methylation and the mean level of estrogen (132±67) in 31 control subjects with normal DNA methylation (P=0.250) (Table 4.4).

Progesterone (pg/ml)

The mean levels of progesterone in subjects with: benign breast tumour and DNA methylation shift (1.1±0.5); benign breast tumour and normal DNA methylation (1.4±0.3); malignant breast tumour and DNA methylation shift (0.5±0.3); control subjects with normal DNA methylation (1.8±1.1) and control subjects with normal DNA methylation (2.1±1.3), were significantly different (F=8.8, P= 0.000) (Table 4.4).

Post Hoc Analysis

The mean level of progesterone (1.1±0.5), in 15 subjects with benign breast tumour and DNA methylation shift, was significantly different compared to the mean levels of progesterone (0.5±0.3) in 19 subjects with malignant breast tumour and DNA methylation shift P=0.004. The mean level of progesterone (1.1±0.5), in 15 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean level of progesterone (2.1±1.3) in 31 control subjects with normal DNA methylation (P=0.008).

The mean level of progesterone (1.1±0.5), in 15 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of progesterone (1.4±0.3) in 6 subjects with benign breast tumour and normal DNA methylation (P=0.619). The mean level of estrogen (1.0±0.5), in 15 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of progesterone (1.8±1.1) in 9 control subjects with normal DNA methylation (P=0.347).

The mean level of progesterone (1.4±0.3), in 6 subjects with benign breast tumour and normal DNA methylation, was significantly different compared to the mean level of progesterone (0.5±0.3) in 19 subjects with malignant breast tumour and DNA methylation shift (P=0.003).

The mean level of progesterone (0.5±0.3), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of progesterone (1.8±1.1) in 9 control subjects with DNA methylation shift (P=0.034). The mean level of progesterone (0.5±0.3), in 19 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of progesterone (2.1±1.3) in 31 control subjects with normal DNA methylation (P=0.000) (Table 4.4).

Table 4.4: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with DNA Methylation Shift and Evidence of Microbial Antigenic Components (Mean ±SD).

	CIC/ mgeq/ml	**NFkB /ng/ml**	**IgG /mg/ml**	**TNFα /pg/ml**	**8OH2DG /ng/ml**	**Estrogen pg/ml**	**Progest ng/ml**
Benign Ab[1] N=15	104±55	0.339±0.042	24.6±4.9	17.3±5.5	16.8±5.8	208±91	1.1±0.5
Benign nr[2] N=6	79±45	0.306±0.056	15.0±4.6	13.4±7.2	13.4±7.2	258±84	1.4±0.3
Malign Ab[3] N=19	145±44	0.377±0.051	28.3±4.0	20.9±2.2	28.1±8.0	179±82	0.5.±0.3
Control nr[4] N=9	68±39	0.310±0.013	19.3±4.0	8.7±2.6	9.3±4.2	137±54	1.8±1.1
F(P-value)	20.4(0.001)	11.0(0.000)	14.8(0.000)	40.5(0.000)	33.8(0.000)	5.4(0.004)	8.8(0.000)
	Post Hoc						
1vs2	0.827	1.641	**0.012**	0.436	0.841	0.756	0.619
1 vs 4	0.378	0.132	0.062	**0.000**	**0.010**	0.148	0.347
1 vs 3	0.163	0.148	0.160	0.183	**0.000**	0.860	**0.004**
3 vs 4	**0.002**	**0.000**	**0.000**	**0.000**	**0.000**	0.511	**0.034**

Key

Benign Ab= Benign tumour with abnormal methylation; Benign nr = Benign tumour with normal methylation

Malign Ab= Malignant tumour with abnormal methylation; Control nr= Control subjects with normal methylation

4.5: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with DNA Methylation Shift without Evidence of Microbial Antigenic Components (Mean ±SD).

Circulating immune complexes (CIC)

The mean levels of Circulating immune complexes (CIC) in subjects with: benign breast tumour and DNA methylation shift (56±17); malignant breast tumour and DNA methylation shift (155±82); control subjects with DNA methylation shift (40±17) and control subjects with normal DNA methylation (44±25), were significantly different (F=16.3, P= 0.000).

Post Hoc Analysis

The mean level of Circulating immune complexes (56±17), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of CIC (40±17) in 8 control subjects with DNA methylation shift P=0.551. The mean level of Circulating immune complexes (56±17), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean levels of CIC (44±25) in 31 control subjects with normal DNA methylation (P=0.668). The mean level of Circulating immune complexes (56±17), in 3 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of CIC (155±44) in 5 subjects with malignant breast tumour and DNA methylation shift (P=0.169). The mean level of Circulating immune complexes (155±44), in 5 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of CIC (40±17) in 8 control subjects with DNA methylation shift (P=0.110). The mean level of Circulating immune complexes (155±44), in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of CIC (44±25) in 31 control subjects with normal DNA methylation (P=0.121) (Table 4.5)

Nuclear Factor Kappa B (NFkB) (ng/ml)

The mean levels of Nuclear Factor Kappa B (NFkB) in subjects with: benign breast tumour and DNA methylation shift (0.335±0.026); malignant breast tumour and DNA methylation shift (0.315±0.048); control subjects with DNA methylation shift (0.328±0.022) and control subjects with normal DNA methylation (0.315±0.018), were not significantly different (F=1.2, P= 0.328).

Post Hoc Analysis

The mean level of NFkB (0.335±0.026), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of NFkB (0.328±0.022) in 8 control subjects with DNA methylation shift P=0.983. The mean level of NFkB (0335±0.026), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean levels of NFkB (0.315±0.018) in 31 control subjects with normal DNA methylation P=0.644. The mean level of NFkB (0335±0.026), in 3 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of NFkB (0.315±0.048); in 5 subjects with malignant breast tumour and DNA methylation shift (P=0.879). The mean level of NFkB (0.315±0.048) in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of NFkB (0.328±0.022) in 8 control subjects with DNA methylation shift (P=0.933). The mean level of NFkB (0.315±0.048) in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of NFkB (0.315±0.018) in 31 control subjects with normal DNA methylation (P=1.000) (Table 4.5).

Immunoglobulin G (mg/ml)

The mean levels of Immunoglobulin G (IgG) in subjects with: benign breast tumour and DNA methylation shift (22.5±2.2); malignant breast tumour and DNA methylation shift (21.6±5.9); control subjects with DNA methylation shift (20.5±5.5) and control subjects with normal DNA methylation (19.5±5.5), were not significantly different (F=0.5, P= 0.718).

Post Hoc Analysis

The mean level of IgG (22.5±2.2), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of IgG (20.5±5.5) in 8 control subjects with DNA methylation shift (P=0.833). The mean level of IgG (22.6±2.2), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean levels of IgG (19.5±5.5) in 31 control subjects with normal DNA methylation (P=0.349). The mean level of IgG (22.6±2.2), in 3 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean level of IgG (21.6±5.9) in 5 subjects with malignant breast tumour and DNA methylation shift (P=0.990). The mean level of IgG (21.6±5.9), in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of IgG (20.5±5.5) in 8 control subjects with DNA methylation shift (P=0.987). The mean level of IgG

(21.6±5.9), in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of IgG (19.5±5.5) in 31 control subjects with normal DNA methylation (P=0.878) (Table 4.5).

Tumour Necrosis Factor-alpha (pg/ml)

The mean levels of Tumour Necrosis Factor-alpha (TNF-α) in subjects with: benign breast tumour and DNA methylation shift (14.5±5.2); malignant breast tumour and DNA methylation shift (19.1±3.2); control subjects with DNA methylation shift (11.1±0.9) and control subjects with normal DNA methylation (9.2±2.0), were significantly different (F=28.9, P= 0.000) (Table 4.5).

Post Hoc Analysis

The mean level of TNF-α (14.5±5.2), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of TNF-α (11.1±0.9) in 8 control subjects with DNA methylation shift (P=0.715). The mean level of TNF-α (14.5±5.2), in 3 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean level of TNF-α (9.2±2.0) in 31 control subjects with normal DNA methylation (P=0.482). The mean level of TNF-α (14.5±5.2), in 3 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of TNF-α (19.1±3.2) in 5 subjects with malignant breast tumour and DNA methylation shift (P=0.584). The mean level of TNF-α (19.1±3.2), in 5 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of TNF-α (11.1±0.9) in 8 control subjects with normal DNA methylation P=0.014. The mean level of TNF-α (19.1±3.2), in 5 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of TNF-α (9.2±2.0) in 31 control subjects with normal DNA methylation (P=0.006) (Table 4.5).

8-hydroxy-2-deoxy Guanosine (ng/ml)

The mean levels of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) in subjects with: benign breast tumour and DNA methylation shift (16.4±1.9); malignant breast tumour and DNA methylation shift (20.7±8.9); control subjects with DNA methylation shift (12.0±4.4) and control subjects with normal DNA methylation (9.4±4.2), were significantly different (F=9.2; P= 0.000 (Table 4.5).

Post Hoc Analysis

The mean level of 8-OH-dG (16.4±1.9), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of 8-OH-dG 12.0±4.4) in 8 control subjects with DNA methylation shift (P=0.156). The mean level of 8-OH-dG (16.4±1.9), in 3 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean levels of 8-OH-dG (9.4±4.2) in 31 control subjects with normal DNA methylation (P=0.015). The mean level of 8-OH-dG (16.4±1.9), in 3 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean level of 8-OH-dG (20.7±8.9) in 5 subjects with malignant breast tumour and DNA methylation shift (P=0.736). The mean level of 8-OH-dG (20.7±8.9), in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of 8-OH-dG (12.0±4.4) in 8 control subjects with DNA methylation shift (P=0.280) The mean level of 8-OH-dG (20.7±8.9), in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean levels of 8-OH-dG (9.4±4.2) in 31 control subjects with normal DNA methylation (P=0.142) (Table 4.5).

Estrogen (pg/ml)

The mean levels of estrogen in subjects with: benign breast tumour and DNA methylation shift (141±138); malignant breast tumour and DNA methylation shift (166±71); control subjects with DNA methylation shift (151±57) and control subjects with normal DNA methylation (132±67), were significantly different (F=0.4, P= 0.742) (Table 4.5).

Post Hoc Analysis

The mean level of estrogen (141±138), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of estrogen (151±57) in 8 control subjects with normal DNA methylation P=0.999. The mean level of estrogen (141±138), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of estrogen (132±67) in 31 control subjects with normal DNA methylation (P=0.999). The mean level of estrogen (141±138), in 3 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean level of estrogen (166±71) in 5 subjects with malignant breast tumour and DNA methylation shift (P=0.989). The mean level of estrogen (166±71), in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of estrogen (151±57) in 8 control subjects with DNA methylation shift (P=0.979). The

mean level of estrogen (166±71), in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of estrogen (132±67) in 31 control subjects with normal DNA methylation (P=0.756) (Table 4.5).

Progesterone (pg/ml)

The mean levels of progesterone in subjects with: benign breast tumour and DNA methylation shift (0.6±0.1); malignant breast tumour and DNA methylation shift (0.7±0.5); control subjects with DNA methylation shift (1.8±1.5) and control subjects with DNA methylation shift (2.1±1.3), were significantly different (F=2.6, P= 0.064) (Table 4.5).

Post Hoc Analysis

The mean level of estrogen (0.6±0.1), in 3 subjects with benign breast tumour and DNA methylation shift was not significantly different compared to the mean level of progesterone (1.8±1.5) in 8 control subjects with normal DNA methylation (P=0.205). The mean level of progesterone (0.6±0.1), in 3 subjects with benign breast tumour and DNA methylation shift was significantly different compared to the mean level of progesterone (2.1±1.3) in 31 control subjects with normal DNA methylation (P=0.000). The mean level of progesterone (0.6±0.1), in 3 subjects with benign breast tumour and DNA methylation shift, was not significantly different compared to the mean level of progesterone (0.7±0.5) in 5 subjects with malignant breast tumour and DNA methylation shift (P=0.956). The mean level of progesterone (0.7±0.5), in 5 subjects with malignant breast tumour and DNA methylation shift, was not significantly different compared to the mean level of progesterone (1.8±1.1) in 8 control subjects with DNA methylation shift (P=0.300). The mean level of progesterone (0.7±0.5), in 5 subjects with malignant breast tumour and DNA methylation shift, was significantly different compared to the mean level of progesterone (2.1±1.3) in 31 control subjects with normal DNA methylation (P=0.003) (Table 4.5).

Table 4.5: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with DNA Methylation Shift without Evidence of Microbial Antigenic Components (Mean ±SD).

	CIC/ mgeq/ml	**NFkB /ng/ml**	**IgG /mg/ml**	**TNFα /pg/ml**	**8OH2DG /ng/ml**	**Estrogen pg/ml**	**Progest ng/ml**
Benign Ab[1] N=3	56±17	0.335±0.026	22.5±2.2	14.5±5.2	16.4±1.9	141±138	0.6±0.1
Malign Ab[2] N=5	155±82	0.315±0.048	21.6±5.9	19.1±3.2	20.7±8.9	166.±71	0.7.±0.5
Contr. Ab[3] N=8	40±17	0.328±0.022	20.5±5.5	11.1±0.9	12.0±4.4	151±57	1.8±1.5
Control nr[4] N=31	44±25	0.315±0.018	19.5±5.5	9.2±2.2	9.4±4.2	132±67	2.1±1.3
F(P-value)	**16.3(0.000)**	1.2(0.328)	0.5(0.715)	**28.9(0.000)**	**9.2(0.000)**	0.4(0.742)	2.6(0.064)
	Post Hoc						
1 vs 2	0.169	0.879	0.990	0.584	0.736	0.989	0.956
1 vs 3	0.551	0.983	0.833	0.715	0.156	0.999	0.205
1 vs 4	0.668	0.644	0.349	0.482	**0.015**	0.999	**0.000**
2 vs 3	0.110	0.933	0.987	**0.014**	0.280	0.979	0.300
2 vs 4	0.121	1.000	0.878	**0.006**	0.142	0.756	**0.003**
3 vs 4	0.967	0.430	1.966	0.006	0.478	0.844	0.964

Key

Benign Ab= Benign tumour with abnormal methylation; Malign Ab= Malignant tumour with abnormal methylation; Control Ab= Control subjects with abnormal methylation; Control nr= Control subjects with normal methylation

4.6: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with DNA Hypomethylation and Evidence of Microbial Antigenic Components (Mean ±SD).

Circulating Immune Complexes (CIC)

The mean levels of Circulating immune complexes (CIC) in 11 hypomethylated subjects with malignant breast tumour (157±35); 5 benign breast tumour (100±36) and 5 control subjects without evidence of microbial antigen (40±12) were significantly different (F=23.6; P= 0.000).

Post Hoc Analysis

The mean level of Circulating immune complexes (157±35), in 11 malignant breast tumour subjects with microbial antigenic components, was significantly different compared to the mean levels of CIC (100±36) in 5 benign breast tumour subjects with microbial antigenic components (P=0.048). The mean level of Circulating immune complexes (100±36), in 5 benign breast tumour subjects with microbial antigenic components, was significantly different compared to the mean level of CIC (40±12) in 5 control subjects without microbial antigenic components P=0.041. The mean level of Circulating immune complexes (157±35), in 11 malignant breast tumour subjects with microbial antigenic components, was significantly different compared to the mean levels of CIC (40±12) in 5 control subjects without microbial antigenic components (P=0.000) (Table 4.6).

Nuclear Factor Kappa B (NFkB) (ng/ml) In Hypomethylation

The mean levels of Nuclear Factor Kappa B (NFkB) in 11 hypomethylated subjects with malignant breast tumour (0.381±0.049); 5 benign breast tumour (0.345±0.045) and 5 control subjects without evidence of microbial antigen (0.333±0.018) were not significantly different (F=2.5, P= 0.107).

Post Hoc Analysis

The mean level of Nuclear Factor Kappa B (0.381±0.049), in 11 malignant breast tumour subjects with microbial antigenic components, was not significantly different compared to the mean level of CIC (0.345±0.045) in 5 benign breast tumour subjects with microbial antigenic components (P=0.364). The mean level of Nuclear Factor Kappa B (NFkB) (0.345±0.045), in 5 benign breast tumour subjects with microbial antigenic components, was not significantly

different compared to the mean level of Nuclear Factor Kappa B (0.333±0.018) in 5 control subjects without microbial antigenic components (P=0.862). The mean level of Nuclear Factor Kappa B (0.381±0.049), in 11 malignant breast tumour subjects with microbial antigenic components, was significantly different compared to the mean level of CIC (0.333±0.018) in 5 control subjects without microbial antigenic components (P=0.035). (Table 4.6).

Immunoglobulin G (mg/ml)

The mean levels of Immunoglobulin G (IgG) in 11 hypomethylated subjects with malignant breast tumour (29.3±3.6); 5 benign breast tumour (20.2±5.2) and 5 control subjects without evidence of microbial antigen (19.0±4.5) were significantly different (F=13.8, P= 0.000).

Post Hoc Analysis

The mean level of Immunoglobulin G (IgG) (29.3±3.6), in 11 malignant breast tumour subjects with microbial antigenic components, was significantly different compared to the mean level of IgG (20.2±5.2), in 5 benign breast tumour subjects with microbial antigenic components (P=0.029). The mean level of Immunoglobulin G (IgG) (20.2±5.2), in 5 benign breast tumour subjects with microbial antigenic components, was not significantly different compared to the mean level of Immunoglobulin G (IgG) (19.0±4.5) in 5 control subjects without microbial antigenic components (P=0.918). The mean level of Immunoglobulin G (IgG) (29.3±3.6), in 11 malignant breast tumour subjects with microbial antigenic components, was significantly different compared to the mean level of IgG (19.0±4.5) in 5 control subjects without microbial antigenic components (P=0.008) (Table 4.6).

Tumour Necrosis Factor-alpha (pg/ml)

The mean level of Tumour Necrosis Factor alpha (TNF-α) in subjects with malignant breast tumour subjects (20.7±2.7), those with benign tumour (17.3±5.8) and control subjects (11.4±1.1) were significantly different (F=12.6, P=0.000).

Post Hoc Analysis

The mean level of Tumour Necrosis Factor-alpha (TNF-α) (20.7±2.7), in 11 malignant breast tumour subjects with microbial antigenic components, was not significantly different compared to the mean level of Tumour Necrosis Factor-alpha (TNF-α) (11.4±1.1) in 5 benign breast tumour subjects with microbial antigenic components (P=0.476). The mean level of Tumour Necrosis Factor-alpha (TNF-α) (17.3±5.8), in 5 benign breast tumour subjects with microbial

antigenic components, was not significantly different compared to the mean level of Tumour Necrosis Factor-alpha (TNF-α) (11.4±1.1) in 5 control subjects without microbial antigenic components (P=0.172). The mean level of Tumour Necrosis Factor-alpha (TNF-α) (20.7±2.7), in 11 malignant breast tumour subjects with microbial antigenic components, was significantly different compared to the mean level of Tumour Necrosis Factor-alpha (TNF-α) (11.4±1.1) in 5 control subjects without microbial antigenic components (P=0.000) (Table 4.6).

8-hydroxy-2-deoxy Guanosine (ng/ml)

The mean level of 8-Hydroxy-2-Deoxy Guanosine (8-OH2DG) in subjects with malignant breast tumour subjects (29.2±9.2), subjects with benign tumour (13.2±4.2) and control subjects (9.7±3.1) were significantly different (F=15.8, P=0.000).

Post Hoc Analysis

The mean level of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) (29.2±9.2), in 11 malignant breast tumour subjects with microbial antigenic components, was significantly different compared to the mean level of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) (9.7±3.1) in 5 control subjects without microbial antigenic components (P=0.001). The mean level of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) (13.2±4.2), in 5 benign breast tumour subjects with microbial antigenic components, was not significantly different compared to the mean level of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) (9.7±3.1) in 5 control subjects without microbial antigenic components (P=0.344). The mean level of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) (29.2±9.2), in 11 malignant breast tumour subjects with microbial antigenic components, was significantly different compared to the mean level of 8-hydroxy-2-deoxy Guanosine (8-OH-dG) (9.7±3.1) in 5 control subjects without microbial antigenic components (P=0.000) (Table 4.6).

Estrogen (pg/ml)

The mean level of estrogen in subjects with malignant breast tumour subjects (195±91), subjects with benign tumour (201±107) and control subjects (135±47) were significantly different F=0.9, (P=0.406).

Post Hoc Analysis

The mean level of (195±91), in 11 malignant breast tumour subjects with microbial antigenic components, was not significantly different compared to the mean level of estrogen (201±107) in 5 benign breast tumour subjects with microbial antigenic components (P=0.995). The mean level

of estrogen (201±107), in 5 benign breast tumour subjects with microbial antigenic components, was not significantly different compared to the mean level of estrogen (135±47) in 5 control subjects without microbial antigenic components (P=0.476). The mean level of (195±91), in 11 malignant breast tumour subjects with microbial antigenic components, was not significantly different compared to the mean level of estrogen (135±47) in 5 control subjects without microbial antigenic components (P=0.232) (Table 4.6).

Progesterone (pg/ml)

The mean level of progesterone in subjects with malignant breast tumour subjects (0.4±0.3), subjects with benign tumour (1.3±0.5) and control subjects (2.0±1.5) were significantly different (F=7.7, P=0.004).

Post Hoc Analysis

The mean level of (0.4±0.3), in 11 malignant breast tumour subjects with microbial antigenic components, was significantly different compared to the mean level of progesterone (1.3±0.5) in 5 benign breast tumour subjects with microbial antigenic components, P=0.028. The mean level of progesterone (1.3±0.5), in 5 benign breast tumour subjects with microbial antigenic components, was not significantly different compared to the mean level of progesterone (2.0±1.5) in 5 control subjects without microbial antigenic components P=0.614. The mean level of (0.4±0.3), in 11 malignant breast tumour subjects with microbial antigenic components, was not significantly different compared to the mean level of progesterone (2.0±1.5) in 5 control subjects without microbial antigenic components (P=0.153) (Table 4.6).

Table 4.6: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with DNA Hypomethylation and Evidence of Microbial Antigenic Components (Mean ±SD).

	CIC/ mgeq/ml	**NFkB /ng/ml**	**IgG /mg/ml**	**TNFα /pg/ml**	**8OH2DG /ng/ml**	**Estrogen pg/ml**	**Progest ng/ml**
Benign Ab[1] N=5	100±36	0.345±045	20.2±5.2	17.3±5.8	13.2±4.2	201±107	1.3±0.5
Malign Ab[2] N=11	157±35	0.381±0.049	29.3±3.6	20.7±2.7	29.2±9.2	195±91	0.4±0.3
Contr. Ab[3] N=5	40±12	0.333±0.018	19.0±4.5	11.4±1.1	9.7±3.1	135±47	2.0±1.5
F(P-value)	**23.6(0.000)**	2.5(0.328)	**13.8(0.000)**	**12.6(0.000)**	**9.2(0.000)**	0.9(0.406)	**7.7(0.004)**
	Post Hoc						
1 vs 2	**0.048**	0.364	**0.029**	0.476	**0.001**	0.995	**0.028**
1 vs 3	**0.041**	0.862	0.918	0.172	0.344	0.476	0.614
2 vs 3	**0.000**	**0.035**	**0.008**	**0.000**	**0.000**	0.232	0.153

Key

Benign Ab= Benign tumour with abnormal methylation; Malign Ab= Malignant tumour with abnormal methylation; Control Ab= Control subjects with abnormal methylation.

4.7. Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with Unmethylated DNA and Evidence of Microbial Antigenic Components (Mean ±SD).

Circulating Immune Complexes (CIC)

The mean levels of Circulating immune complexes (CIC) in subjects with malignant breast tumour (112±43); benign breast tumour (119±67) and control subjects with normal methylation (68±39) were not significantly different (F=2.3, P= 0.128) (Table 4.7).

Post Hoc Analysis

The mean level of circulating immune complexes (112±43), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean levels of CIC (119±67) in 8 subjectss with benign breast tumour (P=0.974). The mean level of circulating immune complexes (119±67), in 8 subjects with benign breast tumour, was not significantly different compared to the mean level of CIC (68±39) in 9 control subjects with normal methylation (P=0.193). The mean level of Circulating immune complexes (112±43), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean levels of CIC (68±39) in 9 control subjects with normal methylation (P=0.208) (Table 4.7).

Nuclear Factor Kappa B (NFkB) (ng/ml) In Unmethylated DNA

The mean levels of Nuclear Factor Kappa B (NFkB) in subjects with malignant breast tumour (0.347±0.030); benign breast tumour (0.323±0.036) and control subjects with normal methylation (0.310±0.013) were not significantly different (F=3.0, P= 0.075) (Table 4.7).

Post Hoc Analysis

The mean level of Nuclear Factor Kappa B (0.347±0.030), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean level of Nuclear Factor Kappa B (0.323±0.036) in 8 subjectss with benign breast tumour P=0.438. The mean level of Nuclear Factor Kappa B (0.323±0.036), in 8 subjects with benign breast tumour, was not significantly different compared to the mean level of Nuclear Factor Kappa B (0.310±0.013) in 9 control subjects with normal methylation (P=0.600). The mean level of Nuclear Factor Kappa B (0.347±0.030), in 5 subjects with malignant breast tumour, was not significantly different

compared to the mean levels of Nuclear Factor Kappa B (0.310±0.013) in 9 control subjects with normal methylation (P=0.102) (Table 4.7).

Immunoglobulin G (IgG) (mg/ml)

The mean levels of Immunoglobulin G in subjects with malignant breast tumour (28.6±2.8); benign breast tumour (27.2±2.9) and control subjects with normal methylation (19.3±4.0) were significantly different (F=16.4, P= 0.000) (Table 4.7).

