ELUCIDATION OF CORRELATION BETWEEN POLY-ADP-RIBOSYLATION OF CELLULAR PROTEINS AND HUMAN CANCERS

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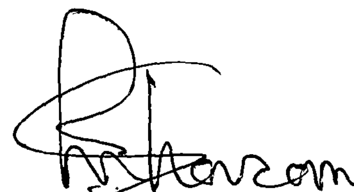

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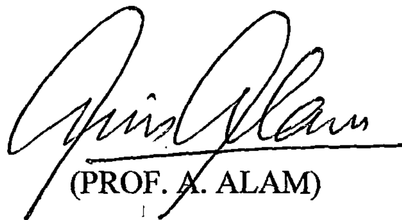

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ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
$\mu g\ \mu l^{-1}$	Microgram per microlitre
µl	Microlitre
AEBN	Aqueous extract of betelnut
AFRO	African region
AIF	Apoptosis inducing factor
ADPRT	ADP-ribosyl transferase
ALL1	Acute lymphoblastic leukemia 1
APC	Anaphase promoting complex
Asp	Aspartate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia-telangiectasia mutated and rad3-related
BAP1	BRCA1-associated protein-1
BARD1	BRCA1-associated RING domain 1
BAX	BCL2-associated X protein
BER	Base vexcision repair
bcl2	B-cell lymphoma 2
Bcr	Breakpoint cluster region
BN	Betel nut
bp	Base pair
BQ	Betel quid
BRCA1	Breast cancer 1, early onset
Brca1	Murine breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
Brca2	Murine breast cancer 2, early onset
BRCT	BRCA1 C-terminal
BUBR1	BUB1 (budding uninhibited by benzimidazoles 1)-related protein kinase
CA	Chromosomal Aberration
Ca^{2+}	Calcium ion
CAF1	Chromatin assembly factor I
cAMP	cyclic AMP
CREBP	cyclic AMP responsive element binding protein
CBP	CREB *(cAMP response element binding)* binding protein
CA-125	Cancer antigen 125
Cdc	Cell division cycle

CDKN2	Cyclin dependent kinase 2
c-erbB	Cellular erythroblastic leukemia oncogene homolog 2
CEA	Carcinoembryonic antigen
CHAF1,CHAF2	Chromatin assembly factor 1 gene, Chromatin assembly factor 2 gene
CIN	cervical intraepithelial neoplasia
c-myc	Cellular myelocytomatosis oncogene
COX-2	Cyclooxygenase-2
CpG	Cytosine-phosphate-guanine
CtIP	CtBp (*C-terminal binding protein*)-interacting protein
CYP17	Cytochrome P450, subfamily XVII (steroid 17-alpha-hydroxylase)
Cys	Cysteine
DEN	Diethylnitrosamine
DNA	Deoxyribonucleic acid
DMN	dimethlynitrosamine
eIF4E	eukaryotic initiation factor
EGFR	Epidermal growth factor receptor
EMRO	Eastern Mediterranean Region
ERCC3	Excision repair cross-complementing rodent repair deficiency, complementation group 3
ERBB2	v-erb-b2 erythroblastic leukemiaviral oncogene homolog 2
ESCC	Esophageal squamous cell carcinoma
ESE	Early sexual exposure
ETS-1	v-ets erythroblastosis virus E26 oncogene homolog 1
FANCG	Fanconi anemia complementation group G
Fas/APO1	TNF (tumor necrosis factor) receptor superfamily, member 6/ Apo-1 antigen
FGF	Fibroblast growth factor
Fig.	Figure
FIHT	Fragile histidine triad
Fos	v-fos FBJ murine osteosarcoma viral oncogene homolog
g	Centrifugal force
g	Gram
G1	Gap 1
G1/S	Gap 1/Synthesis
GDF15/MIC-I	Growth differentiation factor 15/ Macrophage inhibitory cytokine 1
GEF	GTPase exchange factors

GK	Gingival keratinocyes
GST P1	Glutathione S-transferase pi 1
hBUBR1	Human BUBR1
HCF	History of cancer in family
HEBN	Hydrochloric acid extract of betelnut
Hep2	Cell line established from human laryngeal carcinoma
HGF	Human gingival fibroblasts
HMG	High mobility group
hMLH	Human *Mut L* homolog
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HR	Homologous recombination
H-*ras*	Harvey-retrovirus associated DNA sequence
hsp 70	Heat shock protein 70
hUbc9	human homolog of the yeast ubiquitin conjugating enzyme Ubc9
human KB epithelial cell	cell line derived from a human carcinoma of the nasopharynx
IARC	International Agency for Research on Cancer
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IGF-BP	Insulin-like Growth Factor Binding Protein
IGF-BP3	Insulin-like Growth Factor Binding Protein 3
IgG	Immunoglobulin G
int-I	Integrase
kDa	Kilo Dalton
KILLER/ DR5	Tumor necrosis factor receptor superfamily, member 10b /Death receptor 5
Ki-*ras*/ K-*ras*	Kirsten-retrovirus associated DNA sequence
KRAB	Kruppel-associated box
KS3/fgf4	Fibroblast growth factor 4
LFP	Late first pregnancy
LINE	Long interspersed nuclear element
LOH	Loss of heterozygosity
LyF-1	lymphocyte specific factor I
M	Molar
MAP4	Microtubule-associated protein
mdm2	Murine double minute 2
MER	Medium reiteration frequency repeats
MEF	mouse embryonic fibroblast

mg	Milligram
mg ml^{-1}	Milligram per millilitre
min	Minute
ml	Millilitre
MLL	Mixed lineage leukemia
mm	Millimetre
mM	Millimolar
MNPA	Methylnitrosaminopropionaldehyde
MNPN	3-methylnitrosaminopropionitrile
Mos	v-mos Moloney murine sarcoma viral oncogene homolog
MRE11	Meiotic recombination 11 homolog
MW	Molecular weight
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
MYCL	v-myc myelocytomatosis viral oncogene homolog, lung carcinoma derived (avian)
MyoD	myogenic determination factor D
N	Normal
NAD	Nicotinamide adenine nucleotide
NCM	Nitro cellulose membrane
NBS-1	Nijmegen breakage syndrome 1 (nibrin)
NER	Nucleotide excision repair
ng	Nanogram
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Nonhomologous end joining
NLS	Nuclear localization signal
nm	Nanometre
N-myc	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
N-*ras*	Neuroblastoma retrovirus associated DNA sequence viral (v-ras) oncogene
NuMA	nuclear mitotic apparatus proteins
OC	oral contraceptive
OSCC	Oral squamous cell carcinoma
OSF	Oral submucous fibrosis
p14ARF/$p16^{INK4a}$	Cyclin-dependent kinase inhibitor 2A
P16	Cyclin-dependent kinase inhibitor 2A
p21	Cyclin-dependent kinase inhibitor 1A
p300	E1A binding protein p300

p53	Tumor suppressor protein p53
PAR	Poly-ADP-ribosylation
PBL	Peripheral blood lymphocytes
PARG	Poly-ADP-ribose glycohydrolase
PARP-1	Poly-ADP-ribose polymerase family member 1
PARP-2	Poly-ADP-ribose polymerase family member 2
PARP-3	Poly-ADP-ribose polymerase family member 3
PCNA	Proliferating cell nuclear antigen
PBCR	Population Based Cancer Registries
PDGF	Platelet derived growth factor
Pidd	p53-induced protein with a death domain
PIG3	Tumor protein p53 inducible protein 3
PTEN	Phosphatase and tensin homolog
PSA	prostrate-specific antigen
raf-1	v-raf-1 murine leukemia viral oncogene homolog 1
RB	Retinoblastoma
RER	Rough endoplasmic reticulum
RHA	RNA helicase A
RNase	Ribonuclease
ROS	Reactive oxygen species
RPA	Replication protein A
RT	Room temperature
RR	relative risk
SC	Spleen cells
SCC	Squamous cell carcinoma
SCE	Sister chromatid exchange
SD	Standard Deviation
Sec	Second
Ser	Serine
SEARO	South East Asia Region
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SSBR	Single Strand Break Repair
STK11/LKB1	Serine/threonine kinase 11
SW1/SNF	Switch/ Sucrose non fermentable (chromatin remodeling complex)
TIMM23	*mitochondrial membrane translocase 23*
TFIIH	Transcription Factor II H
TGF-α	Transforming growth factor, alpha
TGF-b	Transforming growth factor-b

U	Unit
UV	Ultraviolet
WHO	World Health Organization
WPRO	Western Pacific Region
VEGFR1, VEGFR2 and VEGFR3	Vascular endothelial growth factor receptor 1, 2, 3
w/v	Weight/volume
X	Times
XRCC1	*X*-ray *C*omplementing Factor 1
χ^2	Chi-square

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INTRODUCTION

1.1. CANCER

Cancer is complex disease that includes more than 200 diseases having common characteristics. It is characterized by unregulated cell growth caused by alterations in gene expressions leading to a population of cells that can invade tissues and spread to other parts of the body. The spread of the malignant cells extends outward from the original tumour and has been described as resembling the appearance of a crab. Historically this has been traced back to the origins of the term 'cancer', which was derived from the Latin word for 'crab'. The oldest description of human cancer, referring to eight cases of tumor of the breast, was found in the Egyptian Edwin Smith Papyrus, written around 3000–1500 BC. The oldest specimens of human cancers were detected in the remains of a female skull dating back to the Bronze Age (1900–1600 BC), and in fossilized bones of ancient Egypt (Weber, 2007).

Cancers are originally described as clinical entities characterized by their symptoms and course of disease progression. Cancer is a group of diseases of higher multicellular organisms characterized by alterations in the expression of multiple genes, leading to dysregulation of the normal cellular program for cell division and cell differentiation which favour the growth of a tumor cell population (Ruddon, 2007). These aberrations lead to the abnormal behaviour common to all neoplastic cells. It has been suggested that six alterations in the cell physiology drives it towards malignancy. These are as follows self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Clinically, cancer appears to be many different diseases with different phenotypic characteristics that vary in their age of onset, rate of growth, state of cellular differentiation, diagnostic detectability, invasiveness, metastatic potential, response to treatment, and prognosis. It may appear, particularly at later stages of the disease, that cancer disrupts every aspect of physiology. Likewise, so many differences between transformed and nontransformed cells have been reported in the literature that they may seem to include every component of cell functions.

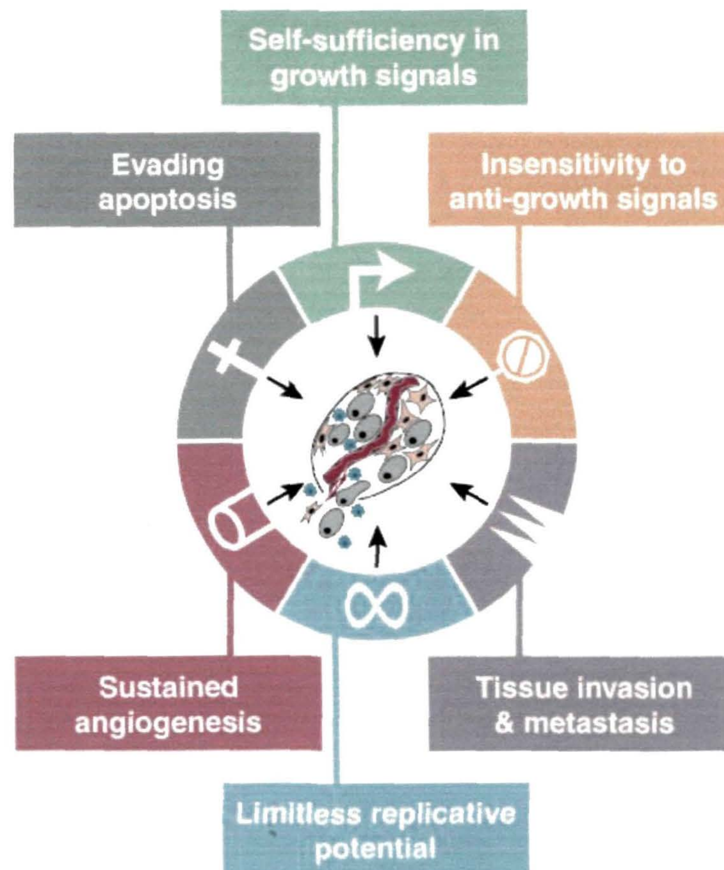


Figure 1: The six distinctive features of a cancer cell. Most of the cancers acquire these abnormalities in their course of development (Adapted from Hanahan and Weinberg, 2000).

1.2. CARCINOGENESIS

Carcinogenesis, the events that turn a normal cell in the body into a cancer cell, are a complex and multifactorial process (Fig. 2). A lot of evidence suggests that, in the majority of cases, cancers arise from a single cell which has acquired some heritable form of growth advantage (Nowell, 1976). Animal models of carcinogenesis, primarily based on models of skin cancer development in mice, have shown that the process of carcinogenesis comprises of the following events *initiation, promotion*, *malignant transformation* and *metastasis*. This initiation step is believed to be caused frequently by some form of genotoxic agent such as radiation or a chemical carcinogen. The cells at this stage, although altered at the DNA level, are phenotypically normal. Further mutational events involving genes responsible for control of cell growth lead to the emergence of clones with additional properties associated with tumor cell progression. Finally, additional changes allow the outgrowth of clones with metastatic potential.

Each of these successive events is likely to make the cell more unstable so that the risk of subsequent changes increases.

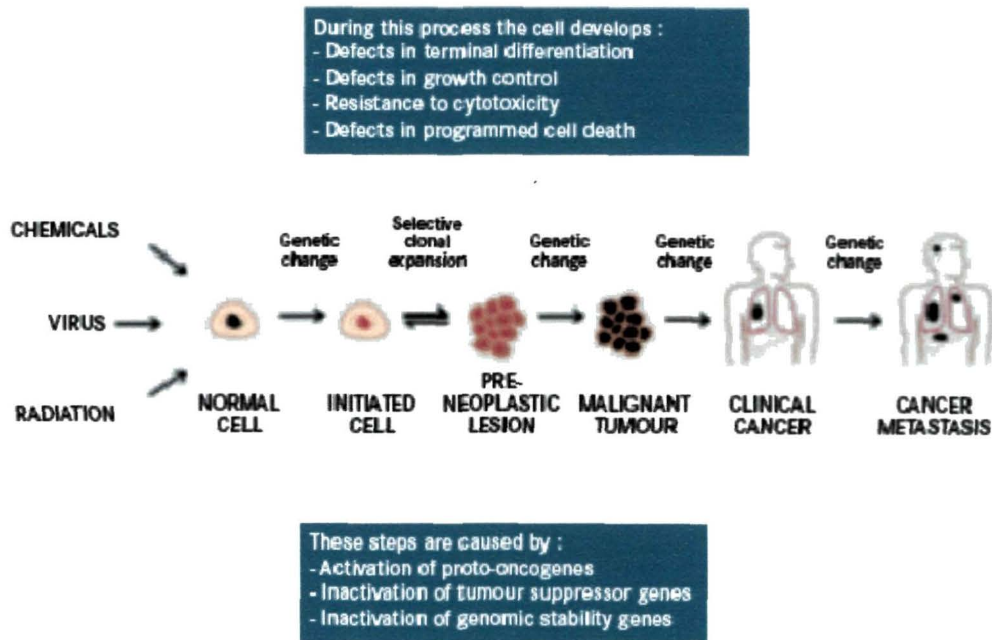


Figure 2: Carcinogenesis a multifactorial process caused by genotoxic agents. It involves genetic alterations, such as activation of protooncogenes and inactivation of tumor suppressor genes. (Adapted from Stewart and Kleihues, 2003)

At molecular level cancer may be a relatively small number of diseases caused by similar molecular defects in cell function resulting from common types of alterations to a cell's genes. Ultimately, cancer is a disease of abnormal gene expression. There is a number of mechanisms by which this altered gene expression occur. These mechanisms may occur via a direct damage to DNA, such as a gene mutation, translocation, amplification, deletion, loss of heterozygosity, or via a mechanism resulting from abnormal gene transcription or translation. The overall result is an imbalance of cell replication and cell death in a tumor cell population that leads to an expansion of tumor tissue. In many cases the causes of cancer are not clearly defined, but both external (e.g., environmental chemicals and radiation) and internal (e.g., immune system defects, genetic predisposition) factors play a role. While any genetic change can take place at any time, only certain order of genetic changes can lead to a successful completion of the cascade of carcinogenesis to generate cancer tumors. This follows from the observation that carcinogenesis is a micro-evolution process in the cell population so that only those genetic changes or epigenetic changes which increase the

fitness of the cells can be fixed and established in the cell population (Hopkin, 1996; Cahill *et al.* 1999). It has been reported that epigenetic changes often lead to loss of heterozygosity and gene mutations, which may underline the importance of epigenetic changes in the initiation and progression of carcinogenesis (Jones and Baylin 2002; Baylin and Ohm 2006).

Carcinogenesis is initiated by genetic changes (Osada and Takahashi, 2002; MacDonald *et al.*, 2004; Pharoah *et al.*, 2004). The genetic changes may either be as small as point gene mutations, or as large as some chromosomal aberrations such as deletion of chromosomal segments, chromosome inversion and chromosomes translocation leading to mutation or deletion of some cancer genes, or activation of some dominant cancer genes, or inactivation of some recessive cancer genes. The cancer genes which contribute to the creation of cancer phenotype are the oncogenes, the suppressor genes and the mis-match repair genes which are involved in DNA synthesis and repair and/or chromosomal segregation.

However, these genetic effects can also be achieved by changes called epigenetic changes which mainly involve activation of oncogenes products or silencing of suppressor genes proteins through DNA methylation of cytosine at *CpG* base pair islands (Belinsky, 2004; Baylin, 2005) or histone acetylation (Ohlsson *et al.*, 2003), or loss of imprinting (Holm *et al.*, 2005; Robertson, 2005), or tissue disorganization and gap junction disruption (Jones and Baylin, 2002; Baylin, 2005).

1.2.1. Cancer status

Cancer affects the entire communities around the globe whether they belong to "developed, developing or under developed" countries. The impact of cancer is far greater than the number of cases alone would suggest. Regardless of prognosis, the initial diagnosis of cancer is still perceived by many patients as a life-threatening event, with over one-third of patients experiencing anxiety and depression. Cancer can be equally if not more distressing for the family, profoundly affecting both the family's daily functioning and economic situation. The economic shock often includes both the loss of income and the expenses associated with health care costs.

Globally, there were an estimated 12.4 million incident cases of cancer in 2008 (6,672,000 in men and 5,779,000 in women) and 7.6 million deaths from cancer (4,293, 000 in men and 3,300,000 in women). Over half of the incident cases occurred in residents of four WHO regions with a large proportion of countries of low and middle – AFRO, EMRO, SEARO and WPRO (World Cancer Report, 2008).

In 2008, five cancers in every ten occurred in residents of four WHO Regions that are mainly constituted of low-resource and medium-resource countries: the African Region (AFRO) (5.4 %), the Eastern Mediterranean Region (EMRO) (3.7 %), the South East Asia Region (SEARO) (12.8 %) and the Western Pacific Region (WPRO) (29.7 %) (Fig. 2). The continued growth and ageing of the world's population will greatly affect the future cancer burden. Given these demographic changes, and factoring in an annual increase in cancer incidence and mortality of 1 %, by 2030 it could be expected that there will be 26.4 million incident cases of cancer and 17.0 million cancer deaths annually. An annual increase of 1 % per annum in the incidence rate seems reasonable, and may well be conservative (World Cancer Report, 2008).

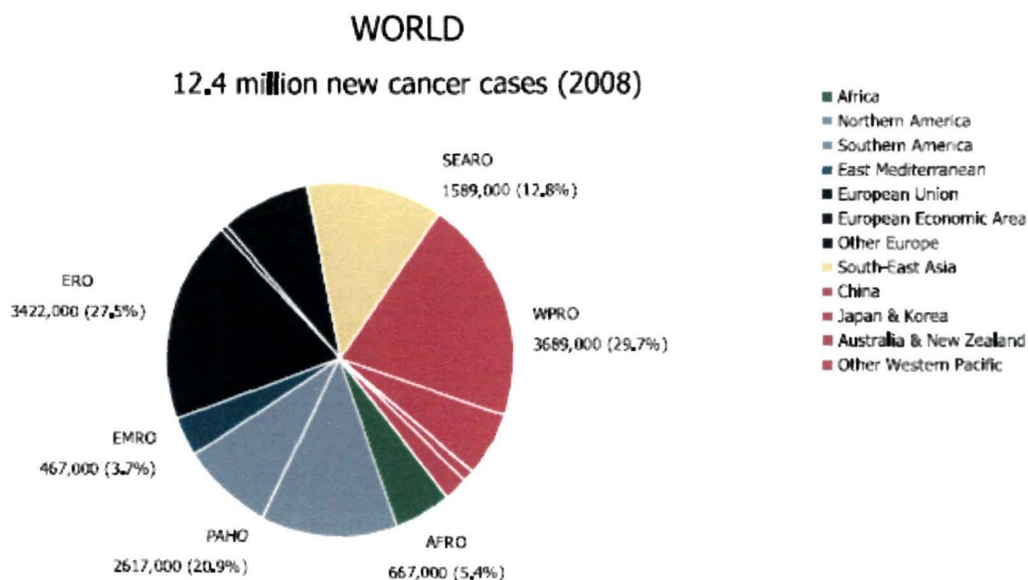
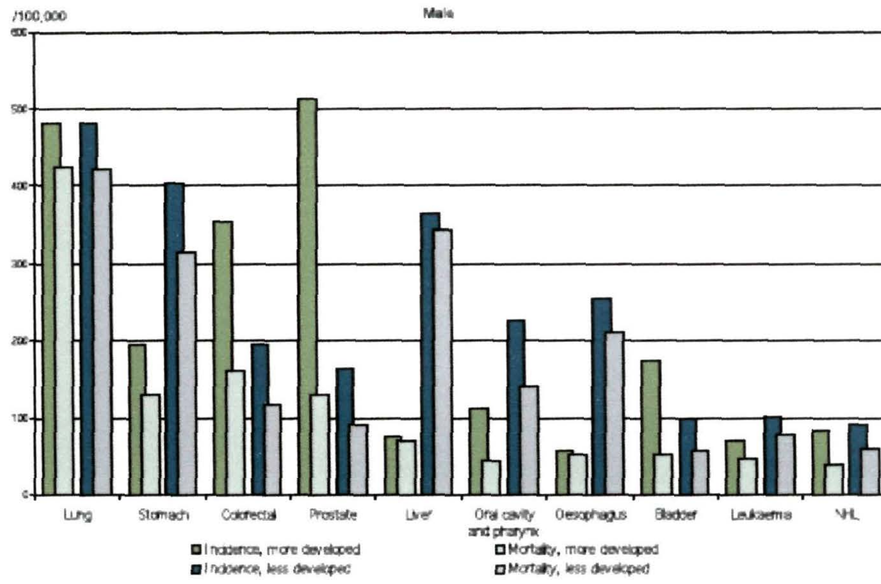


Figure 2: Distribution of global cancer burden by World Health Organisation. (Adapted from World Cancer Report, 2008).

Globally, in 2008 according to the world cancer report lung cancer was the commonest incident cancer and cause of cancer-related mortality in men; in women, the most

common incident cancer and cause of cancer-related death was breast cancer (Fig. 3) (World Cancer Report 2008) in developed and under developed countries.



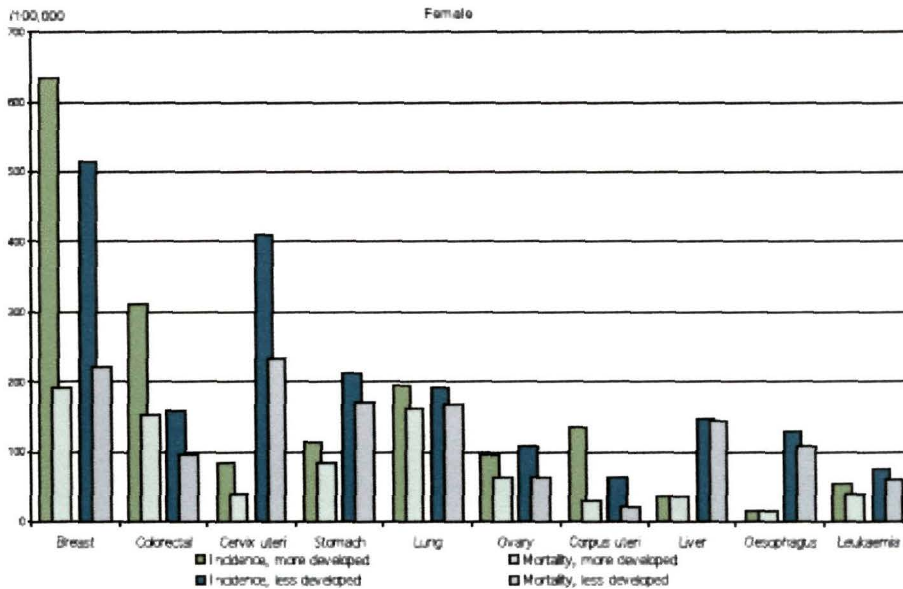


Figure 3: Incidence and mortality of the most common cancers in the world.
Panel A: Shows the incidence and mortality of male in more less developed countries.
Panel B: Shows the incidence and mortality of female in more less developed countries.
(Adapted from World Cancer Report, 2008).