Post Hoc Analysis

The mean level of Immunoglobulin G (28.6±2.8), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean level of Immunoglobulin G (27.2±2.9) in 8 subjectss with benign breast tumour (P=0.692). The mean level of Immunoglobulin G (27.2±2.9), in 8 subjects with benign breast tumour, was significantly different compared to the mean level of Immunoglobulin G (19.3±4.0) in 9 control subjects with normal methylation (P=0.001). The mean level of Immunoglobulin G (28.6±2.8), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean levels of Immunoglobulin G (19.3±4.0) in 9 control subjects with normal methylation (P=0.001) (Table 4.7).

Tumour Necrosis Factor-alpha (pg/ml)

The mean levels of Tumour Necrosis Factor-alpha in subjects with malignant breast tumour (21.6±1.5); benign breast tumour (16.4±5.9) and control subjects with normal methylation (8.8±2.6) were significantly different (F=17.7, P= 0.000) (Table 4.7).

Post Hoc Analysis

The mean level of Tumour Necrosis Factor-alpha (21.6±1.5), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean level of Tumour Necrosis Factor-alpha (16.4±5.9) in 8 subjectss with benign breast tumour (P=0.101). The mean level of Tumour Necrosis Factor-alpha (16.4±5.9), in 8 subjects with benign breast tumour, was significantly different compared to the mean level of Tumour Necrosis Factor-alpha (8.8±2.6) in 9 control subjects with normal methylation (P=0.020). The mean level of Tumour Necrosis Factor-alpha (21.6±1.5), in 5 subjects with malignant breast tumour, was significantly different compared to the mean levels of Tumour Necrosis Factor-alpha (8.8±2.6) in 9 control subjects with normal methylation (P=0.000) (Table 4.7).

8-hydroxy-2-deoxy Guanosine (ng/ml)

The mean levels of 8-hydroxy-2-deoxy Guanosine in subjects with malignant breast tumour (26.2±7.8); benign breast tumour (19.6±5.2) and control subjects with normal methylation (9.1±4.3) were significantly different (F=15.8, P= 0.000) (Table 7).

Post Hoc Analysis

The mean level of 8-hydroxy-2-deoxy Guanosine (26.2±7.8), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean level of 8-hydroxy-2-deoxy Guanosine (19.6±5.2) in 8 subjectss with benign breast tumour (P=0.292). The mean level of 8-hydroxy-2-deoxy Guanosine (19.6±5.2), in 8 subjects with benign breast tumour, was significantly different compared to the mean level of 8-hydroxy-2-deoxy Guanosine (9.1±4.3) in 9 control subjects with normal methylation (P=0.003). The mean level of 8-hydroxy-2-deoxy Guanosine (26.2±7.8), in 5 subjects with malignant breast tumour, was significantly different compared to the mean levels of 8-hydroxy-2-deoxy Guanosine (9.1±4.3) in 9 control subjects with normal methylation (P=0.012) (Table 4.7).

Estrogen (pg/ml)

The mean levels of estrogen in subjects with malignant breast tumour (134±77); benign breast tumour (203±96) and control subjects with normal methylation (137±54) were not significantly different (F=2.0, P= 0.163) (Table 4.7).

Post Hoc Analysis

The mean level of estrogen (134±77), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean level of estrogen (203±96) in 8 subjectss with benign breast tumour (P=0.359). The mean level of estrogen (203±96), in 8 subjects with benign breast tumour, was not significantly different compared to the mean level of estrogen (137±54) in 9 control subjects with normal methylation (P=0.236). The mean level of estrogen (134±77), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean level of estrogen (137±54) in 9 control subjects with normal methylation (P=0.996) (Table 4.7).

Progesterone (pg/ml)

The mean levels of progesterone in subjects with malignant breast tumour (0.6±0.3); benign breast tumour (1.0±0.5) and control subjects with normal methylation (1.8±1.1) were significantly different (F=5.0; P= 0.017) (Table 4.7).

Post Hoc Analysis

The mean level of progesterone (0.6±0.3), in 5 subjects with malignant breast tumour, was not significantly different compared to the mean level of progesterone (1.0±0.5) in 8 subjectss with benign breast tumour (P=0.242). The mean level of progesterone (1.0±0.5), in 8 subjects with benign breast tumour, was not significantly different compared to the mean level of progesterone (1.8±1.1) in 9 control subjects with normal methylation (P=0.108). The mean level of progesterone (0.6±0.3), in 5 subjects with malignant breast tumour, was significantly different compared to the mean level of progesterone (1.8±1.1) in 9 control subjects with normal methylation (P=0.022) (Table 4.7).

Table 4.7: Levels of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant, Benign Breast tumours and Control Subjects with Unmethylation DNA and Evidence of Microbial Antigenic Components (Mean ±SD).

	CIC/ mgeq/ml	**NFkB /ng/ml**	**IgG /mg/ml**	**TNFα /pg/ml**	**8OH2DG /ng/ml**	**Estrogen pg/ml**	**Progest ng/ml**
Benign Ab[1] N=8	119±67	0.323±036	27.2±2.9	16.4±5.9	19.6±5.7	203±96	1.0±0.5
Malign Ab[2] N=5	112±43	0.347±0.030	28.6±2.8	21.6±1.5	26.2±7.8	134±77	0.6±0.3
Contr. nr[3] N=9	68±39	0.310±0.013	19.3±4.0	8.8±2.6	9.1±4.3	137±54	1.8±1.1
F(P-value)	2.3(0.128)	3.0(0.075)	16.4(0.000)	17.7(0.000)	15.8(0.000)	2.0(0.163)	5.0(0.017)
	Post Hoc						
1 vs 2	0.974	0.438	0.692	0.101	0.292	0.356	0.242
1 vs 3	0.193	0.600	**0.001**	**0.020**	**0.003**	0.236	0.108
2 vs 3	0.208	0.102	**0.001**	**0.000**	**0.012**	0.996	**0.022**

Key

Benign Ab= Benign tumour with abnormal methylation; Malign Ab= Malignant tumour with abnormal methylation; Control Ab= Control subjects with abnormal methylation

4.8: Correlation of Circulating Immune Complexes and other Molecules Expressed in Subjects with Malignant Breast Tumour.

In subjects with malignant breast tumour, with mixed antigenic components, there was no significant positive or negative correlation between the expression of circulating immune complexes (CIC), nuclear factor kappa B (NFkB), tumour necrosis factor-alpha (TNF-α), OH2DG, Immunoglobulin G (IgG), estrogen and progesterone (Table 4.8).

In subjects with malignant breast tumour, with evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed strong positive significant association with serum estrogen (r= 0.465; P=0.039). However, no significant positive or negative correlation was seen between the expression of circulating immune complexes (CIC), nuclear factor kappa B (NFkB), tumour necrosis factor-alpha (TNF-α), OH2DG, Immunoglobulin G (IgG), and progesterone.

In subjects with malignant breast tumour, without evidence of microbial antigenic components, expression of circulating immune complexes (CIC), nuclear factor kappa B (NFkB), tumour necrosis factor-alpha (TNF-α), OH2DG, Immunoglobulin G (IgG), estrogen and progesterone. (Table 4.8).

Table 4.8: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Subjects with Malignant Breast Tumour.

	Combined N=25		Presence of MAC N=20		Absence of MAC N=5	
Variables	**(r)**	**P-value**	**(r)**	**P-value**	**(r)**	**P-value**
CIC vs NF-kB	-0.162	0.493	0.029	0.902	-0.606	0.279
CIC vs IgG	-0.231	0.266	-0.014	0.953	-0.561	0.325
CIC vs TNF-α	-0.145	0.491	0.102	0.669	-0.637	0.248
CIC vs OHDG	0.106	0.613	0.142	0.549	0.194	0.755
CIC vs Estrogen	0.309	0.133	***0.465**	**0.039**	-0.129	0.836
CIC vs Progest	0.055	0.793	-0.188	0.428	0.635	0.250
NF-kB vs IgG	0.247	0.233	0.000	0.999	0.069	0.913
NF-kB vs TNF-α	0.110	0.601	-0.104	0.663	0.445	0.435
NF-kB vs OHDG	0.347	0.089	0.377	0.101	-0.381	0.527
NF-kB vs Estrog	0.256	0.216	0.383	0.096	-0.431	0.469
NF-kB vs Progest	-0.273	0.186	-0.212	0.369	-0.421	0.481
IgG vs TNF-α	0.122	0.560	-0.192	0.417	0.461	0.435
IgG vs OHDG	0.216	0.301	0.071	0.767	0.007	0.991
IgG vs Estrogen	0.195	0.349	0.026	0.913	0.855	0.065
IgG vs Progest	-0.054	0.796	-0.076	0.749	0.241	0.696
TNF-α vs OHDG	0.215	0.301	0.038	0.874	0.544	0.343
TNF-α vs Estrog	-0.103	0.623	-0.165	0.488	0.073	0.907
TNF-α vs Progest	-0.359	0.076	-0.368	0.111	0.266	0.665
OHDG vs Estrog	0.122	0.562	0.119	0.616	0.079	0.900
OHDG vs Progest	0.035	0.870	0.056	0.815	0.152	0.807
Estrog vs Progest	0.006	0.976	-0.094	0.892	0.522	0.366

Key

Presence of MAC=Presence of Microbial antigenic components

Absence of MAC=Absence of Microbial antigenic components

*****Moderate Positive significant Correlation

4.9: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Subjects with Benign Breast Tumour.

In subjects with Benign breast tumour, with mixed antigenic components, expression of circulating immune complexes (CIC) showed no significant positive or negative relationship with expression of Immunoglobulin G (IgG), serum expression of OH2DG, estrogen, NF-kB, TNF-alpha and progesterone.

Expression NF-kB showed strong positive significant association with TNF-alpha (r= 0.683; P= 0.000); moderate positive significant association with OH2DG (r= 0.442; P=0.030), but no significant positive or negative association with serum expression of IgG, estrogen and progesterone (Table 4.9).

Expression of Immunoglobulin G (IgG) showed moderate positive significant relationship with serum OH2DG (r= 0.418; P = 0.048), moderate negative significant association with serum progesterone (r= -0.410; P= 0.047) but no significant positive or negative relationship with expression of TNF-alpha and estrogen.

Expression of TNF-alpha showed strong positive significant relationship with expression of OH2DG (r= 0.470, P = 0.020), (Table 4.9), but no significant positive or negative association with estrogen and progesterone.

Expression of OH2DG showed no significant positive or negative relationship with estrogen and progesteorne (Table 4.9).

In benign breast tumour subjects, with evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed strong positive significant association with estrogen (r= 0.487, P=0.025); but no significant positive or negative relationship with expression of Immunoglobulin G (IgG), NFkB, TNF-alpha and progesterone (Table 4.9).

Expression NF-kB showed strong positive significant association with TNF-alpha r= 0.692, P= 0.001; strong positive significant association with OH2DG r= 0.457, P=0.037, but no significant

positive or negative association with serum expression of IgG, estrogen and progesterone (Table 4.19).

Expression of Immunoglobulin G (IgG) showed moderate negative significant association with serum progesterone (r= -0.432, P= 0.050), but no significant positive or negative relationship with expression of TNF-alpha, OH2DG, estrogen (Table 4.9).

Expression of TNF-alpha showed strong positive significant relationship with expression of OH2DG (r= 0.481, P = 0.027), but no significant positive or negative association with estrogen and progesteone.

Expression of OH2DG showed no significant positive or negative relationship with progesterone and serum estrogen (Table 4.9).

Table 4.9: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Subjects with Benign Breast Tumour.

	Combined N=24		Presence of MAC N=21	
Variables	**(r)**	**P-value**	**(r)**	**P-value**
CIC vs NF-kB	0.033	0.878	0.042	0.856
CIC vs IgG	0.334	0.110	0.365	0.104
CIC vs TNF-α	0.195	0.360	0.168	0.466
CIC vs OHDG	0.345	0.099	0.364	0.105
CIC vs Estrogen	-0.298	0.157	***0.487**	**0.025**
CIC vs Progest	0.023	0.914	-0.108	0.643
NF-kB vs IgG	0.244	0.250	0.234	0.307
NF-kB vs TNF-α	****0.683**	**0.000**	****0.692**	**0.001**
NF-kB vs OHDG	***0.442**	**0.030**	**0.457**	**0.037**
NF-kB vs Estrog	0.311	0.139	0.307	0.176
NF-kB vs Progest	-0.224	0.292	-0.223	0.332
IgG vs TNF-α	0.313	0.136	0.329	0.146
IgG vs OHDG	***0.418**	**0.048**	0.430	0.052
IgG vs Estrogen	-0.156	0.466	-0.188	0.415
IgG vs Progest	**-0.410**	**0.047**	**-0.432**	**0.050**
TNF-α vs OHDG	***0.470**	**0.020**	***0.481**	**0.027**
TNF-α vs Estrog	0.121	0.573	0.005	0.983
TNF-α vs Progest	-0.090	0.677	-0.135	0.561
OHDG vs Estrog	0.036	0.869	0.048	0.838
OHDG vs Progest	-0.375	0.071	-0.407	0.067
Estrog vs Progest	0.141	0.512	-0.040	0.864

Key

Presence of MAC=Presence of Microbial antigenic components

****** Srong Positive significant Correlation

***** Moderate Positive significant Correlation

4.10: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Control Subjects without Breast Tumour.

In control subjects, with mixed antigenic components, expression of circulating immune complexes (CIC) showed no association with nuclear factor kappa B, Immunoglobulin G (IgG), tumour necrosis factor-alpha (TNF-α), OH2DG, estrogen and progesterone (Table 4.10).

Expression of NF-kB showed strong positive significant association with serum expression of IgG (r=0.453; P=0.001), strong positive significant association with TNF-alpha (r= 0.501; P= 0.000); strong positive significant association with OH2DG (r= 0.417; P=0.003), but no association with serum estrogen and progesterone (Table 4.10).

Expression of Immunoglobulin G (IgG) showed moderate positive significant relationship with expression of TNF-alpha (r= 0.413; P=0.003), strong positive significant relationship with serum OH2DG (r= 0.668; P = 0.000), but no association with estrogen and progesterone (Table 4.10).

Expression of TNF-alpha showed moderate positive significant relationship with expression of OH2DG (r= 0.402; P = 0.009), but no correlation with estrogen and progesteone (Table 4.10).

Expression of OH2DG showed weak positive relationship with serum estrogen r=0.271, P=0.057 and no relationship with progesteorne (r= -0.064; P= 0.659). Meanwhile expression of serum estrogen showed no relationship with progesterone (r= 0.141; P= 0.328) (Table 4.10).

In control subjects, with evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed strong positive significant association with expression of Immunoglobulin G (IgG) (r= 0.815; P = 0.002), strong positive not significant association with expression of tumour necrosis factor-alpha (TNF-α), (r= 0.456; P = 0.158); strong positive not significant relationship with expression of nuclear factor kappa B (NFkB) (r= 0.481; P = 0.134), but no association with expression of OH2DG, expression of estrogen and progesterone (Table 4.10).

Expression NF-kB showed strong positive not significant association with serum expression of IgG r=0.580, P=0.056, weak positive association with TNF-alpha r= 0.225, P= 0.505; moderate

positive not significant association with OH2DG (r= 0.507; P=0.111), but no association with estrogen (r=0.153; P =0.654) and progesterone (Table 4.10).

Expression of Immunoglobulin G (IgG) showed strong positive significant relationship with serum OH2DG (r= 0.644; P = 0.032), strong positive not significant relationship with expression of TNF-alpha (r= 0.516; P=0.104), but no association with serum estrogen and progesterone (Table 4.10).

Expression of TNF-alpha showed strong positive not significant relationship with expression of OH2DG (r= 0.550; P = 0.080), moderate positive association with estrogen (r= 0.409; P= 0.212) and no relationship with serum progesteone (r= -0.019; P=0.956) (Table 4.10).

Expression of OH2DG showed weak positive relationship with serum estrogen (r=0.240; P=0.478) but no relationship with progesterone (Table 4.10).

In control subjects, without evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed weak positive relationship with expression of nuclear factor kappa B (NFkB) (r= 0.216; P = 0.186), no association with expression Immunoglobulin G, tumour necrosis factor-alpha (TNF-α), expression of OH2DG, expression of estrogen and progesterone (Table 4.10).

Expression NF-kB showed moderate positive significant association with serum expression of IgG (r=0.443; P=0.005), strong positive association with TNF-alpha r= 0.589, P= 0.000; moderate positive significant association with OH2DG (r= 0.400; P=0.012), but no association with estrogen and progesterone (Table 4.10).

Expression of Immunoglobulin G (IgG) showed moderate positive significant relationship with expression of TNF-alpha (r= 0.409; P=0.010), strong positive significant relationship with serum OH2DG (r= 0.679; P = 0.000), but no association with serum estrogen and progesterone (Table 4.10).

Expression of TNF-alpha showed moderate positive significant relationship with expression of OH2DG (r= 0.355; P = 0.026), but no association with estrogen and progesteone levels (Table 4.10).

Expression of OH2DG showed weak positive relationship with serum estrogen (r=0.282; P=0.082) but no association with progesterone. Meanwhile expression of serum estrogen showed no relationship with progesterone (Table 4.10).

Table 4.10: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Control Subjects.

Variables	Combined N=50		Presence of MAC N=11		Absence of MAC N=39	
	(r)	**P-value**	**(r)**	**P-value**	**(r)**	**P-value**
CIC vs NF-kB	0.177	0.218	0.481	0.134	0.216	0.186
CIC vs IgG	0.123	0.393	****0.815**	**0.002**	-0.063	0.704
CIC vs TNF-α	0.072	0.617	0.456	0.158	-0.071	0.669
CIC vs OHDG	0.082	0.547	0.074	0.558	-0.026	0.874
CIC vs Estrogen	0.000	0.999	0.080	0.815	-0.049	0.767
CIC vs Progest	0.082	0.570	-0.101	0.767	0.129	0.433
NF-kB vs IgG	***0.453**	**0.001**	0.580	0.056	***0.443**	**0.005**
NF-kB vs TNF-α	****0.501**	**0.000**	0.225	0.505	****0.589**	**0.000**
NF-kB vs OHDG	**0.417**	**0.003**	0.507	0.111	***0.400**	**0.012**
NF-kB vs Estrog	0.076	0.600	0.153	0.654	0.073	0.660
NF-kB vs Progest	-0.129	0.370	0.193	0.569	-0.174	0.290
IgG vs TNF-α	***0.413**	**0.003**	0.516	0.104	***0.409**	**0.010**
IgG vs OHDG	****0.668**	**0.000**	****0.644**	**0.032**	****0.679**	**0.000**
IgG vs Estrogen	-0.056	0.701	0.081	0.814	-0.077	0.643
IgG vs Progest	0.022	0.879	0.142	0.678	0.001	0.996
TNF-α vs OHDG	***0.402**	**0.004**	0.550	0.080	***0.355**	**0.026**
TNF-α vs Estrog	0.118	0.414	0.409	0.212	0.046	0.782
TNF-α vs Progest	-0.051	0.724	-0.019	0.956	-0.058	0.728
OHDG vs Estrog	0.271	0.057	0.240	0.478	0.282	0.082
OHDG vs Progest	-0.064	0.659	0.139	0.684	-0.104	0.530
Estrog vs Progest	0.141	0.328	-0.045	0.895	0.173	0.292

Key

Presence of MAC=Presence of Microbial antigenic components

Absence of MAC=Absence of Microbial antigenic components

****** Srong Positive significant Correlation

***** Moderate Positive significant Correlation

4.11: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant Subjects with DNA Methylation Shift and Combined Antigenic Components

In malignant subjects with unmethylated DNA and evidence of mixed antigenic components, expression of circulating immune complexes (CIC) showed strong negative significant relationship with expression of OH2DG (r= -0.571; P = 0.048); but showed no significant positive or negative correlation with expression of tumour necrosis factor-alpha (TNF-α), nuclear factor kappa B (NFkB), Immunoglobulin G (IgG), estrogen and progesterone (Table 4.11).

Expression of nuclear factor kappa B (NFkB) showed strong positive significant association with TNF-alpha (r= 0.571; P= 0.048), but no significant positive or negative association with Immunoglobulin G (IgG), OH2DG, estrogen and progesterone (Table 4.11).

Expression of Immunoglobulin G (IgG) showed no relationship with expression of TNF-alpha, no relationship with expression OH2DG, estrogen and progesterone (Table 4.11).

Expression of TNF-alpha showed no significant positive or negative correlation with expression OH2DG, estrogen and progesterone (Table 4.11).

Expression of OH2DG showed no significant positive or negative association with estrogen and progesterone expression. Estrogen expression showed moderate not sgnificant positive correlation with progesterone (r= 0.371; P=0.209) (Table 4.11).

In malignant subjects with hypomethylated DNA and evidence of mixed antigenic components, expression of circulating immune complexes (CIC) showed no significant positive or negative relationship with nuclear factor kappa B (NFkB) transcription, Immunoglobulin G (IgG), tumour necrosis factor-alpha (TNF-α), OH2DG, progesterone (Table 4.11).

Expression of nuclear factor kappa B (NFkB) showed no significant positive or negative relationship with IgG, TNF-alpha, OH2DG, estrogen and progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative association with OH2DG, estrogen and progesterone. Expression of TNF-alpha showed no significant positive or negative correlation with OH2DG, estrogen and progesterone (Table 4.11).

Expression of OH2DG showed moderate positive significant association with serum progesterone (r= 0.457; P=0.044), but no significant positive or negative with estrogen. Expression of estrogen showed no correlation with progesterone (Table 4.11).

In malignant subjects with hypermethylated DNA and evidence of mixed antigenic components, expression of circulating immune complexes (CIC) showed no significant positive or negative correlation with nuclear factor kappa B (NFkB) transcription, Immunoglobulin G (IgG), tumour necrosis factor-alpha (TNF-α) OH2DG, estrogen and progesterone.

Expression of nuclear factor kappa B (NFkB) showed no significant positive or negative relationship with IgG (r=0.000; P=1.000), TNF-alpha, expression of OH2DG, estrogen and progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, OH2DG (r= -0.333; P = 0.479), estrogen and progesterone.

Expression of TNF-alpha showed no significant positive or negative correlation with expression of OH2DG, serum estrogen and progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and progesterone. Estrogen expression showed no significant positive or negative correlation with progesterone (Table 4.11).

Table 4.11: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant Subjects with DNA Methylation Shift and Combined Antigenic Components.

	Unmethylated DNA N=8		Hypomethylated N=12		Hypermethylated N=4	
Variables	**(r)**	**P-value**	**(r)**	**P-value**	**(r)**	**P-value**
CIC vs meth patts	-0.515	0.191	0.334	0.289	-0.775	0.225
CIC vs NF-kB	-0.214	0.458	**-0.455**	**0.040**	-0.333	0.497
CIC vs IgG	-0.357	0.216	0.303	0.170	-0.667	0.174
CIC vs TNF-α	-0.500	0.083	-0.198	0.372	0.000	1.000
CIC vs OHDG	**-0.571**	**0.048**	-0.152	0.493	0.000	1.000
CIC vs Estrogen	0.286	0.322	0.030	0.891	-0.333	0.497
CIC vs Progest	0.371	0.209	0.110	0.627	0.548	0.279
NF-kB vs met patt	0.638	0.089	-0.195	0.544	**0.994**	**0.056**
NF-kB vs IgG	0.143	0.621	-0.364	0.100	0.000	1.000
NF-kB vs TNF-α	**0.571**	**0.048**	-0.046	0.837	0.667	0.174
NF-kB vs OHDG	0.071	0.805	0.273	0.217	0.667	0.174
NF-kB vs Estrog	-0.214	0.458	0.212	0.337	0.333	0.497
NF-kB vs Progest	-0.148	0.615	0.047	0.835	-0.548	0.279
IgG vs met patts	0.591	0.123	0.100	0.758	0.887	0.113
IgG vs TNF-α	0.143	0.621	-0.046	0.837	0.333	0.497
IgG vs OHDG	0.071	0.805	0.121	0.583	-0.333	0.497
IgG vs Estrogen	0.214	0.458	0.061	0.784	0.667	0.174
IgG vs Progest	-0.148	0.615	0.299	0.187	-0.183	0.718
TNF-α vs met pats	0,269	0.520	0.165	0.608	0.540	0.460
TNF-α vs OHDG	0.500	0.083	-0.198	0.372	0.333	0.497
TNF-α vs Estrog	-0.214	0.458	-0.168	0.450	0.667	0.174
TNF-α vs Progest	-0.148	0.615	-0.032	0.889	-0.183	0.718
OHDG vs mpatts	-0.046	0.894	-0.037	0.908	-0.004	0.996
OHDG vs Estrog	-0.286	0.322	0.152	0.493	0.000	1.000
OHDG vs Progest	-0.222	0.451	**0.457**	**0.044**	-0.183	0.718
Prog vs Metpatts	-0872	0.054	0.143	0.657	-0628	0.372
Estrog vs Progest	0.371	0.209	0.047	0.835	0.183	0.718

Key: Met patt= methylation pattern

4.12: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant Subjects with DNA Methylation Shift and Evidence of Microbial Antigenic Components

In malignant subjects with unmethylated DNA and evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed strong positive significant association with serum estrogen (r= 0.937, P= 0.019), but no significant positive or negative association with nuclear factor kappa B (NFkB), Immunoglobulin G (IgG), tumour necrosis factor-alpha (TNF-α), OH2DG, and serum progesterone.

Expression of nuclear factor kappa B (NFkB) showed no significant positive or negative relationship with IgG, TNF-alpha, OH2DG, estrogen expression and progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, expression OH2DG, estrogen and progesterone.

Expression of TNF-alpha showed no significant positive or negative correlation with expression of OH2DG, estrogen and progesterone.

Expression of OH2DG showed no significant positive or negative association estrogen and serum progesterone. Estrogen expression showed moderate sgnificant positive correlation with progesterone (r= 0.537, P=0.042) (Table 4.12).

In malignant subjects with hypomethylated DNA and evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed strong negative significant relationship with nuclear factor kappa B (NFkB) (r= =-0.527; P = 0.024), but no significant positive or negative weak positive relationship with Immunoglobulin G (IgG) tumour necrosis factor-alpha (TNF-α), OH2DG, estrogen and serum progesterone.

Expression of nuclear factor kappa B (NFkB) showed no significant positive or negative relationship with IgG, TNF-alpha, OH2DG, estrogen expression and progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, expression OH2DG, estrogen and progesterone.

Expression of TNF-alpha showed no significant positive or negative correlation with expression of OH2DG, estrogen and progesterone.

Expression of OH2DG showed strong positive significant association with progesterone (r= 0.510; P=0.033), but no significant positive or negative association with serum estrogen. Estrogen expression showed no correlation with progesterone (Table 4.12).

In malignant subjects with hypermethylated DNA and evidence of microbial antigenic components, expression of circulating immune complexes (CIC), nuclear factor kappa B (NFkB), Immunoglobulin G (IgG), tumour necrosis factor-alpha (TNF-α), OH2DG, estrogen and progesterone showed no correlation (Table 4.12).

Table 4.12: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant Subjects with DNA Methylation Shift and Evidence of Microbial Antigenic Components.

	Unmethylated DNA N=5		Hypomethylated N=11		Hypermethylated N=3	
Variables	**(r)**	**P-value**	**(r)**	**P-value**	**(r)**	**P-value**
CIC vs Met patts	0.849	0.069	0.374	0.257	-0.737	0.472
CIC vs NF-kB	0.010	0.987	**-0.527**	**0.024**	-0.548	0.631
CIC vs IgG	0.142	0.819	0.200	0.392	-0.789	0.422
CIC vs TNF-α	-0.785	0.116	-0.110	0.639	0.241	0.845
CIC vs OHDG	-0.842	0.074	0.200	0.392	0.881	0.313
CIC vs Estrogen	**0.937**	**0.019**	-0.091	0.697	-0.224	0.856
CIC vs Progest	0.777	0.122	-0.019	0.937	0.973	0.147
NF-kB vs IgG	-0.868	0.056	-0.382	0.102	0.946	0.209
NF-kB vs TNF-α	0.588	0.297	-0.147	0.532	0.680	0.524
NF-kB vs OHDG	0.219	0.723	0.236	0.312	0.087	0.944
NF-kB vs Estrog	0.120	0.848	0.200	0.392	0.225	0.938
NF-kB vs Progest	0.468	0.427	0.057	0.813	-0.342	0.778
IgG vs Met patts	0.088	0.888	0.077	0.822	**0.997**	**0.051**
IgG vs TNF-α	-0.704	0.184	0.037	0.876	0.407	0.733
IgG vs OHDG	-0.409	0.494	0.164	0.484	-0.404	0.735
IgG vs Estrogen	0.094	0.881	-0.018	0.938	0.776	0.434
IgG vs Progest	0.091	0.884	0.245	0.305	-0.627	0.569
TNF-α vs OHDG	0.798	0.105	-0.220	0.349	0.671	0.532
TNF-α vs Estrog	-0.758	0.137	-0.147	0.523	0.892	0.299
TNF-α vs Progest	-0.306	0.509	0.038	0.874	0.457	0.698
OHDG vs Estrog	-0.777	0.122	0.091	0.697	0.263	0.831
OHDG vs Progest	0.672	0.214	**0.510**	**0.033**	0.966	0.166
Estrog ve Metpatt	**0.874**	**0.053**	0.009	0.978	0.824	0.384
Estrog vs Progest	**0.537**	**0.042**	-0.019	0.937	0.005	0.997

Key: Met patt= methylation pattern

4:13: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Benign Breast Tumour Subjects with DNA Methylation Shift and Combined Antigenic Components

In benign breast tumour subjects with unmethylated DNA and mixed antigenic components, expression of circulating immune complexes (CIC) showed strong positive significant relationship with Immunoglobulin G (IgG) (r=0.667; P = 0.012), no significant positive or negative correlation with nuclear factor kappa B (NFkB), tumour necrosis factor-alpha (TNF-α) expression of OH2DG (r= 0.171; P = 0.527), serum oestrogen and progesterone.

Expression of nuclear factor kappa B (NFkB) showed strong positive significant relationship with IgG r=0.648, P=0.016, strong positive significant relationship with OH2DG (r=0.551; P=0.044), strong negative significant relationship with progesterone (r= -0.771 P=0.004), but no association with TNF-alpha and estrogen.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, expression OH2DG, weak positive relationship with estrogen and progesterone.

Expression of TNF-alpha showed no significant positive or negative correlation with expression OH2DG, oestrogen and progesterone.

Expression of OH2DG showed strong negative significant association with serum progesterone expression (r= -0.725; P=0.008). No significant positive or negative association with serum estrogen (Table 4.13).

In benign breast tumour subjects with hypomethylated DNA and mixed antigenic components, expression of circulating immune complexes (CIC) showed no significant positive or negative relationship with nuclear factor kappa B (NFkB), expression of Immunoglobulin G (IgG), tumour necrosis factor-alpha (TNF-α), OH2DG, estrogen and progesterone.

Expression of nuclear factor kappa B (NFkB) showed strong positive significant relationship with serum estrogen (r= 0.714; P = 0.024), but no significant positive or negative relationship with IgG, TNF-alpha, OH2DG, and progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, OH2DG, estrogen and progesterone.

Expression of TNF-alpha showed no significant positive or negative correlation with expression of OH2DG; estrogen and serum progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and serum progesterone (Table 4.13).

Table 4.13: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Benign Breast Tumour Subjects with DNA Methylation Shift and Combined Antigenic Components.

	Unmethylated DNA N=9		Hypomethylated N=7	
Variables	**(r)**	**P-value**	**(r)**	**P-value**
CIC vs NF-kB	0.366	0.173	-0.048	0.881
CIC vs IgG	**0.667**	**0.012**	0.048	0.881
CIC vs TNF-α	-0.111	0.677	0.333	0.293
CIC vs OHDG	0.171	0.527	0.000	1.000
CIC vs Estrogen	0.056	0.835	-0.143	0.652
CIC vs Progest	-0.254	0.345	0.411	0.210
NF-kB vs IgG	**0.648**	**0.016**	-0.048	0.881
NF-kB vs TNF-α	-0.141	0.600	0.619	0.051
NF-kB vs OHDG	**0.551**	**0.044**	0.195	0.543
NF-kB vs Estrog	0.254	0.345	**0.714**	**0.024**
NF-kB vs Progest	**-0.771**	**0.004**	0.309	0.347
IgG vs TNF-α	-0.111	0.677	0.333	0.293
IgG vs OHDG	0.286	0.292	0.195	0.543
IgG vs Estrogen	0.278	0.297	-0.333	0.293
IgG vs Progest	-0.423	0.116	0.103	0.754
TNF-α vs OHDG	-0.057	0.833	0.390	0.224
TNF-α vs Estrog	0.056	0.835	0.333	0.293
TNF-α vs Progest	0.141	0.600	0.309	0.347
OHDG vs Mpatts	-0.363	0.337	**-0.861**	**0.013**
OHDG vs Estrog	0.229	0.399	0.283	0.362
OHDG vs Progest	**-0.725**	**0.008**	0.264	0.428
Estrog vs Progest	-0.254	0.345	0.309	0.347

4:14: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Benign Breast Tumour Subjects with DNA Methylation Shift and Evidence of Microbial Antigenic Components

In benign breast tumour subjects with unmethylated DNA and evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed strong positive significant relationship with nuclear factor kappa B (NFkB) (r= 0.837; P = 0.004); strong positive significant association with Immunoglobulin G (IgG) (r=0.909; P = 0.002), strong negative significant relationship with serum progesterone (r= -0.593; P=0.044), but no significant positive or negative relationship with expression of tumour necrosis factor-alpha (TNF-α), expression of OH2DG and serum oestrogen.

Expression of nuclear factor kappa B (NFkB) showed strong positive significant relationship with IgG (r=0.857; P=0.003), strong negative significant relationship with progesterone (r= -0.764; P=0.009), but no significant positive or negative association with TNF-alpha, OH2DG and oestrogen.

Expression of Immunoglobulin G (IgG) showed strong negative significant relationship with progesterone (r= -0.618; P= 0.034), but no significant positive or negative relationship with expression of TNF-alpha, expression OH2DG and oestrogen.

Expression of TNF-alpha showed no significant positive or negative correlation with expression of OH2DG, serum oestrogen and progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and progesterone (Table 4.14).

In benign breast tumour subjects with hypomethylated DNA and evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed no significant positive or negative relationship with nuclear factor kappa B (NFkB), Immunoglobulin G (IgG), tumour necrosis factor-alpha (TNF-α), expression of OH2DG, oestrogen and progesterone.

Expression of nuclear factor kappa B (NFkB) showed strong positive significant relationship with serum estrogen (r= 0.800; P = 0.050), but no significant positive or negative relationship with IgG, TNF-alpha, OH2DG and progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, expression OH2DG, oestrogen and progesterone.

Expression of TNF-alpha showed no significant positive or negative correlation with expression of OH2DG, serum oestrogen and serum progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and progesterone. Estrogen expression showed strong positive significant correlation with progesterone (r= 0.800; P=0.050) (Table 4.14).

In benign breast tumour subjects with normal methylated DNA and evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed no significant positive or negative relationship with nuclear factor kappa B (NFkB), Immunoglobulin G (IgG), tumour necrosis factor-alpha (TNF-α), expression of OH2DG, serum estrogen and progesterone.

Expression of nuclear factor kappa B (NFkB) showed strong positive significant association with TNF-alpha (r= 0.938; P= 0.006) and expression of OH2DG (r=0.812; P=0.050), but no significant positive or negative relationship with IgG, estrogen and progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, expression OH2DG, estrogen (r=0.648; P=0.164) and progesterone.

Expression of TNF-alpha showed strong positive significant correlation with expression and OH2DG (r= 0.884; P = 0.019), but no significant positive or negative association with serum estrogen and progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and progesterone (Table 4.14).

4:14: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Benign Breast Tumour Subjects with DNA Methylation Shift and Evidence of Antigenic Components

	Unmethylated DNA N=8		Hypomethylated N=5		Normal Meth N=6	
Variables	**(r)**	**P-value**	**(r)**	**P-value**	**(r)**	**P-value**
CIC vs NF-kB	**0.837**	**0.004**	0.000	1.000	-0.027	0.960
CIC vs IgG	**0.909**	**0.002**	0.400	0.327	-0.367	0.474
CIC vs TNF-α	-0.255	0.383	0.600	0.124	-0.048	0.927
CIC vs OHDG	0.182	0.533	0.105	0.801	-0.155	0.769
CIC vs Estrogen	0.109	0.708	-0.200	0.624	-0.642	0.169
CIC vs Progest	**-0.593**	**0.044**	0.000	1.000	-0.203	0.700
NF-kB vs IgG	****0.857**	**0.003**	-0.200	0.624	0.215	0.682
NF-kB vs TNF-α	0.286	0.322	0.400	0.327	****0.938**	**0.006**
NF-kB vs OHDG	0.357	0.216	0.105	0.801	****0.812**	**0.05**
NF-kB vs Estrog	0.071	0.805	****0.800**	**0.050**	0.482	0.333
NF-kB vs Progest	**-0.764**	**0.009**	0.600	0.142	-0.098	0.854
IgG vs Mpatts	**-0.790**	**0.020**	-0.295	0.630	-0.403	0.428
IgG vs TNF-α	0.286	0.322	0.400	0.327	-0.117	0.826
IgG vs OHDG	0.214	0.458	0.316	0.448	-0.435	0.389
IgG vs Estrogen	0.214	0.458	-0.400	0.327	0.648	0.164
IgG vs Progest	**-0.618**	**0.034**	-0.200	0.624	0.581	0.226
TNF-α vs OHDG	0.071	0.805	0.316	0.448	****0.884**	**0.019**
TNF-α vs Estrog	-0.071	0.805	0.200	0.624	0.448	0.373
TNF-α vs Progest	0.182	0.533	0.400	0.327	0.019	0.972
OHDG vs Mpatts	-0.590	0.124	**-0.939**	**0.018**	0.512	0.299
OHDG vs Estrog	0.143	0.621	0.316	0.448	0.195	0.711
OHDG vs Progest	-0.400	0.170	0.527	0.207	-0.082	0.878
Estrog vs Progest	0.327	0.262	****0.800**	**0.050**	0.397	0.436

**** Srong Positive significant Correlation

4:15: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Control Subjects with DNA Methylation Shift and Combined Antigenic Components

In control subjects with normal methylated DNA and mixed antigenic components, expression of circulating immune complexes (CIC) showed strong positive significant relationship with serum progesterone (r= 1.000; P=0.000), but no significant positive or negative relationship with nuclear factor kappa B (NFkB), expression of Immunoglobulin G (IgG), expression of tumour necrosis factor-alpha (TNF-α), expression of OH2DG and serum oestrogen..

Expression of nuclear factor kappa B (NFkB) showed strong positive significant association with IgG (r=0.590; P=0.000), srong positive significant association with TNF-alpha (r= 0.466; P= 0.002), strong positive significant relationship with OH2DG (r=0.500; P=0.001), but no significant positive or negative relationship with serum oestrogen and progesterone.

Expression of Immunoglobulin G (IgG) showed moderate positive significant relationship with expression of TNF-alpha (r= 0.429; P = 0.006), strong positive significant relationship with expression OH2DG (r=0.829; P = 0.000), strong negative significant relationship with progesterone (r= 0.091; P= 0.577), but no significant positive or negative association with serum oestrogen.

Expression of TNF-alpha showed moderate significant correlation with expression and OH2DG (r= 0.391; P = 0.012); but no significant positive or negative relationship with serum oestrogen and progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and progesterone (Table 4.15).

In control subjects with unmethylated DNA and mixed antigenic components, expression of circulating immune complexes (CIC) showed strong negative significant relationship with expression of OH2DG (r= -0.800; P = 0.050), but no significant positive or negative relationship with nuclear factor kappa B (NFkB), expression of Immunoglobulin G (IgG), expression of tumour necrosis factor-alpha (TNF-α), oestrogen and progesterone.

Expression of nuclear factor kappa B (NFkB) showed no significant positive or negative association with IgG, TNF-alpha, OH2DG, serum estrogen and progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, OH2DG, estrogen (r= -0.400; P=0.327) and progesterone.

Expression of TNF-alpha showed no significant positive or negative relationship with expression of OH2DG serum estrogen and progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and progesterone (r= 0.738; P=0.077) (Table 4.15).

Table 4:15: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Control Subjects with DNA Methylation Shift and Combined Antigenic Components

	Noraml meth N=40		Unmethylated N=5	
Variables	**(r)**	**P-value**	**(r)**	**P-value**
CIC vs NF-kB	0.139	0.391	0.000	1.000
CIC vs IgG	-0.091	0.577	-0.200	0.624
CIC vs TNF-α	-0.098	0.546	0.105	0.801
CIC vs OHDG	0.024	0.883	**-0.800**	**0.05**
CIC vs Estrogen	0.213	0.188	-0.400	0.327
CIC vs Progest	**1.000**	**0.000**	0.527	0.207
NF-kB vs IgG	****0.590**	**0.000**	-0.400	0.327
NF-kB vs TNF-α	***0.466**	**0.002**	0.527	0.207
NF-kB vs OHDG	****0.500**	**0.001**	0.200	0.624
NF-kB vs Estrog	0.084	0.608	0.200	0.624
NF-kB vs Progest	-0.139	0.391	-0.316	0.448
IgG vs TNF-α	***0.429**	**0.006**	0.105	0.801
IgG vs OHDG	****0.829**	**0.000**	0.000	1.000
IgG vs Estrogen	-0.028	0.862	-0.400	0.327
IgG vs Progest	0.091	0.577	0.316	0.448
TNF-α vs OHDG	***!0.391**	**0.012**	0.105	0.801
TNF-α vs Estrog	0.097	0.552	0.105	0.801
TNF-α vs Progest	-0.098	0.546	-0.333	0.435
OHDG vs Estrog	0.222	0.169	0.600	0.124
OHDG vs Progest	-0.024	0.883	0.738	0.077
Estrog vs Progest	0.213	0.188	-0.316	0.448

****** Srong Positive significant Correlation

***** Moderate Positive significant Correlation

***!** Weak Positive significant Correlation

4.16: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Malignant Subjects in Control Subjects with Normal Methylated DNA and Evidence of Microbial Antigenic Components

In control subjects with normal methylated DNA and evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed strong positive significant relationship with expression of Immunoglobulin G (IgG) ($r= 0.890$; $P = 0.001$), but no significant positive or negative relationship with nuclear factor kappa B (NFkB), tumour necrosis factor-alpha (TNF-α), ($r= 0.542$; $P = 0.131$), expression of OH2DG, serum estrogen and progesterone.

Expression of nuclear factor kappa B (NFkB) showed no significant positive or negative association with, TNF-alpha, OH2DG, serum estrogen and serum progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, OH2DG, estrogen and progesterone.

Expression of TNF-alpha showed no significant positive or negative relationship with expression of OH2DG, estrogen and progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and progesterone (Table 4.28).

Table 4:16: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Control Subjects with Normal Methylated DNA and Evidence of Microbial Antigenic Components.

	Noraml meth N=9	
Variables	**(r)**	**P-value**
CIC vs NF-kB	0.530	0.142
CIC vs IgG	**0.890**	**0.001**
CIC vs TNF-α	0.542	0.131
CIC vs OHDG	0.613	0.080
CIC vs Estrogen	0.065	0.868
CIC vs Progest	-0.269	0.484
NF-kB vs IgG	0.484	0.187
NF-kB vs TNF-α	-0.050	0.899
NF-kB vs OHDG	0.476	0.196
NF-kB vs Estrog	0.020	0.959
NF-kB vs Progest	-0.008	0.983
IgG vs TNF-α	0.378	0.316
IgG vs OHDG	0.638	0.065
IgG vs Estrogen	-0.047	0.903
IgG vs Progest	-0.068	0.863
TNF-α vs OHDG	0.520	0.151
TNF-α vs Estrog	0.320	0.402
TNF-α vs Progest	-0.314	0.411
OHDG vs Estrog	0.195	0.615
OHDG vs Progest	0.147	0.706
Estrog vs Progest	-0.220	0.569

4.17: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Control Subjects with DNA Methylation Shift, without Evidence of Microbial Antigenic Components

In control subjects with normal methylated DNA and without evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed no significant positive or negative with nuclear factor kappa B (NFkB), Immunoglobulin G (IgG), expression of tumour necrosis factor-alpha (TNF-α), expression of OH2DG, estrogen and progesterone.

Expression of nuclear factor kappa B (NFkB) showed strong positive significant association with IgG (r= 0.428; P=0.001), TNF-alpha (r= 0.472; P= 0.000), OH2DG (r=0.409; P=0.001), but no significant positive or negative relationship with serum estrogen and progesterone.

Expression of Immunoglobulin G (IgG) showed moderate positive significant relationship with expression of TNF-alpha (r= 0.378; P = 0.003), strong positive significant relationship with expression OH2DG (r=0.708, P = 0.000); but no significant positive or negative association with serum estrogen and serum progesterone.

Expression of TNF-alpha showed weak positive significant association with expression of OH2DG (r= 0.279; P = 0.030); but no significant positive or negative relationship serum estrogen and serum progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and progesterone. Estrogen expression showed weak negative correlation with progesterone (r= 0.251; P=0.052) (Table 4.17).

In control subjects with unmethylated DNA and without evidence of microbial antigenic components, expression of circulating immune complexes (CIC) showed no significant positive or negative association with expression of nuclear factor kappa B (NFkB), expression of Immunoglobulin G (IgG), expression of tumour necrosis factor-alpha (TNF-α), expression of OH2DG, serum estrogen and progesterone.

Expression of nuclear factor kappa B (NFkB) showed no significant positive or negative association with, TNF-alpha, OH2DG, estrogen and progesterone.

Expression of Immunoglobulin G (IgG) showed no significant positive or negative relationship with expression of TNF-alpha, expression OH2DG, serum estrogen and progesterone.

Expression of TNF-alpha showed no significant positive or negative association with expression of OH2DG, serum estrogen and progesterone.

Expression of OH2DG showed no significant positive or negative association with serum estrogen and progesterone. Estrogen expression showed strong negative significant correlation with progesterone (r= -0.800; P=0.050) (Table 4.17).

4.17: Correlation of Circulating Immune Complexes, Pro-inflammatory Molecules, Oxidative Marker and Female Sex Hormones Expressed in Control Subjects with DNA Methylation Shift, without Evidence of Microbial Antigenic Components.

	Noraml meth N=31		Hypomethylated N=5	
Variables	**(r)**	**P-value**	**(r)**	**P-value**
CIC vs NF-kB	0.017	0.892	-0.537	0.207
CIC vs IgG	-0.192	0.130	-0.200	0.624
CIC vs TNF-α	-0.147	0.253	0.105	0.801
CIC vs OHDG	-0.111	0.385	0.600	0.142
CIC vs Estrogen	0.073	0.563	-0.600	0.142
CIC vs Progest	0.227	0.079	0.400	0.327
NF-kB vs IgG	**0.428**	**0.001**	**0.738**	**0.077**
NF-kB vs TNF-α	**0.474**	**0.000**	0.000	1.000
NF-kB vs OHDG	**0.409**	**0.001**	-0.527	0.207
NF-kB vs Estrog	-0.024	0.852	0.105	0.801
NF-kB vs Progest	-0.113	0.384	0.105	0.801
IgG vs TNF-α	**0.378**	**0.003**	0.316	0.448
IgG vs OHDG	**0.708**	**0.000**	-0.200	0.624
IgG vs Estrogen	-0.095	0.454	-0.200	0.624
IgG vs Progest	-0.090	0.484	0.400	0.327
TNF-α vs OHDG	**0.279**	**0.030**	0.527	0.207
TNF-α vs Estrog	-0.013	0.919	-0.527	0.207
TNF-α vs Progest	-0.063	0.632	-0.316	0.448
OHDG vs Estrog	0.004	0.973	0.200	0.624
OHDG vs Progest	-0.055	0.659	0.000	1.000
Estrog vs Progest	0.251	0.052	**-0.800**	**0.05**

4.18: Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Benign, Malignant Breast Tumour and Control Subjects.

Based on the analysis of antigenic components of circulating immune complexes, the statistics showed that distribution of malaria parasites (*Plasmodium falciparum*) in subjects with benign breast tumour 11(45.8%); subjects with malignant breast tumour 10(40%); control subjects 12(24%) was not significantly different (P=0.913). Distribution of Hepatitis C virus (HCV) in subjects with benign breast tumour 10(41.7%); subjects with malignant breast tumour 7(28%); control 1(2%) was significantly different (P=0.030). The frequency distribution of *Helicobacter pylori* in subjects with benign breast tumour 8(33.3%); subjects with malignant breast tumour 5(20%); control 0(0%), was not significantly different P=0.405. The frequency distribution of *Salmonella typhi* in subjects with benign breast tumour 5(20.8%); subjects with malignant breast tumour 8(32%) and control subjects 0(0%), was not significantly different (P=0.405). The frequency distribution of *Hepatitis B virus* in subjects with benign breast tumour 2(8.3%); subjects with malignant breast tumour 2(8%) and control subjects 0(0%) was not significantly different (P=0.1000). The frequency distribution of *Treponema pallidum* in subjects with benign breast tumour 1(4.2%); subjects with malignant breast tumour 2(8%) and control subjects 0(0%) was not significantly different (P=0.564). Only one subject with benign tumour was found to harbour *Mycobacterium tuberculosis* (Table 4.19).

Table 4.18. Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Benign, Malignant Breast Tumour and Apparently Healthy Subjects.