In 2005, cancer killed approximately 826,000 people in India 519,000 of those people were under the age of 70. The WHO has projected that by 2030 there will be 1,462,494 deaths related to cancer (WHO Global InfoBase). The 10 leading causes of cancer death among males in 2005 were cancers of the lung, mouth and oropharynx, oesophagus etc (see Fig.4.A for details) while among females cancers of the cervix, breast, ovary, lymphoma, etc (see Fig.4.B for details)(WHO Global InfoBase). Most of the cancer cases report for diagnosis and treatment in the advance stages of the disease, resulting in poor survival and high mortality rates (Dinshaw *et al*, 1999). In north east India a high prevalence of head and neck cancers has been reported with tobacco related oral cancer being the most common (Bhattacharjee *et al*, 2006). In the first report of population based cancer registries (PBCRs) 2003-2004 of north eastern region of India four states namely, Assam, Manipur, Mizoram and Sikkim were covered in the programme (First Report 2003-2004, Population Based Cancer Registries under North Eastern Regional Cancer Registry, 2006).

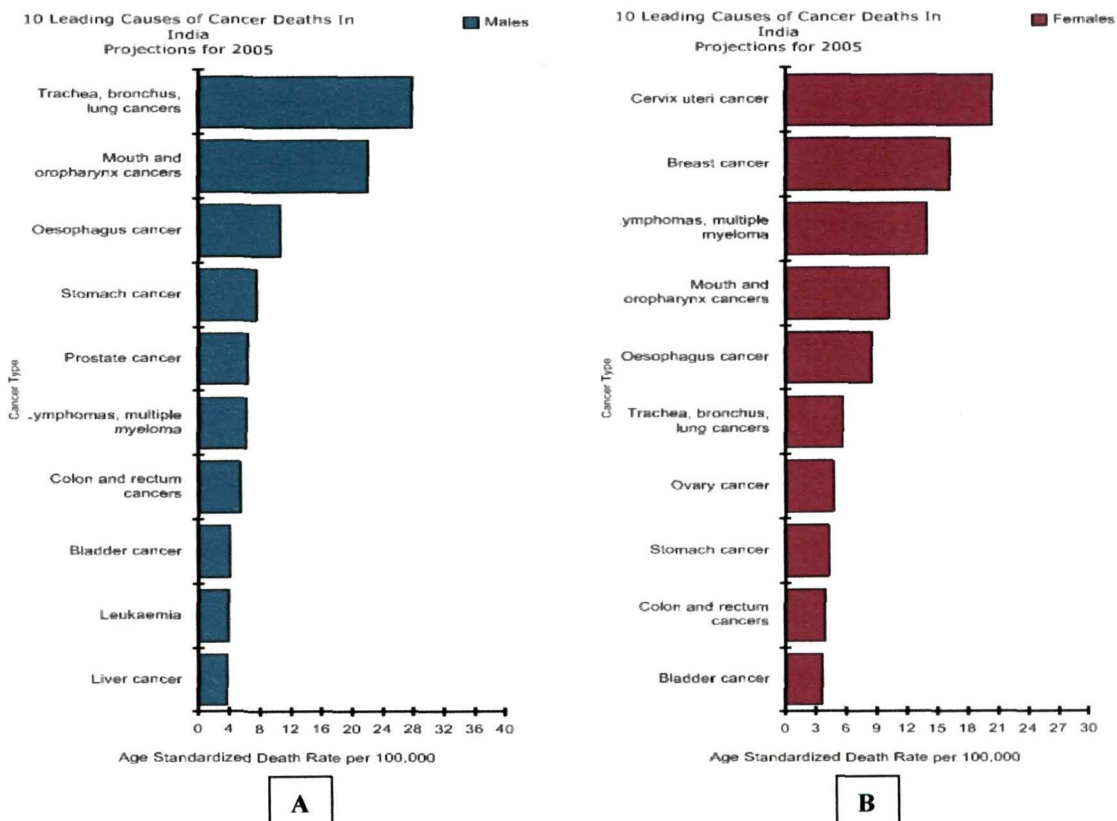


Figure 4.: The 10 leading causes of cancer deaths in India.
Panel A: The 10 leading causes of cancer deaths among males in India.
Panel B: The 10 leading causes of cancer deaths among female cancers in India. (Adapted from WHO Global InfoBase).

Overall, the State of Mizoram as whole and Aizawl district recorded the highest AAR [Age Adjusted or Age Standardised rate] (all sites) reported as yet from the Indian sub continent. Kamrup urban district of Assam state follows closely in having such high incidence rates. The main site of cancer that contributed to the high incidence in males in Mizoram state was stomach cancer. This was followed by cancer of lung, oesophagus and hypopharynx. Among females in Mizoram, apart from stomach cancer, cervix, lung cancer was the leading site comprising nearly 14 % of all cancers in women. In the registries in Assam, among males, cancer of the oesophagus was the leading site in Dibrugarh district and Kamrup Urban district and the third leading site in Silchar town. In Imphal west district of Manipur, lung cancer was also the leading site of cancer in both sexes. In females, it surpassed cervix and breast cancer as the leading site of cancer. Cancer of the nasopharynx was another site of cancer that recorded a high incidence.

In the state of Megahlaya, the district of East Khasi Hills was the only district that had sufficient numbers and rates to describe cancer patterns. In this cancer of the oesophagus was leading site of cancer in both males and females. In males the other sites of cancers that were prevalent in the region are cancer of hypopharynx and stomach. Among the females the other leading sites of cancers were cancer of cervix, breast and stomach (Development of an atlas of cancer in India", First All India Report: 2001-2002, 2004).

1.3. POLY-ADP-RIBOSYLATION (PAR)

Poly-ADP-ribosylation (PAR) is a reversible post translational modification of proteins Althus and Ritcher, 1987; Jacobson and Jacobson, 1989; Poirier and Moreau, 1992; Boulikas, 1993; Sharan, 2009). It was first discovered by Chambon and colleagues in hen liver nuclear extract (Chambon, Weill, and Mandel 1963; Chambon, *et al,* 1963). Over the past four decades research on this subject by different groups has contributed a lot to the understanding of PAR metabolism and its physiological role. PAR is reversible enzyme catalyzed reaction that involves the transfer of ADP-ribose moiety from NAD^+ to glutamate, aspartate and lysine residues of nuclear acceptor proteins. The ADP-ribose polymer formed by sequential attachment of ADP-ribose moieties can

reach very high complexity, with chain lengths of up to 200 units or more in a linear or multiple branching fashions. The ADP-ribose units in the polymer are linked by glycosidic ribose- ribose bonds. Long polymers are branched in an irregular manner. Branching occurs in vitro and in vivo with a frequency of approximately one branch per linear section of 20 to 50 units of ADP-ribose (Miwa *et al*, 1981; Juarez-Salinas *et al*, 1983). The chemical structure of the branching site of poly-ADP-ribose was determined by nuclear magnetic resonance and mass spectroscopy and found to be the same as in the linear regions of the polymer (Miwa *et al*, 1981).

The major enzyme catalysing PAR is poly-ADP-ribose polymerase (PARP). The constitutive levels of poly-ADP-ribose are usually very low in unstimulated cells (D'Amours *et al*, 1999). However in response to mitogenic stimuli or genotoxic stress such as DNA strand breaks, PARP activity and levels of poly-ADP-ribose may increase 10- to 500-fold, while cellular NAD^+ levels are correspondingly low. However, most free or protein-associated poly-ADP-ribose polymers synthesized upon genotoxic stress are rapidly degraded in vivo, with a half-life of >40 s to 6 min (Alvarez-Gonzalez and Althaus, 1989). Till date two different enzymes are known or hypothesized to degrade free (non-protein-bound) or protein-bound linear or branched poly-ADP-ribose (D'Amours, D. *et al*, 1999; Rouleau *et al*., 2004). The major enzyme which carries out degradation of ADP-ribose polymer is poly-ADP-ribose- glycohydrolase (PARG). The major mammalian PARG, has both endoglycosidase and exoglycosidase activities, which are responsible for the hydrolysis of glycosidic ribose-ribose bonds internal to and at the ends of ADP-ribose polymers, respectively (Braun, *et al.*, 1994; Ame *et al.*, 1999). The two metabolic reactions involving PARP and PARG occur simultaneously in opposite direction (Sharan, 2009) (Fig. 5).

1.3.1. Enzymes in PAR metabolism

There three main enzymes that are involved in PAR metabolism.

1.3.1.1. Poly-ADP-ribose polymerase (PARP)

PARP, which is now known as PARP-1, was thought to be the only enzyme with PAR activity in mammalian cells for a long time. But this was challenged recently by the development of mice deficient in the *Parp1* gene (Wang *et al*, 1995) and the

identification of novel poly-ADP-ribosylating enzymes. Primary cells derived from *Parp1*$^{-/-}$ mice can still synthesize poly-ADP-ribose following treatment with DNA-damaging agents (Shieh *et al,* 1998).

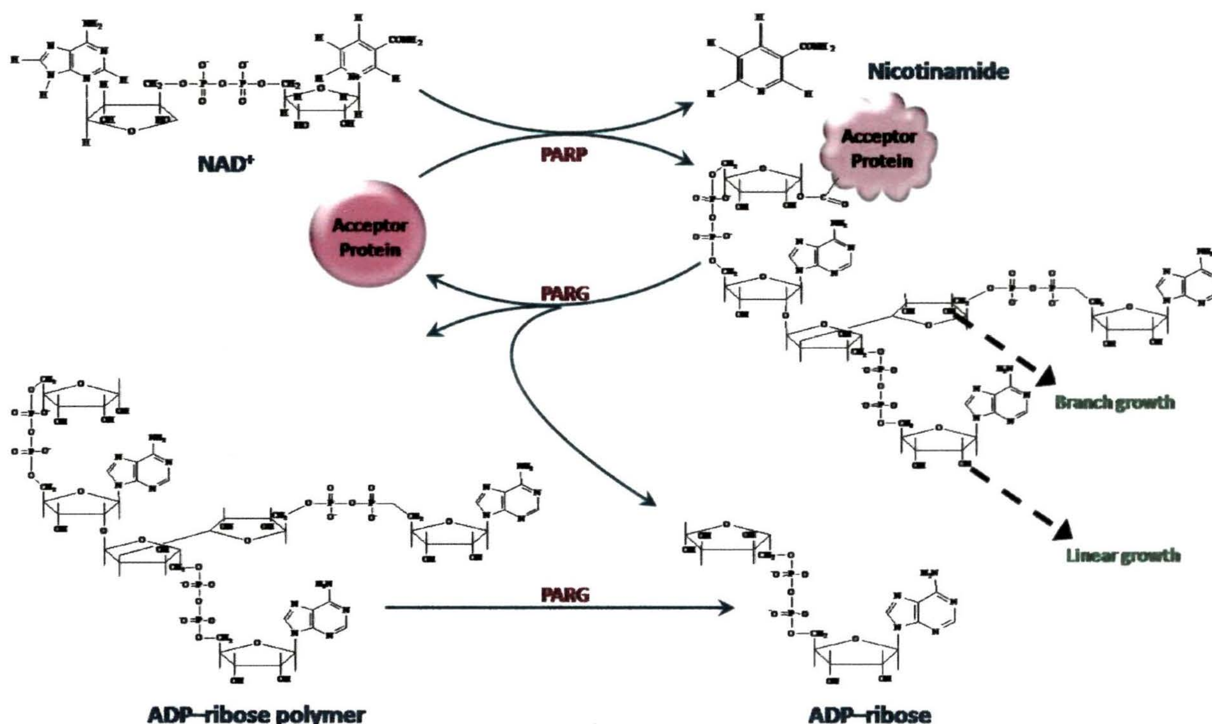


Figure 5. The biochemical pathway depicting the main features of the poly-ADP-ribosylation (PAR) metabolism - the two main enzymes, PARP and PARG, acting in opposite directions (Adapted from Sharan, 2009).

Five new genes encoding PARP enzymes have been identified (Alvarez-Gonzalez and Mendoza-Alvarez, 1995; Smith *et al.,* 1998; Johansson, 1999; Kaminker *et al.*, 2001), indicating that PARP-1 belongs to a family of poly-ADP-ribose polymerases. The six PARP family members can be divided into at least three subgroups according to their domain structures, the sequences of their catalytic domains, and their enzymatic activities. Table I shows a classification of the PARP family based on the literature and on database searches (Burkle, 2005). Subgroup I includes PARP-1; PARP-1b, PARP-2; and PARP-3. Experimental data suggest that both PARP-1 and PARP-2 play a major role in distinct stress response pathways (reviewed in references 20 and 161). Subgroup II contains a single member, PARP-4 (vault-PARP), which is the largest of the family (192.6 kDa) and was identified as a component of the vault complex. Tankyrase 1 and tankyrase 2 belong to subgroup III. All PARP enzymes (PARP-1, PARP-1b, PARP-2,

PARP-3, PARP-4, Tankyrase 1 and tankyrase 2) have automodification activity and most likely covalent auto-ADP-ribosylation activityPARP-1 showed the strongest automodification activity in vitro. The automodified domains were mapped for PARP-1 and PARP-2. Automodification takes place in the DNA-binding domains of PARP-1 and PARP-2 and in the so-called automodification domain of PARP-1 (Desmarais *et al.*, 1991; Schreiber *et al*, 2002). The automodification domain is involved in regulation of the catalytic property of the enzyme itself.PAR of PARP-1 causes the loss catalytic activity of PARP-1, thereby preventing it from poly-ADP-ribosylating target proteins.

Activation of PARP was proposed to be one of the earliest responses of mammalian cells to genotoxic stress (D'Amours *et al.,* 1999). While PARP-1 is constitutively expressed, its characteristic ability of being activated by DNA strand breaks makes PAR an immediate and drastic cellular response to DNA damage as induced by ionizing radiation, alkylating agents and oxidants. In the absence of DNA single and double strand breaks, PAR seems to be a very rare event in live cells, but it can increase over 100-fold upon DNA damage (Alvarez-Gonzalez and Althaus, 1989) Under these conditions about 90 % of poly-ADP-ribose is synthesized by PARP-1(Alvarez-Gonzalez and Jacobson, 1987). PARP-1 is the most abundant (~10^6molecules per mammalian cell) among all its isoforms and accounts for 90 % of total cellular PAR metabolism (Sharan, 2009).

PAR following DNA damage in primary mouse embryos and mouse embryo fibroblasts from *Parp1*$^{-/-}$ knockout mice was observed at 2 to 50 % of wild-type values, depending on the tissue and cell type (Ha and Snyder, 1999), and was drastically reduced only in *Parp1*$^{-/-}$brain, pancreas, liver, small intestine, colon, and testis. Moderate levels of residual poly-ADP-ribose formation were seen in *Parp1* stomach, bladder, thymus, heart, lung, kidney, and spleen (Ha and Snyder, 1999). Only limited data are available regarding the physiological roles and PAR activity of the novel PARP family members. Generally, the contribution of each PARP member to the total cellular PAR activity depends on tissue, cell type, and stimuli and the new PARPs are likely involved in specific nuclear and cytoplasmic functions requiring limited levels of poly-ADP-ribosylation.

Table I: Human enzymes involved in poly-ADP-ribose formation or degradation and their genes. (Adapted from Burkle A., 2005)

Official gene symbol	Name (aliases)	Chromosomal location	Protein size
PARP1	Poly(ADP-ribose) polymerase family, member 1 [PARP-1, ADPRT, ADPRT1, PARP, PPOL, pADPRT-1]	1q41-q42	1014 aa (113kDa)
PARP2	Poly(ADP-ribose) polymerase family, member 2 [PARP-2, NAD+ poly(ADP-ribose) polymerase-2]	14q11.2-q12	583 aa (66 kDa)
PARP3	Poly(ADP-ribose) polymerase family, member 3 [PARP-3, NAD+ poly(ADP-ribose) polymerase-3]	3p22.2-p21.1	532 aa (60 kDa); splice variant *539 aa (60.8 kDa)*
PARP4	Poly(ADP-ribose) polymerase family, member 4 [PARP4, ADPRTL1, PARPL, PH5P, VAULT3, VPARP, p193]	13q11	1724 aa (192.8kDa)
TNKS	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase [PARP-5a, PARP5A, PARPL, TIN1, TINF1, TNKS1]	8p23.1	1327 aa (142 kDa)
TNKS2	Tankyrase-2, TRF1-interacting ankyrin-related ADP-ribose polymerase 2 [PARP-5b, PARP-5c, PARP5B, PARP5C, TANK2, TNKL]	10q23.3	1166 aa (126.9kDa)
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase [PARP-7]	3q25.31	657 aa (75 kDa)
PARP10	Poly (ADP-ribose) polymerase family, member 10 [PARP-10]	8q24.3	1025 aa (150 kDa)
PARG	Poly(ADP-ribose) glycohydrolase [PARG]	10q11.23	Three splice variants 976 aa (111 kDa); 893 aa (102 kDa); 866 aa (99 kDa)

1.3.1. 2. **Poly-ADP-Glycohydrolase (PARG)**

The "classical" poly-ADP-ribose glycohydrolase, PARG, is known to represent the major PARG activity catalyzing the hydrolysis of poly-ADP-ribose polymers to free ADP-ribose in the cell. While at least six genes encode different PARPs synthesizing ADP ribose polymers, only one single gene coding for the "classical" PARG activity has been detected in mammalian cells until recently (Meyer-Ficca *et al.*, 2004; Oka,

Kato and Moss, 2006). The mammalian *Parg* gene encodes at least four isoforms: the low-abundance nuclear isoform PARG-110/ 111 and the three high-abundance cytoplasmic isoforms PARG-102, PARG-99 (characterized mainly in humans), and PARG-59/60 (characterized mainly in mice) (Brochu *et al.*, 1994; Di Meglio *et al.*, 2003; Meyer-Ficca, *et al.*, 2004). Whether all four isoforms simultaneously exist in all mammalian species remains to be investigated. PARG is an enzyme that possesses both endoglycosidic and exoglycosidic activity and is the only protein known to catalyse the hydrolysis of ADP-ribose polymers to free ADP-ribose (Davidovic, 2001). Its products are free poly-ADP-ribose and monomeric ADP-ribose.

1.3.1.3. **ADP-ribosyl protein lyase**

This enzyme is involved in cleaving the protein proximal ADP-ribose-glutamic ester bond (Okayama *et al*, 1978). The removal of the proximal ADP-ribose residue bound to the acceptor protein has been proposed as the rate-limiting step in the catabolism of carcinogen induced polymers (Wielkens *et al.*, 1982).

1.4. TARGET PROTEINS

Until recently, more than 200 nuclear proteins, most of them chromatin associated, have been proposed to be covalently modified by PAR *in vitro*. Target proteins for PAR are mostly nuclear such as topoisomerases I and II, histones, p53, Fos, Ku 70, HMG proteins, DNA polymerases α and β, DNA ligase I and II, RNA polymerases XRCC-1 (X-ray complementing factor-1), PCNA, hUbc9, the human homolog of the yeast ubiquitin conjugating enzyme Ubc9, and the DNA-dependent protein kinase catalytic subunit and PARP itself. The addition of heterogenous ADP-polymers to the target proteins affects the fundamental properties of the proteins in many ways as the net charge on the protein is altered. Such alterations would therefore result in diverse biological consequences (Sharan, 2009).

1.5. PAR AND CELLULAR PROCESSES

There is growing evidence that PAR is critical for many physiological and pathophysiological outcomes. Since ADP-ribosylation can activate or inhibit target

protein function, PAR transduces different signals to cellular machineries that regulate gene expression, DNA damage and repair, apoptosis, ageing, cell cycle, cell differentiation and carcinogenesis and may other cellular and molecular processes. The major cellular processes in which PAR reactions are believed to be involved are shown in Figure 6 (Sharan, 2009). Some of the processes have been discuss in the following sections.

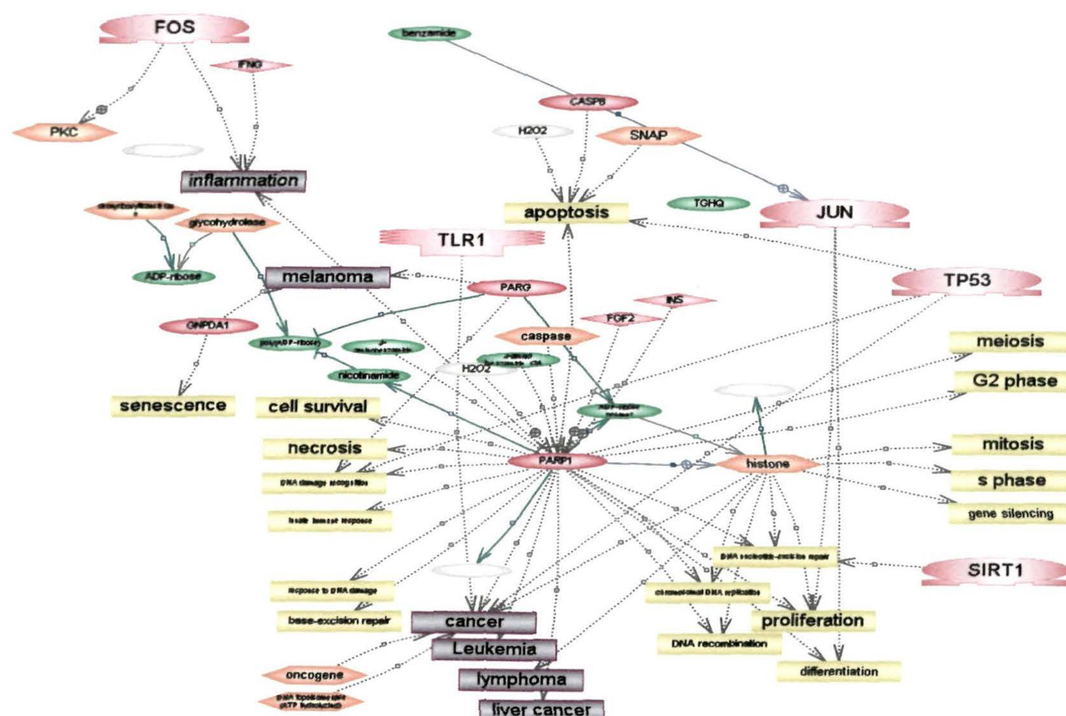


Figure 6. Complex, hypothetical and partial map of metabolic pathways and cellular processes/functions that are influenced by poly-ADP-ribosylation (PAR) metabolism and associated molecules with poly-ADP ribose polymerase (PARP) enzyme at the center. (Adapted from Sharan, 2009)

1.5.1. **PAR in gene expression**

It has long been postulated that PAR could influence the regulation of gene expression via regulation of chromatin remodelling (Tulin and Spradling 2003; Rouleau *et al.*, 2004). Indeed, numerous physical and functional interactions of PARP-1 with transcription factors have been described (Kraus, and Lis, 2003). PARP-1, for example, plays a pivotal role in NF-jB-dependent gene expression, which makes it an important cofactor in immune and inflammatory responses (Hassa. and Hottiger, 2002; Hassa *et*

al, 2003; Carrillo *et al*, 2004). Furthermore, recent data reveal functions of PARP-1 in the CaM kinase IId-dependent neurogenic gene activation pathway (Ju *et al*, 2004), in the NAD+-dependent modulation of chromatin structure and transcription mediated by nucleosome binding of PARP-1 (Kim *et al*, 2004), and in the determination of specificity in a retinoid signalling pathway via direct modulation of Mediator (Pavri *et al*, 2005). The involvement of PAR in long-term potentiation in Aplysia neurones, occurring in the absence of DNA strand breaks, has also been linked with transcriptional effects (Cohen *et al* 2004). In primary fibroblast, PARP deficiency resulted in down regulation of several genes involved in cell cycle progression (Simbulan-Rosenthal *et al*, 2000).

1.5.2. **PAR in DNA damage and repair**

A long list of studies have firmly established that DNA damage-induced PAR contributes to cellular recovery from cytotoxicity in proliferating cells caused with low or moderate levels of DNA damage by alkylation, oxidation or ionizing radiation. PARP-1 as well as the related protein PARP-2 are those members of the PARP family that are responsive to DNA damage and play important roles in DNA repair and maintenance of genomic integrity, thus behaving as 'survival factors (Bouchard *et al.*, 2003; Ishizuka *et al.*, 2003). Specifically, PARP-1 and PARP-2 have been shown to play a crucial role in the base excision repair (BER) pathway. PAR is therefore an immediate and transient post-translational modification of nuclear proteins induced by DNA lesions mainly repaired by the (BER) pathway. PARP-1 is highly efficient in detecting DNA nicks through its two CX2CX28, 30HX2C zinc fingers that define a DNA-break-sensing motif, which is also found in the SSBR/BER enzyme DNA ligase III and the repair enzyme DNA 3′ phosphoesterase from *Arabidopsis thaliana* (Petrucco, 2003). The resulting PAR production has three purposes (Fig.7): first, DNA-damage-induced PAR of the N- and C-terminal tails of histone H1 and H2B or the selective interaction of these proteins with free or PARP-1-bound PAR (Poirier *et al.*, 1982; Realini *et al.*, 1992) contributes to the relaxation of the 30-nm chromatin fibre and increases the access to breaks; second, it signals the occurrence and the extent of DNA damage so that the cell can establish an adaptive response according to the severity of the injury (DNA repair or cell suicide); third, it mediates the fast recruitment of SSBR/BER factors to the site of the lesion. PARP interacts with X-ray repair cross complementing factor-1(XRCC1) (Caldecott *et al* 1996; Masson *et al*, 1998), an adaptor

protein which has also two interfaces with two important base excision repair enzymes, DNA ligase III (Nash *et al.*, 1997) and DNA polymerase b (Kubota *et al.*, 1996). As a consequence of these interactions, XRCC1 stabilizes DNA ligase III (Caldecott *et al*, 1995), but negatively regulates PARP activation following oxidative stress, presumably in a transient manner (Masson *et al.*, 1998). Therefore, PARP is probably associated with a multifunctional complex including, at least, XRCC1, DNA polymerase b and DNA ligase III. Both enzymes are involved in the BER, the most frequently solicited DNA repair pathway in mammalian cells (Seeberg *et al.*, 1995).

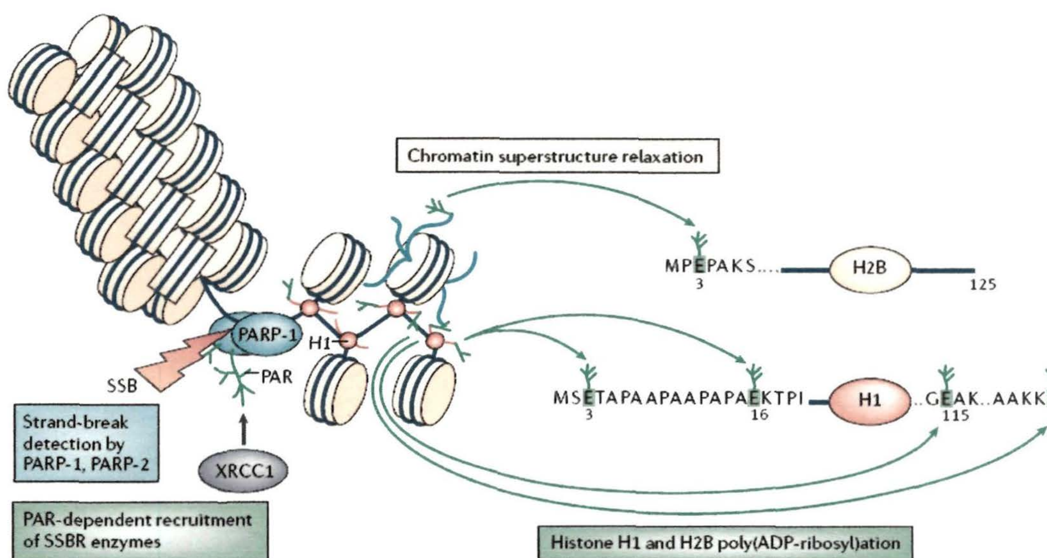


Figure 7: PARP-1 in the repair of single-strand breaks. Following the detection of single-strand-DNA breaks (SSBs) by PARP-1 and PARP-2, poly-ADP-ribose synthesis at the DNA-damage site triggers both the immediate recruitment of the SSB repair (SSBR) scaffold protein XRCC1 and the relaxation of the chromatin superstructure as mediated by the PAR of histone H1 and H2B tails. Together, this facilitates access to the DNA lesion of repair enzymes that interact with XRCC1 (Adapted from Khorasanizadeh, S., 2004).

In cells in which single-strand breaks and double-strand breaks were locally introduced by irradiation, the recruitment of XRCC1 to the damage site was abolished due chemical inhibition of PAR (Okano *et al.*, 2003; Ame *et al.*, 2004).

A similar conclusion was reached following the irradiation of *Parp-1–/–* cells (Schreiber *et al.*, 2006). Prevention of XRCC1 recruitment affects the subsequent repair process, as XRCC1 conducts the SSBR/BER machinery by stimulating most of the

repair enzymes (Caldecott, 2003). This probably explains the delay in strand-break rejoining that is observed in *Parp-1–/–* cells and its deleterious impact on genome integrity and cell survival (Trucco *et al*, 1998).

1.5.3. PAR in apoptosis

Apoptosis, also called "programmed cell death" or "cell suicide", is considered a controlled biochemical pathway of cell demise. The apoptotic process is generally divided in three main phases (initiation, effector, and execution) and requires ATP (Gonzalez *et al.*, 2001). In the 1990s, cell biologists used the expression "death substrate" (Tewari *et al.*, *1995*) to name PARP-1, one of the first identified substrates of caspases, the main executioners of apoptosis (Kaufmann *et al.*, 1993). Therefore, a role for PARP-1 in the regulation of apoptosis has been suggested. During apoptosis, a precocious and transient stimulation of PARP-1 causes poly-ADP-ribose accumulation in early apoptotic cells (Donzelli *et al.*, 1997; Rosenthal *et al.*, 1997; Soldani *et al.*, 2001). Excessive NAD consumption is prevented by the cleavage of PARP-1 by caspases (Soldani and Scovassi, 2002). PARP-1 cleavage generates two inactive fragments of 24 and 89 kDa. The N-terminal fragment (p24) remains in the nucleolus, retains its DNA-binding activity and inhibits the catalytic activity of uncleaved PARP-1, and also impairs DNA repair (Alvarez-Gonzalez, *et al.*, 1999; D'Amours *et al.*, 2001). The p89 fragment migrates from the nucleus to the cytoplasm in late apoptotic cells with advanced nuclear fragmentation (Soldani *et al.*, 2001; Mi, *et al.*, 2003), becoming a potential target of autoimmunity (Rodenburg *et al.*, 2000). A further evidence for the involvement of PAR in cell death machinery is provided by the observation that PARP-1 is required for the translocation of the AIF (Apoptosis-Inducing Factor) protein from the mitochondria to the nucleus, with the consequent activation of a caspase-independent apoptotic pathway (Yu *et al.*, 2002). The molecular mechanisms triggering the release of AIF are still obscure (Cregan *et al.*, 2004), even if the recent evidence of an intramitochondrial PARP could support its direct effect on AIF (Du, *et al.*, 2003). Remarkably, during apoptosis PAR regulates the activity of DNAS1L3, a Ca^{2+}/Mg^{2+}dependent nuclease that is normally repressed by poly-ADP-ribose. PARP-1 inhibition was shown to promote its release, with a consequent DNA fragmentation and cell death (Yakovlev *et al.*, 2000; Boulares *et al.*, 2002).

1.5.4. **PAR in ageing**

Ageing is a multi-factorial process and has been defined as time-dependent general decline in physiological function of an organism, associated with a progressively increasing risk of morbidity and mortality. It is apparent that during ageing different organs are losing their functional reserve and plasticity and become less able to fulfil their physiological function, especially under conditions of stress. Fifteen years ago, Grube and Burkle showed that DNA strand break-dependent PARP activity in permeabilized peripheral blood mononuclear cells (PBMC) correlates positively with the maximal life span in mammalian species (Grube and Burkle, 1992). Human PBMC displayed a 5-fold increase in PAR production compared to rat PBMC, yet expressed the same amount of PARP-1 protein. Subsequent experiments with recombinant PARP-1 proteins from man and rat revealed a 2-fold higher automodification level in human PARP-1 although classical enzymatic parameters like Vmax and Km were not different (Beneke *et al.*, 2000). The interplay with other proteins either relaying the PAR signal or being directly influenced by it probably enhances evolutionary divergences. Interestingly, both in humans and rats, PARP activity declined with donor age. On the other hand, a study using immortalized lymphocytes proved that PARP activity was higher in cells from centenarians than in appropriate controls (Muiras *et al.,* 1998). ADPRT gene expression was also reported to be down regulated in proliferating fibroblasts from old human donors and from progeria patients compared to normal young donors (Ly *et al.*, 2000). Viewed together, various pieces of evidence from a variety of systems and experimental approaches are now converging in support of a close link between PAR and the control of the ageing process.

1.5.5. **PAR in cell cycle**

The subcellular localization of several PARPs hints at a possible physiological role of PAR in the regulation of cell division. The first important observation was that PARP-1 and PARP-2 localize to centromeres where they interact with the kinetochore proteins CENPA, CENPB and BUB3 (Saxena *et al.*, 2002; Saxena *et al.*, 2002). Interestingly, the ablation of the *Parp-2* gene in mice causes significant DNA damage-induced chromosome mis-segregation that is associated with kinetochore defects, which indicates that PARP-2 has a crucial guardian function in pericentric heterochromatin integrity (Menissier de Murcia *et al.*, 2003). Another role of PAR in cell division relates to the association of both PARP-1 and PARP-3 with centrosomes (Augustin *et*

al., 2003). Although the precise significance of this observation is currently unclear, it links the DNA-damage-surveillance network with the mitotic fidelity checkpoint. This link is further indicated by the inhibition of the chromosomal passenger protein Aurora-B by PAR in response to DNA damage and by the interaction of PARP-1 with the spindle checkpoint component BUBR1 (Monaco *et al*., 2005; Fang, *et al*., 2006). More recent evidence for the role of PAR in the regulation of microtubule organization has been provided by the enrichment of PAR and PARG at mitotic spindle microtubules, spindle poles and kinetochores in *Xenopus laevis* egg extracts (Chang *et al*., 2004). Tankyrase-1 seems to be the enzyme that is required for the polymerization of spindle-associated PAR (Chang *et al*., 2005). The PAR activity of tankyrase-1 might be crucial for the accurate formation and maintenance of spindle bipolarity, partly through the covalent modification of the spindle pole protein nuclear mitotic apparatus proteins (NuMA), which cross links microtubule ends at the spindle poles. Furthermore, PARP activity of tankyrase-1 was shown to be required for normal telomere separation before anaphase. The PAR of NuMa by tankyrase-1 might help to coordinate sister-telomere resolution with mitotic progression (Dynek and Smith, 2004). Taken together, these studies clearly indicate the importance of PAR and PARP enzymes in the regulation of chromosomal segregation. PARP-1 is also involved in cell-cycle check-point control after DNA damage. After γ-irradiation, p53-dependent induction of the *p21* and *mdm2* genes is attenuated by PARP inhibitors and suppression of G1 arrest and enhancement of G2 arrest are observed (Nozaki *et al*., 1994; Masutani *et al*., 2005). After treatment with neocarzinostatin, an increased level of γ-H2AX, a marker of DSB, was observed, accompanied by augmented p53 phosphorylation at the ser 18 residue in *PARP*-$1^{-/-}$ mouse embryonic fibroblast (MEF). This accompanied enhancement of kinase activity of the ATM protein (Watanabe *et al*., 2004). In addition, S-phase entry from G0 phase was found to be delayed in several cell types by *PARP-1* deficiency (Masutani *et al*., 2005).

1.5.6. **PAR in cell proliferation and differentiation**

To fulfill specific tasks and to function as specialized building blocks of tissues, cells need to undergo a series of proliferative steps during which they gain new functions and lose others. This strictly controlled process requires concerted gene activation and repression and results in differentiation into specialized cells functioning as hepatocytes, neurons, renal tubular cells, and so forth. Furthermore, many fully

differentiated cell types such as lymphocytes, fibroblasts, and hepatocytes retain the ability to proliferate, such as in the course of immune response, wound healing, or liver regeneration, respectively. Moreover, after DNA damage, it is of primary importance to stop replications at certain check points to allow for the repair of DNA damage. From our current knowledge of PARP function, it is now widely accepted that PARP-1 is involved in the regulation of DNA replication, differentiation, and gene expression. Involvement of PARP-1 in the regulation of replication is supported by observations that poly-ADP-ribose metabolism is accelerated in the nuclei of proliferating cells (Tanuma *et al.*, 1978; Kanai *et al.*, 1981; Leduc *et al.*, 1988; Bakondi *et al.*, 2002). Several lines of evidence suggest that PARP-1 is part of the MRC (Simbulan-Rosenthal *et al.*, 1996). PARP-1 co-purifies with DNA polymerase α and δ, DNA primase, DNA helicase, DNA ligase, topoisomerases I and II, and key components of MRC (Simbulan-Rosenthal *et al.*, 1996; Dantzer *et al.*, 1998; Bauer *et al.*, 2001). Furthermore, several centromere proteins (Saxena *et al.*, 2002) and replication factors such as DNA polymerase α, topoisomerase I and II, and proliferating cell nuclear antigen have been shown to be poly-ADP-ribosylated (Simbulan-Rosenthal *et al.*, 1996). Moreover, PAR of histones was also proposed to facilitate the assembly and deposition of histone complexes on DNA during replication (Boulikas, 1990). Nonetheless, the exact role of PARP-1 in the regulation of replication is still controversial. Inhibition of PARP by pharmacological or molecular biological means (anti-PARP-1 antisense, knockout cells, dominant- negative PARP inhibition by over expression of the DNA binding domain of PARP) has been shown to inhibit replication, cell proliferation, and differentiation in various experimental models (D'Amours *et al.*, 1999). However, PARP-1 has also been proposed to be a negative regulator for the initiation of DNA replication (Eki, 1994). Given that replication and differentiation are closely coupled processes, the above-mentioned experimental data may provide rationale for a differentiation-modifying effect of PARP. Indeed, inhibition of PARP has been shown to interfere with differentiation in various cellular models. In NB4 acute promyelocytic leukemia and HL-60 acute myelocytic leukemia cells, PARP levels were dramatically modulated during monocyte / macrophage and neutrophilic differentiation (Bhatia *et al.*, 1995). PARP inhibitors (5-methylnicotinamide, 3-methoxybenzamide, and 3-aminobenzamide) were found to inhibit differentiation of human granulocyte-macrophage progenitor cells to the macrophage lineage (Francis *et al.*, 1983). Differentiation to the neutrophil-granulocyte

lineage was much less affected (Francis *et al.*, 1983). In other studies, over expression of PARP arrested NB4 cells and blocked all trans-retinoic acid-induced terminal neutrophilic differentiation (Bhatia *et al.*, 1996). Furthermore, plasmacytic differentiation of Daudi lymphoma cells was impaired in the presence of PARP inhibitors (Exley *et al.*, 1987). Importance of cell type-specific differences is also underlined by observations that benzamide PARP inhibitors induced melanogenesis and differentiation of melanoma cells (Durkacz *et al.*, 1992). PAR has also been implicated in erythroid differentiation (Rastl and Swetly, 1978; Morioka *et al.*, 1979; Terada *et al.*, 1979; Sugiura *et al.*, 1984), chicken limb bud mesenchymal cell differentiation (Nishio *et al.*, 1983; Cherney *et al.*, 1985) and trophoblastic cell differentiation during tumorigenesis (Masutani *et al.*, 2001).

1.6. PAR IN CARCINOGENESIS

Carcinogenesis involves multiple steps and pathways with functional alterations in a variety of genes. There is accumulating evidence that a deficiency of PARP-1 leads to DNA repair defects, genomic instability, failure of induction of cell death and modulation of gene transcription. PARP-1 also supports the growth of tumor cells in certain situations. The earliest report of that linked PAR with carcinogenesis was the up regulation of PARP in malignant or transformed cell in vitro when compared to control (Miwa *et al.,* 1977) and in leukemia and ovarian cancer (Singh, 1991). In human cancers, increased expression of the PARP-1 gene has been reported in Ewing's sarcomas, (Masutani *et al,* 2005) and in malignant lymphomas (Masutani *et al,* 2005). In contrast, decreased expression has been observed in several gastric and colon cancer cell lines, (Masutani *et al,* 2005) as well as in some breast cancers (Bieche *et al.,* 1996). The importance of a proficient PAR system for the maintenance of genomic stability and thus the prevention of cancer is also mirrored in the finding that the V762A genetic variant of PARP-1 (Van Gool *et al.*, 1997; Cottet *et al.*, 2000), which is associated with diminished enzyme activity, contributes to prostate cancer susceptibility (Lockett *et al,* 2004). Previous work done in our laboratory shown strong negative correlation between PAR of cellular proteins and initiation, promotion and progression of cancer (Saikia, 1996; Pariat, 1997; Devi, 2001; Kma, 2003). A novel immunoprobe assay has been developed to determine the link between PAR and carcinogenesis (Sharan *et al,*

1998; Sharan *et al,* 2005). PAR of total cellular proteins as well as of histone proteins of liver and spleen cells were significantly lowered during initiation of carcinogenesis in mice exposed to DMN (dimethlynitrosamine)(Devi BJ *et al.,* 2005; Kma and Sharan, 2006; Kma and Sharan, 2008). Aqueous extract of betel nut was also found to lower PAR of different proteins in mice (Saikia *et al,* 1998; Pariat and Sharan, 2002). In Dalton's lymphoma ascites tumorigenesis, PAR of histone proteins was progressively lowered during the later stage of cancer development (Devi and Sharan, 2005). It has also been suggested that lowering of PAR is a hallmark during both the initiation phase of carcinogenesis as well as tumorigenesis (Devi BJ et al, 2005. Blood lymphocyte proteins of mouse exposed to DMN also mirrored lowering of total PAR (Kma and Sharan, 2006; Kma, 2003). In normal peripheral blood lymphocytes of laryngeal cancer patients diminished PARP activity has been reported (Rajaee-Behbahani *et al.,* 2002).

The human PARG gene has been mapped to chromosome10q11.23 (Shimokawa *et al.,* 1998) and is organized into 18 exons (Meyer *et al.,* 2003). Exons9–14 forms the catalytic center, while exons 1–4 encode the putative regulatory domains. Notably, PARG structure variants, some of which may be due to polymorphisms, are clustered from exons 1 to 3 (Meyer *et al,* 2003), although the relevance of these polymorphisms to cancer still needs careful analysis. The PARG gene shares a 470-bp common promoter region with the *mitochondrial membrane translocase 23* (*TIMM23*) gene. A bidirectional mode of promoter activity was demonstrated (Meyer *et al.,* 2003). The PARG promoter contains three overlapping Sp1 binding sites, binding sites for lymphocyte specific factor I (LyF-1), MyoD (myogenic determination factor D), ETS-1 binding sites and a putative inhibitory 120-kDa cyclic AMP (cAMP) responsive element binding protein (CREBP) (Meyer *et al.,* 2003). Because the activity of these transcription factors might change during carcinogenesis, resultant alteration in expression levels of the PARG gene may cause the alteration in PAR of proteins.

1.7. CANCERS OF HEAD AND NECK REGION

Head and neck cancer encompasses a wide range of malignant tumours arising from many diverse and complex structures in this region of the body, and which have major physiological and aesthetic importance. Most head and neck cancers begin in the cells that line the mucosal surfaces in the head and neck area, e.g., mouth, nose, and throat.

Mucosal surfaces are moist tissues lining hollow organs and cavities of the body open to the environment. Normal mucosal cells look like scales (squamous) under the microscope, so head and neck cancers are often referred to as squamous cell carcinomas. Some head and neck cancers begin in other types of cells. For example, cancers that begin in glandular cells are called adenocarcinomas.

Cancers of the head and neck are further identified by the area in which they begin: (www. cancer. net.html)

- Oral cavity. The oral cavity includes the lips, the front two-thirds of the tongue, the gingiva (gums), the buccal mucosa (lining inside the cheeks and lips), the floor (bottom) of the mouth under the tongue, the hard palate (bony top of the mouth), and the small area behind the wisdom teeth.
- Salivary glands. The salivary glands produce saliva, the fluid that keeps mucosal surfaces in the mouth and throat moist. There are many salivary glands; the major ones are in the floor of the mouth, and near the jawbone.
- Paranasal sinuses and nasal cavity. The paranasal sinuses are small hollow spaces in the bones of the head surrounding the nose. The nasal cavity is the hollow space inside the nose.
- Pharynx. The pharynx is a hollow tube about 5 inches long that starts behind the nose and leads to the esophagus (the tube that goes to the stomach) and the trachea (the tube that goes to the lungs). The pharynx has three parts:
  - Nasopharynx. The nasopharynx, the upper part of the pharynx, is behind the nose.
  - Oropharynx. The oropharynx is the middle part of the pharynx. The oropharynx includes the soft palate (the back of the mouth), the base of the tongue, and the tonsils.
  - Hypopharynx. The hypopharynx is the lower part of the pharynx.

- Larynx. The larynx, also called the voicebox, is a short passageway formed by cartilage just below the pharynx in the neck. The larynx contains the vocal cords. It also has a small piece of tissue, called the epiglottis, which moves to cover the larynx to prevent food from entering the air passages.

- Lymph nodes in the upper part of the neck. Sometimes, squamous cancer cells are found in the lymph nodes of the upper neck when there is no evidence of cancer in other parts of the head and neck. When this happens, the cancer is called metastatic squamous neck cancer with unknown (occult) primary.

Each year there are approximately 400000 cases of cancer of the oral cavity and pharynx, with 160 000 cancers of the larynx, resulting in approximately 300 000 deaths (Franceschi *et al.*, 2000). Regions with a high incidence include much of Southern Asia as well as parts of Central and Southern Europe (World Cancer report, 2008). The main risk factors for these cancers are tobacco and alcohol use. Established risk factors specifically for oral cavity cancer are betel quid and areca nut in India and Taiwan (IARC 2004). Human papilloma virus (HPV) is a recognised cause of some head and neck cancers, with substantial evidence for a role of HPV16E6 from large case-control studies (Kreimer *et al.*, 2005).

A number of specific genetic events have been identified in the progression of head and neck squamous cell carcinoma (HNSCC). Identification of the critical genetic changes that drive the neoplastic process has provided a preliminary progression model for head and neck cancer. Statistical analysis based on the age-specific incidence of head and neck cancer suggests that HNSCC tumors arise after the accumulation of six to ten independent genetic events (Renan, 1993). Cytogenetic approaches have given us some insights into potential areas of deletion and amplification involved in the progression of head and neck cancer. Karyotypic studies have already demonstrated consistent chromosomal abnormalities and the presence of important alterations (Van Dyke *et al.*, 1994). Loss of chromosomes 3p, 5q, 8p, 9p, 18q, and 21q has been commonly identified. Preliminary data also suggest that loss of 18q may indicate the presence of a tumor with a poor prognosis (Van Dyke *et al.*, 1994). Additionally, multiple chromosomal breakpoints including those on 1p22, 3p21, 8p11, and distal 14q have correlated with decreased radiosensitivity (Cowan *et al.*, 1993). Proto oncogenes were initially identified as activated cellular genes specifically altered in some human neoplasms (Bishop, 1991). Definitive studies suggested that the 11q13 amplification was associated with amplification of a critical protooncogene termed cyclin D1 (PRAD1; CCND1). Although other genes were also co-amplified in the same region, only cyclin D1 was consistently amplified in approximately 30 % of HNSCC and most

other neoplasms (Berenson *et al.*, 1989; Callender *et al.*, 1994). Moreover, amplification of this region correlated with increased expression of the cyclin D1 gene and may indicate a likelihood of progression in primary HNSCC (Jares *et al.*, 1994).

As previously noted, amplification of 3q has been noted in many squamous cell carcinomas, including head and neck cancer. A p53 homologue (p40 /p51 /p63) has been cloned and localized to the distal arm of 3q (Trink *et al.*, 1998). The role of cyclin D1 in the progression of human cancer is now well established (Hunter and Pines, 1994). Other tumor suppressor genes, including Rb and p16, are negative regulators of the cyclin D1 pathway and often are inactivated in human neoplasms. In head and neck cancer, p16 appears to be a major target of inactivation. Thus, abnormal cycling through this critical G1/S checkpoint may be a consistent genetic alteration in a majority of primary HNSCC. Although p16 and Rb inactivation are almost always exclusive, cyclin D1 amplification is independent of p16 inactivation in head and neck cancers (Okami *et al.*, 1999).

Like other epithelial neoplasms, the role of other protooncogenes has been much less definitive. Few mutations in ras have been identified in primary head and neck tumors. Although epidermal growth factor receptor (EGFR) has been an interesting candidate, increased levels of the receptor at the RNA or protein level rarely correlate with primary DNA amplification (Grandis and Tweardy, 1993). New evidence suggests that activation of signaling through Stat-3 leads to EGFR-mediated cell growth and that antisense suppression of EGFR protein leads to apoptosis (programmed cell death) (He *et al*, 1998; Grandis *et al.*, 1998). The protein eukaryotic initiation factor (eIF4E) binds to messenger RNA during initial protein synthesis, and its overexpression can result in the up-regulation of proteins essential for cell growth and division. Over expression of eIF4E has been found in HNSCC, and there has been some evidence of gene amplification and protein over expression in these tumors (Sorrells *et al.*, 1999a; Sorrells *et al.*, 1999b). Over expression of the protein in cells can lead to oncogenic transformation and may facilitate the synthesis of angiogenic factors such as vascular epidermal growth factor by enhancing their translation. In at least one study, there was a correlation between increasing eIF4E and vascular epidermal growth factor levels, suggesting its possible role in angiogenesis (Nathan *et al.*, 1999).

Additionally, several other genes or gene products have been found to be over expressed in head and neck tumors. High levels of cyclooxygenase (COX)-2, have been seen in squamous cell carcinomas by a competitive reverse transcription assay (Chan *et al.*, 1999). GST P1 messenger RNA levels are found to be high in most moderately and poorly differentiated tumors (Wang *et al.*, 1997) but only a fraction of these had specific gene amplification. Newer cytogenetic techniques may lead to the identification of important protooncogenes more commonly involved in the progression of HNSCC. In addition to growth factors with "positive" regulation and augmentation of tumor growth, other growth factor pathways may suppress cell growth. Transforming growth factor-b (TGF-b) is among these growth factors that have been implicated almost universally with suppression of tumor growth.Initially, some head and neck cancer cell lines were also found to harbor TGF-b receptor mutations, and mutations in the conserved serine/threonine kinase domain were found in 6 of 28 primary tumors (Markowitz *et al.*, 1995; Garrigueantar *et al.*, 1995).