	Benign N=24	**Malignant N=25**	**Control N=50**	**P-value**
MP (Pf)	**8//*3 11(45.8%)	**9//*1 10(40%)	*11//**1 12(24%)	0.913
HCV	**8 // *2 10(41.6%)	**5 // *2 7(28%)	**1 1(2%)	0.030
H.pylori	**5 // *3 8(33.3%)	**3 // *2 5(20%)	0(0%)	0.405
Samonella typhi	**3 // *2 5(20.8%)	**7 // *1 8(32%)	0(0%)	0.405
HBV	**2 2(8.3%)	**2 2(8%)	0(0%)	0.1000
T.pallidum	*1 1(4.2%)	*2 2(8%)	0(0%)	0.564
Mtb	*1 1(4.2%)	0(0%)	0(0%)	1.000

KEY:

**Number of individuals with heterogeneity of microbial antigenic components

*Number of individual with homogeneity of microbial antigenic components

MP(Pf)= Malaria parasite (*Plasmodium falciparum*); T.pallidum= *Treponema pallidum*; TB(Mtb)=Tuberculosis (*Mycobacterium tuberculosis*); HBV= Hepatitis B virus; HCV= Hepatitis C virus; H.pylori= *Helicobacter pylori*

4.19: Percentage Distribution of Different Types of Microbial Antigenic Components of Circulating Immune Complexes in Various Stages of Malignant Breast Tumour

Based on the analysis of antigenic components of circulating immune complexes, the result showed that in stages 3b, and 3c, Malaria parasites (*Plasmodium falciparum*) was detected in 5 (20.0%) and 5 (20.0%) respectively, but was not detected in subjects in stages 2b and 4. *Treponema pallidum* was detected in 1 (4.0%) of the 5 subjects in stage 2b and 1 (4.0%) 3c respectively, but was not detected in stages 3b and 4. *Hepatitis B. Virus* was detected in 1 (4.0%) of the 9 subjects in stage 3b, 1 (4.0%) of the 7 subjects in stage 3c, but was not detected in stages 2b and 4. *Hepatitis C. Virus* was detected in 3 (12.0%) of the 9 subjects in stage 3b, 2 (8.0%) of the 7 subjects in stage 3c and 2 (8.0%) of the 4 subjects in stage 4, but was not found in stage 2b *Helicobacter pylori* was detected in 1 (4.0%) of the 5 subjects in stage 2b, 1 (4.0%) of the 9 subjects in stage 3b, 2 (8.0%) of the 7 subjects in stage 3c and 1 (4.0%) of the 4 subjects in stage 4. *Mycobacterium tuberculosis* was not detected in any of the stages. *Salmonella typhi* was detected in 1 (4.0%) of the 5 subjects in stage 2b, 1 (4.0%) of the 9 subjects in stage 3b, 4 (16.0%) of the 7 subjects in stage 3c and 2 (8.0%) of the 4 subjects in stage 4.

Table 4.19: Percentage Distribution of Different Types of Microbial Antigenic Components of Circulating Immune Complexes in Various Stages of Malignant Breast Tumour

	Stage 2b N=5	**Stage 3b N=9**	**Stage 3c N=7**	**Stage 4 N=4**
MP (Pf)	0(0%)	*1//**4 5 (20.0%)	**5 5 (20.0%)	0(0%)
T. pallidum	*1 1 (4.0%)	0(0%)	*1 1 (4.0%)	0(0%)
HBV	(()%)	**1 1 (4.0%)	**1 1 (4.0%)	0(0%)
HCV	0(0%)	*1//**2 3(12.0%)	*1//**1 2(8.0%)	**2 2 (8.0%)
H.pylori	*1 1 (4.0%)	**1 1 (4.0%)	*1**1 2 (8.0%)	**1 1 (4.0%)
TB	0(0%)	0(0%)	0(0%)	0(0%)
Samonella typhi	*1 1(4.0%)	**1 1 (4.0%)	**4 4 (16.0%)	**2 2(4.0%)

KEY:

**Number of individuals with heterogeneity of microbial antigenic components

*Number of individual with homogeneity of microbial antigenic components

MP(Pf)= Malaria parasite (*Plasmodium falciparum*); T.pallidum= *Treponema pallidum*; TB(Mt)=Tuberculosis (*Mycobacterium tuberculosis*); HBV= Hepatitis B virus; HCV= Hepatitis C virus; H.pylori= *Helicobacter pylori*

4.20: Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Subjects with Benign, Malignant Tumours and Apparently Healthy Subjects, with Hypomethylated DNA.

Based on the analysis of antigenic components of circulating immune complexes, the occurance of malaria parasites (*Plasmodium falciparum*) in hypomethylated subjects with benign breast tumour 3(42.9%); hypomethylated subjects with malignant breast tumour 7(58.3%) and hypomethylated control subjects 0(0%) was not significantly different (P=0.096). Also the occurance of *Hepatitis C virus* (HCV) in hypomethylated subjects with benign breast tumour 3(42.9%); malignant breast tumour subjects 3(25%) and hypomethylated control subjects 0(0%) was not significantly different (P=0.705). The occurance of *Salmonella typhi* in hypomethylated subjects with benign breast tumour 2(28.6%); malignant breast tumour subjects 6(50%); was not significantly different (P=0.157). The occurance of *Hepatitis B virus* (HBV) in hypomethylated subjects with benign breast tumour 2(28.6%); hypomethylated subjects with malignant breast tumour 2(16.7%); was not significantly different (P=0.1000). *Helicobacter pylori* was detected only in 2(16.7%) hypomethylated subjects with malignant breast tumour. *Treponema pallidum* and *Mycobacterium tuberculosis* were not detected in subjects with hypomethylated DNA (Table 4.20).

Table 4.20. Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Benign, Malignant Tumour and Apparently Healthy Subjects, with Hypomethylated DNA

	Benign N=7	**Malignant N=12**	**Control N=5**	**P-value**
MP (Pf)	** 1 // *1 2(28.6%)	**6 // *1 7 (58.3%)	0(0%)	0.096
HCV	**2 // *1 3(42.9%)	**2// *2 4(33.3%)	0(0%)	0.705
H.pylori	0(0%)	**3 3(25%)	0(0%)	1.000
Samonella typhi	**1//*2 2(28.6%)	**5//*1 6(50%)	0(0%)	0.157
HBV	**2 2(28.6%)	**2 2(16.7%)	0(0%)	0.1000
T.pallidum	0(0%)	0(0%)	0(0%)	0.1000
Mtb	0(0%)	0(0%)	0(0%)	1.000

Key:

**Number of individuals with heterogeneity of microbial antigenic components

*Number of individual with homogeneity of microbial antigenic components

MP(Pf)= Malaria parasite (*Plasmodium falciparum*); T.pallidum= *Treponema pallidum*; TB(Mtb)=Tuberculosis (*Mycobacterium tuberculosis*); HBV= Hepatitis B virus; HCV= Hepatitis C virus; H.pylori= *Helicobacter pylori*

4.21. Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Benign, Malignant Tumour and Apparently Healthy Subjects, with Hypermethylated DNA

The analysis of antigenic components of circulating immune complexes, showed that the occurance of malaria parasites (*Plasmodium falciparum*) in hypermethylated subjects with benign breast tumour 2(100%); hypermethylated subjects with malignant breast tumour 2(50%), was not significantly different (P=0.1000). Also the occurance of *Hepatitis C virus* (HCV) in hypermethylated subjects with benign breast tumour 1(50%); hypermethylated subjects with malignant breast tumour 3(75%); was not significantly different (P=0.046). *Helicobacter pylori* was detected only in 1(50%) hypermethylated subjects with benign breast tumour. The oocurance of *Salmonella typhi* in hypermethylated subjects with benign breast tumour 1(50%); hypermethylated DNA subjects with malignant breast tumour 2(50%); was not significantly different (P=0.564). *Hepatitis B virus* (HBV), Treponema pallidum and Mycobacterium tuberculosis were not detected in subjects with hypermethylated DNA. Meanwhile hypermethylation was not detecteded in control subjects (See table 4.21)

Table 4.21. Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Benign, Malignant Tumour and Apparently Healthy Subjects, with Hypermethylated DNA

	Benign **N=2**	**Malignant** **N=4**	**Control** **N=0**	**P-value**
MP (Pf)	**2 2(100%)	**2 2(50%)	----	0.1000
HCV	**1 1(50%)	**3 3(75%)	----	0.317
H.pylori	**1 1(50%)	0(0%)	----	1.000
Samonella spp	**1 1(50%)	**2// 2(50%)	----	0.564
HBV	0(0%)	0(0%)	----	1.000
T.pallidum	0(0%)	0(0%)	----	1.000
Mtb	0(0%)	0(0%)	----	1.000

Key

**Number of individuals with heterogeneity of microbial antigenic components

*Number of individual with homogeneity of microbial antigenic components

MP(Pf)= Malaria parasite (*Plasmodium falciparum*); T.pallidum= *Treponema pallidum*; TB(Mtb)=Tuberculosis (*Mycobacterium tuberculosis*)**;** HBV= Hepatitis B virus; HCV= Hepatitis C virus; H.pylori= *Helicobacter pylori*

4.22. Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Benign, Malignant Tumour and Apparently Healthy Subjects, with Unmethylated DNA

Based on the analysis of antigenic components of circulating immune complexes, the occurance of malaria parasites (*Plasmodium falciparum*) in benign breast tumour subjects with unmethylated DNA 4(44.4%); malignant breast tumour subjects with unmethylated DNA 1(12.5%); control subjects with unmethylated DNA 0(0%) was not significantly different (P=0.368). Also the occurance of *Hepatitis C virus* (HCV) in benign breast tumour subjects with unmethylated DNA 4(44.4%); malignant breast tumour subjects with unmethylated DNA 1(12.5%); control subjects with unmethylated DNA 0(0%) was not significantly different (P=0.180). The occurance of *Helicobacter pylori* in benign breast tumour 5(55.6%); malignant breast tumour subjects with unmethylated DNA 2(25%); control subjects with unmethylated DNA 0(0%) was not significantly different (P=0.102). *Salmonella typhi* was detected only in one benign breast tumour subject with unmethylated DNA 1(11.1%). The occurance of *Treponema pallidum* in benign breast tumour 1(11.1%); malignant breast tumour 2(25%); unmethylated control subjects with unmethylated DNA 0(0%) was not significantly different (P=0.564). *Hepatitis B virus* (HBV) and Mycobacterium tuberculosis were not detected in benign malignant and control subjects with unmethylated DNA (Table 4.22).

Table 4.22. Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Benign, Malignant Tumour and Apparently Healthy Subjects, with Unmethylated DNA

	Benign N=9	**Malignant N=8**	**Control N=5**	**P-value**
MP (Pf)	**4 4(44.4%)	*1 1(12.5%)	**2 2(40%)	0.368
HCV	**3//*1 4(44.4%)	*1 1(12.5%)	0(0%)	0.180
H.pylori	**3//*2 5(55.6%)	*1 2(25%)	0(0%)	0.102
Samonella spp	*1 1(11.1%)	0(0%)	0(0%)	1.000
HBV	0(0%)	0(0%)	0(0%)	1.000
T.pallidum	**1 1(11.1%)	*2 2(25%)	0(0%)	0.564
Mtb	0(0%)	0(0%)	0(0%)	1.000

Key

**Number of individuals with heterogeneity of microbial antigenic components

*Number of individual with homogeneity of microbial antigenic components

MP(Pf)= Malaria parasite (*Plasmodium falciparum*); T.pallidum= *Treponema pallidum*; TB(Mtb)=Tuberculosis (*Mycobacterium tuberculosis*); HBV= Hepatitis B virus; HCV= Hepatitis C virus; H.pylori= *Helicobacter pylori*

4.23. Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Benign, Malignant Tumour and Apparently Healthy Subjects, with Normal methylated DNA

The analysis of antigenic components of circulating immune complexes, indicated that the occurrence of malaria parasites (*Plasmodium falciparum*) in benign breast tumour subjects 3(50%) with normal methylated DNA and control subjects 9(22.5%) with normal methylated DNA was not significantly different (P=0.052). Also the occurance of *Hepatitis C virus* (HCV) in benign breast tumour subjects 2(22.2%) with normal methylated DNA and control subjects 1(2.5%) was not significantly different (P=0.564). *Helicobacter pylori* was detected only in 2(22.2%) benign breast tumour subjects with normal methylated DNA. *Salmonella typhi* and *Mycobacterium tuberculosis* was detected only in one benign breast tumour subject each 1(16.7%) with normal methylated DNA. *Treponema pallidum* and *Hepatitis B virus* (HBV) were not detected benign, malignant and control subjects with in normal methylated DNA subjects (Table 4.23).

Table 4.23. Distribution of Microbial Agents based on the Analysis of Antigenic Components of Circulating Immune Complexes in Benign, Malignant Tumour and Apparently Healthy Subjects, with Normal methylated DNA

	Benign N=6	**Malignant N=1**	**Control N=40**	**P-value**
MP (Pf)	**1//*2 3(50%)	0(0%)	**1//*8 9(22.5%)	0.052
HCV	**1 // *1 2(33.3%)	0(0%)	**1 1(2.5%)	0.564
H.pylori	**1//*1 2(33.3%)	0(0%)	0(0%)	1.000
Samonella spp	*1 1(16.7%)	0(0%)	0(0%)	1.000
HBV	0(0%)	0(0%)	0(0%)	1.000
T.pallidum	0(0%)	0(0%)	0(0%)	1.000
Mtb	*1 1(16.7%)	0(0%)	0(0%)	1.000

Key

**Number of individuals with heterogeneity of microbial antigenic components

*Number of individual with homogeneity of microbial antigenic components

MP(Pf)= Malaria parasite (*Plasmodium falciparum*); T.pallidum= *Treponema pallidum*; TB(Mtb)=Tuberculosis (*Mycobacterium tuberculosis*); HBV= Hepatitis B virus; HCV= Hepatitis C virus; H.pylori= *Helicobacter pylori*

4.24: Cut off Values of Circulating Immune Complexes and other Molecules in Healthy Female Subjects (Mean+2SD).

The following borderline values were used in this study for circulating immune complexes and other molecules expressed: Circulating immune complexes 56.47ugeq/ml; Nuclear factor kappa B 0.33ng; Immunoglobulin G 24.7mg/ml; Tumour necrosis factor alpha 12.2pg/ml; OH2DG 14.1ng/ml; estrogen 400pg/ml; progesterone 0.70ng/ml

Table 4.24: The Cut off Values of Circulating Immune Complexes and other Molecules in Healthy Female Subjects (Mean+2SD).

	CIC ugeq/ml	Nfkb ng/ml	IgG mg/ml	Tnf-alpha pg/ml	8-OH2DG ng/ml	Estrogen pg/ml	Proges ng/ml
Cut off Value	**56.47**	**0.33**	**24.70**	**12.20**	**14.10**	**400**	**0.70**

4.25. Pattern Global DNA Methylation in Subjects with Breast Tumours and Apparently Healthy Subjects

DNA methylation pattern was determined in 24 benign tumour subjects, 25 malignant tumour subjects, and 50 control subjects. The rate of occurrence of DNA hypomethylation in subjects with benign breast tumour 7 (7.1%); malignant breast tumour 12 (12.1%) and control subjects 5 (5.1%) was statistically significant P=0.000. The rate of occurrence of unmethylated DNA in subjects with benign breast tumour 9 (9.1%); malignant breast tumour 8(8.1%) and control subjects 5 (5.1%) was statistically significant P=0.000. The rate of occurrence of DNA hypermethylation in subjects with benign breast tumour 2 (2.0%) and malignant breast tumour 4 (4.0%), was statistically significant P=0.014. Also the occurrence of normal methylated DNA detected in subjects with benign breast tumour 6 (6.1%); malignant breast tumour 1(1.0%) and control subjects 40(40.4%) was statistically significant P=0.000 (Figure 4.1).

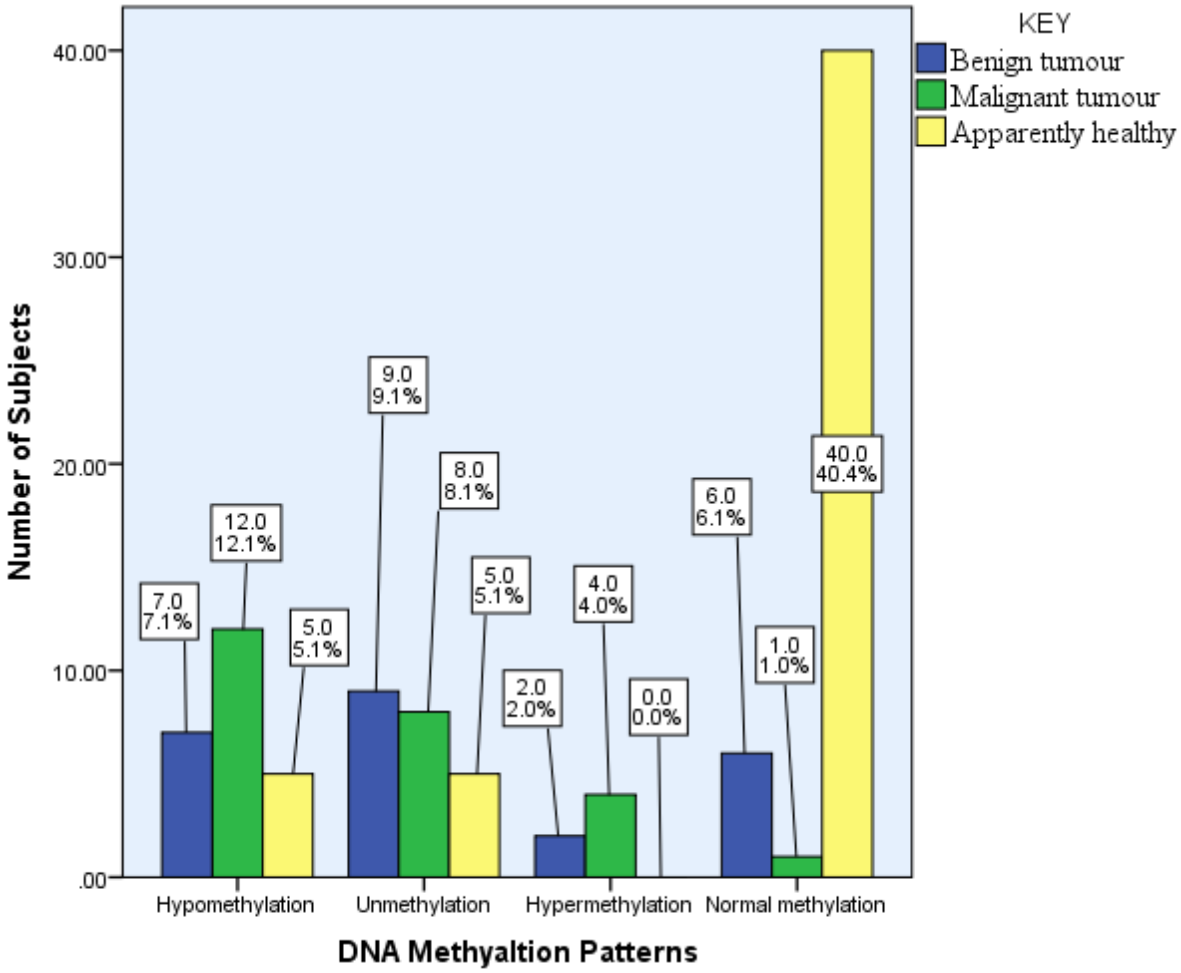

Figure 4.1: Pattern of Global DNA Methylation in Subjects with Breast Tumours and Apparently Healthy Subjects

4.25. Distribution of Benign and Malignant Breast Tumours and Apparently Healthy Subjects with and without Microbial Antigens.

Distribution of microbial antigenic components of circulating immune complexes was determined in 24 subjects with benign breast tumour, 25 subjects with malignant breast tumour and 50 apparently healthy subjects. Out of the 99 subjects, 21 (21.2%) subjects with benign breast tumour, had microbial antigenic components of circulating immune complexes, while 3 (3.0%) were free from the microbial antigenic components. In subjects with malignant breast tumour, 20 (20.2%) had microbial antigenic components while 5 (5.1%) were free from the microbial antigenic components. In apparently healthy subjects, 12 (12.1%) had microbial antigenic components, while 38 (38.4%) were free from the microbial antigenic components (Figure 4.2).

The distribution of microbial antigenic components in subjects with benign breast tumour 21 (21.2%); malignant breast tumour 20 (20.2%) and apparently healthy subjects 12 (12.1%) was significantly different P=0.000. Also the difference in number of subjects with benign breast tumour 3 (3.0%); malignant breast tumour 5 (5.1%) and apparently healthy subjects 38 (38.4%) without evidence of the microbial antigenic components of circulating immune complexes was statistically significant P=0.000.

In subjects with benign breast tumour, the number expressing microbial antigenic components 21 (21.2%) is significantly higher compared to the number without the microbial antigenic components 3 (3.0%) P=0.000. In subjects with malignant breast tumour, the number expressing microbial antigenic components 20 (20.2%) is significantly higher compared to the number without the microbial antigenic components 5 (20%) P=0.000. In control subjects, the number expressing microbial antigenic components 12 (12.1%) is significantly lower compared to the number without microbial antigenic components 38 (38.4%) P=0.000 (Figure 4.2).

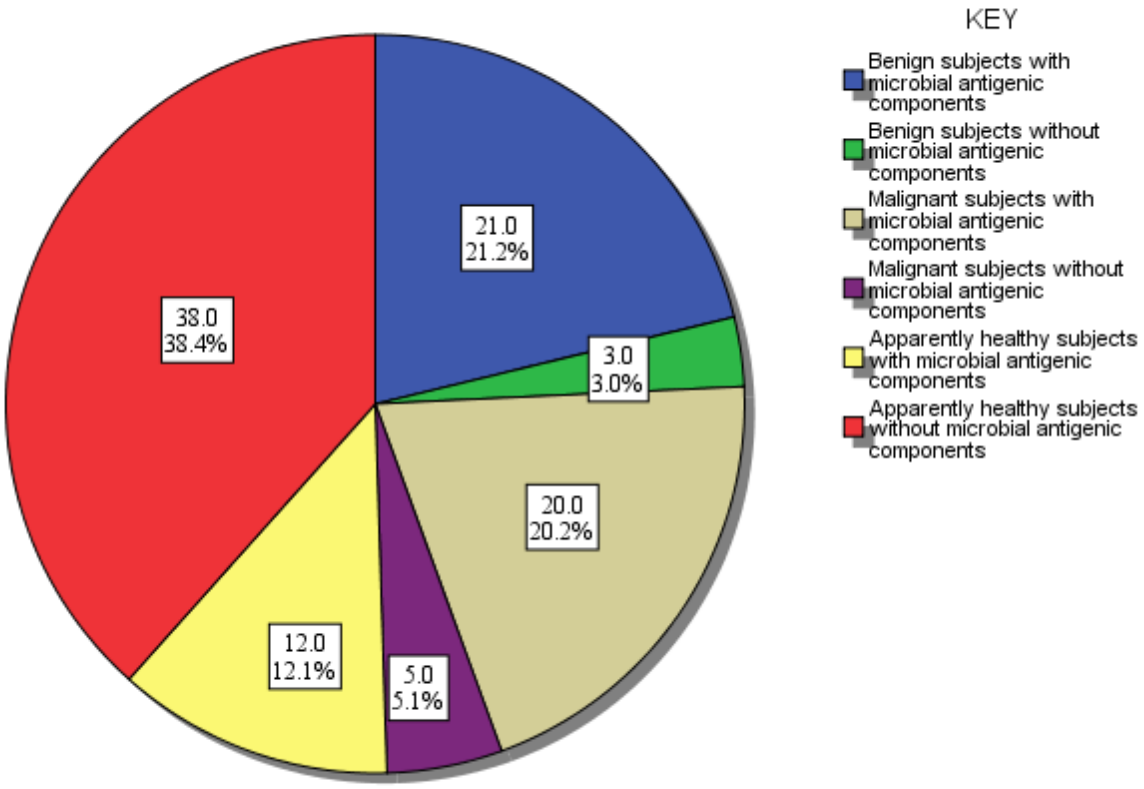

Figure 4.2: Distribution of Benign and Malignant Breast Tumours and Apparently Healthy Subjects with and without Microbial Antigens.

4.26. Distribution of Microbial Antigenic Components of Circulating Immune Complexes in DNA Methylation Patterns in Subjects with Benign and Malignant Breast Tumours and Apparently Healthy Subjects.

Out of the 7 hypomethylated subjects with benign breast tumour 5 (78%) had evidence of microbial antigenic components of circulating immune complexes, while 2(28%) showed no evidence of microbial antigenic components. Out of the 9 unmethylated subjects with benign breast tumour 8(89%) had evidence of microbial antigenic components, while 1(11%) showed no evidence of microbial antigenic components. Out of the 2 hypermethylated subjects with benign breast tumour both had evidence of microbial antigenic components. Out of the 6 normal methylated subjects with benign breast tumour 6(100%) had evidence of microbial antigenic components (Figure 4.3a)

Out of 12 hypomethylated subjects with malignant breast tumour, 11(92%) had evidence of microbial antigenic components, while 1(8%) showed no evidence of microbial antigenic components. Out of 4 hypermethylated subjects with malignant breast tumour, 3(75%) had evidence of microbial antigenic components, while 1(25%) showed no evidence of microbial antigenic components. Out of 8 unmethylated subjects with malignant breast tumour, 5(63%) had evidence of microbial antigenic components, while 3(37%) showed no evidence of microbial antigenic components. Only 1 subject with malignant breast tumour presented with normal methylation but showed no evidence of microbial antigenic component (Figure 4.3b).

In control subjects, 5 subjects presented with hypomethylation and none showed evidence of microbial antigenic components Out of the 5 control subjects with unmethylation, 3 (6%) had evidence of microbial antigenic components, while 2(4%) showed no evidence of microbial antigenic components In control subjects, there was no methylation shift resulting to hypermethylation. Out of the 40 control subjects with normal methylation, 10(25%) had evidence of microbial antigenic components, while 30(75%) showed no evidence of microbial antigenic components (Figure 4.3c).