Molecular analysis has now revolutionized the ability to look at primary neoplasms. It is now generally believed that these allelic losses (or chromosomal deletions) are markers for inactivation of critical tumor suppressor genes contained within the regions of loss (Knudson, 1971). Testing of highly polymorphic microsatellite markers (small 2– to 4–base pair repeats) from a specific chromosomal region allows rapid assessment of allelic loss by comparing the alleles in tumor DNA to normal DNA. Perhaps the best example of this association is derived from loss of chromosome 17p. These losses led to characterization of p53 as a candidate gene within the deleted area and subsequent identification of point mutations within the remaining allele. Inactivation of p53 now represents the best described and most common genetic change in all of human cancer (Hollstein *et al.*, 1991). Approximately 50 % of all primary HNSCCs harbor p53 mutations in the conserved regions (exons 5 to 9) (Boyle *et al.*, 1993; Brennan *et al.*, 1995).

A comprehensive allotype of head and neck cancer has now been completed and refined (Nawroz *et al.*, 1994; Ahsee *et al.*, 1994). The most commonly deleted region in head and neck cancer is located at chromosome 9p21-22 (Nawroz *et al.*, 1994). Loss of chromosome 9p21 occurs in the majority of invasive tumors and is also present at a high frequency in the earliest definable lesions, including dysplasia and carcinoma in

situ (Van der Riet *et al.*, 1994). Furthermore, homozygous deletions in this region are frequent in HNSCC and represent one of the most common genetic changes identified in all human neoplasms. p16 (CDKN2) is contained within this critically deleted region and is a potent inhibitor of cyclin D1/CDK4 complexes. Thus, p16 has emerged as an excellent candidate tumor suppressor gene within the deleted area (Kamb *et al.*, 1994).

Although initial enthusiasm for p16 as a target gene in head and neck cancer was diminished when sequence analysis revealed rare point mutation (approximately 10 % to 15 % of HNSCC tumors) (Zhang *et al.*, 1994; Cairns *et al.*, 1994) alternative mechanisms of inactivation were identified suggesting that abrogation of p16 function may be a common occurrence in head and neck cancer. Homozygous deletion (deletion of both gene copies) and methylation of the 5' CpG region of p16 have been identified, each detected in approximately one-fourth of primary head and neck cancers (Merlo *et al.*, 1995; Cairns *et al.*, 1995). This methylation is associated with complete block of p16 transcription and appears to be a common mechanism for p16 inactivation. The notion that p16 inactivation is directly involved in the progression of primary tumors has been strengthened. Lack of p16 protein was detected by immunostaining in most primary invasive lesions, and tumors with absent p16 protein contained a homozygous deletion, methylation, or point mutation of p16.60 Loss of p16 protein has been observed in most advanced premalignant lesions (Papadimitrakopoulou *et al.*, 1997).
A second commonly deleted locus occurs on chromosome 3p. Several studies have suggested that this region of loss is complex in head and neck cancer and may in fact be composed of three distinct suppressor regions juxtaposed to one another (Maestr *et al.*, 1993; Wu *et al.*, 1994). As for 9p21, analysis of 3p21 losses in HNSCC has revealed frequent loss in early lesions. Loss of chromosome 17p is a frequent occurrence in most human cancers, and head and neck cancer is no exception (occurring in 60 % of invasive lesions) (Boyle *et al.*, 1993). Although p53 inactivation correlates closely with loss of 17p in invasive lesions, p53 mutations are quite rare in early lesions that contain 17p loss. Some evidence from cell lines also suggests that a distal breakpoint to p53 occurs in head and neck cancer. Together, these data suggest that a second tumor suppressor gene on 17p may be involved early in the progression of this neoplasm. p53 mutations, as in most tumors, generally rise in frequency between the preinvasive to the invasive state.

Loss of chromosomal arm 10q is not uncommon in HNSCC and lung cancer. The PTEN gene was cloned and found to be homozygously deleted and inactivated in a variety of different cancers. Homozygous deletion and rare point mutation inactivation have been seen in HNSCC (Okami *et al.*, 1998; Shao *et al.*, 1998). Although only 10 % of these cancers have inactivated gene, it seems to be more common in advanced tumors and may harbor a poor prognosis (Okami *et al.*, 1998; Gasparotto *et al.*, 1999). Loss of 13q also occurs in approximately 60 % of primary tumors and the minimal area of loss includes the tumor suppressor gene Rb. However, immunohistochemical analysis of Rb (which detects most Rb alterations) revealed inactivation of Rb in only small percentages of tumors with loss of 13q (Yoo *et al.*, 1994). Again, as in many other chromosomal regions, there appears to be another tumor suppressor gene near Rb, putatively inactivated in the progression of head and neck cancer.

1.8. CANCER OF THE BREAST

Breast cancer is the most common cancer among women worldwide. It was estimated that 636,000 incident cases occurred in developed countries and 514,000 in developing countries during 2002 (Parkin *et al.*, 2005). Breast cancer is also the most important cause of neoplastic deaths among women; the estimated number of deaths in 2002 was 410,000 worldwide. Breast cancer is a term broadly applied to infiltrating ductal, infiltrating lobular, medullary, tubular, and mucinous carcinomas. A number of risk factors have been associated with development of the disease, including the dose and duration of estrogen exposure, early menarche, late menopause, age at first childbirth, nulliparity, fat in the diet, postmenopausal hormone replacement therapy, alcohol consumption, cigarette smoking and ionizing radiation (Field and Spandidos, 1990).Family history of breast cancer is associated with a 2-3-fold higher risk of the same disease, and risk increases with the number of affected first-degree relatives (Familial breast cancer, 2001).

Although relatively little is known about the molecular mechanisms leading to breast cancer development, breast cancers have probably been studied more than any other tumor type with regard to oncogene expression. *MYC, ERBB2* or one of the *RAS* family have been found to be expressed in over 60 % of cases. Mutations in highly

susceptibility genes are estimated to underlie only 5–10 % of breast cancers yet these are important as individuals carrying such mutations are at very high risk of developing breast cancers, of having more than one primary cancer, and of passing these altered genes to their offspring (Ang and Garber, 2000). Following the genomic localization and subsequent identification of the breast cancer susceptibility genes *BRCA1* and *BRCA2* (considered being tumor suppressor genes), the basic pattern of cancer risk associated with these genes has been defined (Rahmanand and Stratton, 1998). Very few examples of rearrangements of the *MYCN* or *MYCL* genes have been found in breast cancer. On the other hand, there is considerable evidence for MYC amplification although the reported incidence varies from 4 % to 52 % (Bieche and Lidereau, 1995; Nass and Dickson, 1997). Three large studies of *MYC* amplification in breast cancer have produced different conclusions as to the value of this marker in prognosis (Field and Spandidos, 1990).

Numerous studies have shown the importance of the *p53* tumor suppressor gene in cancer, including breast cancer (Warren *et al.*, 1992; Delmolino *et al.*, 1993). In addition to mutations, a second mechanism is believed to inactivate wild-type p53. In some types of breast cancer, the wild-type p53 can lose its tumor-suppressive function by being sequestered in the cytoplasm and prevented from entering the nucleus, its normal site of action (Moll *et al.*, 1992). This has been suggested as a possible explanation for the development of breast cancer in cases that do not show any *p53* mutations. Most breast cancer cases are believed to be sporadic, the results of a spontaneously arising mutation. However, a small proportion of cancers result from an inherited predisposition. Evidence for inherited disease includes early age of onset and extensive bilateral breast tumors clustered within families. For example, the Li-Fraumeni syndrome is a familial predisposition to the early childhood development of soft tissue sarcomas as well as early-onset breast cancer in parents and relatives (Warren *et al.*, 1992). Breast tumors from Li-Fraumeni cancer patients are associated with inherited mutations within exon 7 of the *p53* gene. These mutations are primarily CG-TA transitions at CG dinucleotides (Warren *et al.*, 1992; Coles *et al.*, 1992). Sporadic breast tumors are associated with mutations clustered within exons 5, 6, 7 and 8. However, most other inherited breast cancer cases are rarely caused by germ-line point mutations in *p53*. Several studies have shown the possible involvement of a number of other tumor suppressor genes and several oncogenes in the development of

the disease (Coles *et al.,* 1992). Although mutations within *p53* are significantly associated with breast cancer development, several other factors can play a role in the disease process. These include estrogen receptor status, accumulation of p53 protein and the p53-binding murine double minute-2 (MDM2) gene product which has been identified as a negative regulator of *p53. MDM2* over expression may be another mechanism by which cancer cells overcome p53-regulated growth control without selecting for p53 mutations as such (Ozbun and Butel 1995).

The breast cancer susceptibility genes *BRCA1* and *BRCA2* encode multifunctional proteins, the mutant phenotype of which predisposes to both breast and ovarian cancer (Rahman and Stratton, 1998; Welsch *et al.,* 2000). The combined contribution to overall breast cancer incidence of strongly predisposing mutations in *BRCA1* and *BRCA2* which confer individual risks of about 60 % by age 70 years is less than 5 %. By contrast, a predisposing allele with a relative risk of 2 and a frequency of 20 % could account for up to 20 % of breast cancer incidence (Ponder, 2001). The risk to close relatives of a case, averaged across all ages, is about twofold. Most of this familial risk is probably genetic in origin (Peto, 2001). The risk is about the same for the mother, sisters or daughters of a case, suggesting dominant rather than recessive effects. Large population-based studies indicate that only 15–20 % of overall familial risk is attributable to mutations in *BRCA1* and *BRCA2* and that *BRCA1* and *BRCA2* mutations are rare in the general population (Ponder, 2001; Anglia Breast Cancer Study Group, 2000).

1.9. CANCER OF THE CERVIX

Cervical cancer is an important global public health problem. It accounted for an estimated 493,000 incident cases, 1.4 million prevalent cases and 273,000 deaths in the world in 2002, constituting approximately 8 % of the global burden of cancer among women and the second most common cancer among women worldwide. Developing countries accounted for four fifths of this global burden. It is a major cause of mortality and premature death among women in their most productive years in low- and medium-resource countries in Asia, Africa and Latin America (World Cancer report, 2008).

Cervical cancers arise in the epithelium covering the uterine cervix, particularly at the junction between the columnar epithelium of the endocervix and the squamous epithelium of the ectocervix, a site of continuous metaplastic change, especially in utero, at puberty and after first pregnancy through to menopause. Persistent infection of the cervical epithelium with one or more oncogenic types of human papilloma viruses (HPV) lead to the development of precancerous lesions therein, a proportion of which, if not detected and treated, progress to invasive cervical cancer over a period of 10 – 20 years. Squamous-cell carcinomas are the most common type of epithelial tumours of the cervix, accounting for 85–90 % of the epithelial cancers. Adenocarcinomas and adenosquamous cancers, among others, constitute the remaining 10–15 %. Adenocarcinoma cases constitute a quarter of cervical cancer cases in western countries. Persistent infection with one or more of the oncogenic types of HPV is the central and necessary cause of cervical cancer (Bosch *et al.*, 2002; IARC 1995). Other exogenous or endogenous factors acting in conjunction with HPV may be necessary for pro have identified a number of other risk factors that contribute to the development of cervical cancer precursors and cervical cancer. These include sexual intercourse at an early age, multiple sexual partners, multiparity, long-term oral contraceptive use, tobacco smoking, low socioeconomic status, infection with Chlamydia trachomatis and micronutrient deficiency in vegetables and fruits (Bosch *et al.*, 2002; IARC, 1995; Munoz *et al.*, 2003).

Molecular and epidemiologic studies have demonstrated a strong relationship between human papilloma virus (HPV), cervical intraepithelial neoplasia (CIN), and invasive carcinomas of the cervix (Durst *et al.*, 1983 and Bergeron *et al.*, 1992). HPV DNA has been identified in more than 95 % of cervical carcinomas (Bosch *et al.*, 1995), HPV DNA transcripts and protein products have also been identified in invasive cervical carcinomas (Arends *et al.*, 1998; Pillai *et al.*, 1996 and Sano *et al.*, 1998). In high-grade CIN and invasive carcinoma, papillomavirus DNA is typically integrated into the human genome rather than remaining in an intact viral capsid (Winkler and Richart, 1987). It has been theorized that integration of HPV DNA in the human genome, possibly at the E2 site, causes persistent transcription of the E6 and E7 genes. Functional inactivation of p53 by E6 protein or of Rb by E7 protein disrupts normal cell-cycle control mechanisms (Choo *et al.*, 1987; Kessler, 1990 and Scheffner *et al.*, 1990).

1.10. ETIOLOGICAL FACTORS

Most cancer types vary in incidence and mortality among different populations in different parts of the world. When populations move from one country to another, the rates for many cancers tend toward that of the local population rather than that of their country of origin. The huge geographic and temporal variation in many types of cancer provides strong evidence that cancer is not an intrinsic consequence of life, but is affected by lifestyle and environmental factors in addition to inherited genes. In etiologic research, epidemiologic studies have been crucial in identifying and characterizing the carcinogenicity of tobacco, radiation, and many other occupational, environmental, and infectious agents.

Table II: Estimated Percentage of Total Cancer Deaths Attributable to Established Causes of Cancer. (Adapted from International Agency for Research on Cancer, 1982)

Risk Factor	Percentage
Tobacco	30
Adult diet and obesity	30
Sedentary lifestyle	5
Occupational factors	5
Family history of cancer	5
Viruses and other biologic agents	5
Perinatal factors and growth	5
Reproductive factors	3
Alcohol	3
Socioeconomic status	3
Environmental pollution	2
Ionizing and ultraviolet radiation	2
Prescription drugs and medical procedures	1
Salt, other food additives, and contaminants	1

To evaluate the many substances / agents suspected of carcinogenicity, International Agency for Research on Cancer (IARC) has established a formal process that systematically evaluates known and suspected carcinogens. These agents are generally divided into three categories: occupational, medical, and social (IARC, 1982).Although the information presented in Table II suggests that most human cancer is caused by occupational or medical exposures, this is not the case. Rather, the cause of the bulk of human cancers is unknown, and only after certain discrete exposures are well documented can cancer causation be laid at the feet of a particular agent.

1.10.1. **Tobacco (Smoke and Smokeless)**

Epidemiologists have attributed as many as 30 % of all cancer deaths to tobacco use, primarily cigarette smoking. In 2005, for example, approximately 163,000 deaths due to lung cancer were expected to occur in the United States (90,000 men and 73,000 women), making up about 31 % of all deaths from cancer in men and 27 % in women(Jemal *et al*., 2005). Although the most direct correlation is between cigarette smoking and lung cancer, tobacco use has also been implicated in cancers of the mouth, pharynx, larynx, esophagus, urinary bladder, pancreas, kidney, and more recently, stomach and liver (Peto, 2001) and perhaps colorectal cancer(Bradbury, 2000). Smoking of pipes or cigars has been implicated in the occurrence of cancers of the mouth, pharynx, larynx, and esophagus, but this form of tobacco use is generally considered much less dangerous because the smoke is usually not inhaled. An enormous amount of research on the relationship between tobacco smoking and cancer has been carried out over a number of years. The vast majority of these studies indicate cigarette smoking as a major cause of lung cancer.

Numerous mutagens and carcinogens have been identified in the particulate or vapor phasesof tobacco smoke; these include benzo(a)-pyrene, dibenza(a)anthracene, nickel, cadmium, radioactive polonium^{210}Po, hydrazine, urethan, formaldehyde, nitrogen oxides, and nitrosodiethylamine (United States Public Health Service, 1982). Moreover, mutagenic activity is 5- to 10- fold higher in the urine of smokers than that of non-smokers (Putzrath *et al.,* 1981). An increased incidence of chromosomal abnormalities has been observed in smokers' peripheral blood lymphocytes compared to lymphocytes of non-smokers (Obe *et al*., 1982; Vijayalaxmi and Evans, 1982). A meta-analysis of DNA adducts present in peripheral white blood cells or tissue of smokers who have cancer compared to smokers that don't showed that current smokers with high levels of adducts have an increased risk of lung and bladder cancers(Veglia *et al.*, 2003). However, only a fraction of smokers develop lung cancer, thus suggesting individual variability in susceptibility. A study (Wei *et al*., 2000) found that a low DNA repair capacity correlated with increased risk of lung cancer in a population of smokers.

During most of the 20th century, use of oral and nasal smokeless tobacco products has been significant in India and other Asian countries, as well as in some parts of Africa.

Smokeless tobacco is consumed without burning the product, and can be used orally or nasally. Globally, a wide variety of different smokeless tobacco products are used. These may be used on their own, mixed with other products, such as slaked lime (khaini) or as ingredients to other products, such as betel quid. The prevalence of use of smokeless tobacco varies substantially not only across countries, but also within countries, by gender, age, ethnicity and socioeconomic characteristics.

In India, a large variety of commercial or home-made smokeless tobacco products exist. The use of chewing tobacco (often chewed with betel quid or other preparations including areca nut) is more prevalent than the use of snuff; applying smokeless tobacco products as a dentifrice is also common. According to a 1998–99 survey, 28.1 % of adult men and 12.0 % of women reported chewing tobacco (Rani *et al*, 2003). Smokeless tobacco products are also widely used in other countries in Southeast Asia. Betel quid without tobacco, as well as areca nut, the common ingredient of betel quid, have been classified as human carcinogens; they cause cancers of the oral cavity, the pharynx and the oesophagus (IARC, 2004). Several case–control studies from India, Pakistan and Sudan provide strong and consistent evidence of an increased risk of oral cancers. During most of the 20th century, use of oral and nasal smokeless tobacco products has been significant in India and other Asian countries, as well as in some parts of Africa, (IARC, 2007). Additional evidence comes from ecological studies showing positive correlations between use of smokeless tobacco products and high rates of oral cancer. A few studies from India and North Africa support the hypothesis of an association between nasal snuff use and risk of cancer of the oral cavity, the esophagus and the lung (IARC, 2007). There are over 30 carcinogens in smokeless tobacco, including volatile and tobacco specific nitrosamines, nitrosamino acids, polycyclic aromatic hydrocarbons, aldehydes, metals (IARC, 2007). Smokeless tobacco use entails the highest known non-occupational human exposure to the carcinogenic nitrosamines, NNN and NNK Exposure levels are 100 to 1000 times greater than in foods and beverages commonly containing nitrosamine carcinogens.

1.10.2. **Alcohol**

An association has been established between alcohol drinking and cancers of the oral cavity, pharynx, larynx, esophagus, liver, colon, rectum and, in women, breast (Boffetta and Hashibe, 2006). An association is suspected for lung cancer. Some studies have

shown an increased risk of pancreatic cancer with heavy drinking, but the epidemiologic evidence for this is weak. For squamous-cell carcinomas of the upper aerodigestive tract (oral cavity, pharynx, larynx and esophagus), a causal relationship was first demonstrated in the mid-1950s (IARC, 1988). In epidemiological studies of this group of tumours, an effect of heavy alcohol intake and a linear relationship with amount of drinking has been consistently shown. A carcinogenic effect of alcohol drinking independent from that of smoking (i.e. an increased risk of head and neck cancers in non-smokers) was first reported in 1961 (IARC, 1988), and replicated in a recent large scale pooled analysis (Hashibe et al, 2007).

In a meta-analysis of 28 case–control and 10 cohort studies, comparing drinkers and nondrinkers, the risk of breast cancer increased 24 % with consumption of two drinks per day (Longnecker, 1994). Another study, pooling data from six prospective studies, reported a 9 % increase in breast cancer incidence with each 10 grams of alcohol consumed per day (Smith-Warner *et al.*, 1998). These data were mostly obtained from postmenopausal women. In another case–control study, recent alcohol consumption of 13 grams per day (about equivalent to 3 drinks of 100 proof whiskey) was associated with a 21 % increased risk of breast cancer, but in the age group less than 30 years of age there was no increased risk (Byrne *et al.*, 2002). The mechanisms by which alcohol drinking exerts its carcinogenic effects are not fully elucidated, plausible hypotheses include a genotoxic effect of acetaldehyde (the main metabolite of ethanol), an increase in estrogen levels (relevant for breast carcinogenesis), a role as a solvent for other carcinogens, the production of reactive oxygen and nitrogen species and the alteration of folate metabolism.

1.10.3. **Betel Nut**

Betel quid (BQ) without tobacco, as well as areca nut, the common ingredient of betel quid, have been classified as human carcinogens; they cause cancers of the oral cavity, the pharynx and the oesophagus (IARC, 2004; Choudhury and Sharan, 2009). BQ without tobacco causes oral cancer, while BQ with tobacco causes cancers of the oral cavity, pharynx and oesophagus (IARC, 2004). In India, a large variety of commercial or home-made smokeless tobacco products exist. The use of chewing tobacco (often chewed with betel quid or other preparations including areca nut) is more prevalent

than the use of snuff; applying smokeless tobacco products as a dentifrice is also common.

In hamsters, administration of *Areca* nut and application of its aqueous or dimethyl sulphoxide extracts to the cheek-pouch mucosa resulted in squamous cell carcinomas (SCC) of the cheek pouch and carcinomas of the forestomach (IARC 1985). While Betel quid has various components (IARC, 1985; Sharan, 1996), the habit of chewing betel quid has been associated with high risk of oral cancers in several studies (IARC, 1985). Aqueous extract of betel nut (AEBN) exerts higher genotoxic effects than arecoline (Dave et al., 1992; Sharan, 1996). AEBN has been shown to induce double strand breaks in DNA of mouse kidney cells in vitro (Wary and Sharan 1988; Sharan and Wary, 1992). AEBN has been shown to induce conformational changes in mouse liver high mobility group (HMG) proteins leading to the development of preneoplastic nodules in the liver (Pariat and Sharan, 1998 a; Pariat and Sharan, 1998 b).

1.10.4. **Diet**

The incidence of most cancers varies worldwide and cancers of the breast, colorectum, prostate, endometrium, ovary and lung are generally much more frequent in the developed countries. These cancers are a major burden in countries of Europe, North America and in Australia. They are markedly less frequent in developing countries of Asia and Africa. In contrast, some cancers of the digestive system, including those of the stomach and liver, are more frequent in developing countries of Central and South America, Africa and Asia than they are in the developed world (Doll *et al.*, 1966; Doll *et al.*, 1970). The most consistent finding on diet as a determinant of cancer risk is the association between consumption of vegetables and fruit and reduced risk of several cancers. Consumption of vegetables and fruit is associated with reduced risk of cancers of the pharynx, larynx, lung, oesophagus, stomach and cervix uteri, while only vegetables, but not fruit, seem to protect against cancers of the colon and rectum. During the last 30 years, over 250 epidemiological studies (case-control, cohort or ecological correlations) have been conducted around the world to investigate the relationship between fruit and vegetable consumption and cancer risk. About 80 % of these studies found a significant protective effect of overall consumption of vegetables and/or fruit, or at least of some types of vegetables and fruits (WCRF/AICR, 1997). Consumption of salt added to food and salt-preserved foods has been investigated

mainly in relation to cancers of the stomach, colorectum and nasopharynx. Several studies conducted in Europe, South America and Eastern Asia have reported increased relative risks of stomach cancer in relation to the consumption of salt and salt-preserved foods, particularly in populations with high stomach cancer incidence and high salt intake. Salted, smoked, pickled and preserved food (rich in salt, nitrite and preformed N-nitroso compounds) are associated with increased risk of gastric cancer. Such high salt intake, together with Helicobacter pylori infection, may contribute to the development of atrophic gastritis, and hence gastric cancer. Consumption of Chinese-style salted fish has been specifically associated with increased risk of nasopharyngeal cancer in South-East Asia (IARC, 1993). Epidemiological studies on meat consumption and cancer risk support the existence of a specific association with colorectal cancer risk. This association, however, seems to have been found more consistently for consumption of red meat (beef, lamb and pork) and processed meat (ham, salami, bacon and other charcuterie) for which consumption of 80 g per day may increase colorectal cancer risk by 25 and 67 %, respectively (Norat *et al.*, 2002). Food additives are chemicals added to food for the purpose of preservation or to enhance flavour, texture or colour. Less than comprehensive toxicological data are available for most additives, although some have been tested for mutagenic or carcinogenic activity. In in vitro assay systems, some additives, such as dietary phenolics, have both mutagenic and antimutagenic effects (Ferguson, 1999).

In the 1960s, ecological observations pointed at several intriguing relationships between intake of fats and mortality from colorectal cancer or breast cancer. An example of such a correlation was found between incidence of colon cancer in women and mean individual daily meat consumption (Armstrong and Doll, 1975). Additionally, studies in migrants showed that subjects moving from areas with a low incidence of several cancers, including colorectal and breast cancer, tend to acquire the cancer incidence levels of the host populations (Tomatis *et al.*, 1990; McCredie *et al.*, 1999a; McCredie *et al.*, 1999b; Maskarinec and Noh, 2004). An IARC Working Group (IARC, 2002) found that overweight and obesity were consistently associated with (in both men and women) adenocarcinoma or the esophagus, kidney cancer, (in men) colon cancer, (in women) breast cancer and endometrial cancer in post-menopausal women.