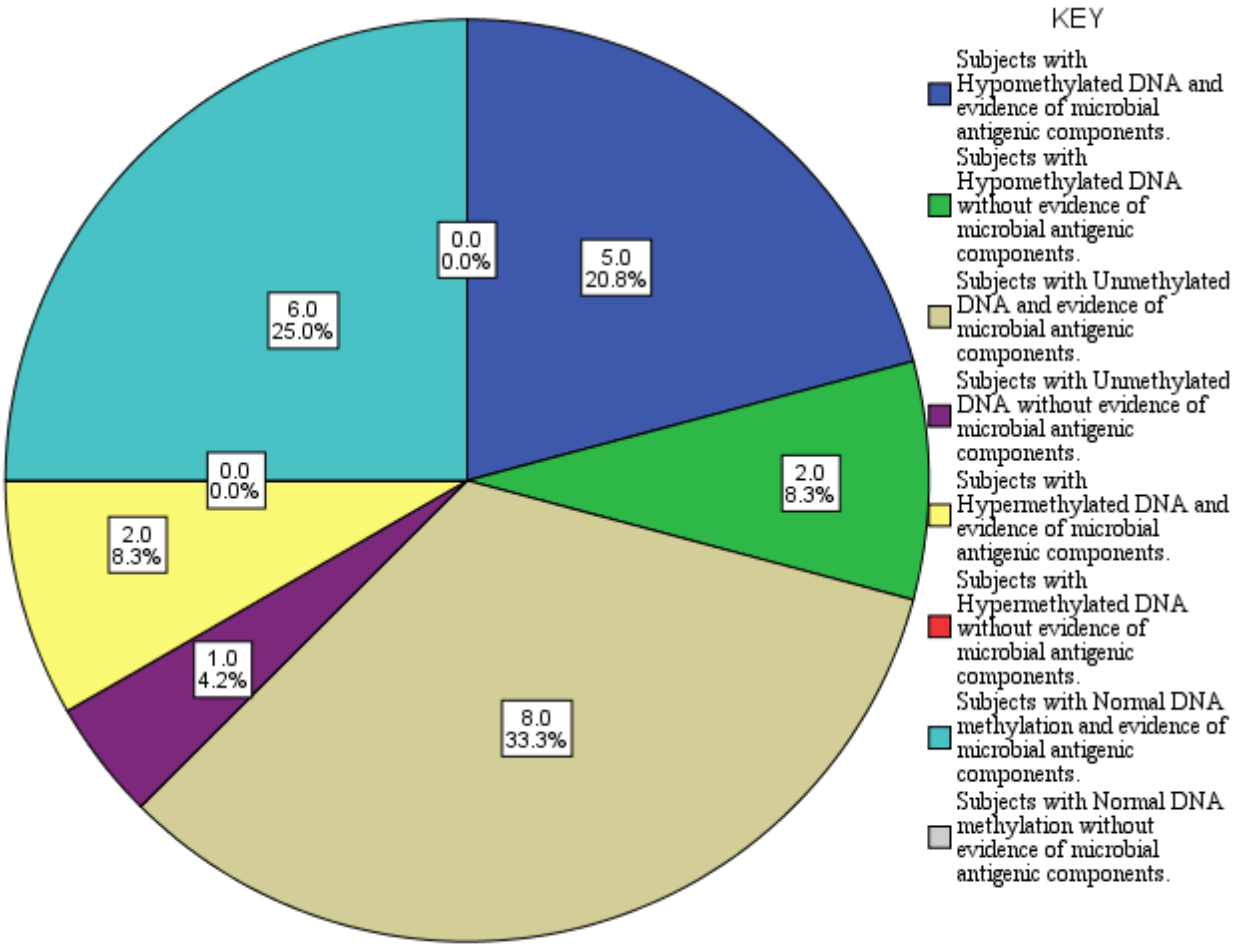

Figure 4.3a: Distribution of Microbial Antigenic Components of Circulating Immune Complexes in DNA Methylation Patterns in Subjects with Benign Breast Tumour

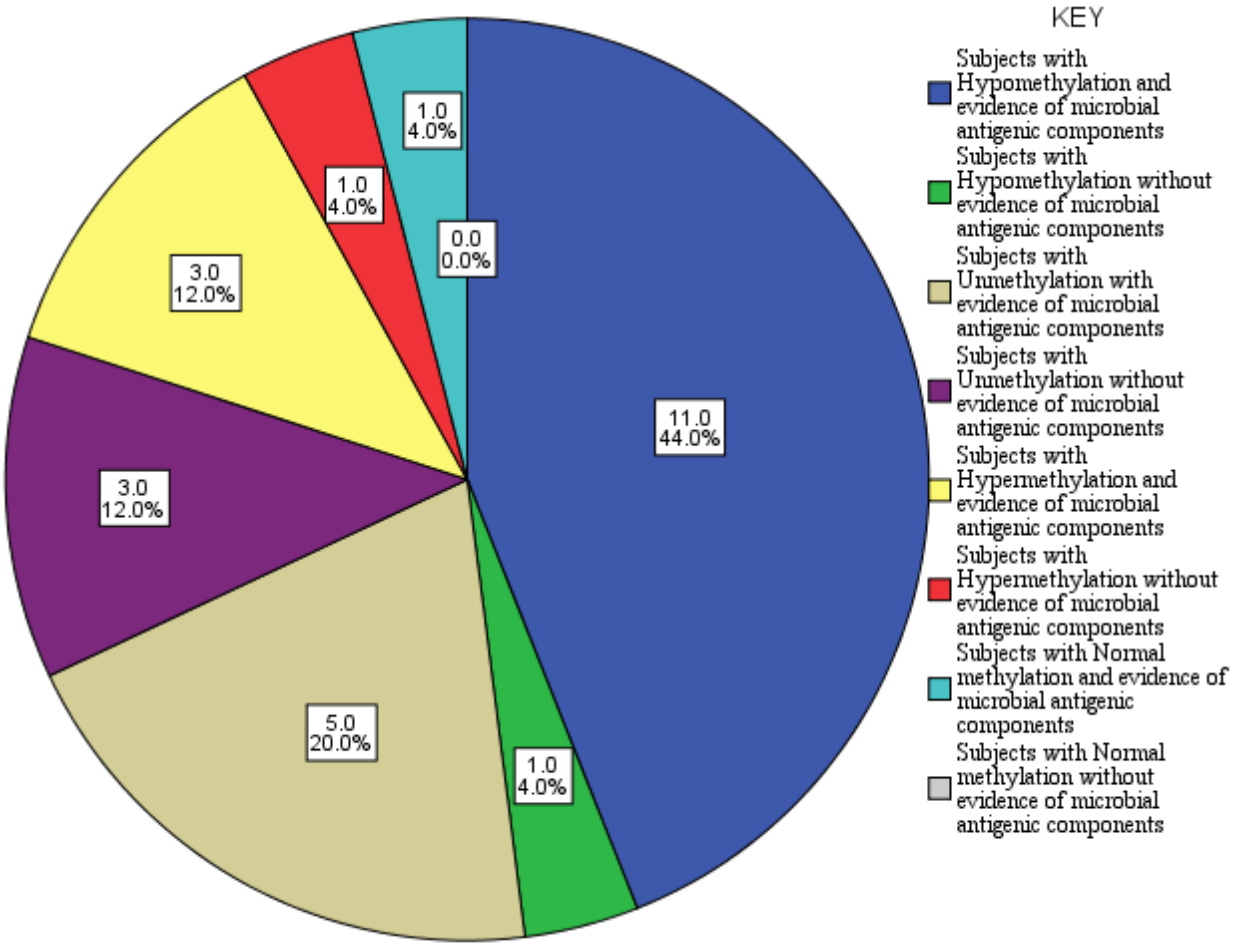

Figure 4.3b: Distribution of Microbial Antigenic Components of Circulating Immune Complexes in DNA Methylation Patterns in Subjects with Malignant Breast Tumour

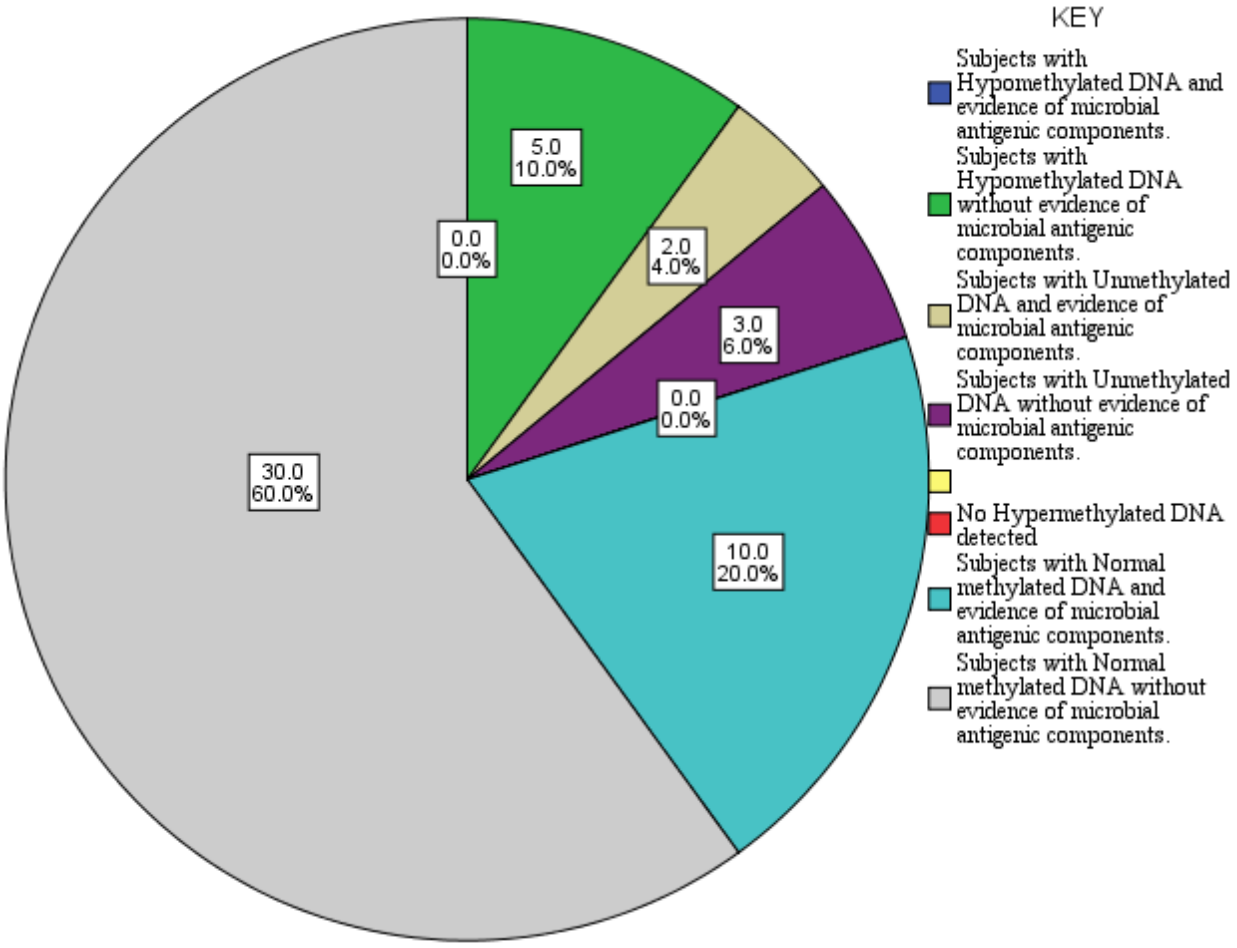

Figure 4.3c: Distribution of Microbial Antigenic Components of Circulating Immune Complexes in DNA Methylation Patterns in Apparently Healthy Subjects.

4.27: Out of the 5 subjects in stage 2b, DNA hypomethylation was detected in 1 (4.0%) subject, DNA unmethylation was detected in 3 (12.0%) subjects, normal DNA methylation was observed in 1 (4.0%) and DNA hypermethylation was detectesd in 0 (0%). Out of the 9 subjects in stage 3b, DNA hypomethylation was detected in 6 (24.0%) subjects, DNA unmethylation was detected in 1 (4.0%) subjects, normal DNA methylation was observed in 0 (0%) and DNA hypermethylation was detectesd in 2 (8.0%). Out of the 7 subjects in stage 3c, DNA hypomethylation was detected in 4 (16.0%) subjects, DNA unmethylation was detected in 2 (8.0%) subjects, normal DNA methylation was observed in 0 (0%) and DNA hypermethylation was detected in 1 (4.0%) subject. Out of the 4 subjects in stage 4, DNA hypomethylation was detected in 1 (4.0%) subject, DNA unmethylation was detected in 2 (8%) subjects, normal DNA methylation was detected in 0 (0%) and DNA hypermethylation was detectesd in 1 (4.0%) (Figure 4.4).

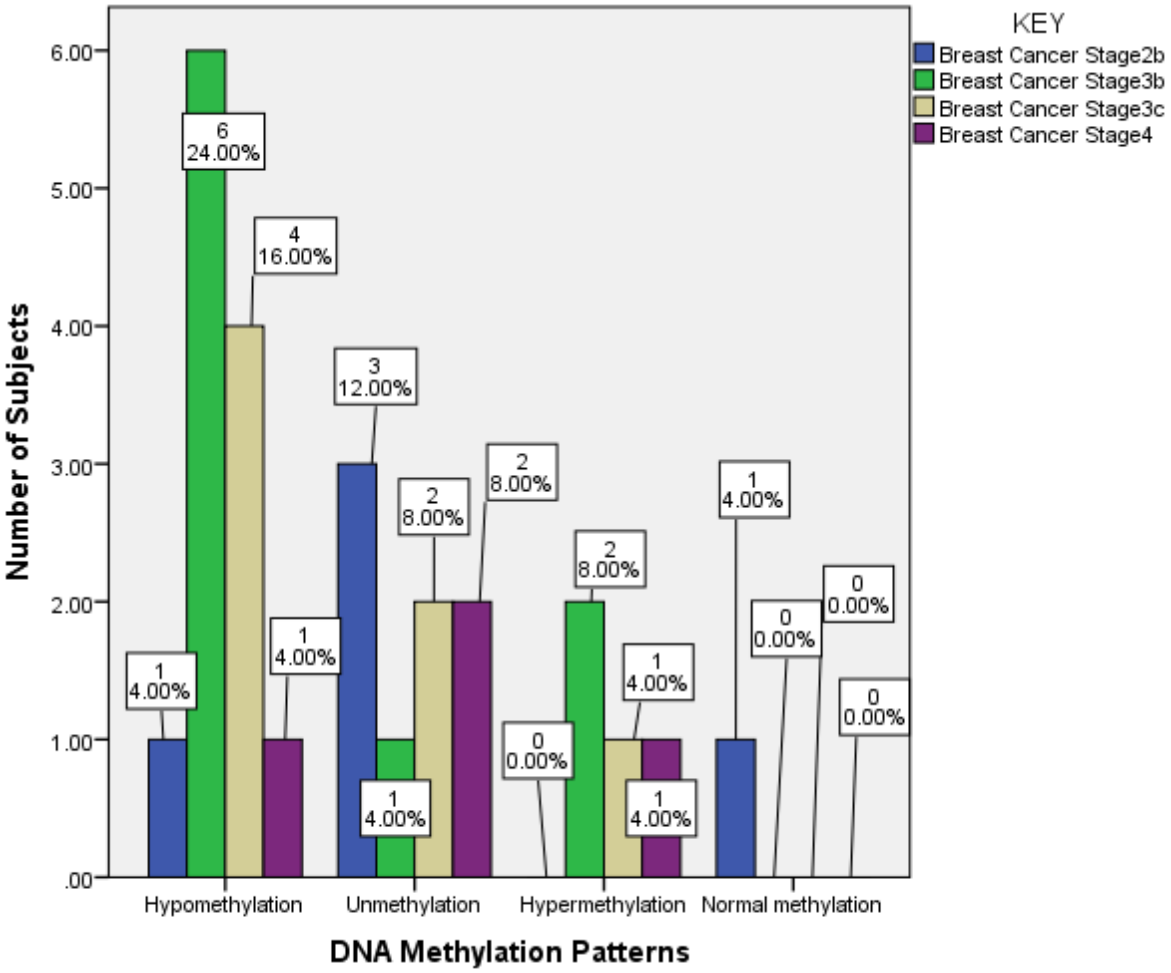

Figure 4.4: Stages of Malignant Breast Tumour and their Patterns of DNA Methylation

4.28: Out of 5 subjects with malignant breast tumour in stage 2b, 4 (16.0%) had CIC with microbial antigenic component (MAC) while 1 (4.0%) had CIC without microbial antigenic component. Prevailing number of malignant subjects with MAC was detected in stage 3b totalling 9 subjects. Out of these 9 subjects, 7 (28.0%) had CIC with MAC while 2 (8.0%) had CICs without MAC. Stage 3c had 7 subjects and all the 7 (28.0%) subjects had MAC, while stage 4 had 4 subjects 3 (12.0%) of the subjects had CICMAC while 1 (4.0%) had CIC without MAC (Figure 4.5).

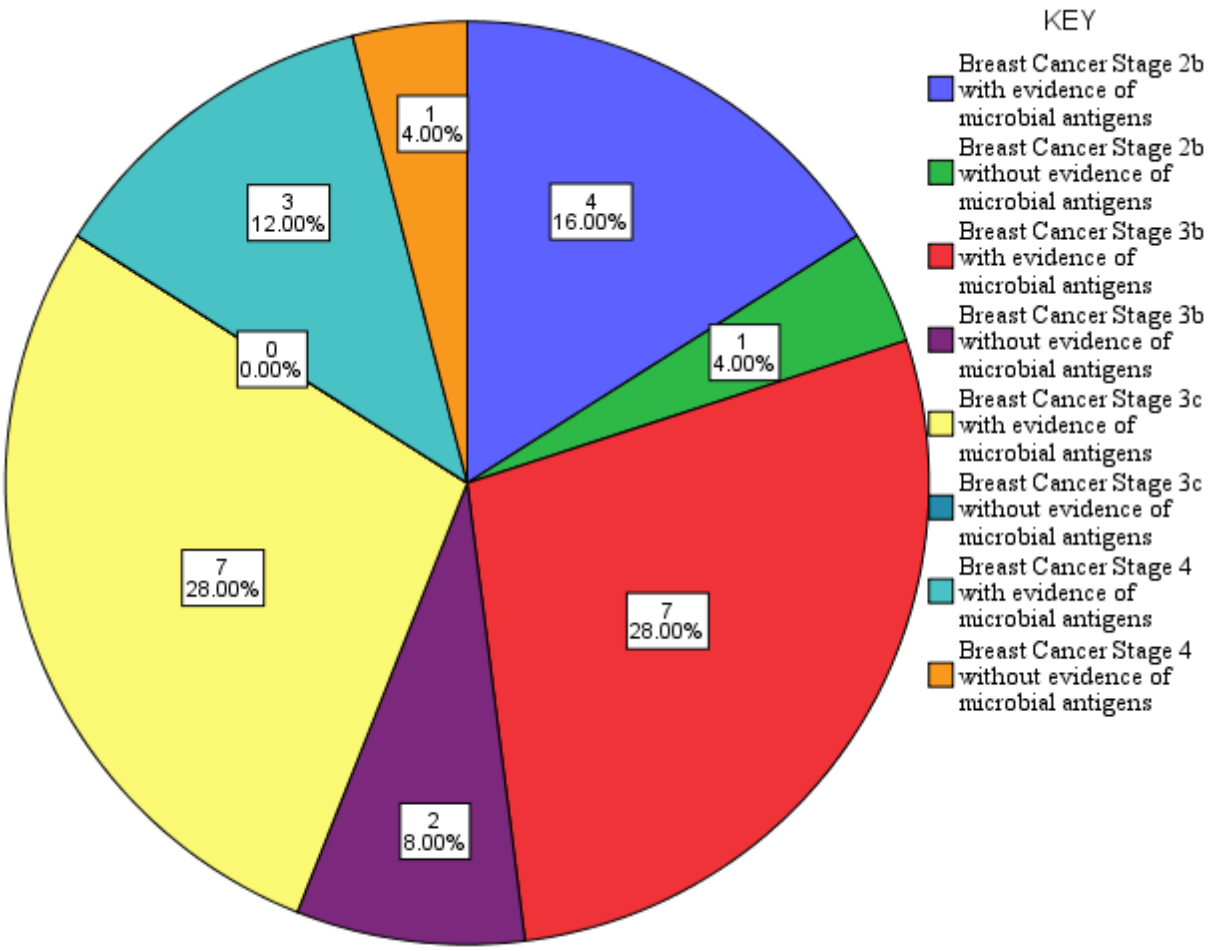

Figure 4.5: Percentage Distribution of Microbial Antigenic Components in Different Stages of Malignant Breast Tumour.

CHAPTER FIVE

5.0. DISCUSSION

This study looked into Global DNA methylation-based signatures in determining the pattern of methylation in benign breast tumour, malignant breast tumour and apparently healthy female subjects. Different types of antigens have been isolated from immune complexes including exogenuous antigens such as microbial antigens, toxins and chemical antigen and endogenous antigens such as auto-antigens, dead cell associated molecular pattern (DAMP) and cancer cell antigens (Tolle and Standiford, 2013). However this study, focused on identification of microbial antigenic components because of emerging significance of microbial association with breast cancer and other carcinomas. Furthermore, this study, evaluated various molecular expression pathways such as NFkB translocation, proinflammatory responses (TNF-alpha), immunoglobulin pathway (IgG), oxidative stress (OH2DG) and female sex hormone (estrogen and progesterone), in subjects with evidence of microbial antigenic components and those without such evidence. This aided to find out the association between these molecules sequel to their expressions and pleotropic nature in the process of tumour development. By the analysis of the characterized immune complexes, it was possible to specifically identify and link microbial infection to expression of various molecules and possible influences their association may have on DNA methylation, as well as study their prevalence in various groups of subjects with tumour and healthy subjects without tumour.

This work revealed that 75–96 percent of breast tumour development could develop during individual's life time and attributable to epigenetic cell alteration. About 75% of the subjects with benign tumour had epigenomic aberration or irregularities (DNA methylation), while 96% of the subjects with malignant tumour had epigenomic aberration. This is in line with the report that only 5-10% of cancers are due to gene abnormalities inherited from parents and that about 90-95% of cases are attributed to epigenetic factors, 'wear and tear' of life in general (Annand et al., 2008). DNA methylation is vital during development, and aberrant DNA methylation, both hypermethylation and demethylation, have been associated with aging, cancer and other diseases (Jung and Pfeifer, 2015). Therefore, methods to study DNA methylation are important tools in biological research. DNA methylation shift seem to be an important epigenetic event in breast tumourigenesis and by using global methylation assay, it is possible to identify these markers for diagnostic and therapeutic purposes in this disease.

Tumor node metastasis (TNM) which is a measure of stages of cancer has been used to measure cancer spread and prognosis (American Cancer Society, 2016), and is commonly used in clinical setting. The stages range from 0 to IV, with stage 0 being in situ, stage I being early stage invasive cancer, and stage IV being the most advanced disease. Larger size, nodal spread, and metastasis have a larger stage number and a worse prognosis (Edge et al., 2010). Stage 0 is a pre-cancerous or marker condition, either ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS). Stages 1–3 are within the breast or regional lymph nodes. Stage 4 is 'metastatic' cancer that has a less favorable prognosis (American cancer society 2016). Based on these, this study revealed 4 different stages of breast cancer viz-a-viz stages 2b, 3b, 3c and 4. This indicated that majority of the breast cancer cases did not present themselves for medical attention at the early stage. Therefore, the prevailing nature of demethylation in subjects with malignant breast tumour could be a source of evidence to nodal spread and progression, as higher numbers of hypomethylation/unmethylation were found in stages 3b and 3c. Thus this work considers stages 3b and 3c very clinically important stages of breast cancer stages in this locality in terms of physiological changes. In stage 3 of breast cancer, the cancer is lager, growing into nearby tissues (the skin over the breast or the muscle underneath), or has spread to many nearby lymph nodes indicating advanced clinical development and requiring both local and systemic therapy (American Cancer Society, 2017).

DNA methylation changes in cancer cells include the loss of methylation at normally methylated sequences (hypomethylation) and the gain of methylated sequences at sites usually unmethylated (hypermethylation) (Lisanti et al., 2013). In this study, presentation of normal DNA methylation is low in subjects with breast tumours, while DNA methylation shift such as hypomethylation unmethylation and hypermethylation were significantly high in subjects with malignant and benign breast tumours. Szyf, (2012) reported that in vertebrates almost every CpG site is methylated, with the exception of those in CpG islands where transcription takes place and that specifically, nearly 80% of CpG sites are methylated in the human genome. However, in cancer cells, dramatic and opposing changes to the epigenetic landscape often occur and these changes consist of global hypomethylation of the DNA and localized hypermethylation of CpG island-associated promoters. It is important to note that presentation of hypermethylation may not be peculiar with CpG islands associated promoter regions as this work revealed high level of hypomethylation and low level of hypermethylation in subjects with breast tumours using global methylation assay. Earlier, the work of Tan et al., (2009) though in pancreatic cancer, detected hypermethylation and hypomethylation using global methylation profiling assay.

Specifically shift in DNA methylation was more profound in tumour subjects with only 4% of malignant breast tumour and 25% of benign breast tumour subjects having normal DNA methylation unlike in control subjects with 80% having normal DNA methylation (p=0.000). The prevailing state of demethylation (unmethylation and hypomethylation) in subjects with benign and malignant tumour (66.6%) and (80%) respectively and the occurrence of unmethylation in malignant subjects and benign subjects call for attention. However, as this work was designed to access the influence of retained circulating immune complexes in general and the influence of microbial antigens as antigenic components of the circulating immune complex specifically, it was discovered that majority of the unmethylated/hypomethylated subjects harboured microbial antigens.