1.10.5. **Reproductive factors**

Evidence is accumulating in the literature on the implication of endogenous hormones (particularly sex steroids and growth factors) in the etiology and in the development of several human cancers, especially breast cancer and those of the female reproductive organs (World Cancer Report2008). The incidence of breast cancer is very low in females below the age of 15, and increases very steeply (in the order of about a hundredfold) by the age of 45. After menopause, the production of estrogens and progesterone from the ovaries ceases, and the increase in breast cancer incidence rates with age slows down compared to pre-menopausal women. This suggests a significant implication of hormones in the etiology of breast cancer. In vitro experiments have shown that estrogen increases mammary cell proliferation and in vivo experiments in animals have demonstrated that estrogen increases tumour development. Further elements strengthen the association between endogenous sex steroids and breast cancer: an early age at menarche, a late age at menopause and the use of hormone replacement therapy in post-menopausal women have been repeatedly associated with an increase in breast cancer risk (Key *et al.*, 2001). Increases in breast cancer risk are generally explained by the longer lifetime exposure of women to high levels of endogenous sex steroids, especially estradiol, that increase the proliferation and inhibit apoptosis of mammary epithelium In addition, overweight and obesity in post-menopausal women not taking exogenous hormones have also been associated with an overall 40 % increase in breast cancer risk, and the most widely accepted explanation is again related to the exposure to elevated levels of sex steroids, since in post-menopausal women the ovaries stop producing estrogens, which are instead produced by the aromatisation of androgens in the adipose tissues. Early age at first pregnancy, high parity and prolonged breast feeding have been associated with decreased risk of breast cancer (Key *et al.,* 2001), mainly explained by the differentiation of mammary tissue induced by pregnancy-related hormones. Pregnancy has, however, a double effect on breast cancer risk: a short-term increase and a long-term reduction in risk. Most information on the relation between breast cancer and oral contraceptive (OC) use is derived from a collaborative reanalysis of individual data including 53297 women with breast cancer and 100239 controls from 54 epidemiological studies (Breast cancer and hormonal contraceptives, 1996). This provided definitive evidence that current and recent users of combined OCs have a small increase in the relative risk (RR) of breast cancer. Although chronic human papilloma virus (HPV) infection is a necessary cause of

cervical cancer (Walboomers et al., 1999), other factors are likely to have a role in cervical carcinogenesis. Among these are tobacco smoking and exogenous female hormones, including OCs (Schiffman et al., 1996). Several epidemiological studies have reported an increased risk of invasive cervical carcinoma in relation to ever OC use, and a stronger risk for a longer duration of use. The evidence of an association between OC use and adenocarcinoma of the cervix is based on more limited data (IARC, 1999). The RR of cervical cancer was significantly elevated among long-term OC users in a study from Morocco (Chaouki et al., 1998) and in three studies from the Philippines (Ngelangel et al., 1998), Thailand (Chichareon et al., 1998) and the UK (Deacon et al, 1998).

1.10.6. **Radiation**

Natural and man-made sources generate radiant energy in the form of electromagnetic waves. Their interaction with biological systems is principally understood at the cellular level. Electromagnetic waves are characterised by their wavelength, frequency or energy. Effects on biological systems are determined by the intensity of the radiation, the energy in each photon and the amount of energy absorbed by the exposed tissue. It has been known for a long time that exposure to UV or ionizing irradiation can cause cancer in humans. The evidence for the association of skin cancer and UV radiation is compelling. Both malignant melanoma and non-melanoma skin cancers are associated with exposure to UV radiation, although the dose–response curve is less steep for melanoma (Scott and Straf, 1977). The most common types of skin cancer are basal cell carcinoma, which may be locally invasive but is almost never metastatic; squamous cell carcinoma, which is more aggressive than basal cell carcinoma, invades locally, and may rarely metastasize; and melanoma, which is less common than the other forms of skin cancer but is often highly malignant and rapidly metastatic, with an average 5-year survival rate of over 90 % if detected and treated early but only 14 % if metastatic.

The carcinogenic effects of ionizing radiation were discovered from studies of pioneer radiation workers who were occupationally exposed, individuals who were exposed to diagnostic or therapeutic radiation, and atomic bomb survivors. Malignant epitheliomas of the skin were observed in the earliest experimenters with X-rays and radium within a few years after their discovery in 1895 and 1898, respectively. By 1914, a total of 104

case reports of radiation cancers had been noted and analyzed. In 1944, the role of ionizing radiation in the increased incidence of leukemia among radiologists was recognized (March, 1944). Exposure to ionizing radiation is unavoidable (IARC, 2000). Humans are exposed both to X-rays and gamma-rays from natural sources (including cosmic radiation and radioactivity present in rocks and soil) and typically, to a much lower extent, from man-made sources. On average, for a member of the general public, the greatest contribution comes from medical X-rays and the use of radiopharmaceuticals, with lower doses from fallout from weapons testing, nuclear accidents (such as Chernobyl) and accidental and routine releases from nuclear installations. Medical exposures occur both in the diagnosis (e.g. radiography) of diseases and injuries and in the treatment (e.g. radiotherapy) of cancer and of some benign diseases. Occupational exposure to ionizing radiation occurs in a number of jobs, including the nuclear industry and medicine. Airline pilots and crew are exposed to cosmic radiation. The risk projections suggest that (by 2006) Chernobyl may have caused about 1000 cases of thyroid cancer and 4000 cases of other cancers in Europe, representing about 0.01 % of all incident cancers since the accident.

1.10.7. **Ageing**

Cancer is a disease of aging. The average age at diagnosis is 67 and the median age of patients with cancer in the United States is 70 years (Ershler and Longo, 1997). The incidence of cancer rises exponentially with age from ages 40 to 80 years. This age related increase in cancer probably relates to the combined effects of accumulated genetic alterations (mutations, translocations, etc.), increased epigenetic gene silencing, telomere dysfunction, and altered tissue stroma as tissues age. There is evidence for each of these factors playing a role (DePinho, 2000). Increased somatic mutations have been observed in aged cells and tissues from humans and mice. This process most likely occurs as a result of accumulated DNA damage due to exogenous and endogenous agents such as oxygen free radical–forming agents .Such mutations can also result from error-prone repair during DNA replication. Whether decreasing DNA repair capacity with aging is the culprit is not clear. However, in cells from aged mice and humans, an increased level of chromosomal abnormalities has been observed. In addition, an age-related decline in repair of UV-induced DNA damage has been found in cultured primary skin fibroblasts and lymphoblastoid cell lines, when comparing normal donors up to 10 years of age with normal donors in their 80s or 90s (DePinho,

2000). There is also an age-associated decrease in cellular levels of proteins involved in DNA repair, such as ERCC3 for excision repair, replication protein A, and p53.

1.10.8. **Genetic factors**

A number of inherited traits are related to causation of cancer. A few cancers have a definite inheritance, whereas others may arise in individuals with a genetic defect that makes them more susceptible to potentially carcinogenic agents. A list of inherited cancer syndromes is shown in Table III. These neoplasms represent a small fraction, perhaps 1 % to 2 %, of total cancers (Knudson, 1977).

Table III: Inherited Cancer Syndromes Caused by a Single Genetic Defect. (Adapted from Stewart and Kleihues, 2003)

Syndrome	Gene	Location	Cancer site and Cancer type
Multiple endocrine neoplasia II	RET	10q11	Medullary thyroid carcinoma, pheochromocytoma
Multiple endocrine neoplasia I	MEN1	11q13	Adrenal, pancreatic islet cells
Neurofibromatosis type I	NF1	17q11	Neurofibromas, optic gliomas, pheochromocytoma
Neurofibromatosis type II	NF2	22q2	Bilateral acoustic neuromas, meningiomas, cerebral astrocytomas
Bloom syndrome	BLM	15q26	Leukemia, lymphoma
Familial adenomatous polyposis	APC	5q21	Colorectal, thyroid
Von Hippel-Lindau	VHL	3p25	Renal cell carcinoma, pheochromocytoma
Familial Wilm's tumor	WT1	11q	Wilms tumor (kidney)
Xeroderma pigmentosum	XP(A–D)	9q,3p,19q,15p	Basal cell carcinoma, squamous cell carcinoma, melanoma (skin)
Fanconi anemia	FAC	16q, 9q, 3p	Acute leukemia
Li-Fraumeni syndrome	p53	17p13	Breast and andrenocortical carcinomas, bone and soft-tissue sarcomas, brain tumors, leukemia
Cowden syndrome	PTEN	10q22	Breast, thyroid
Gorlin syndrome	PTCH	9q31	Basal cell carcinoma
X-linked proliferative disorder	XLP	Xq25	Lymphoma
Peutz-Jeghers syndrome	LKB1	19p	Breast, colon
Ataxi telangiectasia	ATM	11q22	Leukemia, lymphoma

Table IV lists some of the high-risk cancer susceptibility genes, their chromosomal location, and the associated cancers. Some of these genes are involved in genome

integrity (brca1 and brca2), some are cell cycle regulator genes (p16 and CDK4), and some are DNA mismatch repair genes (e.g., hMLH1, hMSH2, and hMSH6).These genetic mutations usually demonstrate incomplete pentrance. For example, a woman carrying a mutated brca1 gene has about a 70 % - 80 % lifetime risk for developing breast cancer. It is clear that other cofactors are involved in this risk. The so-called high-risk susceptibility genes may also be involved in sporadic cancers for which no clear gene association has been found. The inherited cancer syndrome genes usually have a very high degree of penetrance and are relatively rare (e.g., the incidence of p53 gene mutations involved in the Li-Fraumeni syndrome is 1 in 10,000 individuals) (World Cancer Report, 2003). The inherited susceptibility gene mutations are more common and are seen in common types of cancer and, as noted, in sporadic cancers. However, the distinction between the rarer inherited cancer syndromes and those mutations found in the more common cancers is somewhat arbitrary. For example, rb1, apc, p53, and PTEN mutations are involved in both inherited and sporadic cancers.

Table IV: High-risk Susceptibility Genes and Their Chromosomal Location (Adapted from Stewart and Kleihues, 2003)

Gene	Location	Associated tumors
BRCA1	17q	Breast, ovary, colon, prostrate
BRCA2	13q	Breast, ovary, pancreas, prostrate
P16 INK4A	9p	Melanoma, pancreas,
CDK4	6p	Melanoma
hMLH1	3p	Colorectal, endometrial, ovarian cancer
hMSH2	2p	Colorectal, endometrial, ovarian cancer
hMSH6	2p	Colorectal, endometrial, ovarian cancer
PMS1	2q	Colorectal
PMS2	7p	Colorectal
HPC2	17p	Prostrate

1.10.9. **Occupational exposures**

The first reports of associations between risk of cancer and employment in particular occupations appeared during the 18th century (scrotal cancer among chimney sweeps (Pott, 1775) and 19th century (bladder cancer in workers exposed to dyes) (Rhen, 1895). However, the majority of studies establishing a link between an increased risk of cancer and a particular working environment were published between 1950 and 1975 (Monson, 1996). Relatively few occupational carcinogens have been identified in the

last 25 years. The chemicals and industrial processes that have a known or suspected etiologic role in the development of cancer are listed in Table V.

Table V: Chemicals classified as probably carcinogenic to humans for which exposures are mostly occupational (Adapted from Stewart and Kleihues, 2003)

Agent	Cancer/ Cancer site	Main industry / use
Acrylonitrile	Lung, prostate lymphoma	Plastics
Benz[a]anthracene	Lung, skin	Combustion fumes
Benzidine-based dyes	Bladder	Paper, leather, textile dyes
Benzo[a]pyrene	Lung, skin	Combustion fumes
1,3-Butadiene	Leukaemia, lymphoma	Plastics, rubber, monomer
Chlorinated toluenes	Lung	Chemical intermediates
para-Chloro-ortho-toluidine	Bladder	Dye/pigment manufacture, textiles
4-Chloro-ortho-toluidine	Bladder	Dye/pigment, manufacture, insecticide
Creosotes	Skin	Wood preservation
Dibenz[a,h]anthracene	Lung, skin	Combustion fumes
Formaldehyde	Nasopharynx	Plastics, textiles, laboratory agent
4,4'-Methylenebis(2-chloroaniline)	Bladder	Rubber manufacture
ortho-Toluidine	Bladder	Dye/pigment manufacture
Polychlorinated biphenyls	Liver, bile ducts, leukaemia, lymphoma	Electrical components
Tetrachloroethylene	Oesophagus, lymphoma	Solvent, dry cleaning
Trichloroethylene	Liver, lymphoma	Solvent, dry cleaning, metal

As noted above, about 2 %–5 % of all cancer deaths are attributed to occupational hazards. Of those agents listed as carcinogenic for humans, a number were identified because of their close association between an abnormal clustering of certain cancers and exposure to an industrial chemical or process. For example, epidemiologic studies of workers occupationally exposed to industrial levels of 4-aminobiphenyl have a higher incidence of bladder cancer (Melamed, 1978). Occupational exposure to

asbestos fibers results in a higher incidence of lung cancer, mesotheliomas, gastrointestinal tract cancers, and laryngeal cancers (IARC, 1980).

1.11. CANCER BIOMARKERS

In 1987 the National Research Council of the National Academy of Sciences defined biological markers as "indicators signaling events in biological samples" (Biomarkers: The Clues to Genetic Susceptibility, 1994). Our understanding of a biomarker's role in cancer will lead to an earlier and more accurate diagnosis of the disease. By identifying people at high risk of developing cancer, it would be possible to develop intervention efforts on prevention rather than treatment. There has been much interest in biomarkers of cancer risk in predicting future patterns of disease, especially as cancer treatment has made such positive strides in the last few years.

A number of types of tumor markers have been identified and employed as clinical cancer markers (Sidransky, 2002). These include nucleic acid–based markers such as mutations, loss of genetic heterozygosity, microsatellite instability, and gene expression microarrays, as well as protein markers and protein pattern recognition profiles, circulating tumor cells, and circulating endothelial cells. Some of these markers can be detected in the circulation or in body fluids, and some require tumor tissue.

Biomarkers can be present in the serum, or be genetic testing factors, and all are being studied acutely to find out how they can be of more use in cancer screening. Serum biomarkers are produced by body organs or tumors and measure antigens on cell surfaces. When detected in high amounts in blood, they can be suggestive of tumor activity. Serum biomarkers are nonspecific for cancer and can be produced by normal organs as well.

One of these serum biomarkers in wide use is prostrate-specific antigen (PSA). PSA is produced by normal prostate cells in small amounts, but the higher the PSA is in the serum, the higher the correlation is toward the existence of prostate cancer.PSA is probably the only serum biomarker currently used consistently in primary care. There are reasons other than cancer that can cause rises in PSA. Infections within the prostate gland (prostatitis), increased exercise with irritation of the affected area, can cause a

PSA rise. Factors such as the degree of elevation, the rapidity of increase, and the fraction of free non-bound PSA (higher in benign causes) are all factored in to determine a next step. Cancer antigen 125 (CA-125) can be a biomarker of ovarian cancer risk or an indicator of malignancy, but it has low sensitivity and specificity. Levels of this marker can be high in people who have pancreatitis, kidney or liver disease, making its accuracy as a cancer diagnostic tool very limited. However, it can be used to follow the progress of treatment of cancer, and predict a treatment failure when levels rise despite the use of chemotherapeutic agents. Sometimes, a combination of several tumor markers can give risk predictions in someone whose family history for the disease is quite high. Carcinoembryonic antigen (CEA) is another biomarker that is elevated in patients with colorectal, breast, lung, or pancreatic cancer. As a screening test, it can be elevated by many other factors than cancer; smoking for instance raises CEA levels. Following CEA post-surgery for colon cancer however is an effective way of determining the adequacy of postoperative therapy.

A number of genetic modifications have been detected in cancer cells and some of these have been useful in cancer diagnosis and staging. The discovery of free DNA in plasma and urine has provided a way to assess the presence of cancer in patients. Tumors release substantial amounts of genomic DNA as cancer cells, which often have a high cell turnover rate, undergo necrosis or apoptosis. Tumor-derived DNA can be detected in plasma, urine, or stool samples (Sidransky, 2002). Alterations in DNA can be assessed by loss of heterozygosity, mutations, microsatellite DNA alterations, and DNA methylation patterns (Chang *et al.*, 2002). When genetic or epigenetic alterations are detected in circulating DNA samples, they can be specific to the primary tumor of origin, but plasma DNA is a mixture of neoplastic and non-neoplastic DNA. Elevated circulating DNA levels are also seen in patients with severe infections or autoimmune diseases, for example. In addition, plasma DNA is often degraded to a variable extent, which can be a problem for assessing allelic imbalance and microsatellite markers (Chang *et al.*, 2002). Detection of cancer-associated mutations in body fluids were first observed when fragments of the p53 gene were detected in the urine of bladder cancer patients and ras gene mutations were found in stool samples from patients with colorectal cancer (Sidransky, 2002).

1.12. AIMS AND OBJECTIVES

Previous reports in literature on PAR metabolism have dealt more with the expression of PARP gene and with the level or activity of the enzyme itself. Such studies were taken as an indicator of DNA damage, repair and carcinogenesis (Miwa, M., *et al.*, 1977; Singh, 1991). Even among these studies, some have reported an increased expression of PARP gene (Masutani *et al.*, 2005) while others have reported decreased gene expression (Bieche *et al.*, 1996). Therefore such contradictory results do not reveal the clear picture of the PAR level. On the other hand studies have reported low PAR level in PBL of laryngeal cancer patients based on the PARP activity (Rajaee-Behbahani *et al.*, 2002). Monitoring only PARP activity will not give us the true metabolic level of ADP-ribose polymers since both the biosynthesizing and biodegrading enzymes i. e. PARP and PARG will act in opposite direction respectively. Previous PAR work done in our laboratory on mice has shown a strong negative correlation between PAR of cellular proteins and initiation, promotion and progression of cancer (Saikia, 1996; Pariat, 1997; Devi, 2001; Kma, 2003). A novel ELISA based slot and Western blot immunoprobe based method to assay the true metabolic PAR level has been developed (Sharan *et al.*, 1998; Sharan *et al.*, 2005). Moreover, recent results on blood lymphocyte proteins of mouse exposed to DMN, revealed a significant lowering of total PAR level.

With this view in mind it would be of significant importance to extend the study to human cancer situation. Elucidating this correlation between PAR of cellular protein and different human cancer using PBL of cancer patients will be tremendously valuable. It is envisaged that this immunoassay shall give the correct estimation of metabolic level of PAR in human cancer cells. Thus in this study three types of human cancers which are the most prevalent in this region were selected. They are cancers of the head and neck region, breast cancer and cervix cancer. The specific aims and objectives of this study were as follows

(1) To find the correlation between PAR of human blood lymphocyte proteins and different cancers of:

- **a.** the head and neck
- **b.** breast
- **c.** cervix

(2) To find the correlation between PAR of human blood lymphocyte proteins and various etiological factors.

MATERIALS & METHODS

2.1. CHEMICALS

All chemicals used were of analytical grade and were used without further purification. Nitrocellulose membrane (NCM), Histopaque 1077, ethylenediamine tetraacetic acid (EDTA), sephadex G-25, 2-mercaptoethanol, phenylmethanesulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), Freund's complete adjuvant, Freund's incomplete adjuvant and bovine serum albumin (BSA) fraction V, DNA type 1: Sodium salt from calf thymus were obtained from Sigma Chemical Company, St. Louis, USA. Alkaline-phosphatase labeled goat anti-rabbit IgG, 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (BCIP/NBT), Tween-20, agarose were purchased from Bangalore Genei Pvt. Ltd., Bangalore, India. Methanol, tris base (hydroxymethyl) methylamine, glycine, potassium chloride, sucrose, acetic acid glacial, urea, ammonium sulphate, D-glucose, babituric acid, trichloroacetic acid (TCA), orcinol and diphenylamine purchased were from Qualigens Fine Chemicals, Mumbai, India. While Coomassie brilliant blue G250 and heparin were from Sisco Research Laboratories (SRL) Pvt. Ltd, Mumbai, India, ortho-phosphoric acid, sodium acetate, D-sorbitol, magnesium chloride, ammonia solution, ferric chloride and calcium chloride were from S.D. Fine-chem limited, Mumbai, India and Sodium hydroxide, di-sodium hydrogen phosphate and sodium dihydrogen phosphate were from Merck, Mumbai, India. Dehydrated alcohol was obtained from Bengal Chemicals and Pharmaceuticals Ltd, Kolkata, India and India ink came from Rotring Zeichentusche Drawing Ink, Hamburg, Germany. RPMI-1640 was a product of HyClone, Logan, Utah, USA.

2.2. INSTRUMENTS

The main instruments used in this investigation were UV-Vis Spectrophotometer 119 (Systronics, India), Bio-Dot SF Microfiltration Apparatus (Bio-Rad, USA), Mini-Protean II Electrophoretic Cell (Bio-Rad, USA), Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA), Heto Lyolab 3000 (Heto-Holten A/S, Allerød Denmark), Laboratory Centrifuges 3K30 (Sigma Chemical Company, St. Louis, USA), R8C Laboratory Centrifuge (Remi, India) and Scanjet 7400C (HP).

2.3. EXPERIMENTAL SYSTEMS

(A) Small experimental animals

Swiss albino mice (Balb/c) were used for preparation of ADP-ribose polymer antigen. Same age group (6-8 weeks) male mice were used. Russian Chinchilla strain rabbit was used to raise polyclonal antibodies against mouse ADP-ribose polymers. The rabbit was purchased from Pasteur Institute, Shillong, India. These animals were housed in an animal room maintained at temperature of 22-23 °C. They were fed with standard mice and water *ad labitum.*

(B) Human experimental groups

Control group

The control group consisted of normal healthy volunteers with no indication whatsoever of being inflicted by cancer (age range 25-80 years). None of the subjects in this category was subjected to any medical test to ascertain whether or not they have cancer. Their recruitment for the study was based on their responses. A large number of control subjects were in their twenties to thirties. Attempts were made to include subjects with different ethnicity from the north eastern region of India in this group. Overwhelming number of subjects under control group were mostly research scholars of NEHU who volunteered to participate in the study. Some subjects were also foreign nationals visiting NEHU for short courses or research training who volunteered for the study. Some control subjects were also recruited from the institute, BBCI. They included relatives of patients, attendants and staff of the institute.

Experimental group

The human experimental group comprised cancer patient visiting Dr. B. Barooah Cancer Institute (BBCI), Guwahati. Samples were collected only from the patients who volunteered and consented to participate in the study following detailed information about the research work and strictly in accordance with the Ethics Committee recommendations. This group consisted of patients with confirmed cancers (stage III/ IV) of different sites of head and neck region, breast and cervix. They have henceforth been referred to as 'cases' in this thesis. The patients were mainly from the north eastern part of India with different ethnic backgrounds. Certain exclusion criteria were applied while recruiting patients for the study. For example, for this study only those

cancer patients were selected who had (i) cancers as listed in Table I, (ii) had not undergone prior treatment, such as chemotherapy or radiation therapy and (iii) had no prior surgical intervention for the cancer.

Different cancers of the head and neck region covered in this study included cancer of the esophagus, tongue, tonsil, nasopharynx, pharynx, buccal mucosa, alveolo, pyriform sinus, larynx, lip, nasal cavity, epiglottis, oral cavity and vocal chord (see Table VI). Besides, patients with cancers of breast and cervix were also studied. Therefore, a total of 16 different types of cancers have been examined in this study.

Table VI: Table lists different cancers of humans that have been covered in this study.

Category/Type/Site of cancer
Cancers of head & neck region
Cancer of oesophagus
Cancer of alveolo
Cancer of pyriform sinus
Cancer of nasopharynx
Cancer of larynx
Cancer of tongue
Cancer of tonsil
Cancer of buccal mucosa
Cancer of pharynx
Cancer of lip
Cancer of nasal cavity
Cancer of oral cavity
Cancer of epiglottis
Cancer of vocal chord
Cancer of breast
Cancer of cervix

2.4. COLLECTION OF HUMAN BLOOD SAMPLES

The blood samples from cancer patients and controls were obtained after informed consent (see format of consent form in Annexure I). Samples were collected at BBCI as well at North-Eastern Hill University (N.E.H.U.) health center by trained medical staff using all sterile equipments.

2.5. QUESTIONNAIRE

In order to collect detailed clinical information of each cancer patient as well as control subjects, a questionnaire was developed in this study in consultation with the collaborating institute, Dr. B. Barooah Cancer Institute, Guwahati. The questionnaire contained questions addressing different aspects that are known to be associated with carcinogenesis. The questionnaire consisted of eight main sections, each with many questions. A brief description of organization of the questionnaire is given below while the full questionnaire may be seen at Annexure II.

Section 1. Background information included questions on:

a. Personal data: age, gender, height, weight, address etc

b. Demographic information: marital status, income, education etc

c. Ethnicity

Section 2. Family history of cancer:

This section attempts to ascertain this aspect by asking questions on history of cancer within the family, type of cancer, status of the patient etc. In case of female subject questions regarding their reproductive and pregnancy history were also included.

Section 3. Social / Lifestyle habits and addiction included questions on:

a. History of tobacco use: smoke and smokeless

b. History of betel nut use

c. History of alcohol use

d. History of drug abuse

Section 4. Nutritional history included questions on:

Dietary patterns, vegetarian or non vegetarian etc.

Section 5. Medical history included questions on:

History of surgery, usage history of prescribed and non prescribed drugs, etc.

Section 6. Radiation exposure history.

Section 7. Occupational history.

Section 8. Other information that may be relevant.

The details of each of the respondents were filled as far as practicable after blood collection. Some of the respondents were very cooperative in providing the detailed information while others were not willing to provide information either due to lack of information or due other reasons, such as privacy or embarrassment.