The importance of DNA methylation events associated with bacterial infections is becoming increasingly appreciated. Studies have shown that certain infectious agents can contribute to the host epigenetic changes resulting in the onset and progression of some diseases, especially in malignancies (Gilbert et al., 2010). Moreover, pathogenic bacteria have been considered as potential epimutagens able to reshape the epigenome (Bierne et al., 2012). While in cancer, epigenetic states are deregulated and thought to be of significance in cancer development and progression (Stefansson et al., 2013). Thus, from this study it seems that global hypomethylation or unmethylation would positively influence tumourigenesis. Furthermore, DNA hypomethylation can cause the undesirable activation of dormant repeat elements and lead to altered expression of associated genes. DNA hypomethylation can cause genomic instability and may contribute to mutations and chromosomal recombinations (Lisanti et al., 2013). DNA demethylation has an important role in cancer by turning on the expression of pro-metastatic genes, such as the heparanase gene, *MMP2* (which encodes matrix metalloproteinase-2) and *uPA* (which encodes urokinase plasminogen activator) (Szyf, 2012). A causal role for demethylation in cancer metastasis is supported by the fact that treatment of non-metastatic breast cancer cells with demethylating agents increases their invasiveness (Chik and Szyf, 2011), and that treatment of invasive breast cancer and liver cancer cell lines with agents that reverse unmethylation results in inhibition of invasiveness and metastasis (Stefanska et al., 2011).

Detection of methylation shift in the present study amongst the healthy subjects is suggested to be an indication that DNA methylation would be an early indicator for breast tumour development in women. Aberrant methylation can begin very early in tumor development and mediate most of the important pathway abnormalities in cancer development including loss of

cell cycle control, altered function of transcription factors, altered receptor function, disruption of normal cell–cell and cell–substratum interaction, inactivation of signal transduction pathways, loss of apoptotic signals and genetic instability (Baylin and Jones 2011). Thus the use of circulating DNA (cfDNA) from whole blood (liquid biopsy) in this study, highlighted the importance of early tumour detection and thus auguments early DNA methylation aberration detection, as the use of solid biopsy can only be possible when tumour must have developed (Ignatiadis & Dawson 2014). Using methylation haplotypes Guo *et al* (2017), demonstrated quantitative estimation of tumor load and tissue-of-origin mapping in the circulating cell-free DNA of 59 patients with lung or colorectal cancer. Thus, this study has shown that a blood test could be used for early tumour detection and early enough to detect pre-tumour genomic aberration even before the tumour develops. Circulating tumour DNA (CtDNA) can be distinguished from circulating DNA from healthy cells by the presence of genomic aberrations that correspond to those found in the tumor, such as tumor-specific mutations or methylation (Warton et al., 2016).

Many research works earlier done on immune complexes, emphasized on quantification of immune complexes in cancer patients (Perveen et al., 2010, Rai and Mody 2012). However, this study ventured into specific detection of presence of microbial proteins that have been complexed with antibody and remain in circulation. The consequence of this is presumed to be enablement of immunopathologically mediated tumorigenesis or tumour metastasis via sustenance of chronic inflammation. Additional investigations were done to determine whether the presence of microbial antigenic components of immune complexes contributes in accessing of prognosis as have been previously done extensively (Bierne et al., 2012), or whether it is also helpful in accessing the course of tumour development and cancer metastasis. The quantitative analysis of CIC in previous studies could not indicate the peculiar influence CIC may have in tumourigenesis instead they were used to monitor progress of cancer treatments. Thus dessociation and characterization of the complexes in this study gave room for more detailed analysis by spoting the differential influences in the presence of microbial antigenic components (MAC) of the circulating immune complexes (presence of MAC) and in its absence (absence of MAC). The majority of benign (87.5%) and malignant (80%) breast tumour subjects harbour microbial antigenic components in lieu of other antigenic components (benign 12.5% and malignant 20%). This was considered to be abnormal and possible trait to tumour progression based on the findings that healthy control counterparts harbour significant low level of microbial

antigenic components. This work has revealed that microbial antigens are prevalent in breast tumours compared to other antigens.

Notable microbial agents detected in this study through the analysis of components of the immune complex, include: *Plasmodium falciparum*, *Treponema pallidum*, *Hepatitis B virus*, *Hepatitis C virus*, *Helicobacter pylori* and *Salmonella typhi*. Previous researches have implicated *Helicobacter pylori*, *Streptococcus bovis, Chlamydia pneumoniae, Campylobacter rectus, Epstein-Barr* virus, hepatitis viruses, Human papilloma virus, polyomaviruses, etc.), to contribute to the host epigenetic changes resulting in the onset and progression of some diseases, especially in malignancies (Gilbert et al., 2010). Further detailed analysis of this work detected those subjects with only a particular complexed microbial antigen, reffered to as homogeneity of microbial antigenic component of immune complexes. This pattern of antigenic retention was found to be prevailing in healthy subjects, with respect to *Plasmodium falciparum* infection. Additionally, mixed microbial antigen reffered to as heterogeneity of microbial antigen, was also detected which prevalence was significantly greater in subjects with breast tumour.

Considerably, dealing with microbial antigens in this study may not justify the role a microbial agent may play in the infected system. However, while presence of microbial agent would mediate pathophysiological damages, the retention of their antigens would mediate immunopathological consequences as well as fuel chronicity. The presence of microbial antigenic components (MAC) or its absence in immune complex can induce enzymatic/chemical hydrolysis of DNA by activation and persistent stimulation of immune cells and molecular pathways. This agrees with the work of Chinnusamy and Zhu (2009), that the first step in any epigenetic study is global DNA methylation analyses which rely mostly on a prior enzymatic/chemical hydrolysis of DNA to obtain 2′-deoxymononucleosides. This has justified the approach of correlating the presence of the microbial antigens and their absence to different molecular pathways that may lead to development of tumour microenvironment in this study.

Additionally, in this study, presence of microbial antigenic components (MAC) in lieu of absence of MAC was found to be synonymous with various patterns of DNA methylation, noting that 71.4% of benign tumour subjects with hypomethylation had MAC while 28.6% had other antigenic components. Also 91.7% of the malignant tumour subjects with hypomethylation had MAC while only 8.3% had other antigenic components. Such large numbers were also found to harbour MAC amongst unmethylated and hypermethylated subjects with breast tumour. It is

suggested from this work that presence of MAC is prevalent in subjects with tumour and could adversely influence normal DNA methylation and thus induce a shift in pattern of methylation in some subjects. This present finding is in line with the finding of Gilbert et al (2010), which reported that presence of some infectious agents can contribute to the host epigenetic changes resulting in the onset of and progression of some diseases, especially in malignancy. Chronic bacteria infection and inflammation may be related to appearance of aberrant genomic DNA methylation which at least, partly may explain the mechanism of infection associated carcinogenesis. However this work also revealed that not all presence of MAC can induce or influence DNA methylation, considering the presence of MAC in 6 normal methylated subjects with benign tumour. This is in line with the report that in contrast to the pathogenic nature of *Fusobacterium* in colon cancer, the bacterium *Bacteroidetes fragilis* exerts a protective effect against colitis by modulating inflammatory immune responses in the gut (Mazmanian et al., 2008, Xuan, et al., 2014). From these and other recent studies, it is becoming increasingly apparent that both community composition and discrete bacterial species can exert either pathogenic effects that encourage disease development or probiotic effects that maintain health status (Xuan, et al., 2014). However, the pathophysiological or immunopathological importance of the presence of microbial antigens in epigenomic process may include enhancement of the chances of methylation shift by stimulating immune cells and cytokine activations and thus increasing their activities and subsequent perturbation of the DNA. Previous studies have shown that bacteria can affect the chromatin structure and transcriptional program of host cells by influencing diverse epigenetic factors (i.e. histone modifications, DNA methylation, chromatin-associated complexes, noncoding RNAs, and RNA splicing factors). Bacterial-induced epigenetic deregulations may affect host cell function either to promote host defense or to allow pathogen persistence. Thus, pathogenic bacteria can be considered as potential epimutagens able to reshape the epigenome. Their effects might generate specific, long-lasting imprints on host cells, leading to a memory of infection that influences immunity and might be at the origin of unexplained diseases (Bierne et al., 2012). This phenomena would depend on types of microbial antigen as well as the distribution pattern (heteogeneity or homogeneity of microbial antigen), of the microbial antigens as detected in this study, being factors to consider. This is because immunological activities of a particular antigenic component may not be enough for epigenomics while the existence of immune complexes due to 2 different microbial agents may be antagonistic or synergistic toward epigenomic development.

The association of global DNA hypomethylation with the early stages of carcinogenesis or with tumour progression would provide cancer markers that would be very useful in the clinic (Ehrich, 2009, Dehbid and Hamidreza 2016). In addition, the knowledge that metastatic genes are activated by hypomethylation will be necessary for development of personalized treatment programs that are molecularly based. Translation of research findings to the clinical setting will be facilitated by the recent developments in high-throughput and high-resolution methods for DNA methylation analysis (Kristensen and Hansen 2009). Besides since DNA methylation is subject to environmental influences, its study should be wholesome to include the prevalence of microbial agents in its analysis depending however, on the locality.

Mechanisms of direct and indirect carcinogenesis by which infections may contribute to cancer development have been suggested. Immunosuppression or induction of reactive oxygen species via inflammatory reactions have also been suggested as the two most prominent pathways for indirect infectious carcinogens. Unlike the immunosuppression induced by HIV, the mechanism by which Hepatitis B and C viruses contribute to cancer still remains obscure (Zur, 2009). Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are both hepatotropic viruses. Their coinfection is associated with clinically and histologically more severe liver disease and higher risk of the development of hepatocellular carcinoma (Shepard *et al*., 2005, Su *et al*., 2011). However, HCV infection has been reported to predispose patients to extrahepatic disorders involving renal, dermatologic, hematologic, and rheumatologic systems as well as autoimmune abnormalities (Su *et al*., 2011). Extrahepatic manifestations may result from immunologically triggered mechanisms as well as virus invasion and replication that affect extrahepatic tissues and organs. Only HCV has the lymphotropic character that is assumed to be the cause of HCV-associated extrahepatic manifestation. This may explain why Su *et al* (2011), found that HCV, but not HBV, was associated with breast cancer. This present study up holds this report to certain extent but differs as well because the rate of occurence of HCV was very high in subjects with benign and malignant breast tumours. HCV was also detected in hypomethylation, hypermethylation and unmethylation both in subjects with benign and malignant breast tumour and only in benign tumour under normal methylation. However, by analysis of antigenic components of CICs, detection of HBV antigens in subjects with benign and malignant tumours in this study suggests that HBV infection may also be implicated in extra-hepatic involvements such as breast tumourigenesis. Thus, this work deviates at this point with the report of Su *et al*., (2011). The diagnostic approach may also contribute to the differences in the results. Thus the use of dissociated antigen-antibody complex as embraced in this study, is a rare approach but has

proven reliable in microbial detection where microbial agent could not be isolated or the antigen opsonised (Bruner and Sigal, 2001, Ezeani *et al*., 2012). Further prospective cohort studies may be required to verify this result. The potential link between HCV infection and the risk of developing malignancy other than hepatocarcinoma has been raised in several studies (Malaguarnera *et al*., 2006, Schöllkopf *et al*., 2008). This suggested that HCV chronic infection may not only promote hepatocarcinoma, but also other solid tumors (Larrey *et al*., 2010). Although hepatitis C (HCV) is the most common blood-borne infection in the United States, little information exists about treatment of breast cancer in the presence of chronic HCV infection. As the majority of these breast cancer patients completed the initial chemotherapy plan, study indicates that breast cancer patients with HCV can be treated with cytotoxic therapy (Morrow *et al*., 2010). In the course of HBV, research has shown that HBV-carrier patients could present, during their course of life in other malignancies unrelated to hepatitis viruses for which chemotherapy treatment is indicated. Furthermore, chemotherapy-induced immunosuppression can lead to HBV reactivation and may cause hepatitis and liver failure, discontinuation of anticancer treatment and death (Bozza *et al*., 2016). Additionally, a more recent metanalysis detected HBsAg-positive breast cancer patients (Liu *et al*., 2015).

Presence of microbial antigenic components was significantly higher than its absence in all stages of breast cancer, and the burden seem to be more in subjects in stages 3b and 3c. This indicates notable persistence of these microbial antigens in malignancy and could be contributing to escalation of the disease condition. Homogeneity of microbial antigenic distribution was prevalent in stage 2b. On the other hand, heterogeneity of microbial antigenic components was prevalent in stage 3c, followed by stages 3b and 4, with mixed infection of *Plasmodium falciparum*/HCV/*Salmonella typhi* and HCV/*Salmonella typhi*, leading the course of presentation. No heterogeneity of microbial antigenic circulation was found in stage 2b. This suggests that mixed microbial infection is an important pathological phenomenon in breast cancer progression and metastasis. It was observed in this study that *H.pylori* had the widest distribution because its antigens were detected in all status of DNA methylation and it is the only bacteria which antigen was detected in all stages of breast malignancy. The work of Na and Woo (2014) and Banerjee *et al* (2015) earlier implicated *H. Pylori* infection in eliciting hypermethylation and as major contributors to human cancers as etiological agents. The burden of *Salmonella typhi* antigen could be associated with demethylation (hypomethylation) in malignant subjects based on the out come of these results and as such may worsen the situation. The burden of heterogeneity of circulating microbial antigens is differently distributed in

subjects with benign and malignant tumours, but found to be higher compared to the heterogenous microbial antigenic circulation in control subjects. Only one subject harbour heterogeneity of microbial antigens (*Plasmodium falciparum*/hepatitis C virus) in control subjects while others harbour homogeneity of *Plasmodium falciparum*. This appears to indicate that heterogeneity of microbial antigens in circulation may be a significant factor in tumour initiation and progression. In line with the significance of heterogeneity of microbial antigens detected in this study, Vedham *et al* (2014), reported that co-infections are relatively common in areas with a high prevalence of infectious agents, especially in developing countries. These co-infections can cause an imbalance in the host immune system by encouraging persistence of and susceptibility to malignancy. Also Cabrera-Rubio *et al* (2012) revealed that the breast milk of healthy women has been shown to harbour abundance of bacterial species including commonly found skin bacteria.

Homogeneity of *Treponema pallidum* was found in 2 subjects with malignant breast tumour and unmethylated DNA and out numbers other microbial antigens in these subjects. The inability to cultivate *T. pallidum* in vitro, coupled with the lack of a suitable inbred animal model for immunological studies, has greatly hindered efforts to elucidate the basic immunobiological aspects of syphilis. As a consequence, many questions remain unanswered about the cultural characteristics of the spirochete, most importantly, *T. pallidum* which can persist for extended periods in tissues, despite evoking vigorous cellular and humoral immune responses (Salazar et al., 2007). These have justified the importance of the immunological and post infectious analysis in this study because because previous work has shown that during late latency, inflammatory foci may progress at sites of persistent infection, but the individual is no longer infectious (Militz and Hungerer 2015). Implication of *Treponema pallidum* in breast cancer is not yet a popular finding and needs to be further investigated. However, recently, Militz and Hungerer (2015) submitted a case report implicating *Treponema pallidum* infection in breast lesion. The presence of these microbial antigens could represent the distinct microbiological signatures associated with DNA methylation in breast tumours, in this locality. Homogeneity of *Plasmodium falciparum* was detected in control subjects with normal methylation where it was found to be prevalent followed by subjects with benign tumour and with unmethylated DNA.

All these microbial involvements may have been encountered following some factors reported to predispose to infection, such as those that are host associated and those that are treatment associated. Host-associated factors include underlying immune deficiencies, medical co-

morbidities, past infections, poor nutritional status, and psychological stress. Treatment-associated factors include surgery, radiation, immunosuppressant therapies, antimicrobial use, and invasive procedures (Maschmeyer and Haas, 2008). This study has revealed the need to characterize and grade circulating immune complexes in relation to pathogenicity. The components of the immune complexes should be identified before CIC is implicated in any disease condition. This indicates that some components of immune complexes may be more influential in certain disease than others. Components of CIC could be microbial antigens, toxins, chemical components as well as host cell debris (autoantigens), tumour antigens. Presence of all these different components may exert different changes in physiological and pathological conditions. All these were collectively implicated in certain disease conditions without detecting actually one that is playing a significant role in the disease condition. The detection is important because it would create better understanding for treatment and control of such disease condition.

The persistent cell activation in the context of chronic infection might promote cell transformation via DNA damage or production of pro-inflammatory factors that sustain chronic inflammation and may support tumour growth (Kidane et al., 2014). The immune system which recognizes pathogens or cell demage activates an influx of neutrophil and macrophages that take up the bacteria, dead cells and debris which include proteins nucleic acid and other molecules released by the damaged cells. In response, the cells release highly reactive chemicals (ROS) that mediate degradation of the bacteria as well as diffuse into the tissue and may cause its injury. Persistence of such activities would sustain chronic inflammation which may provide appropriate microenvironment for the transformation of cells by insertion of oncogenes and inhibition of tumour suppressor genes leading to initial tumour development. The prostaglandins, reactive oxygen species (ROS), Nitrogen species, Micro RNAs which are all key mediators of inflammatory induced cancer, may cause changes in cell proliferation, cell death, DNA methylation and DNA mutation, that also contribute to carcinogenesis (Schetter et al., 2010).

Detection of various microbial antigens in subjects with breast tumours, is in line with recent developments with focus on microbial involvement in tumourigenesis or carcinogenesis. Previous works have shown that infection with one or more viruses or microorganisms is the third highest contributor to the development of cancer accounting for at least 20% of tumors

discovered (de Martel et al., 2012, Sawyer et al., 2013). Furthermore, justification of the analysis of microbial infection as carried out in this study, in search of scientific reasons behind tumourigenesis is backed by several immunological developments. Pathogens attacking hosts are confronted by the immune system, and often the immune responses stimulated by one pathogen interact with those stimulated by a coinfecting pathogen. This type of interaction can be either synergistic or antagonistic, depending on pathogen identity and type of immune response (Cabrera-Rubio et al., 2012). Research has shown that when coinfection occurs, one or both pathogens may suppress the immune response. This suppression may facilitate the spread of drug-resistant mutants of the coinfecting pathogen via several routes. First, reduced immune-mediated killing of pathogens may lead to higher pathogen replication, which can increase the probability of the emergence of *de novo* resistance (e.g., HIV–malaria coinfection). Second, the reduced efficacy of the immune system may increase the frequency of symptomatic infections (in the absence of immunopathology) and hence the use of antimicrobials (e.g., HIV–herpes simplex virus 2 coinfection, which will increase the selective pressure for resistant mutants and potentially the spread of resistant pathogens). Third, reduced immune-mediated killing may allow the replication of drug-resistant strains bearing a high fitness cost (which would otherwise be outcompeted by fitter sensitive strains, e.g., HIV–TB coinfection) (Cabrera-Rubio et al., 2012). Similarly, impaired immune control may increase the danger of a recrudescence of partially resistant pathogen populations after therapy has ended. Such partially resistant pathogen populations are often selected for during therapy, and might be present after treatment; with an effective immune response they would be rapidly eliminated, but an immunosuppressive coinfection may allow for their proliferation. Interaction with the immune system does not necessarily lead to synergistic interactions between coinfecting pathogens. If the two coinfecting species or strains are antigenically or immunologically similar enough, the immune response to one strain or species may suppress the other. In such cases, the immune system mediates ‘apparent competition’, that is, the abundances of two pathogens are inversely correlated, as is the case for classic competition (Bienne et al., 2012).

There is also convincing evidence that infectious agents are capable of influencing the methylation pattern. Bienne et al., (2012), revealed that bacterial-induced epigenetic deregulations may affect host cell function either to promote host defense or to allow pathogen persistence. These observations are easily linked with outlined central principles reported by Blaser, (2008), that although the microbial agents and tumours they promote are quite varied; several principles are conserved and can be used to guide future considerations. First, oncogenic

microbes generally persist in their hosts for long periods (years or longer). The mean duration of tumour presentation in subjects with benign tumour was 18.83±20.86 and malignant tumour 36.19±53.0 months. Acute infections may be resolved but also may leave scars that promote neoplasia. Much more common are persisting microbes that the host cannot eliminate and which engage the host in an ongoing battle that damages tissues (Blaser and Kirschner 2007) and promotes tumour formation and subsequent malignancy. This study has practicalised this theory by using the analysis of immune complexes to implicate microbial agents to tumourigenesis. Most of the microbial agents implicated are known for chronicity. It was also discovered in this study that one of the best approaches to studying persistent infection is through antigenic left over complexed by its corresponding antibody in the system. For example, squamous cell carcinomas follow the long-term inflammatory consequences of chronic osteomyelitis with sinus tract formation (Blaser and Kirschner 2007). A group of colonizing organisms rather than a single pathogen has been recognized as provoking this chronic inflammation (Blaser and Kirschner 2007). In this study, different microbial antigens were detected in many subjects in homogenous or heterogenous forms, with recorded persistent characters. Secondly, variation in oncogenic potential exists within microbial species. This variation has been well established for Papiloma virus (HPV), certain types of which cause most cancers, and for the hepatitis viruses and *H. pylori*, in which particular genotypes are most virulent (Blaser, 2008). Third, microbial load often matters (Yu et al., 2005). Fourth, the interactions between microbial genotype and load may be synergistic, leading to markedly enhanced disease risks (Blaser et al., 2007). Fifth, host genotypes and phenotypes (e.g., host age and age on acquiring an organism) are part of the microbial genotype-load interaction and modulate risk (Chang et al., 2006, Blaser et al., 2007). Sixth, because microbes are communicable agents, their prevalence in individuals in a prior generation influences their prevalence in the next. Therefore, secular trends toward intergenerational amplification (of prevalence or virulence) can develop in a population without any known increase in exposure, in contrast to chemical carcinogenesis, for example. As obligate intracellular parasites, viruses have developed numerous ways of hijacking cell processes to facilitate the completion of their life cycle and sometimes to evade the immune responses of their host. Viruses that cause persistent (often latent) infections are likely to benefit from heritable epigenetic changes in host transcription that produce an environment for their latent or persistent state without having to continuously express the initiating effectors (Paschos and Allday 2010). Host genes involved in cell cycle progression, senescence, survival, inflammation and immunity are prime candidates as targets for such epigenetic control. Upon a microbial attack, host cells undergo massive changes in their transcriptional program, mobilizing

genes involved in key processes (e.g, immunity, cell death/survival, and adhesion/motility) to trigger an appropriate response (Jenner and Young 2005, Bierne et al., 2012). It is thus not surprising that successful pathogens have developed specific mechanisms to deregulate the expression levels and/or kinetics of these defense genes. Host transcription factors are first obvious targets to reprogram the genome and bacteria use diverse tricks to alter their function. For instance, bacterial factors can hijack cellular signaling pathways that activate or sequester transcription factors (e.g., NF-κB, IRF/STATs, or AP-1) in the cytosol of targeted cells, or manipulate their half-lives via posttranslational modifications (Ribet and Cossart 2010; Perrett et al. 2011). Furthermore, *H. pylori*-mediated inflammation triggered lymphocyte and macrophage infiltration, appears to have a key role in induction of methylation (Hur et al. 2011). Thus, although the mechanisms by which *H. pylori* induces DNA hypermethylation are still unclear, the infection-associated inflammatory response is a tempting explanation. Among signals resulting from chronic inflammation, elevated levels of IL1β and nitric oxide (NO) are proposed to contribute to influence the recruitment of DNMTs at specific loci (Ding et al. 2010; Ushijima and Hattori 2012).

The discoveries made in this study are strongly backed up with the reports made by Raid, (2013): that genomes are regularly targeted by epigenetic regulatory mechanisms in infected cells. In addition, proteins encoded by microbial genomes may disturb the action of a set of cellular promoters by interacting with the same epi-regulatory machinery. The outcome of this may result in epigenetic dysregulation and subsequent cellular dysfunctions that may manifest in or contribute to the development of pathological changes. How epigenetic methylation decorations on DNA and histones are started and established remains largely unknown. The inherited nature of these processes in regulation of genes suggests that they could play key roles in chronic diseases associated with microbial persistence; they might also explain so-called hit-and-run phenomena in infectious disease pathogenesis (Raid, 2013). Microbes infecting mammals may cause diseases by causing hyper-methylation of key cellular promoters at CpG di-nucleotides and may induce pathological changes by epigenetic reprogramming of host cells they are interacting with, elucidation of the epigenetic consequences of microbe–host interactions may have important therapeutic implications because epigenetic processes can be reverted and elimination of microbes inducing patho-epigenetic changes may prevent disease development (Raid, 2013). The attachment of importance to presence of microbial proteins in circulation is in line with many studies making effort to discover the role of microbial agents to tumour or cancer development.

This work looked at the molecular expressions and their interactive or expression correlation under three different possible influences such as presence of combined state of antigenic components: involving subjects with evidence of microbial antigen and subjects with other antigens; presence of Microbial antigenic components only and absence of microbial antigen. In subjects with combined antigenic components, expression of circulating immune complex was detected in benign and malignant subjects at significantly raised levels. Brunner and Sigal (2000) reported that under normal physiological condition, immune complexes are undetectable and that detectable levels are found in chronic infections, persistent exposure to foreign substances and ongoing acute infection. In this study, circulating immune complex level of 56.47ugeq/ml was being used as reference, above which pathological conditions are suspect. This can be understood against the background that in tropical environments such as in Nigeria, people are exposed to much environmental pollutions including microbial agents capable of inducing immune response (Tanyigna et al., 2004, Ezeani et al., 2011a). This may also be sequelled to peculiarities to individuals' approach to life, with due consideration to hygiene, environmental exposure, nutritional status and attitude to medications, and nature of work. This study is in full agreement with earlier findings of Perveen et al., (2010) indicating higher values of Circulating immune complexes in patients with cancer.