2.6. ISOLATION OF ADP-RIBOSE POLYMERS

Heterogeneous ADP-ribose polymers isolated from spleen cells of mice were used as antigen to raise polyclonal antibody in rabbit. The ADP-ribose polymer was isolated essentially following a method, which has been standardized in the laboratory and described earlier (Saikia, 1996; Devi, 2001; Kma, 2003; Sharan *et al*, 2005). A brief description of the method is provided below.

2.6.1. Required materials.

1. Phosphate buffered saline (PBS), pH 7.4: This buffer consisted of the following components:

Sl. No.	Components	Molarity
a	NaCl	140 mM
b	KCl	2 mM
c	Na_2HPO_4	10 mM
d	KH_2PO_4	1 mM

The above chemicals were dissolved in the required volume of Millipore water. The pH recorded was approximately 7.4, and the pH was not adjusted further. The buffer was kept refrigerated at RT.

I. 10 % TCA
II. 0.1M Tris-glycine buffer, pH 10.5
III. Sephadex G-25
IV. 0.03 M Tris-barbiturate buffer, pH 7.2 containing 6 M urea and 0.1 % SDS

2.6.2. ***Methodology***

Spleens from 6-8 weeks old healthy Swiss albino mice were excised. Spleen cell suspension was prepared in pre-chilled PBS. The suspension was homogenized and centrifuged at 1,300 x g for 15 min at 4 °C. The cell pellet was collected, resuspended in 10 % TCA, incubated for 30 min on ice and centrifuged at 20,000 x g for 15 min. The pellet was dissolved in 0.1 M Tris-glycine buffer, pH 10.5, incubated at 37 °C for 1 h and centrifuged at 20,000 x g for 1 h. The supernatant fraction containing the ADP-ribose polymers was desalted on Sephadex G-25 gel by elution with 0.03 M Tris-barbiturate buffer, pH 7.2 containing 6 M urea and 0.1 % SDS at a flow rate of 0.2 ml min^{-1}. The fractions showing peak absorbance at 490 nm were pooled. The eluent was dialyzed extensively against double distilled water for 48 h. The dialysate was frozen, lyophilized and stored for used as antigen. Protein, DNA and RNA concentrations were checked by standard methods of Bradford, Diphenylamine and Orcinol, respectively (§ 2.12, 2.13, 2.14).

2.7. RAISING POLYCLONAL ANTIBODIES AGAINST POLY-ADP-RIBOSE

The immunization method was followed as described earlier (Humtsoe, 2000; Devi, 2001; Kma, 2003; Sharan *et al*, 2005)

2.7.1. *Required materials*

I. Freund's complete adjuvant
II. Freund's incomplete adjuvant

2.7.2. *Methodology*

An emulsion of the antigen: Freund's complete adjuvant (1 : 1) was prepared. A young healthy rabbit was sub-cutaneously immunized at different spots on the back, which was shaved clean. Secondary immunization was done 4 weeks later using the same emulsion. An emulsion of antigen: Freund's incomplete adjuvant (1 : 1) was used for the first booster. The second booster was given after two weeks of the first. After 10 days of the second booster dose, the rabbit was bled from the marginal veins of the ears.

Blood was collected and serum was separated and stored. Henceforth, the rabbit was bled every two weeks of the last booster injection. The process continued until 2 months after which the rabbit was killed and all blood from veins and heart were drained.

2.8. PREPARATION OF POLYCLONAL ANTIBODY

The serum immunoglobulins were precipitated using ammonium sulphate as described earlier (Devi, 2001; Sharan *et al* 2005).

2.8.1. ***Required materials***Ammonium sulphate saturated solution:

It was prepared by dissolving ammonium sulphate at a concentration of 800 g l^{-1} in distilled water at pH 7. It was pre-cooled to 4°C and stored in presences of ammonium crystals.

II. Dialysis buffer:

The dialysis buffer was made up on 100 mM Tris-Cl buffer at pH 8.

2.8.2. Methodology

A known volume of serum was transferred into a beaker on ice. All subsequent steps were performed on ice. An equal volume of saturated ammonium sulphate solution was added drop by drop while gently stirring the serum. Precipitation was allowed for around 1 h with constant but mild stirring. The solution was finally centrifuged at 10,000 x g for 10 min at 4 °C. The pellet was resuspended in cold saturated ammonium sulphate solution (5 % of the original volume). Finally, the resulting solution was dialyzed against three changes of the dialysis buffer over 48 h. The dialyzed solution was centrifuged at 10,000 x g for 10 min at 4 °C and the supernatant containing IgG fraction of polyclonal antibody was recovered and frozen. It was lyophilized and stored in the freezer. Protein concentrations were checked by standard methods of Bradford (Sections 2.12).

The weight of the lyophilized powder containing IgG fraction of polyclonal antibody was usually 10-15 mg per rabbit with a protein concentration of 0.80 to 1.0 $\mu g\ \mu l^{-1}$.

2.9. SPECIFICITY OF THE POLYCLONAL ANTIBODIES

The specificity of the polyclonal antibody against ADP-ribose polymer antigen was tested by Ouchterlony immunodiffusion (Ouchterlony, 1949).

2.9.1. Required material

I. Agarose
II. 0.9 % NaCl
III. Sodium azide
IV. BSA

2.9.2. Methodology

Immunodiffusion assay was performed using 1 % agarose gel. One % agarose solution in 0.9 % NaCl solution containing 0.02 % sodium azide was prepared. It was poured onto clean glass slides and allowed to polymerize. Three wells were punched on the gel each 1 cm apart. The antibody solution was loaded in the middle well, while the antigen (ADP-ribose polymer) and BSA solutions were loaded in the other two peripheral wells. The slides were kept in humid conditions at 37 °C until a clear precipitin line was visible. It usually took 24 h to see the precipitin line. Several slides were prepared and used for serially diluted antigen and antibody solutions.

2.10. PREPARATION OF PERIPHERAL BLOOD LYMPHOCYTES

Peripheral blood lymphocytes (PBL) were recovered from the whole blood collected from human cancer patients and control volunteers by the method of Bøyum (1968a & 1968b) and as previously described (Kma, 2003; Kma and Sharan, 2006) with some modifications. A brief description of the methodology is given below.

2.10.1. *Materials required*

1. Balanced salt solution (BSS) pH 7.6, consisted of the following components:-

Sl. No.	Components	Molarity
a	Anhydrous glucose	5.5 mM
b	$CaCl_2$	5 mM
c	$MgCl_2$	0.098 mM
d	KCl	5.4 mM
e	Tris-HCl	145 mM

The above chemicals were dissolved in Millipore water, and the pH of the solution was adjusted with concentrated HCl before making up the necessary volume with water.

2. Heparin solution: - 4 mg ml^{-1} solution of heparin sodium salt in Millipore water.

3. Histopaque 1077 for recovery of viable mononuclear cells from whole human blood.

4. RPMI-1640 medium: - RPMI-1640 obtained in powder form was dissolved in the required volume of Millipore water as per the manufacturer's recommendations, and sterilized by autoclaving before use.

5. Siliconized glass centrifuge tubes and Pasteur pippettes: - Glass centrifuge tubes and Pasteur pippettes were siliconized in order to retard clotting of blood. For this the tubes were by coated with a thin layer of Sigmacote used as supplied by the manufacture without further dilution, and allowed to dry.

2.10.2. *Methodology*

Blood was collected in tubes containing small volume of heparin. The heparinized blood was then mixed with an equal volume of BSS. The blood-BSS mixture was carefully layered over Histopaque 1077 in the ratio 4:3 (v/v). Following centrifugation (400 x g) for 40 min at room temperature (RT), the lymphocyte layer formed at the interphase was recovered using a siliconized Pasteur pipette, and washed twice with 3 volumes of BSS (100 x g) for 10 min at room temperature. The lymphocytes were then resuspended in 1 ml of RPMI-1640 and stored at -80°C till further use.

2.11. PREPARATION OF CELL LYSATE OF PERIPHERAL BLOOD LYMPHOCYTES

The recovered PBLs were lysed to obtain a PBL lysate using the method of Rosenberg (1996) with some modifications, as previously described (Kma, 2003; Kma and Sharan, 2006)

2.11.1. *Materials required*

1. Phosphate buffered saline (PBS) pH 7.4: - This buffer was prepared as described in § 2.6.1.

2. Cell lysis buffer: - This buffer consisted of the following components:

Sl. No.	Components	Molarity
a	Tris-HCl, pH 8.0	20 mM
b	NaCl	10 mM
c	Triton X-100	0.5 %
d	EDTA	5 mM
e	$MgCl_2$	3 mM
f	PMSF	10 mM

To prepare 100 ml of Tris-Cl buffer, 0.24 g of Tris base was dissolved in Millipore water, the pH was adjusted to 8 with concentrated HCL, and the final volume was raised to 100 ml with Millipore water. The remaining chemicals were dissolved in the appropriate volume of Tris-Cl buffer. PMSF was prepared as a 100 mM stock solution in isopropanol by heating to 60°C until the PMSF flakes had dissolved completely. The required volume was added to the cell lysis buffer, and the buffer was stored refrigerated at 4°C.

2.11.2. *Methodology*

For lysis of lymphocytes, RPMI-1640 was removed by centrifuging (250 x g) for 10 min at 4°C, and the supernatant discarded. The pellet was washed once with 1 ml of cold PBS by centrifuging (200 x g) for 10 min at 4°C, and the supernatant again discarded. The pellet was resuspended in 1 ml of cold cell lysis buffer and kept at -20°C for 30 min, followed by centrifugation (5000 x g) for 15 min at 4°C. The supernatant was collected and its protein content determined by the method of Bradford (1976).

2.12. QUANTIFICATION OF PROTEIN

The total protein content of PBL lysate was estimated by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.12.1. *Materials required*

I. Solution of BSA in water at a concentration of 1 mg ml^{-1}.
II. Stock solution of Bradford reagent: - This reagent consisted of the following components:

[a] Coomassie brilliant blue (CBB) G-250 100 mg

[b] 95 % ethanol 50 ml

[c] 85 % orthophosphoric acid 100 ml

The CBB was completely dissolved in ethanol and then mixed with orthophosphoric acid. This solution was stored refrigerated in a dark bottle, at 4°C.

III. Working solution of Bradford reagent: - The following materials were 100 ml of working solution

[a] Stock solution of Bradford reagent 15 ml

[b] Millipore water 85 ml

[c] Whatmann Filter Paper No. 1

Working solution was prepared by mixing the stock solution with water just before use. This solution was filtered through Whatmann Filter Paper No. 1.

IV. Millipore water

2.12.2. *Methodology*

A standard calibration curve was prepared, using varying concentration of BSA. The amount of B.S.A. in each tube varied from 10 μg to 100 μg i.e. the concentartion varied form 0.1 μg μl^{-1} to 1 μg μl^{-1}. 5 ml of working solution was added to each tube. A blank solution was prepared by adding 5 ml of working solution to 100 μl of Millipore water. Test samples were prepared in triplicate in the same way by taking 20 μl of PBL lysate. The absorbance of standard as well test samples was read at 595 nm against the blank solution, with the help of a spectrophotometer. A standard calibration curve was plotted by plotting the different concentration of BSA versus the corresponding absorbance at 595 nm, and, this curve was used to determine the concentration of protein in the test samples.

2.13. QUANTIFICATION OF DNA

The total DNA content of the antigen was estimated by the method of Burton (1968) using commercially available DNA type 1: Sodium salt from calf thymus as a standard.

2.13.2. *Materials required*

1. Diphenylamine reagent: 1.5 g of diphenylamine was dissolved in 100 ml of glacial acetic acid and 1.5 ml of concentrated sulphuric acid. This reagent was stored at RT.

2. DNA solution

2.13.2. *Methodology*

A standard calibration curve was prepared, using varying concentration of DNA. The samples to be assayed were taken and its volume was raised to 0.1 ml with distilled water. To this 2 ml of diphenylamine reagent was added and mix thoroughly. The mixture was incubated in a boiling water-bath for 10 min. On cooling, the absorbance of the solution was measured at 595 nm.

2.14. QUANTIFICATION OF RNA

The total RNA content of the antigen was estimated by the method of Merchant *et al* (1969) using commercially available RNA (Type III from Bakers Yeast) as a standard.

2.14.1. *Materials required*

1. Orcinol reagent: 1 g of Orcinol was dissolved, immediately before used, in 100 ml of concentrated HCl containing 0.5 g of ferric chloride.

2. RNA solution:

2.14.2. *Methodology*

A standard calibration curve was prepared, using varying concentration of RNA. The samples to be assayed were taken and its volume was raised to 0.1 ml with distilled water. To this 2 ml of Orcinol reagent was added and mix thoroughly. The absorbance of the solution was measured at 660 nm after cooling down to RT.

2.15. SLOT-BLOTTING OF PROTEIN SAMPLES

Slot-blotting was performed, following the method previously described (Sharan et al., 2005) with some modifications.

2.15.1. *Materials required*

1. Bio-Dot SF Microfiltration Apparatus, Bio-Rad.
2. Nitrocellulose (NC) membrane pore size 0.45 μm
3. Whatman Filter Paper No 1.
4. Tris buffered saline (TBS) pH 7.5: - This buffer consisted of the following components:

Sl. No.	Components	Molarity
a	Tris-Cl, pH 7.5	10 mM
b	NaCl	500 mM

To prepare 100 ml of Tris-Cl buffer, 0.12 g of Tris base was dissolved in 100 ml of Millipore water, the pH was adjusted to 7.5 with concentrated HCL, and the final volume was adjusted to 100 ml with Millipore water. TBS buffer was prepared by dissolving the required amount of NaCl in Tris-Cl buffer, and the buffer was stored at RT.

2.15.2. *Methodology*

After estimation of total protein, samples were prepared for slot-blotting by serial dilution of PBL lysate with Millipore water to obtain a concentration of 500 ng total protein per 100 μl of sample. The samples were boiled for 3-4 min to eliminate endogenous alkaline phosphatase activity, as the secondary antibody used was labeled with alkaline phosphatase. NC membrane was cut to the required size and soaked in TBS at RT for 20 min, taking care to avoid touching the membrane with bare fingers. 3 sheets of Whatman Filter Paper, cut to appropriate size were also soaked in TBS. The apparatus was assembled by placing the NC membrane over the filter paper. An equal volume of the samples (100 μl containing 500 ng total proteins) was loaded into each

slot. Each sample was applied in 4-5 replicates along successive slots in one column. Care was taken to ensure that sample in all the slots dried simultaneously, following which the membrane was washed once with TBS. The apparatus was then disassembled, and the membrane removed to be used for immunoprobing or staining with India ink.

2.16. INDIA INK STAINING OF SLOT-BLOT

Protein blots can be stained for total protein with India ink, in order to ascertain equal loading of protein sample for further quantitative studies. The method previously standardized in the laboratory (Kma, 2003 and Sharan et. al, 2005) was followed for staining of slot-blots with India ink, with a few modifications.

2.16.1. *Materials required*

1. Phosphate buffered saline (PBS) buffer, pH 7.4: This buffer was prepared as described in § 2.6.1.

2. Phosphate buffered saline- Tween 20 (PBST)/ Tween solution: This solution consisted of 0.3 % Tween-20 (v/v) in PBS buffer, pH 7.4 prepared as described in § 2.8.1. To prepare 1 L of PBST solution 3 ml of Tween-20 was dissolved in 1L of PBS buffer by continuous stirring.

3. Staining solution: The staining solution was a 0.2 % solution of India ink in PBST, and 100 ml of staining solution was prepared by mixing 200 µl of India ink with 100 ml of PBST solution by continuous stirring. India ink was used directly from the bottle as supplied by the manufacturer. This solution was prepared immediately before use.

2.16.2. *Methodology*

India ink stain for total protein the membrane was stained with using the following procedure:

2.16.2.1. Washing at RT: The membrane was washed by completely immersing it in 10 ml of PBST solution, with shaking on a rocker at medium speed. Washing was performed with three changes of PBST solution for 30 min each at RT.

2.16.2.2. Washing at 37°C: The membrane was washed by completely immersing it in 10 ml of PBST solution, with mild shaking. Washing was performed with three changes of PBST solution for 30 min each at 37°C.

2.16.2.3. Staining: The membrane was immersed in 10 ml of staining solution and incubated at 37°C for 1-2 hours.

2.16.2.4. Destaining: The membrane was destained at RT with 2-3 changes of PBST solution, for 10 min each, by shaking on a rocker at medium speed.

2.17. IMMUNOPROBING OF SLOT-BLOT

The slot-blots were immunoprobed with specific antibody using the method standardized in the laboratory (Kma, 2003 and Sharan et al. 2005), with some modifications.

2.17.1. *Materials required*

1. Tris buffered saline (TBS) buffer, pH 7.5: This buffer was prepared as described in § 2.15.1.

2. Tween-20- tris buffered saline (TTBS) solution: This solution consisted of 0.05 % Tween-20 (v/v) in TBS buffer. To prepare 1 L of TTBS solution, 50 µl of Tween-20 was dissolved in 1 L of TBS buffer by continuous stirring.

3. Blocking solution (BS): BS was a 5 % solution of fat free milk in TBS buffer.

4. Primary antibody (polyclonal anti ADP-ribose) solution: Anti-ADP-ribose polyclonal antibody raised in rabbit and stored as lyophilized powder was dissolved in PBS at a concentration of 2 mg ml^{-1}. The effective dilution used for immunoprobing was 1:1000.

5. Secondary antibody solution: Anti-rabbit IgG-alkaline phosphatase conjugate at a dilution of 1:10,000 was used.

6. BCIP/NBT solution: This was used directly from the bottle as supplied by the manufacturer.

2.17.2. *Methodology*

After completion of slot blotting, the membrane was immunoprobed using the following procedure:

2.17.2.1. Blocking: The NC membrane was blocked by immersing in 10 ml BS, and kept shaking on a rocker at medium speed, for 45 min.

2.17.2.2. Incubation in primary antibody solution: The BS was discarded and 10 ml of fresh BS is added. Primary antibody was added to the BS, followed by shaking on a rocker at medium speed for 5 min. The NC membrane was then incubated for 1h in primary antibody solution at 37°C.

2.17.2.3. Washing: The primary antibody solution was discarded, and the membrane washed with 20 ml of TTBS for 10 min, by shaking on a rocker at medium speed.

2.17.2.4. Incubation in secondary antibody solution: After washing with TTBS, the membrane was incubated in secondary antibody for 1h at 37°C.

2.17.2.5. Washing: The secondary antibody solution was discarded, and the membrane washed with 20 ml of TTBS for 10 min, by shaking on a rocker at medium speed.

2.17.2.6. Washing: The TTBS was discarded, and the membrane washed with 20 ml of TBS for 10 min, by shaking on a rocker at medium speed.

2.17.2.7. Developing: The TBS was discarded. Appropriate volume of BCIP/NBT solution was added to cover the NC membrane, and then incubated at 37°C, until optimum color developed, usually for 10-15 min.

2.18. QUANTIFICATION AND ANALYSIS OF SLOT-BLOT

Following staining with India ink for total protein and immunoprobing with specific antibody, the slot-blots were digitized by scanning the NCM using HP Scanjet 7400C. The digitized image was used for densitometric analysis using KDS-1D software (Kodak). The software measured the signal of the selected area which was one slot and expressed in terms of mean net intensity.

Slot-blotting of each sample was performed thrice, with each slot-blot comprising 5 replicates of the sample which were then either immunoprobed by anti poly-ADP-ribose polymer or stained with India ink for total protein. Thus, data point presented in this study is mean of a total of 15 replicates. Each data point is then corrected in terms of total amount of protein slotted.

2.19. STATISTICAL ANALYSIS

Statistical calculations and plotting of graphs were done with the help of GraphPad Prism 5. A χ^2 test was used to examine the distribution of age, gender and etiological factors such as tobacco consumption, alcohol consumption, betel nut chewing, etc between control and cases i.e. cancer patients. A Fisher's exact test was applied when necessary. An unpaired t-test with Welch's correction was also used to analyze the distribution of mean age. The test assumes unequal variance. A linear regression analysis with 95 % confidence bands was performed to examine PAR level within the control group. A One way ANOVA was used to compare PAR level between control group and patients with cancers of the head and linear neck region. A test that compares the differences among two or more groups. An unpaired t-test with Welch's correction was used to compare PAR level between control group and cases i.e. patients with cancer of the breast and cervix as well as head and neck cancer. The variation parameter analysis in the control and cases was performed using Mann-Whitney rank sum test. A non parametric test that compares two unpaired groups. Spearman rank correlation test was performed to evaluate the correlation between PAR level and age. Mann-Whitney rank sum test was also performed to compare PAR level in control and cases matched for age and gender. P value of <0.05 were regarded as statistically

significant. In the entire test a two-tailed P value was used. Cancer incidence among cancer patients was expressed in terms of percentage.

Table VII: Table showing the summary of P value

P value	**Wording**	**Summary**
>0.05	Not significant	ns
0.01 to 0.05	Significant	*
0.001 to 0.01	Very significant	**
<0.001	Highly significant	***

RESULTS

3.1. STUDY SUBJECTS

The study included 112 patients with cancer of different regions of the head and neck (H & N), breast and cervix (henceforth also referred to as 'cases') with an age range 25-80 years. In the head and neck region the sites of cancer included oesophagus, alveolo, pyriform sinus, nasopharynx, larynx, tongue, tonsil, buccal mucosa, pharynx, lip, nasal cavity, oral cavity, epiglottis and vocal cord. The patients were in advance stages of cancer, i. e. stage III or IV. The control group consisted of 68 individuals with no known history of cancer with an age range 25-80 years.

Table VIII: Table showing total number of individuals as well as their numbers in each gender category (control volunteers and cancer patients) covered in the study. It includes 14 different sites of cancer in neck and head region along with the cancers of breast and cervix.

Category/Type/Site of cancer	Number of subjects		
	Male	Female	Total
Controls	**41**	**27**	**68**
Cancers of head & neck region	**49**	**16**	**65**
Cancer of oesophagus	12	7	19
Cancer of alveolo	4	2	6
Cancer of pyriform sinus	4	0	4
Cancer of nasopharynx	8	2	10
Cancer of larynx	1	0	1
Cancer of tongue	2	2	4
Cancer of tonsil	4	1	5
Cancer of buccal mucosa	3	0	3
Cancer of pharynx	2	1	3
Cancer of lip	3	0	3
Cancer of nasal cavity	2	0	2
Cancer of oral cavity	1	0	1
Cancer of epiglottis	3	1	4
Cancer of vocal chord	1	0	1
Cancer of breast	**0**	**22**	**22**
Cancer of cervix	**0**	**24**	**24**

Table VIII shows the breakup of number of subjects covered (total as well as in two genders) under controls as well as different category of cases (cancer patients). Detailed clinical information of each patient as well as control subjects were also collected.

3.2. ASSAY OF TOTAL PAR OF PBL PROTEINS

The samples from controls and cases were subjected to quantitative detection of PAR using slot blot immunoprobing followed by densitometric analysis. The results have been expressed as mean net intensity with standard deviation in a graph. Fig 3.1 shows a typical set of results of assay of PAR. The India ink stained slot blot (left panel) shows five replicates each of control sample (A) and sample from cancer patient (cases) (B) slotted on NCM. A cursory visual comparison of the two ink-stained slot blots indicate that equal amount of protein has been slotted. Their replica slot was immunoprobed (right panel). Again visual examination clearly indicates that the intensity of bands of control samples (A) in all replicates was significantly higher than that of the samples from cancer patients (cases) (B). As has been explained in § 2.18-2.19, densitometric quantification of these bands with appropriate statistical calculation finally gave mean ± SD values that have been used to plot graphs (see below).

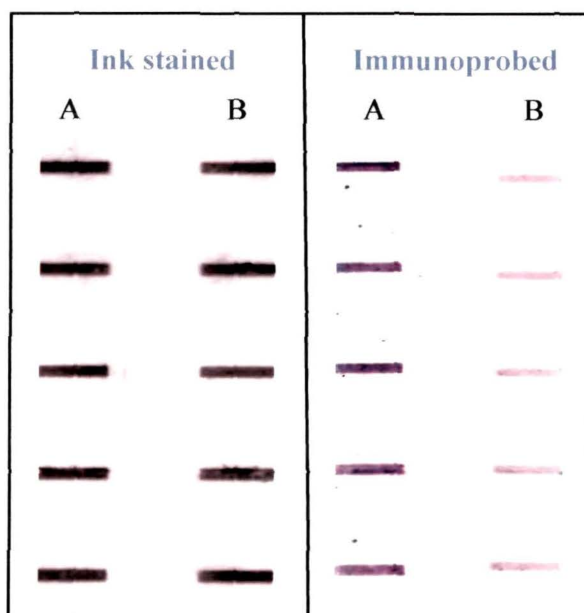


Figure 3.1: India ink stained (left panel) and immunoprobed (right panel) slot blots on nitrocellulose membrane showing total protein and poly-ADP-ribosylated proteins, respectively. (Panels A = control and B = one of the cancer samples).

3.3. TOTAL PAR OF PBL PROTEINS IN CONTROL SUBJECTS

The control group consisted of 68 subjects or individuals. Replica slot of samples from each subject was slotted and immunoprobed for quantitative detection of PAR in five

replicates. The mean net intensity of each subject was then determined as detailed in § 2.18, which represents the total PAR level of PBL proteins of the individual. Value so obtained for each individual subject has been concentration corrected for total amount of protein slotted (see § 2.18). The concentration corrected PAR level of each subject has been plotted as a dot in Fig. 3.2.

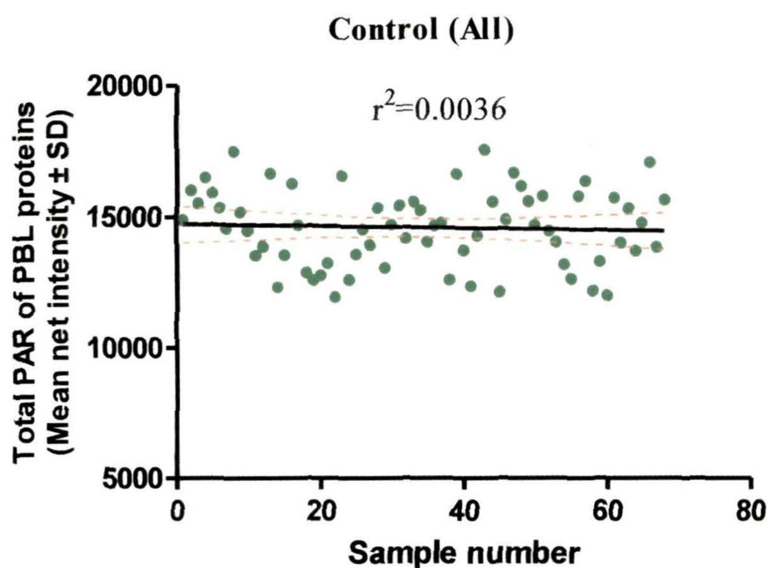


Figure 3.2: A linear regression plot (mean ± SD) showing total PAR level of PBL proteins in controls with P = 0.6233 and r^2 = 0.0036. The total number of subjects was 68. Each point (dot) represents one control subject (individual).

A linear regression test performed on the data yielded a P value of 0.6233 and r^2 of 0.0036 (Fig 3.2). The mean PAR level in control was 14573 ± 1452, with a minimum value of 11950 and maximum of 17540. The data passed the D'Agostino & Pearson omnibus normality test yielding a P value of 0.1067.

3.4. TOTAL PAR OF PBL PROTEINS IN CANCER PATIENTS (CASES)

The cases (cancer) group consisted of 112 patients with cancer of H&N region (n = 68), breast (n = 22) and cervix (n = 24). As done in the control group, samples from cancer patients were identically handled, subjected to replica slotting in replicates of five on NCM and India ink stained as well as immunoprobed. In the section below, results of each group of cancer has been discussed separately.

3.4.1. PAR level in cancers of the H&N region

Among the cancers of head and neck region, cancers of different sites examined in this study were oesophagus, tongue, tonsil, nasopharynx, pharynx, buccal mucosa, alveolo, pyriform sinus, larynx, lip, nasal cavity, epiglottis, oral cavity and vocal chord (details in Table I). The samples from each of the cancer site were compared to controls in terms of total PAR level. A One-way ANOVA performed between PAR levels of control and cancers of different sites of head and neck region, yielded a statistically highly significant lowering of PAR level (P <0.0001) as shown in Table IX below.

Table IX: Table showing one-way ANOVA between total PAR (mean ± SD) of PBL proteins of control and different cancers of head and neck region. ($P < 0.0001$, $r^2 = 0.893$).

Category/Type/Site of cancer	Number of cases	PAR Level Mean ± SD	r^2	P
Control	**68**	**14573 ± 1452**	0.893	<0.0001
Cancers of head & neck region	**66**	**8059 ± 304**		
Cancer of oesophagus	19	8007 ± 463		
Cancer of alveolo	6	8173 ± 651		
Cancer of pyriform sinus	4	8196 ± 258		
Cancer of nasopharynx	10	7890 ± 833		
Cancer of larynx	1	7537		
Cancer of tongue	4	8340 ± 885		
Cancer of tonsil	5	7879 ± 1081		
Cancer of buccal mucosa	3	8172 ± 327		
Cancer of pharynx	3	8177 ± 261		
Cancer of lip	3	7801 ± 906		
Cancer of nasal cavity	2	8554 ± 440		
Cancer of oral cavity	1	7681		
Cancer of epiglottis	4	8553 ± 440		
Cancer of vocal chord	1	7873		

In addition, the PAR level of controls and each of the sites of head and neck cancer was further analyzed using an unpaired t test with Welch corrections as shown in Table X. Here also we find that lowering of PAR was generally highly significant in all cancer types covered in this study. The statistical verification for some cancers (e.g., cancers of larynx, nasal cavity, oral cavity, etc.) was not possible as only one patient with these types of cancer was covered in this study.

Table X: Table showing an unpaired t test with Welch corrections between total PAR level (mean ± SD) of PBL proteins in control and each site of cancer of head and neck. Each of the sites of cancer was compared to control separately. NA - test not applicable as n was one (single case).

Category/Type/Site of cancer	Number of cases	PAR Level Mean ± SD	r^2	P
Control	**68**	**14573 ±1452**		
Cancers of head & neck region	**66**	**8059 ± 304**		
Cancer of oesophagus	19	8007 ± 463	0.9247	<0.0001
Cancer of alveolo	6	8173 ± 651	0.9761	<0.0001
Cancer of pyriform sinus	4	8196 ± 258	0.9759	<0.0001
Cancer of nasopharynx	10	7890 ± 833	0.9611	<0.0001
Cancer of larynx	1	7537	NA	NA
Cancer of tongue	4	8340 ± 885	0.893	0.002
Cancer of tonsil	5	7879 ± 1081	0.9713	<0.0001
Cancer of buccal mucosa	3	8172 ± 327	0.9903	<0.0001
Cancer of pharynx	3	8177 ± 261	0.9870	<0.0001
Cancer of lip	3	7801 ±906	0.9869	0.0066
Cancer of nasal cavity	2	8554 ± 440	NA	NA
Cancer of oral cavity	1	7681	NA	NA
Cancer of epiglottis	4	8553 ± 440	0.9849	<0.0001
Cancer of vocal chord	1	7873	NA	NA

The data for each individual control subject and patient has been plotted as dots in Fig. 3.3 to visually show the distribution of level of total PAR of PBL proteins of each subject under control as well as cancers of different sites of H & N region.

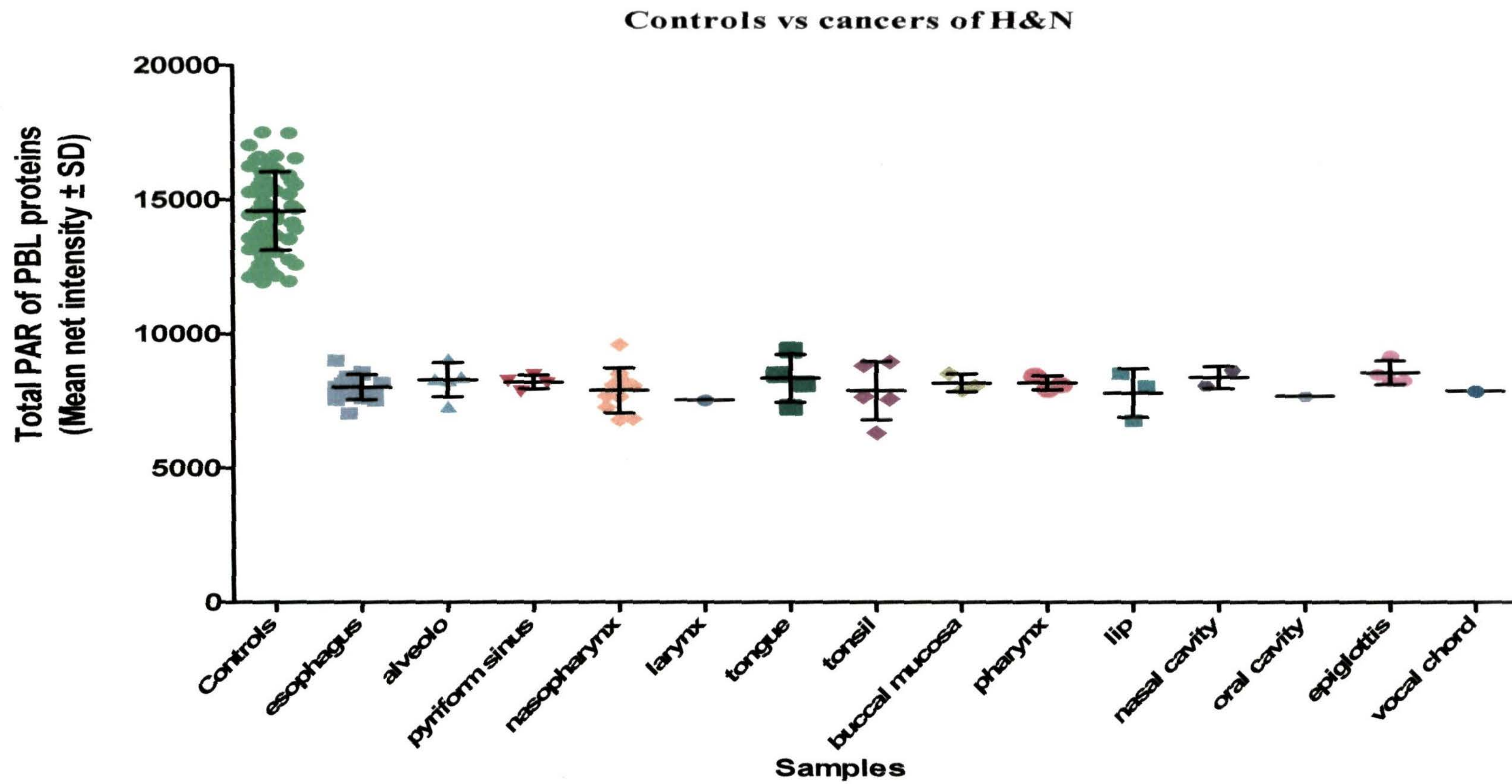


Figure 3.3: A plot showing total PAR level (mean ± SD) of PBL proteins of control and different cancers in the head and neck (H & N) region. Each point represents the mean net intensity of one subject. Number of subjects: Control - 68, Ca esophagus - 19, Ca alveolo - 06, Ca pyriform sinus - 04, Ca nasopharynx - 10, Ca larynx - 01, Ca tongue -04, Ca tonsil - 05, Ca buccal mucosa - 03, Ca pharynx - 03, Ca lip - 03, Ca nasal cavity - 02, Ca oral cavity - 01, Ca epiglottis - 04 and Ca vocal chord - 01.

3.4.2. PAR level in cancer of breast

Twenty two patients with cancer of the breast were covered in this study. An unpaired t test with Welch corrections was performed to test the significance of difference between PAR level of controls - female patients only (n = 27) and the breast cancer patients. A statistically highly significant reduction of PAR level was observed in the patients (P <0.0001) as shown in Table XI. Similarly, even when the entire control population was taken into consideration - that is, PAR of male + female control subjects together - a similar trend was observed (P <0.0001).

Table XI: Table shows results of an unpaired t test with Welch corrections between PAR of control and cancer of the breast. Number of subjects: Ca breast - 22, Control (females only) - 27 & Control (all, i.e., male + female] - 68.

Category/Type/Site of cancer	Number of cases	PAR Level Mean ± SD	P
Cancer of breast	22	7594 ± 852	
Control (females only)	27	14125 ± 1431	<0.0001***
Control (all: males + females)	68	14573 ± 1452	<0.0001***

The reduction in the level of PAR in cancer of breast was in the range of 52 to 54 % as compared to the controls (Table XI). Fig. 3.4 gives a graphically representation of total PAR of PBL proteins expressed as mean net intensity ± SD for each individual subject for control and the breast cancer patient as dots.

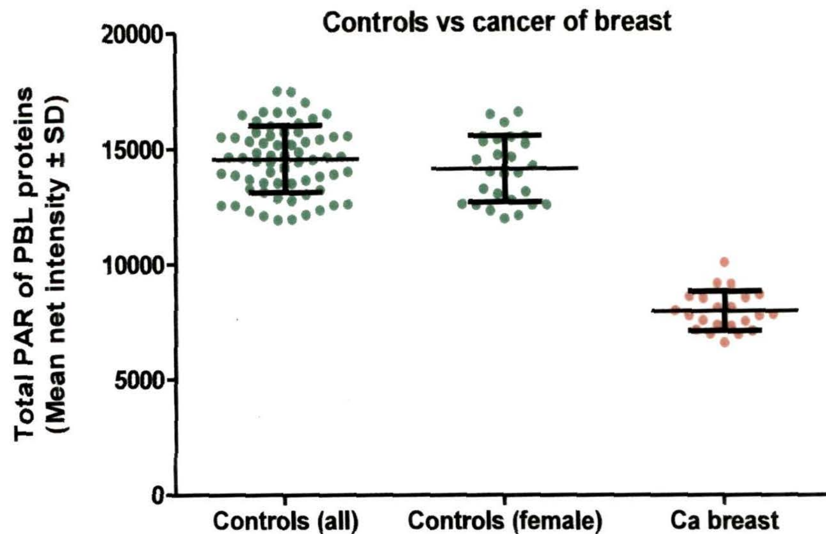


Figure 3.4: A plot comparing total PAR level (mean ± SD) of PBL proteins of control (female), control (all) versus cancer of the breast (both at P <0.00001***). Each point represents the mean net intensity of one subject (*** highly significant). Number of subjects: Ca breast - 22, Control (all) - 68, Control (female) – 27.

3.4.3. PAR level in cancer of cervix

In the group of cancer of the cervix, there were 24 patients. A statistically highly significant reduction of PAR level was observed in the patients (P <0.0001) when an unpaired t test with Welch corrections was performed between PAR level of control female (n = 27) and the cervical cancer patients as shown in Table XII. A similar trend was also seen when the entire control group i.e., PAR of male + female control subjects together, was compared to the cervical cancer patients in terms of PAR level.

Table XII Table shows results of an unpaired t test with Welch corrections between PAR level of control and cancer of the cervix. Number of subjects: Ca cervix - 24, Control (all) - 68, Control (female) - 27.

Category/Type/Site of cancer	Number of cases	PAR level Mean ± SD	P
Cancer of cervix	24	8139 ± 799.1	
Control	68	14573 ± 1452	<0.0001***
Control (female)	27	14125 ± 1431	<0.0001***

The reduction in the level of PAR in cancer of cervix was in the range of 55 to 57 % as compared to the controls (Table XII). Fig. 3.5 presents a graphical representation of the comparison between total PAR of PBL proteins expressed in terms of mean net intensity

± SD for individual subject belonging to control and patients with cancer of the cervix as dots.

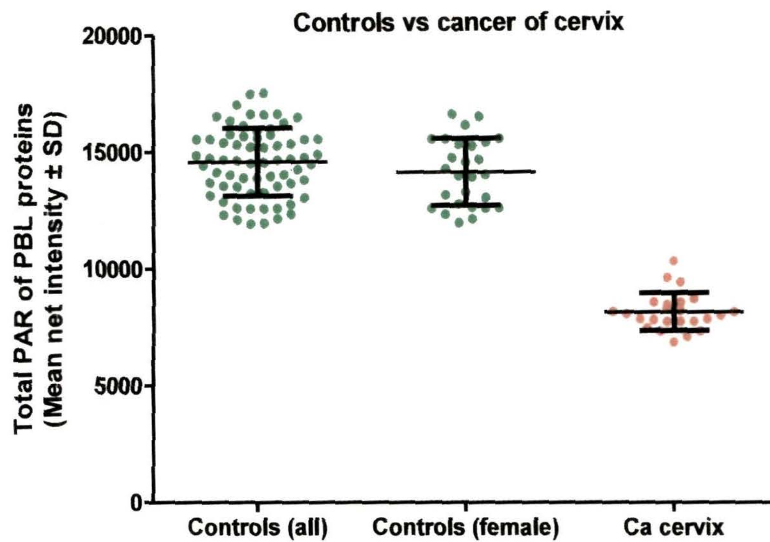


Figure 3.5: A plot comparing total PAR level (mean ± SD) of PBL proteins of control (all), control (female) versus cancer of the cervix (both at P <0.00001***). Each point represents the mean net intensity of one subject (*** highly significant). Number of subjects: Ca breast - 24, Control (all) [i.e. male + female] - 68, Control (female) - 27.

3.5. ANALYSIS OF AGE OF CONTROL AND CASE SUBJECTS

Age is known to be a critical factor in human carcinogenesis. Therefore, it was important to analyze it in the subjects covered in this study. To do this, the age of each individual subject for controls and cases was plotted to visually see the spread of age in the two groups. This is shown in Fig. 3.6.

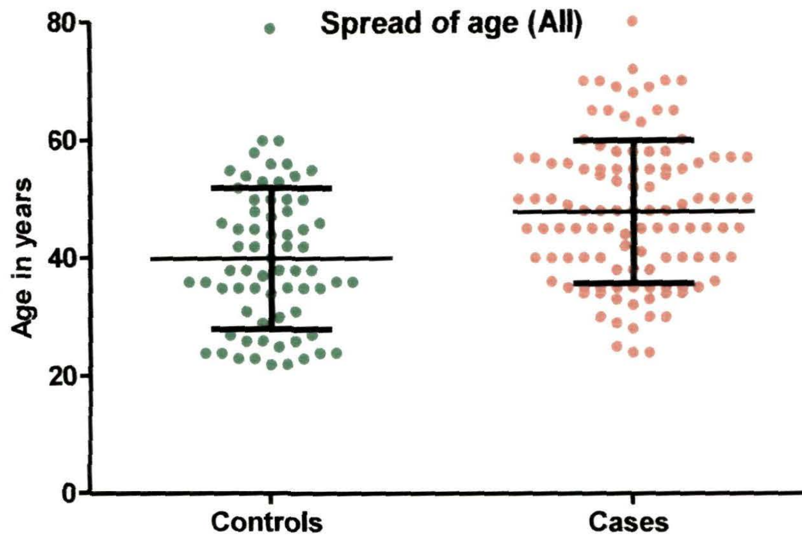


Figure 3.6: A plot showing the distribution of age in the control as well as cases group. In control age range was 25 - 80 years while cases it was 25 - 80 years with a (mean ± SD) 39.9 ± 11.97 years and 47.7 ± 12.09 years, respectively.

Fig. 3.7 represents the plot of spread of age of all male subjects covered in this study. It also shows the difference in the mean age of male subjects. The number of male subjects was approximately the same in both groups.

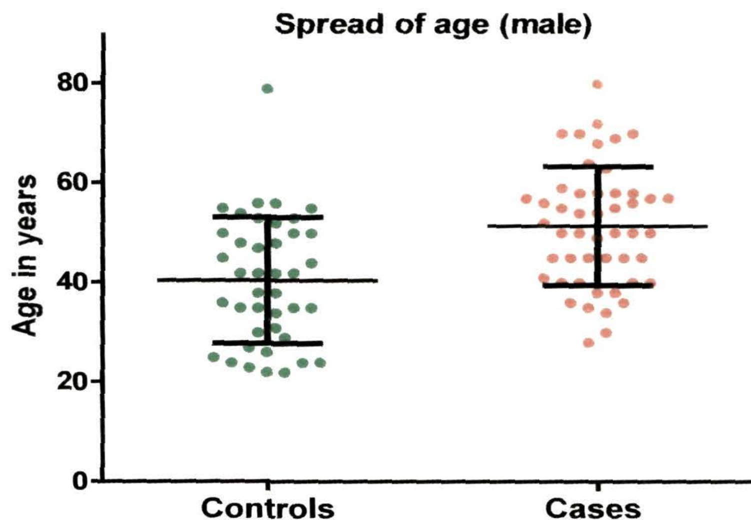


Figure 3.7: A plot showing the distribution of age of male control and case subjects ($P < 0.0001$). Number of males: Controls - 41 and Cases - 49 with a (mean ± SD) age of 40.39 ± 12.69 years and 51.33 ± 11.96 years, respectively.

An entirely different situation is seen with regard to age of the female subjects covered in the study which shows a similar plot for all female subjects (Fig. 3.8).The average age of

subjects in both groups was essentially similar. We had larger number of female subjects in case group as compared to control group.

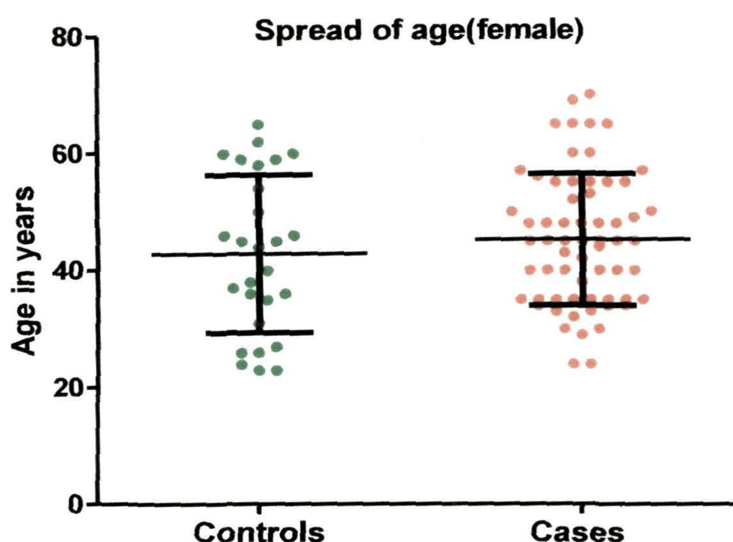


Figure 3.8.: A distribution of female age in control and cases (P = 0.425). Number of females: Control – 27 and Cases - 63 with a mean ± SD age of 42.77 ± 13.48 years and 45.16 ± 11.23 years, respectively.

The results have been summed up for all subjects as well as for male and female subjects in Table XIII to get a clearer picture of difference, if any, among two groups (controls and cases) in terms of age. As unpaired t test with Welch's corrections was applied to compare the two groups. We find that the mean age of control was 39.9 ± 11.97 years and that of cancer patients was 47.7 ± 12.09 years when all subjects (males + females) were considered together. In this case, there exists a highly significant difference between the mean age of controls and cases (P <0.0001) covered in this study. Splitting the subjects in two genders, the male subjects were statistically older in the case (cancer) group than their controls (P <0.0001). Among the female subjects, on the other hand, the mean age of cases and controls were statistically the same (P = 0.4250).

Table XIII: Table shows the difference in mean age of controls and cases covered in this study. An unpaired t test with Welch's correction of the mean male age yielded a highly significant difference between mean ages of controls (all) and cases as well as controls (male) and cases. No difference was observed amongst the female subjects.

Group	Age (years)		
	Controls (*n* = 68)	Cases (*n* = 112)	P
Age (all: male + female)	39.9 ± 11.97	47.7 ± 12.09	<0.0001***
Male age (Mean ± SD)	40.39 ± 12.69	51.33 ± 11.96	<0.0001***
Female age (Mean ± SD)	42.77 ± 13.48	45.16 ± 11.23	0.4250

To further analyse the results in terms of the frequency of occurrence of cancer expressed as percentage in different age groups, the study subjects of cases were grouped into four age groups as shown in Table XIV.

Table XIV: Table shows the cancer incidence among the different age groups of the subjects within cancer patient group (cases).

Group (Age range in years)	Number of subjects	Cancer incidence (%)
Group I (25-34)	15	14 %
Group II (35-44)	27	24 %
Group III (45-54)	32	29 %
Group IV (55 +)	37	33 %

When the subjects were grouped according to their age, their number in each group varied considerably as shown in Fig 3.9. In Group I the number of subjects in control was more when compared to case group, while in Group II, III and IV, the number of subjects in control was less than that in cancer (case) groups.

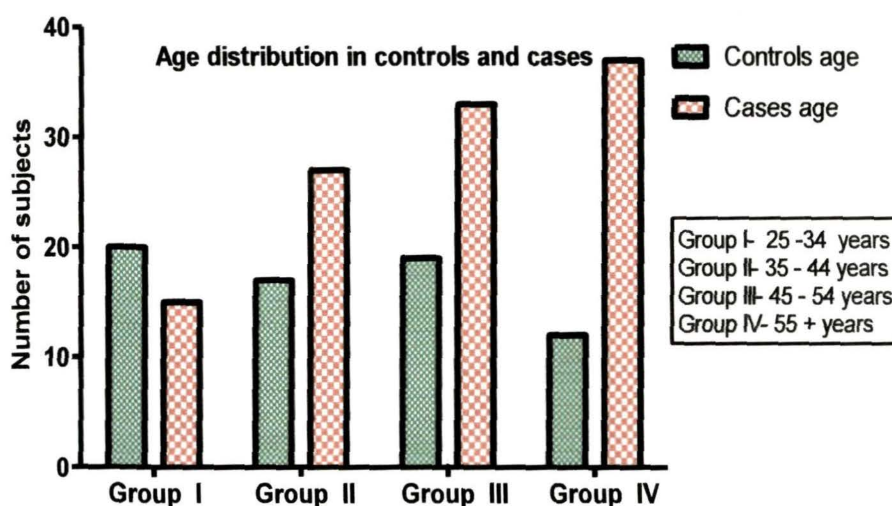


Figure 3.9: Number of subjects among different age groups of control and cases - The subjects from control as well as cases was grouped according to their age. Number of subjects: Group I – Control - 20, Cases - 15; Group II – Control -17, Cases - 27; Group III - Control - 19, Cases - 32; Group IV – Control - 12, Cases - 37.