In subjects with evidence of microbial antigenic components, the mean level of circulating immune complexes was raised in all the groups indicating that the capacity to mediate immune complex formation was more in the presence of microbial antigens. It is possible that other antigenic components may induce raised level of immune complexes, but the number of subjects with microbial antigenic components, was overwhelming. The importance of immune complexes (IC) in accessing tumourigenesis bothers on its ability to induced inflammatory responses, which are significant contributors to the pathogenic mechanisms responsible for tissue destruction. The initiation of this inflammatory response by IgG-containing immune complexes may be triggered by either activation of the soluble proteins of the complement system, or engagement of the cellular receptors for IgG (the Fc receptors) (Merle et al., 2015). Immune Complex injury may also result from activation of resident tissue inflammatory cells and/or the recruitment of circulating monocytes or neutrophils to sites of deposition. As an example, the fragment crystalizable gamma receptor (FcγR)-bearing resident cells in the lung, responsible for initiating the inflammatory response, is likely to be the alveolar macrophage. These cells elaborate several inflammatory mediators critical to Immune Complex (IC)-induced alveolitis, including the cytokines IL-1, TNF-α, IL-6 and several chemokines, including MIP-1 and C-X-C cytokines

(Higham et al., 2016). There is paucity of data supporting such molecular activation or interactive process in the breast tissues. The present study evaluated the interaction of several molecules responsible for initiating inflammatory response by correlating their expressions in the system under the proven evidence of combined antigenic component, evidence of microbial antigenic components alone and without evidence of microbial antigenic components. This is sequel to evidence firmly supporting a link between chronic inflammation and cancer that occurs in various organs including breast (Higham et al., 2016). Moreover, sequel to the research highlights of Ledoux and Perkins (2014), that inflammation-associated cancer consists of white blood cells, notably tumor-associated macrophages (TAM) and T lymphocytes; increased generation of reactive oxygen species (ROS)/reactive nitrogen species (RNS); altered cytokine/chemokine expression; and augmented molecular signaling via nuclear factor kappa B (NFκB), signal transducer and activator of transcription proteins (STATs), cyclooxygenase-2 (COX-2), this work also upholds that retention of increased expression of these inflammatory molecules may have a link to tumour development. The correlation study done in this work helped to access the suspected influence of immune complexes in stimulating the expression of these molecules.

The out come of this study in malignant subjects showed that of all the expressed molecules, expression of CIC would enhance oestrogen expression in the presence of microbial antigenic components. This could be a viable link to cancer progression and metastasis in malignancy. Little is known about the link between inflammation and oestrogen expression in the development of breast cancer. But report has shown that inflamed white adipose tissue (WAT) within the breast is associated with elevated levels of proinflammatory mediators, enhancing expression of aromatase (the rate-limiting enzyme for estrogen biosynthesis), and increased oestrogen receptor-α (ER-α)-dependent gene expression. Circulating oestrogens, such as oestradiol, are known to stimulate the proliferation of breast epithelial cells and potentially exert a mutagenic effect. Higher levels of circulating oestradiol as a result of increased adiposity and aromatase expression are thought to contribute, in part, to the greater risk of ER/PR-positive breast cancer in obese postmenopausal women (Cleary and Grossmann 2009, Iyenger et al., 2013). Thus positive associtation of CIC with oestrogen reveals another possible source of oestrogen stimulation via immune complexes that harbour microbial antigens in subjects with malignant tumour. Previously, the work of Hotamisligil, (2010) which showed that activation of oestrogen signaling pathways via inflammation-mediated up regulation of aromatase promotes breast cancer. Virchow first postulated that cancer originates at sites of chronic inflammation, in

part based on his hypothesis, that some classes of irritants causing inflammation also enhance cell proliferation. This work agrees with Virchow's report in that expression of oestrogen via CIC mediated inflammatory pathway, would enhance tumour cell proliferation and growth.

In subjects with malignant breast tumour, this work reveals high expression of NFkB, IgG, TNF-α, OH2DG, but reduced serum levels of progesterone, especially in subjects with presence of microbial antigenic components. Chaturvedi et al., (2011), indicated that one strategy to evaluate the relationship of cancer with chronic inflammation is to measure circulating levels of inflammatory markers. Most previous epidemiologic investigations of circulating inflammatory markers and cancer have included a narrow range of markers (e.g., C-Reactive Protein, IL-6, IL10, TNF-alpha etc.) (Chaturvedi et al., 2011). Furthermore, Chaturvedi et al., (2011), also indicated that the process of inflammation is complex and involves multiple key mediators, including chemokines, pro-inflammatory cytokines, anti-inflammatory cytokines, growth factors. Thus, indicating possible variations in molecule expressions in breast cancer. However the expression correlation between these molecules was weak or negligible in subjects with malignant tumour, except the association between circulating immune complexes and estrogen as mentioned above. It seems that in malignancy, the influential role of some molecules in immune response is depreciated. This may be attributed to degenerative stage of the cancer in which the cancer cell development overwhelmes the process of normal immune response. It is important to note that the polarization of immune cells and molecules, which may enable tumour cells to take over the normal cells activities may alter normal cell-cell and intermolecular associations as observed in this study. On the the other hand, in subjects with benign tumour, though with lower expression of CIC, NFkB, IgG TNF-alpha OH2DG and higher expression of Progesterone level than in subjects with malignant tumour, higher number of molecular expression correlation was observed. This may be attributed to the expected active immunologic response to cub tumour development and quell invasion. Increase in circulating immune complexes with microbial antigenic components, could be an influential factor to raised serum oestrogen levels based on the positive correlation of CIC and estrogen in both subjects with benign and malignant breast tumours. Positive correlation of other molecules such as NF-kB and TNF-alpha; NF-kB and OH2DG; IgG and OH2DG as well as TNF-alpha and OH2DG, could be other possible immuno-molecular links for tumour suppression or tumour progression, expecially in subjects with benign tumour.

The choice of these molecules was supported by the reported involvement of array of mechanisms under which microbes become oncogenic, categorized within four general processes: (1) direct effects on signal transduction pathways in host cells, (2) chronic inflammation, (3) changes in host physiology, and (4) effects on other microbes. These processes are not exclusive because many important microbial agents are known to have several mechanisms belonging to more than one process. An example of the first process (signal transduction pathways) is microbes that invade cells that ultimately are sloughed, which often have a selective advantage if they can delay turnover of the host cell. Microbial promotion of cell longevity could involve inhibition of apoptosis via effects on p53 (Oliviera, 2007), up-regulation of cell cyclins (Chang et al., 2006, Blaser, 2008), or effects on stroma (Pillinger et al., 2007). This is in line with the correlation of NF-kB and TNF-alpha; NF-kB and OH2DG. The second process; chronic inflammation, promotes neoplasia per se through many mechanisms (Medzhitov, 2010) including increased host cell turnover that increase the probability of mutagenic events and genotoxic effects of reactive oxygen and nitrogen species stemming from inflammation (Blaser, 2008). This is in line with the correlation of IgG and OH2DG as well as TNF-alpha and OH2DG. Tumour necrosis factor (TNF)-α is a marker of chronic inflammation. It is a proinflammatory cytokine with pleiotropic downstream effects (Aya et al., 2012). Research has shown that TNF-α is sufficient to induce DNA damage or geneotoxicity in line with tumour necrosis factor receptor (TNFR) signalling (Aya et al., 2012). Aya et al., (2012) also reported that in the wild-type mice, 500 ng per mouse of TNF-α was sufficient to induce DNA damage to multiple cell types and organs 1-hour post-administration. The third process; changes in host physiology due to microbes and the host responses they promote, can occur at a tissue, organ, or cellular level (Francois et al., 2008). Phenomena such as immunosuppression, hormonal perturbation and effects on motility in organs where flow is critical are examples of these physiologic changes. This is in line with the correlation of CIC and estrogen, IgG and OH2DG as well as TNF-alpha and OH2DG. The fourth process, microbes affecting other microbes, can involve any and all of the previously described processes and mechanisms (Blaser, 2008).

The persistent stimulation of pro-inflammatory inflammatory molecules in presence of microbial antigenic components as observed in this study may push the inflammatory response out of proportion to the threat it is dealing with or may be directed against inappropriate targets. In the first case, the immunopathological effect can be more damaging to the body than the pathological effect of the microbial agent itself. Research has shown that the inflammatory

responses necessary for enabling an immune reaction may however, set the stage for promoting neoplastic disease (Hanahan and Weinberg 2011). The exact initiation process of breast cancer is unknown, although several hypotheses have emerged. Inflammation has been proposed as an important player in tumor initiation, promotion, angiogenesis, and metastasis, all phenomena in which cytokines are prominent players (Esquivel-Velázquez et al., 2015). Virchow described leukocyte infiltrates within tumours, which provided the first evidence for what is now commonly considered a hallmark of cancer. These leukocyte infiltrates were initially thought to be solely indicative of tumour immune surveillance and antitumour immune responses but it is now clear that they can exert both tumour-suppressive and tumour-promoting effects, and the underlying mechanisms are starting to be understood (Hanahan and Weinberg 2011). When tissues are injured or are exposed to irritants, damaged cells are removed by the induction of cell death pathways, while cell proliferation is enhanced to facilitate tissue regeneration in an attempt to re-establish tissue homeostasis (Hanahan and Weinberg 2011). Proliferation and inflammation resolve only after insulting agents are removed or tissue repair is completed. In contrast, when insulting agents such as immune complexes persist over time, sustained cycles of cell proliferation and death in environments rich in inflammatory cells and their bioactive products may increase neoplastic risk and foster tumor progression (Karin and Clevers 2016). Accumulating evidence shows that chronic inflammation can promote all stages of tumorigenesis, including DNA damage, limitless replication, apoptosis evasion, sustained angiogenesis, self-sufficiency in growth signaling, insensitivity to anti-growth signaling, and tissue invasion/metastasis (Karin and Clevers 2016)**.**

The normal expression of Nf-KB found in control subjects with microbial antigens, as opposed to the high expression found in subjects with benign and malignant tumours may be attributed to the difference between the types of microbial agent evidenced in the tumour subjects and control subjects. In this study, the subjects with tumour, harbour a lot of mixed infections while the control subjects harbour single infection of *Plasmodium falciparum* only. Suffice it to say that in malaria infection, there could be a mechanism for inhibiting NFkB translocation to the neuclus therefore suppressing its expression. This agrees with the work of Labbé et al., (2010) which reported that presence of malaria parasite can induce increased expression of caspase-12 which has the ability to suppress the inflammatory response to malaria. Mechanistically, caspase-12 presence competed with NF-kB essential modulator (NEMO) for association with IkB kinase (IKK)-a/b, effectively preventing the phosphorylation of the IKK complex and inhibiting downstream transcriptional activation by NF-kB (Labbé et al., 2010). This present study has

raised the importance of synagystic roles of microbial infections in regulating immune responses and tumour progression. NF-κB exerts effects on almost all cell types in the body, playing an important function in inflammation, immune responses, cell cycle, and cell survival (Ledoux and Perkins, 2014). Thus the increased expression of NF-kB in benign tumour may as well induce chronic inflammation and subsequent tumour microenvironment if the translocation process persists.

Observed significant increase in expression of IgG in subjects with evidence of microbial antigenic components, may encourage more Fc binding by the polymorphs leading to generation of more reactive oxygen species and subsequent oxidative stress markers. Immunoglobulin G (IgG) is a chronic marker during which Th cells have polarized to Th2, which secretes cytokines that stimulate B cell activation and subsequent antibody production (Bassaganya-Riera et al., 2012). Th2 cytokines such as IL-4, IL-5, IL-10, IL-13, inhibit cytotoxic T cell activities which are required to destroy tumour cells (Bassaganya-Riera et al., 2012). Persistent production of these cytokines due to continuous need to generate antibody, would enable tumour invasion, progression and metastasis (Yang et al., 2013). Conditionally, this present study agrees with the report of Chen et al., (2010) which indicated a significant increase in IgG light chain protein expressed in malignancy compared to benign soft tissue tumors. The out come of this present study suggests that the situation may differ under certain conditions such as retention of circulating immune complexes and their component antigens as well as under different methylation status. In subjects that harbour immune complexes with microbial antigenic components, there was significant difference in expression of IgG between benign and malignant breast tumours but no significant difference was found in presence of immune complexes without microbial antigenic components. Moreso significant increase in IgG expression was found in malignancy in subjects with hypomethylated DNA but not in hypermethylation and unmethylation. This may be involving in defining the role of IgG expression in tumourignesis. Thus the persistent presence of microbial antigen may utilize IgG expression pathway to enhance tumour development by inducing suppression of cytotoxicity of tumour cell even at the earliest part of its development. Increased expression of serum IgG was observed in this study to be a more regular and stably stimulated pathway in the presence of MAC compared to its absence. In consideration of the supposed healthy control subjects and the persistent presence of complexed IgG, complement would continually be activated, macrophages and neutophils continually attracted and as such reactive oxygen species would continually be released with gradual slow or unnoticable rate of DNA oxidative damages taking place. While such

persistence would induce invasion in benign subjects, it would enable progression and metastasis in malignant subjects and possibly induce initiation of tumour with respect to formation of tumour microenvironment in supposed healthy subjects. This report is in line with the work of Yang et al., (2013), which reported positive correlation of plasma IgG levels to breast cancer development. The increased plasma IgG level could be derived from the patients' immune response to microbial antigens or from cancer-produced IgG in response to tumour specific or tumour associated antigens. This is in line with the work of Yang et al., (2013), which reported correlation of IgG expression with malignancy of the breast diseases and histologic subtypes of carcinoma. However, the works of Chen et al., (2010) and Yang et al., (2013), did not consider microbial involvement and methylation status being the difference with this present study. On the other hand, since report has shown that Immunoglobulin G (IgG) immune complexes, target Fc-gamma receptors on dendritic cells (receptor mediated cross presentation) to shuttle exogenous antigens efficiently into the cross-presentation pathway, a well-described route for the induction of strong $CD8^+$ T cell responses (Baker et al., 2013). Increased expression of IgG could turnout to be a potent source of immunotherapy by enhancing cytotoxic T lymphocytes pathway. (Baker et al., 2013, Guilliams et al., 2014).

Based on positive significant correlation of OH2DG with IgG in subjects with benign breast tumour and control subjects both in the presence and absence of microbial antigens, this study, yielded to the ealier research showing initiation of inflammation through adaptive immune response and could be a strong pathway towards chronic inflammatory induced oxidative stress (Yang et al., 2013). Yang et al., (2013), found correlation of plasma IgG levels to breast cancer development. The increased plasma IgG level could be derived from the patients immune response to microbial antigens or from cancer-produced IgG in response to tumour specific or tumour associated antigens. It is believed that recognition of these immune complexes in tissues by cells having Fc receptors initiates or increases tissue destruction by macrophages and possibly other cells such as polymorphonuclear leukocytes (Yang et al., 2013). Andreu et al., (2010), found that IC stimulation of leukocyte FcRγ is critical for establishing a protumor microenvironment in premalignant tissue that directs not only recruitment of leukocytes from peripheral blood but also induces bioeffector functions once within neoplastic tissue. As such, proangiogenic and protumorigenic functions of mast cells and macrophages are differentially regulated by humoral immunity and functionally contribute to squamous carcinogenesis (Andreu et al., 2010). These findings have broad clinical implications as they reveal critical signaling pathways regulated by humoral immunity and FcRγ to target therapeutically in patients at risk

for cancer development, e.g. patients suffering from chronic inflammatory diseases, as well as individuals harboring premalignant lesions where chronic inflammation compromises tissue integrity and enhances risk of malignancy (Andreu et al., 2010). This is in line with the correlation of CIC with IgG in control subjects in this study and could be a potent pathway in collaboration with significant positive association of TNF-alpha with 8-OH2DG as well as IgG with TNF-alpha for initiation of tumour in supposed healthy subjects. Earlier, Mazlan et al., (2012) indicated that engagement of the Fc portion of the complexed IgG is capable of up-regulation of TNF-alpha thereby stimulating inflammatory response. Ig expressed in breast cancer or other epithelial cancers is likely to be useful as prognostic marker to guide therapy, especially immunotherapy (Yang et al., 2013). Prevalent expression of IgG in human carcinomas and its growth-promoting functions may have important implications in growth regulation and targeted therapy of human cancers. Blockade of tumor-derived IgG by either antisense DNA or antihuman IgG antibody increased programmed cell death and inhibited growth of cancer cells in vitro. More importantly, administration of antihuman IgG antibody also suppressed the growth of an IgG-secreting carcinoma line in immunodeficient nude mice (Qiu et al., 2003; Yang et al., 2013).

Increase in expression of tumour necrosis factor alpha (TNF-α) and 8-OH2DG was very remarkable in subjects with malignant and benign breast tumour, with evidence of microbial antigenic components. Research has shown that several proinflammatory genes, for example, tumor necrosis factor (*TNF*) and members of its superfamily, IL-1a, IL-1b, IL-6, IL-8, IL-18, chemokines, VEGF, MMP-9, 5-LOX, and COX-2, play critical role in the control of apoptosis, angiogenesis, cell proliferation, invasion, and metastasis and that overexpression of transcription factors like NF-κB, which becomes constitutively active in most tumors, is principally responsible for the expression of these genes (Dong et al., 2010). Furthermore, the survival and growth of the inflammatory initiated cells are prerequisite for tumor development, adding that many inflammatory mediators such as interleukins, eicosanoids, and chemokines are able to motivate the propagation of both normal and cancer cells (Yasmin et al., 2015). When TNF-α administration was combined with interleukin (IL)-1β, another proinflammatory cytokine, DNA damage persisted for up to 24 hours. When combined with IL-10, an anti-inflammatory cytokine, decreased genotoxicity was observed *in vivo* and *in vitro*. TNF-α/TNFR-mediated signalling is therefore sufficient and plays a large role in mediating DNA damage to various cell types (Aya et al., 2012). This therefore supports the significant positive association of TNF-alpha and OH2DG observed in this study. This information has justified the clinical importance of TNF-α

expression, however depending on the circulating level of the molecule but it is important to appreciate the possible gradual accumulation due to chronic situation. The work of Aya et al., (2012), also justifies the importance of correlating NF-κB and TNF-α in this study considering the pleiotropic (Phosphorylating effect) effect of TNF-α on NF-κB **(**Connelly et al., 2011). Connelly et al., (2011) reported that inhibition of TNF-α and NF-kappaB (NF-κB) transcription factor is proved to be protective with respect to chemical induced mammary gland carcinogenesis. Further, the in vitro activation of the TNF-α/NF-κB pathways has induced an invasive and malignant behaviour in breast cancer cells and during an infection, pathogen clearance is dependent on proper regulation of TNF- α, but aberrant expression of TNF-α can lead to significant morbidity and mortality (Connelly et al., 2011). Indeed, investigation carried out by Kamel et al., (2012), strongly suggests that the chronic expression of TNF-α in breast tumors actually supports tumor growth. The tumor-promoting functions of TNF-α may be mediated by its ability to induce proangiogenic functions, to promote the expression of matrix metalloproteinases (MMP) and endothelial adhesion molecules, and to cause DNA damage via reactive oxygen (Kamel et al., 2012). Furthermore, with interest in origination of tumour, it is important to report the slight increase in the mean levels of TNF-α and 8-OH2DG in control subjects without evidence of microbial antigenic components. Thus such slight increase should not be overlooked especially when it persisted, considering the clinical importance of the molecues.

The significant increase in level of 8-OH2DG in malignant and benign breast tumour subjects compared to the level in control subjects, suggests that the DNA of the cells of the breast tissues in the tumour subjects are facing more oxidative damages. However, the burden of the expression may be higher in subjects with malignant breast tumour. The expression of this 8-OH2DG molecule in malignant breast tumour was more prominent in subjects with microbial antigenic component. In this study, it was observed that the slight increase in molecular expression seen in control subjects is prevalent in the presence of immune complexes without microbial antigenic components as opposed to the prevalence of molecular expression in the presence of immune complexes with microbial antigenic complexes observed in tumour subjects. Suffice it to say that immune complexes generally (with presence of microbial antigenic component or not) can induce raised inflammatory molecular expression and that microbial infections and their effects is highly dominant in subjects with tumour, thus, playing dominant influential role in tumour development or progression. This slight increase in oxidative stress marker detected in apperantly healthy control individuals, would better be analysed by the

Immune Complexes engaging Fcγ receptors on the surface of the neutrophil, triggering degranulation and production of ROS into the tissue. Activation of primed neutrophils, either by phagocytosis of opsonized bacteria or by frustrated phagocytosis, generates the rapid production of reactive oxygen species (ROS) via the action of NADPH oxidase (Wright et al., 2010, Natallia et al., 2015). Activation of the respiratory burst generates ROS production (O_2^-, $HO^\bullet$, $^1O_2$ and H_2O_2) by NADPH oxidase, and hypochlorous acid (HOCl) via the action of myeloperoxidase. Oxygen radicals cause damage to DNA, oxidation of lipids, proteins and lipoproteins, and may be implicated in gene mutations. 8-OH2DG is the product of such oxidative DNA damage and remains the major marker for oxidative DNA damage (Wright et al., 2010). Increase in oxidative stress and TNF-alpha molecules in healthy control subjects and subsequent molecular activities as described above give room to say that their persistence could cause epigenomic cell alteration due to constant cellular perturbation and thus enhance tumour initiation. The ability of TNF-alpha to cause the release of NF-kB from its IkB complex in the cytosol and enable the translocation of NF-kB to the nucleus, is a significant factor in this study. This is in line with the work of Aya et al., (2012) which reported potential mechanisms of tumour necrosis factor-receptor (TNFR) signalling-induced DNA damage. TNF-α acts by binding to the TNFRs, which results in recruitment of various signal transducers activating caspases, AP-1 and NF-κB. TNFR signalling induces nicotinamide adenine dinuleotide phosphate (NADPH) oxidase and inducible nitric oxide synthase (iNOS), alters glutathione levels and causes mitochondrial disruption, all of which have the capacity to produce RONS and induce DNA damage. They also demonstrated that NF-κB signalling is involved in induction of DNA damage (Aya et al., 2012). Furthermore, as NF-κB signalling inhibitors decreased genotoxicity, downstream mediators of NF-κB such as cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinuleotide phosphate (NADPH) oxidase may drive TNFR-mediated DNA damage. Persistence of these molecular activities and interactions could perturb cell epigenomic activities and induce methylation shift resulting to hypomethylation or hypermethylation.

Increased serum level of oestrogen was noted in subjects with benign breast tumour compared to the levels in malignant breast tumour subjects and control subjects. Specifically, this study revealed that presence of immune complexes with microbial antigenic components, can significantly up-regulate serum level of estrogen in subjects with benign and malignant breast tumours. In this study, the interest in estrogen regulation is focused on presence of immune

complexes generally with consideration to age. Oestrogen is a female hormone that is mainly produced by the ovaries (Files et al., 2011). Oestradiol (E2) is the most important estrogen in non-pregnant females who are between the menarche and menopause stages of life. The steroid 17β-estradiol is the most potent and prevalent endogenous oestrogen, but several metabolites of oestradiol also have oestrogenic hormonal activity. The mean age of the subjects with benign breast was 37.9±10; malignant breast tumour, 49.6±11 and control subjects 35.9±9 years. Files et al., (2011), reported that the normal range of oestrogen varies depending upon the patient's age. Typically women aged 20 to 29 will have an average level of 149 pg/ml (pictograms per milliliter), women aged 30 to 39 years will have an average level of 210 pg/ml. And those over 40 but not in menopause will have an average level of 152pg/ml. These average levels can vary day to day depending on each female's menstrual cycle. Estrogen levels are considered low when the range is 10 to 20 pg/ml (Files et al., 2011). Using this as a panancea, this work revealed that subjects with benign tumour had mean estrogen level of 212±97pg/ml but when seperated, those who harbour microbial antigenic component had higher serum level of estrogen (222±90pg/ml), at the mean age of 37.5±11, while those without microbial antigenic components had lower level of estrogen (141±138pg/ml) at mean age of 41±7. Furthermore, subjects with malignant breast tumour had mean estrogen level of 173±78. Physiologically a lower level of oestrogen is expected in these subjects at the mean age of 49.6±11 when menopausse is expected to be setting in or have set in. Otolorin et al., (1989), Ellen, (2011), reported that the average age of menopause in Nigeria is 52.8 years. Due to the depletion of primordial follicles, ovarian estradiol secretion ceases at menopause and is followed by very low levels of circulating estradiol generating the typical menopausal symptoms (Labrie, 2015). Moreover, those who harboured immune complexes with microbial antigenic component had higher serum level of estrogen (175±81pg/ml), at the mean age of 50.1±12, while those without microbial antigenic components had lower level of estrogen (166±71pg/ml) at mean age of 47.6±4.2. At the mean age of 31.1±7.6, the control subjects who harbour microbial antigenic components had estrogen level of (142±50pg/ml), while those without microbial antigen had estrogen level of 136±65 at the mean age of 37.2±9.0. This analysis indicates that immune complexes with microbial antigenic component may upregulate estrogen level irrespective of age or physiological status while immune complexes without microbial antigen may down regulate estrogen level. Once inside the cell, estrogen binds to and activates oestrogen receptors (ERs) which in turn modulate the expression of many genes. It has since become clear that human breast cancers are dependent upon oestrogen and/or progesterone for growth and that this effect is mediated through ERs and progesterone receptors (PRs). Oestrogen has a proliferative effect on breast tissue. They are

related to increased mitotic activity and believed to enhance tumour growth. (Roy and Vadlamudi 2012, Mahor et al., 2015).

On the other hand, serum level of progesterone was significantly reduced in subjects with malignant breast tumour compared to benign subjects and control subjects in the presence of microbial antigenic components, while reduced levels were observed in the presence of immune complexes without microbial antigenic components. The pattern of expression of progesterone indicates that the lower the progesterone levels in breast tumour the poorer the prognosis. Homeostasis of the human immune system is regulated by multiple factors whose alterations may result in pathological conditions. These factors include the sex hormones that affect both phenotype and function of immune cells through interaction with specific receptors expressed by these cells. In particular, activation of sex hormone receptors by hormone binding may impact many biological processes such as immune cell differentiation and maintenance of immune homeostasis. In turn, they are involved in the pathogenesis of a wide spectrum of diseases, including autoimmune disorders and cancer. Although it has been suggested that estrogens may enhance immune reactions, while androgens and progesterone may reduce immune system function, the mechanisms underlying this scenario are far from being elucidated. Understanding the effects of the sex hormones on immune-mediated diseases could lead to the identification of innovative and readily available therapeutic interventions, such as hormone antagonists or agonists, to manage cancer and prevent tumourigenesis. The low level of serum progesterone and raised level of serum estrogen in subjects with malignant breast tumour, that harbour immune complexes with microbial antigen in this study may suggest the role of microbial agents in influencing the molecular status of breast cancer. Research has shown that raised estrogen or progesterone level would augument development of estrogen receptor positive (ER+) or progesterone receptor positive (PR+) by providing the enabling binding sights for the receptors (Elana et al., 2015). The hypothalamus-pituitary-adrenal (HPA) serves as a key element of this communication pathway. Activation of the HPA axis is known to serve the body's response to stress, which can be defined as any physical or psychological stimulus that disrupts the body's homeostasis (Hafner et al., 2013). Additionally research has shown that elevated concentrations of progesterone during pregnancy inhibit the development of Th1 (helper T-cell immune type 1) responses and the production of proinflammatory cytokines such as IFN-γ, while promoting Th2 immune responses, including the synthesis of anti-inflammatory cytokines such as IL-4, IL-5, and IL-10 which enhances a pathway to immunoglobulin secretion (Dionne and Sabrea 2012).

This development may not be the same in female with tumour as significant negative correlation of immunoglobulin G with progesterone was observed in this study.

This work revealed negative association between progesterone and NF-kB suggesting a pathway for supresssion of NF-kB signalling. Ealier research had shown that progesterone is typically an immunosuppressive agent since it inhibits the activation of NFκB and increases the expression of the suppressor of cytokine signaling protein (SOCS1) in macrophages (Hafner et al., 2013). Murphy et al., (2010) demonstrated that pre-treatment of human macrophages with estradiol/progestrone attenuates lipopolysaccharide (LPS)-induced TNF-a expression through the inhibition of NF-kB activation, showing that activation of macrophages with LPS inhibits the expression of kB-Ras2 but that pretreatment with estradiol abrogates this inhibition, leading to enhanced expression of kB-Ras2 (Murphy et al., 2010). This work has revealed that there are numerous pathways to induction of chronic inflammation and DNA oxidative stress. Moreover, Good knowledge of these molecular interactive pathways could be the proper channels for blocking the generation of more epigenetic cell alterations and cancer cells and their spread (Mazlan et a., 2012).

In this study, demethylation (unmethylation and hypomethylation) is most indicated for pathological consequencies amongst other methylation patterns and thus regarded in this study as most influencial pathways to tumourigenesis especially in subjects who harbour CICs with microbial antigenic components. DNA methylation is an important process during normal development and homeostasis, and frequently becomes deregulated during disease pathogenesis, including carcinogenesis **(**Reik, 2007, Weiguo et al., 2013). This work accesed the expression of inflammatory and oxidative markers, immunoglobulin G and sex hormones in different patterns of DNA methylation to associate them with different methylation shifts. Research has shown that DNA methylation profiles at the level of individual CpGs exhibit substantial variation across molecules, cells, tissues, and phenotypes within the normal human population (Lister et al., 2011). So far, little is known about the functional implications of the precise spatial distribution patterns of DNA methylation among tissues/individuals. Functional analysis of individual CpG dinucleotides is desirable for better understanding of normal cellular physiology, inter-individual variation of DNA methylation, disease pathogenesis and for the optimization of cancer biomarker searches for robustness and precision (Weiguo et al., 2013)

In this study, it was discovered that the increase in CIC in presence of microbial antigenic components, was aligned with abnormal DNA methylation in subjects with benign and malignant tumour. Based on this, it is suggested that increased level of circulating immune complexes (CICs) with microbial antigenic components could be synonymous with abnormal DNA methylation (specifically hypomethylation) in subjects with benign and malignant breast tumour. But in control subjects, hypomethylatin was found only in subjects without microbial antigenic components. This indicates that other antigenic components other than microbial, may influence individuals' methylation status. Thus the specific influence of *Plasmodium faciparum* on DNA methylation is questioned because almost all the control subjects harbour only *Plasmodium falciparum*.

This present study revealed upregulation of NFkB, IgG, TNF-alpha, OH2DG and estrogen in subjects with benign and malignant breast tumours in the presence of abnormal DNA methylation (hypomethylated DNA). Suffice it to say that up regulation of these molecules is synonymous with DNA methylation shift. It is important to report that CIC with microbial antigens and oestrogen found in significant positive correlation may represent an important link to DNA methylation shift and possible tumour development, considering a positive correlation of oestrogen and DNA methylation (unmethylation) in subjects with malignant breast tumour. This present report is in line with the submission that DNA methylation can regulate gene transcription as indicated by Rappe et al., (2015), which also justifies the positive correlation of some molecules in different pattern of DNA methylation as well as negative correlation of IgG, OH2DG and unmethylation and hypomethylation respectively, as observed in this study. High expression of NF-kB in hypomethylated control subjects without microbial antigenic components is supported by the research report that there are several lines of evidence that relate NF-*k*B to carcinogenesis (Aggarwal and Gehlot 2009). NF-*k*B regulates most of the genes linked to inflammation – some of those may have protumor effects; NF-*k*B regulates anti-apoptotic genes, genes related to proliferation, invasion and angiogenesis. NF-*k*B may be activated by various exogenous and endogenous factors including proinflammatory cytokines, T-and B-cell mitogens, biological, physical and chemical stressors. Rappe et al., (2015) had reported that all cells may contain the same genes, but their expression and function may differ widely from one tissue to another and that expression of tissue and cell specific genes is regulated by complex interactions involving tissue and cell specific transcription factors, extracellular signals, chromatin packing and epigenetic changes including DNA methylation of cytosine residues. This report justifies the upregulation of the molecules in this study. Suffice it to suggest that

while presence of microbial or none microbial antigenic components of immune complexes may be inducing DNA methylation shift, the resulting abnormal DNA methylation, also induces abnormal gene regulation and thus altering normal cell activities. This is based on the molecular expression correlations observed in subjects with different patterns of DNA methylation in this study. There is clear indication that aberrations in DNA methylation would cause aberrant regulation of pro-inflammatory molecules and female sex hormones as well as induce oxidative stress as opposed to the situation where DNAs are normally methylated (Rappe et al., 2015). Despite the general trend for CpGs throughout the genome to be methylated, CpG sites in CpG islands and especially those associated with gene promoters are usually unmethylated which correlates with their potential for active gene transcription (Stearns et al., 2007, Jovanovic et al., 2010). In cancer cells, CpG islands that are normally unmethylated may become methylated, which might result in silencing of important genes, such as tumor-suppressor genes. At the same time, CpG dinucleotides in other regions may become unmethylated, leading to poor transcriptional repression of normally silenced genes such as oncogenes or retrotransposons. It was also observed that different patterns of methylation would regulate molecular expression differently (Jovanovic et al., 2010). In unmethylated condition, significant positive expression correlations of CIC and NFkB; CIC and IgG as well as NF-kB and IgG in this study, are suggested to be complex interactive pathways that can inducing abnormalities in regulation of cell activities in subjects with benign tumour, while CIC and oestrogen as well as oestrogen and progesterone are suggestive complex interactive link to progression in malignancy. Under hypomethylation, NF-kB and esstrogen as well as estrogen and progesterone associations were prominent in subjects with benign breast tumour while OH2DG and progesterone as well as negative significant association of CIC and NFkB were prominent in subjects with malignant breast tumour. It is important to report that CIC and progesterone; NF-kB and progesterone as well as IgG and progesterone found in significant negative correlation may represent the important inhibitory pathways to tumour or disease progression. These molecular interaction or the expression correlation, serves the bases for understanding the development activities in normal tissues and tissues already exposed to tumour microenvironment. Research has shown that stimulation of cells with a variety of ligands, such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), or pathogen-associated molecular patterns (PAMPs), leads to the rapid phosphorylation of IκB by the IκB kinase complex (IKK). Ea and Baltimore (2009), blocked methylation reactions in cells with a broad methylation inhibitor, 5′-deoxy-5′-methylthioadenosine (MTA) and observed that it inhibited the TNFα-induced response of an NF-κB-driven luciferase reporter in a dose-dependent manner. MTA does not affect IκBα

degradation or p65 nuclear translocation (Ea and Baltimore, 2009). Suffice it to say that the use of such methylation inhibitors as 5′-deoxy-5′-methylthioadenosine could serve a good immunotherapeutic purpose for blocking or checking chronic inflammatory pathways that involves p65 nuclear translocation, thus blocking tumour initiation or progression.

In hypomethylation, there is increased expression of TNF-α in all the groups irrespective of antigenic components, indicating possible persistent inflammatory reponses. But in unmethylated condition, TNF-α and OH2DG were up regulated in subjects with benign and malignant breast tumour. The burden of these molecular expressions in the presence of hypomethylation and unmethylation is suggested to be significantly higher in malignancy especially in subjects with microbial antigenic components. This indicates increased oxidative DNA damage in malignancy and may encourage metastatic process as more DNA are being damage. Persistence of such damages on tumour suppressor gene in benign and healthy subjects would encourage hypomethylation and thus, switch off the gene thereby encouraging abnormal cell proliferation and subsequent tumour development. This also indicates the dominant and clinical important role of microbial infections in breast cancer initiation and progression. Thus, suggesting microbial involvement in DNA methylation shift to hypomethylation and unmethylation through chronic inflammation and DNA oxidative damage pathways. 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a modified or oxidized nucleoside of DNA base, which is the most commonly, studied and detected product of DNA damage that is excreted in urine upon DNA repair. So the occurrence of 8-OHdG (8-hydroxy-2'-deoxyguanosine) has been used to study damaging effects on DNA of ROS (Szymańska-Chabowska et al., 2009, Sova et al., 2010). The present findings linking presence of microbial antigens to DNA methylation shift and tumourigenesis, is in line with previous report from clinical and epidemiological studies suggesting a strong connection between cancer, inflammation, and chronic infection (Yesmin et al., 2015) and that proinflammatory mediators can contribute to tumor promotion and progression when produced intolerably and persistently because of events like aberrant epigenetic changes (Ben-Baruch, 2006, Yesmin et al., 2015). Overexpression of TNF-α in subjects with hypomethylated DNA and malignant breast tumour is evident and agrees with the report that inflammatory cytokines and chemokines, for example, tumor necrosis factor-alpha (TNF-α) as detected in this study as well as IL-1, IL-6 and interferon gamma (IFN-γ) (Kuo et al., 2012, Yesmin et al., 2015), which can be produced by the tumor cells and/or tumor-associated leukocytes and platelets, may add directly to the development of malignancy. Though, report has also shown that cytokines can also mediate the activities of immune cells in the fight against

malignant cells (Yesmin et al., 2015). Thus, such interactions may either be in immunotherapeutic direction trying to suppress tumour development especially if the condition is acute and the antigens removed, or immunosuppressive trying to initiate tumour especially in chronic condition when thee antigens persist..

Conclusion

Elevated Serum levels of Nuclear Factor kappa B (NFkB), Immunoglobulin G (IgG), Tumour Necrosis Factor-alpha (TNF-α), 8-hydroxy-2-deoxy Guanosine (8-OH2DG) and oestrogen were expressed in subjects with benign and malignant breast tumours and increased level of circulating immune complexes. Higher serum expression of these molecules was seen in subjects with benign and malignant breast tumours and with evidence of microbial antigenic components of the CIC. Serum level of progesterone was found to be significantly lower in subjects with malaignant tumour.

This study has established cut-off values for circulating immune complexes (CIC), Nuclear Factor kappa B (NFkB), Immunoglobulin G (IgG), Tumour Necrosis Factor-alpha (TNF-α), 8-hydroxy-2-deoxy Guanosine (8-OH2DG).

Unmethylation and Hypomethylation were the predominant global DNA methylation patterns detected in subjects with benign and malignant breast tumours followed by hypermethylation. These abnormal patterns of DNA methylation were found in higher number of subjects with breast tumours and microbial antigenic components of circulating immune complexes than in subjects without evidence of microbial antigenic components.

Hepatitis C virus, Hepatitis B virus, *Helicobacter pylori, Treponema pallidum, Salmonella typhi, and Plasmodium falciparum*, were detected in subjects with benign and malignant tumours. The pattern of exposure showed high heterogeneous pattern of infection in the tumour subjects and homogeneous pattern of infection in apparently healthy subjects.

Association of circulating immune complexes, pro-inflammatory molecules, oxidative marker, oestrogen and progesterone was more in apparently healthy subjects and subjects with benign benign tumour. Circulating immune complexes (CIC) was found in association with oestrogen in subjects with malignant breast tumour and microbial antigenic components of the CIC.

Recomendations

By detection of retained microbial antigens in circulation, it is recommended that analysis of antigenic components of circulating immune complexes would aid in accessing the burden of microbial infections in immuno-pathological conditions and management of tumour cases.

DNA methylation test as routine test and the use of cell free DNA (cfDNA-Liqiud Biopsy) for detection of hypomethylation, unmethylation and hypermethylation could be used for pre-tumour detection enabling prevention of tumourigenesis as well as in early detection of tumour prior to tissue biopsy for adequate establishment of tumour development.

Limitations of the Study

The overwhelming presentation of microbial antigenic component in subjects with benign and malignant breast tumours hindered the comparison of molecular expression in the presence of microbial antigen and its absence.

Application of specific invitro studies was stalled due to lack of fund. This could have determined the specific effect microbial antigenic components may have on specific breast tumour suppressor gene for tumour initiation, progression or metastasis.

The small sample size due to prevalence rate in the area of this study hindered further specific data analysis to determine specific influential role the detected antigens may have on molecular expressions

Contribution of this Study to Knowledge

Detection of methylation patterns could be used as a pre-tumour marker and for early detection of tumour development. Moreover, using whole blood (liquid biopsy) with easily accessible and unsophisticated technique such as Enzyme Linked Immuno-sorbeent Assay (ELISA) would encourage routine check and as such enable detection of pre-tumour development.

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Appendix i

Ehtical Committee Approval

Appendix ii

Participant Enlightenment Sheet

Principal Investigator: Ezeani Michael Chukwudi

Immunology Department, Faculty of Medicine,

Nnamdi Azikiwe University

TITLE: Global DNA Methylation, Pro-Inflammatory Molecules and Circulating Immune Complexes in Subjects with Breast Tumours in Nnewi, Nigeria'

What is the purpose of this study?

The essence of this study is to look into the Epigenetic and molecular expressions in cancer participants with respect to immune complex retention. This study is targeted to expose and enhance immuno-therapeutic and diagnostic measures as well as molecular pathways expressed at different types breast tumour and stages of cancer. This work would not expose the participants to any form of health risk or worsen their health condition.

Risks and Effects of Study Intervention

Main intervention is a venous and tissue puncture as required in routine care. The drawing of the blood sample is a 'calculated injury' sometimes leading to a small hematoma and rarely causing discrete pain around the venipuncture site which resolves within days without any residues. A hematoma can be avoided under most circumstances by pressing gently with a piece of cotton on the puncture site immediately following the procedure. Should the venipuncture result in any injury to the patient, the required care will be provided by the study physician in charge without any fee.

Data Protection/Data Bank

Data submission to the data pool should be as close as possible to patient management. All data entry, submission or transfer, will be done electronically. Reports on confirmed or suspected serious adverse events (SAE's) have to be reported immediately to the Principal Investigator via the network coordination site. All patient data are considered as strictly confidential. All data required for study purposes will be stored electronically in an anonymized format.

Freedom of Participation

After the participant have read the information and asked any questions she may have, I (the Principal investigator), will ask the potential participant to complete an Informed Consent Form, however if at any time, before, during or after the sessions the participant wishes to withdraw from the study the participant should just contact the main investigator. she can withdraw at any time, for any reason and will not be asked to explain the reasons for withdrawing.

Will participants be required to attend any sessions and where will these be

Only the normal clinical visit is required and they are needed as long as their clinical appointment is still on.

Is there anything the participants would do before the sessions

Participants are expected to decide whether she would participate in the study. If her decision is yes, she would be required to sign the consent form after reading the information and agree to participate in the study. Participants are also expected to answer some questions from the main investigator. Those under the age of 18, are required to seek for permission from their parents before taking part.

Is there anything the participants need to bring?

The participants are not expected to come with anything but are expected to maintain their normal and or advised diet if any.

What personal information will be required from the participants

They may be asked questions on the following matters: some risk factors such as age, eating, smoking, alcohol intake, activities, weight, number of children, age at which first child was delivered, first menstrual period, infections. Under any hormonal drug therapy or contraceptive. Other disease conditions, antibiotic intake, family history of breast cancer.

What would the participants get for participating?

Any information obtained from this study that is beneficial to your health and longevity would be communicated to you, (under the statutory obligations of the institution which the researchers are working with)

Should the participants have more questions who should they contact?

They should Please ask the investigator

Appendix iii

Immunology Department

Faculty of Medicine

Nnamdi Azikiwe University

Nnewi Campus

January 11th 2013

Dear Participant

LETTER OF INVITATION

I am Ezeani Michael Chukwudi of Immunology Department, Faculty of Medicine. Hereby invite you to participate in this study haven read through the research information. The participants are assured that medical information from this study would be treated as confidential.

Please read the consent form and append your signature at its column to indicate your consent

Ezeani Michael Chukwudi

Investigator

Appendix iv

Consent Form

Principal Investigator: Ezeani, Michael Chukwudi

Immunology Department, Faculty of Medicine,

Nnamdi Azikiwe University

TITLE: Global DNA Methylation, Pro-Inflammatory Molecules and Circulating Immune Complexes in Subjects with Breast Tumours in Nnewi, Nigeria'

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Nnamdi Azikiwe University Teaching Hospital (NAUTH) Ethical Advisory Committee.

I have read and understood the information sheet and this consent form and I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in the study and that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.

I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant.

Expected intervention in a way of hazard, is a venous and tissue puncture as required in routine care while drawing blood sample.

I understand that the harzard is a 'calculated injury' sometimes leading to a small hematoma and rarely causing discrete pain around the venipuncture site which resolves within days without any residues.

I also understand that this hematoma can be avoided under most circumstances by pressing gently with a piece of cotton on the puncture site immediately following the procedure.

Should the venipuncture result in any injury, the required care will be provided by the study physician in charge without any fee.

Any information obtained from this study, that is beneficial to my health and longevity would be communicated to me, (under the statutory obligations of the institution which the researchers are working with). In this way, I will benefit in this study.

I agree to participate in this study.

Name of participant ----------
Surname first

Date ---------- **Signature** ----------

Phone ----------

Namee of Investigator ----------
Surname First

Date ---------- **Signature** ----------

Phone ----------

Name of Witness ----------
Surname First

Date ---------- **Signature** ----------

Phone ----------

Appendix v

ORAL QUESTIONNAIRE FOR A STUDY PROPOSAL

Project Title

TITLE: Global DNA Methylation, Pro-Inflammatory Molecules and Circulating Immune Complexes in Subjects with Breast Tumours in Nnewi, Nigeria

Investigator
Ezeani, Michael Chukwudi PGD Imm, M.Sc.
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Nnewi Campus
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Name

Sex

How old are you ?

What type of food do you eat often?

How often do you eat snacks ?

Do you smoke ?

How many sticks of cigarette do you take in a day?

Do you drink alcohol

What type of alcohol do you take – ethanol or beer

How many shuts of ethanol do you take in a day ?

How many bottles of beer do you take in a day?

What type of job do you do?

At what time do you normally start work in a day ?

At what time do you normally close work in a day?

What other things do you do apart from your Job?

Please mount the weight so that we know your weight and height

Are you married?

How many children do you have?

At what age did you deliver your first child?

Did you lactate properly at that time ?

How long did you breast feed the child?

At what age did you see your first menstrual flow ?

Are you under any hormonal drug?

Are you taking any contraceptive ?

Do you have any infection ?

Have you had infection

How long has the infection been with you ?

Are you under any antibiotic or cancer drug?

How long have you been taking the antibiotic?

Do have HIV ?

What other disease(s) do you have (diabetes, hypertension, rheumatism or arthritis)?

Does your mother or father have cancer ?

Does any of your relative have cancer (breast cancer) from your mother's side or father's side

Appendix vi

Immunology Department
Nnamdi Azikiwe University
Nnewi Campus
11th January, 2013

Dear Sir/Ma,

LETTER OF COLLABORATION

In apropos of my PhD proposal titled: **Global DNA Methylation, Pro-Inflammatory Molecules and Circulating Immune Complexes in Subjects with Breast Tumours in Nnewi, Nigeria'**, I request for your collaboration in area of some clinical records of the patients and blood sample collection. The study requires about 10mls of blood from 50 patients with breast tumours.

Participation would be voluntary as each subject is expected to sign a consent form after being informed about the study. There is a questionnaire designed to ascertain the suitability of the patients for participation in the study.

Thank you for your anticipated cooperation.

Ezeani Michael Chukwudi
Principal Investigator
Phone: 08035522824

Appendix vii

Preparation of 0.01M Borate buffer at pH 8.4 and 10.2

4.76 g Boric Acid
2.54 g Borax
1000 mL of dH_2O
pH to 8.4 or 10.2 with NaOH

The solution was stored in plastic container at room temperature (18° to 26° C). If desired, the solution may be stored at 4° C or less. Note: some salts may precipitate out of solution at lower temperature. Allow buffer to equilibrate to room temperature (18° to 26° C) to restore solubility of some salts and shake to mix before use.

Preparation of 4.166% PEG

4.166 g of Polyehtylene glycol (PEG 6000) dissolved in 100ml of distilled water

Preparation of the Grades of Alcohol 70% and 90%

70% (v/v): mix 70ml of absolute ethanol with 30 ml of sterile H2O.
90% (v/v): of absolute ethanol with 30 ml of sterile H2O.
Prepared as needed but can be stored at -20 O^C.

Appendix viii

Determination of Immune Complex Values

Peg Index formula: Peg Index= OD450 with Peg – OD450 with BBS without PEG x 1000

1. **Calculation of Cut Off Values Mean +2SD (at 95% confidence interval)**

CIC= 32.19 + 2 x 12.14 = 56.47

NF-kB= 0.296 + 2 x 0.016 = 0.328

IgG= 16.9 + 2 x 3.9 = 24.7

TNF-alpha= 7.79 + 2 x 2.2 = 12.2

OH2DG= 7.9 + 2 x 3.1 = 14.1

2. **Quality Controls**

2.1. Chromatographic immuneassays

Internal procedural controls were included in the test. It confirmed the sufficient specimen volume and correct procedural technique.

2.2. Prescision of the Test

Prescision of the test was ascertained by determining the coefficient of variation (CV). The CV= (Std/Mean x 100 of 2 sets of the samples) of each control supplied and/or (where the supplied control was small) three different levels (low, normal and high) of pool control sera secured during determination of cut off values was determined. Used for comparism of the variability of two or more series. The series of data for which the coefficient of variation was large indicated that the group was more variable and it was less stable or less uniform, but when a coefficient of variation was small it indicated that the group was less variable and it was more stable or more uniform.

Appendix ix

Pre-recruitment Screening Tests

HIV Screening

Rapid HIV testing was performed using Determine HIV-1/2 Ag/Ab Combo and Uni-Gold HIV Test (HIV Rapid Testing Algorithm), following a modified version of the testing algorithm. The Determine Combo test was used which detects both HIV antibody and p24 antigen, the latter being a marker of acute infection (UNAIDS 2010).

Reumathoid Factor (RF)

Study Design

The design of this study involves both analytical and non-analytical. The non-analytical study involves the descriptive study showcasing the DNA methylation patterns and exposure to microbial agents in female subjects.

The analytical study involves analytical-observational study in which the subjects who have been exposed to microbial agents were studied for possible outcome. Since the study of the outcome was done sometime after the exposure, it is refered to as **cohort study.**

Note: Analytical could be experimental (when the investigator is involved in exposure or intervention of the subjects) or observational (when the investigator is not involved in exposure and intervention of the subjects but studies the outcome of the exposure or intervention)

Appendix ix

Demographic characteristics of subjects with benign tumour, malignant tumour and control subjects

		Benign N=24	Malignant N=25	Control N=50
Mean Age		37.9±10.1	49.6±10.8	35.9±9
Mean Body mass		29.3±3.9	25.8±6.9	29.1±3.0
Alcohol intake	**Yes**	12	10	25
Alcohol intake	**No**	12	15	25
Smoking	**Yes**	0	0	0
Smoking	**No**	24	25	50
Mean Age of Menarche		12.8±1.3	9.1±6.6	11.3±3.8
Infection	**Yes**	10	10	0
Infection	**No**	14	15	50
Taking Snacks|:	**Often**	9	5	24
	Occasionally	5	7	11
	No	10	13	15
Family History:	**Yes**	1	3	0
Family History:	**No**	23	22	50

	Benign	Malignant	Control
Oral Contraceptive: Yes	0	0	0
Oral Contraceptive: No	24	25	50
Mean Urine PH	4.4±2.4	5.1±1.7	4.8±2.4
Urine Protein: Yes	9	16	14
Urine Protein: No	15	9	36