A χ^2 test shows that statistically this difference in number of subjects in control and case categories in four age groups is only weakly significant (P = 0.0254) and may not seriously influence the results (Table XV).

Table XV: Number of subjects covered in different groups of control and cases. A χ^2 test analysis showed a weakly significant difference (P = 0.0254*) in the distribution of number of subjects in the different age groups of control and cases.

Group (Age range in years)	Number of subjects (% of total subjects)		P
	Controls	Cases	
Group I (25-34)	20 (29 %)	15 (14 %)	0.0254 *
Group II (35-44)	17 (25 %)	27 (24 %)	
Group III (45-54)	19 (28 %)	32 (29 %)	
Group IV (55 +)	12 (18 %)	37 (33 %)	

3.6. ETIOLOGICAL FACTORS AND INCIDENCE OF CANCER

The data for this section was collected from the questionnaire (Annexure II) maintained for each of individual subject from the control and case groups. In this, many important etiological factors, such as gender, tobacco consumption, betel nut mastication, alcohol consumption, history of cancer in the family and diet were considered. Tobacco consumption included use of both smoking and smokeless forms of tobacco. In addition, data regarding the reproductive history of the female subjects, such as age at first sexual exposure and late first pregnancy were also analyzed.

3.6.1. Analysis based on distribution of gender among control and case subjects

The distribution of gender among the control and cases group was looked into to see if they were similar or different among the subjects covered in the study. Fig. 3.10 shows the distribution of male and female subjects in controls and cases. A mildly significant difference in distribution of gender amongst controls and cases is apparent (P = 0.0330). The total number of cancer subjects involved in this study was 112; the number of male subjects was 49 (44 %) while the number of female subjects was 63 (56 %). The frequency of cancer incidence in this study when expressed in percentage is, therefore, higher in females.

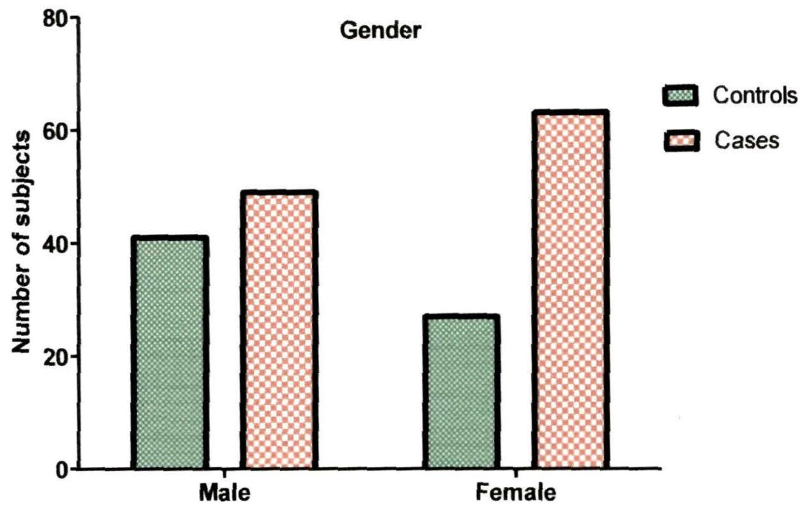


Figure 3.10: Distribution of gender in control and case subjects - Number of subjects ~ Control: male - 41, female - 27; Cases: male - 49, female - 63.

While number of males was higher among controls, it was female subjects that outnumbered males among the cancer patients (cases).

3.6.2. Tobacco consumption

When the subjects were grouped based on tobacco consuming (tobacco +) or non-consuming (tobacco -) habit among the controls and cases, a different picture emerged (Fig. 3.11).

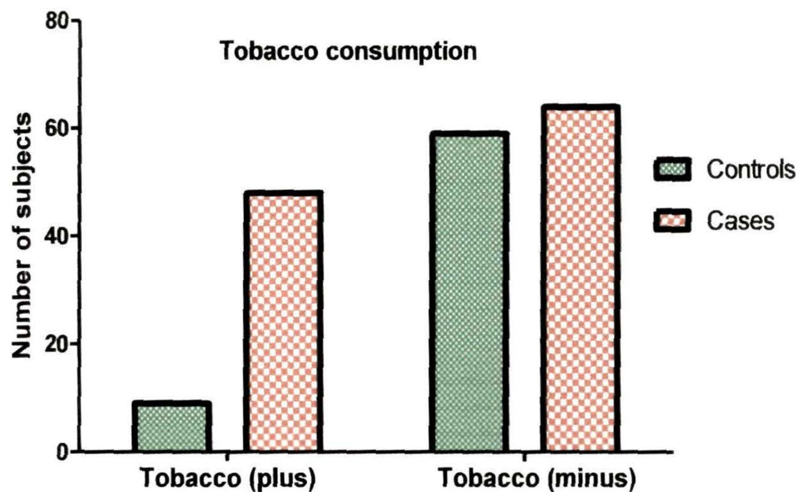


Figure 3.11: Distribution of etiological factors in control and cases - A bar diagram showing consumers and non-consumers of tobacco in control and cases ($P < 0.001$). Number of subjects ~ Control: tobacco consumers - 09, non-consumers of tobacco - 59; Cases: tobacco consumers - 48, non-consumers of tobacco - 64.

In this study tobacco consumption included both smoking and smokeless (chewing) tobacco as well as former and current users of tobacco (for details see Annexure II). A

statistically highly significant difference was observed in the distribution of tobacco consumption among the controls and cases. In cases group, the number of tobacco consumers was 48 (43 %) while non-consumers of tobacco were 64 (57 %). The cancer incidence when expressed in percentage was higher among the non-tobacco consumers with 57 %. However, within each group, i.e., tobacco + and tobacco -, the relative frequency of incidence of cancer was higher in tobacco + group than tobacco -.

3.6.3. Alcohol consumption

The subjects were grouped based on alcohol consuming (alcohol +) or non-consuming (alcohol -) population among controls and cases. A significant difference in the distribution of consumers and non-consumers of alcohol among the controls and cases was observed (P = 0.020). It has to be noted that in alcohol consumption group only moderate and heavy consumers were included (for details see Annexure II). The number of alcohol consumers was 34 (30 %) and non-alcohol consumers was 78 (70 %) among the cancer patients. Therefore, the frequency of cancer incidence expressed in percentage was higher among the alcohol - than alcohol +.

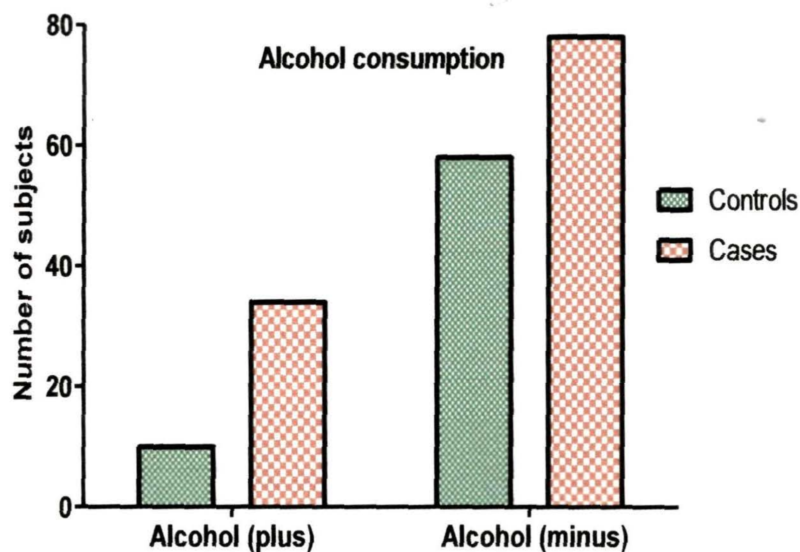


Figure 3.12: A bar diagram showing consumers and non-consumers of alcohol in control and cases (P = 0.020*). Number of subjects ~ Control: alcohol consumers - 10, non-consumers of alcohol - 58; Cases: alcohol consumers - 34, non-consumers of alcohol - 78.

However, within each group, i.e., alcohol + and alcohol -, the relative frequency of incidence of cancer was slightly higher in alcohol + group than alcohol - group.

3.6.4. Betel nut chewing

When the subjects were grouped based on prevalence of betel nut chewing (betel nut +) or non-chewing (betel nut -) habit among controls and cases, the following picture emerged (Fig. 3.13). Betel nut chewing habit, prevalent in the north eastern part of the country, seems to be prevalent among both cases as in controls. The number of betel nut chewers was comparable in cases as well as controls when all forms of betel nut chewing were considered. In the cancer patients group, the number of consumers and non-consumers of betel nut was 68 (61 %) and 44 (39 %), respectively. Betel consumers, therefore, had a higher percentage of cancer incidences.

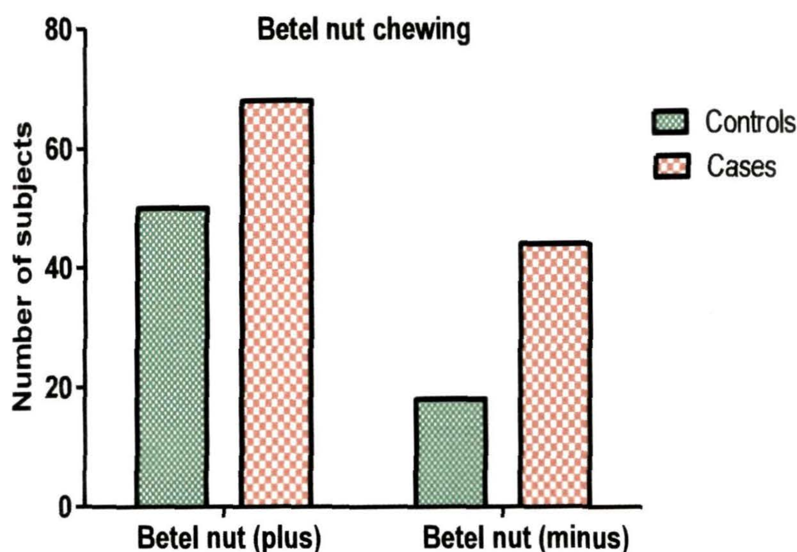


Figure 3.13: A bar diagram showing consumers and non-consumers of betel nut in control and cases. Number of subjects ~ Control: betel nut consumers -50, non-consumers of betel nut - 18; Cases: betel nut consumers - 68, non-consumers of betel nut - 44.

In contrast, relative to respective controls in betel nut + and betel nut - groups, the relative frequency of incidence of cancer was statistically the same in both groups.

6.5. Diet

Another factor examined in this study was the diet of the subjects. The diet only refers to the broad category of vegetarian and non-vegetarian meal habit in this study. It also included the use of spices as well as processed food (see Annexure II for details). Fig. 3.14 depicts the results. As is apparent, both kinds of diet were nearly evenly distributed among cases and controls. Among the cases, the number of subjects with vegetarian as

well non-vegetarian diets was 56 each. Within each group also there appears to be essentially no influence of dietary habit on the frequency of occurrence of cancer.

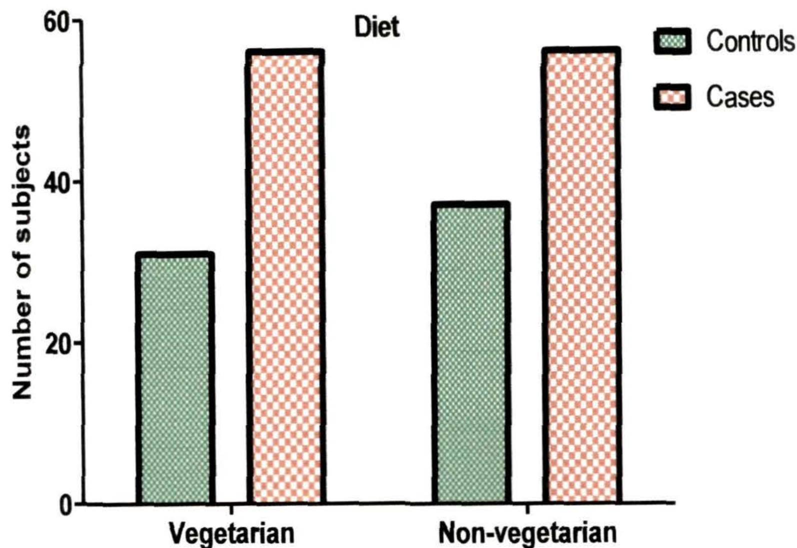


Figure 3.14: A bar diagram showing vegetarian and non-vegetarian diet habit among controls and cases. Number of subjects ~ Control: vegetarian - 31, non-vegetarian - 37; Cases: vegetarian - 56, non-vegetarian - 56

3.6.6. History of cancer in family (HCF) among female subjects

Attempt was also made to ascertain if history of cancer in family (HCF) was common among women in this study. The number of female subjects with a history of cancer in the family among the controls was 3 while in cases it was 8. As apparent from Fig. 3.15, the distribution of HCF and non-HCF were similar in both control and case groups. The total number of female subjects in cases was 63; females with HCF were 8 (12 %) and with non-HCF were 55 (88 %). The incidence of cancer was higher among non-HCF female cases.

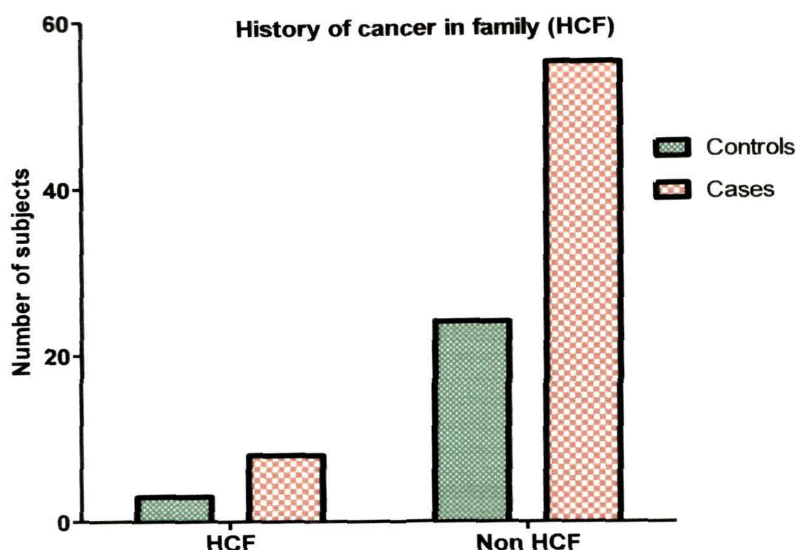


Figure 3.15: A bar diagram showing history of cancer in family (HCF) of female control and cases. Number of females ~ Control: HCF - 03, non HCF - 24; Cases ~ HCF - 08, non-HCF - 55.

Table XVI summarizes the results of these etiological factors and their likely influence on human cancers covered in this investigation.

Table XVI: Table shows the influence of various etiological factors on the incidence of cancer among the cancer patients covered in the study.

Factors	Defined groups	Sample size	Cancer incidence (%)
Gender	male	49	44 %
	female	63	56 %
Tobacco	yes	59	43 %
	no	64	57 %
Alcohol	yes	34	30 %
	no	78	70 %
Betel nut	yes	68	61 %
	no	44	39 %
Diet	Vegetarian	56	50 %
	Non-vegetarian	56	50 %
History of cancer	yes	08	12 %
	no	55	88 %

Table XVII shows the summary of the distribution of the various etiological factors among the controls and cases.

Table XVII: Table showing the χ^2 test analysis of the distribution of various factors in control and cases. A significant difference was observed in the distribution of gender and number of alcohol and tobacco consumers between control and cases.

Factors	Defined groups	Sample size	P
Gender	Control male/female	41/27	0.0330*
	Cases male/female	49/63	
Tobacco	Control yes / no	9/59	<0.001***
	Cases yes / no	59/64	
Alcohol	Control yes / no	10/58	0.020*
	Cases yes / no	34/78	
Betel nut	Control yes / no	50/18	0.1054
	Cases yes / no	68/44	
Diet	Control Vegetarian / Non-vegetarian	31/37	0.6449
	Cases Vegetarian / Non-vegetarian	56/56	
History of cancer	Control yes / no	03/24	1.00
	Cases yes / no	06/57	

The results suggest that the gender of the cases had a significant influence in terms of cancer incidence with females having a higher percentage of cancer incidences (56 %). The pattern of tobacco (43 %) and alcohol (30 %) consumptions among controls and cancer patients covered in this investigation show that both these etiological factors were unequally distributed. Nonetheless, there was a significant influence of betel nut chewing only on the frequency of cancer among the betel nut consumers (61 %). Alcohol + group exhibited a slightly higher relative frequency of cancer. The diet (vegetarian and non-vegetarian diet) of controls and cases also did not seem to have statistically significant influence. Similarly, the family history of cancer also did not seem to have any significant influence on incidence of cancer among controls and cases covered in this study.

The gender of subjects covered in the study as a factor was found to be significantly different (P = 0.0330) as males were overrepresented in the control group while females were overrepresented among cancer patients (controls: 60 % male and 40 % female; cancer patients: 43 % male and 57 % female). The pattern of tobacco and alcohol consumption among controls and cancer patients covered in this investigation show that both these etiological factors were distributed unequally. There was a statistically highly significant difference in the distribution pattern of tobacco consumption between the

control and cases (P <0.001), but only weakly significant difference for alcohol consumption (P = 0.020). On the other hand, there was no significant difference in the distribution of betel nut consumers among the two groups (P = 0.1054). The diet (vegetarian and non-vegetarian diet) of controls and cases also did not seem to have statistically significant difference in distribution (P = 0.6449). Similarly, the family history of cancer also did not seem to have any statistically significant difference in the distribution (P = 1.00) among controls and cases covered in this study.

Two additional factors, relevant especially to female subjects, were also analysed based on the data available under the study. These two factors are early sexual exposures and the age at first pregnancy.

3.6.7. Early sexual exposure (ESE)

Fig 3.16 shows the frequency of early sexual exposure (ESE) among female subjects in control and case groups. None of the control subjects belonged to this category while 8 female subjects among cancer patients had ESE. The distribution pattern of this factor was not significantly different between the two groups. Incidence of occurrence of cancer does not seem to be influenced by ESE in cases since the number of ESE was 8 (13 %) and non-ESE was 55 (87 %) (Fig 3.16).

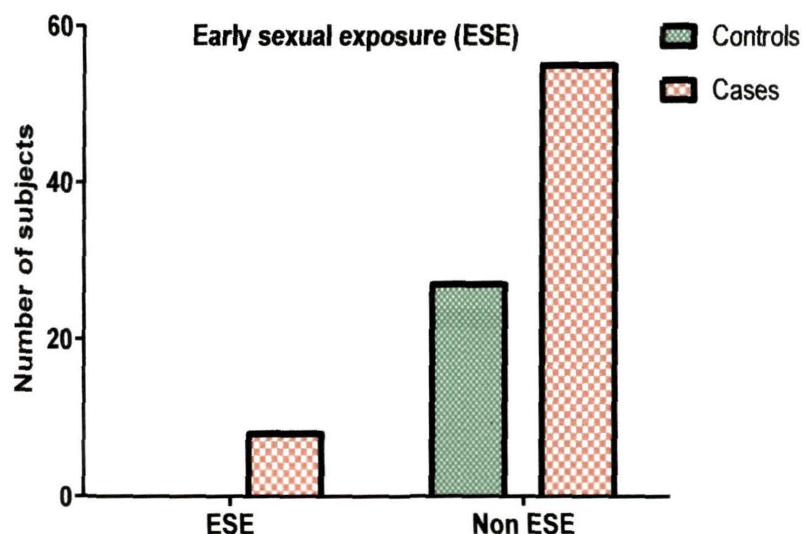


Figure 3.16: A bar diagram showing female number of female subjects with early sexual exposure (ESE) among controls and cases. Number of females: Control: ESE-0, non ESE-27; Cases: ESE - 08, non ESE - 55.

3.6.8. Late first pregnancy (LFP)

The other important etiological factor among female subjects is late first pregnancy (LFP) i.e. first pregnancy at over the age of 30 years. There was no significant difference in the distribution of this factor in the subjects in either of the two categories as shown in Figure 3.17. In terms of cancer incidence expressed as percentage, this factor did not show any influence on the frequency of cancer among the LFP (13 %) and non-LFP (87 %) of cases.

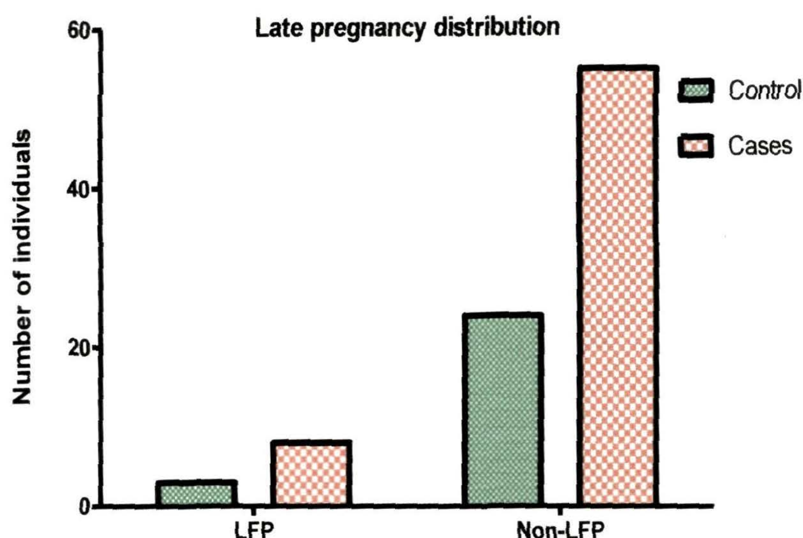


Figure 3.17: A bar diagram showing female subjects with late first pregnancy (LFP) in control and cases. Number of females: Control: LFP - 3, non-LFP - 24; Cases: LFP - 08, non-LFP - 55.

Table XVIII below summarizes the results of these two factors for female subjects only. As is apparent, there was no significant influence of these two factors on the outcome and frequency of occurrence of cancer.

Table XVIII: Table shows the influence of various etiological factors on the incidence of cancer among the subjects in the cases.

Factors	Defined groups	Sample size	Cancer incidence (%)
Early sexual exposure	Yes	08	13 %
	No	55	87 %
Late first pregnancy	Yes	08	13 %
	No	55	87 %

Table XIX summarizes the distribution of these two factors among the female subjects of control and cases.

Table XIX: Table shows a χ^2 test analysis of the distribution of two factors amongst the female control and cases. A significant difference was observed in the distribution of early sexual exposure between female control and cases.

Factors	Defined groups	Sample size	P
Early sexual exposure	Control female	00/27	0.0995
	Cases female	08/55	
Late first pregnancy	Control female	03/24	0.7360
	Cases female	08/55	

3.7. ETIOLOGICAL FACTORS AND TOTAL PAR OF PBL PROTEINS

Statistical analysis of inter-individual factors such as age, gender, habits of tobacco, alcohol, and betel nut consumption, and diet for the control and cases group were performed to understand their influences on total PAR of PBL proteins. Among the female subjects other parameters, such history of history of cancer in the family (HCF), late first pregnancy (LFP) were also analyzed.

3.7.1. In control group

The effect of age of subject under investigation on the total PAR level of PBL proteins was examined. The total PAR level in the controls was in the range of 11950 to 17590. The spread of PAR level in different subjects under Spearman correlation test showed no significant correlation (P = 0.0945) with a r value of 0.2044. The PAR level of each individual control subject is shown in Fig. 3.18.

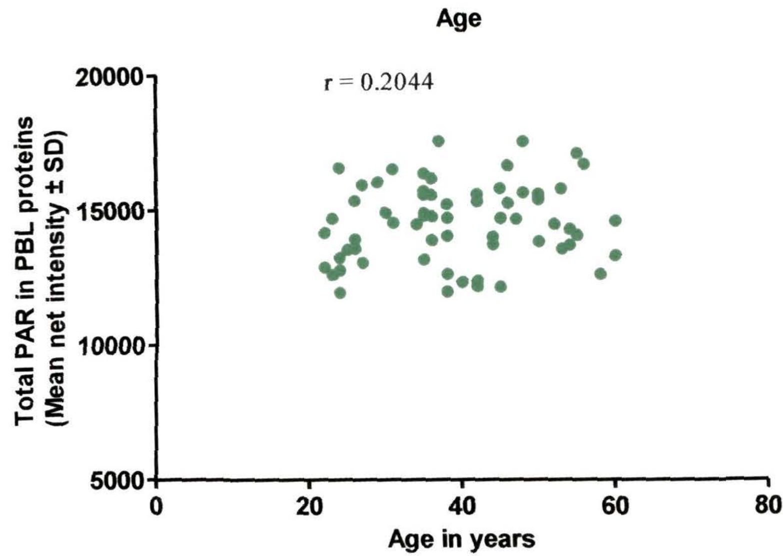


Figure 3.18: A Spearman rank correlation test of control age versus total PAR in PBL proteins. (P = 0.0945; r = 0.2044). Total number of Control subjects - 68. Age range was 25 - 80 years. Total PAR level in Controls: Minimum – 11950, Maximum - 17590.

However, gender did exhibit a mildly significant influence (P = 0.0463) on total PAR of PBL proteins. Total PAR level was statistically a bit higher in controls males than in females as shown in Fig. 3.19.

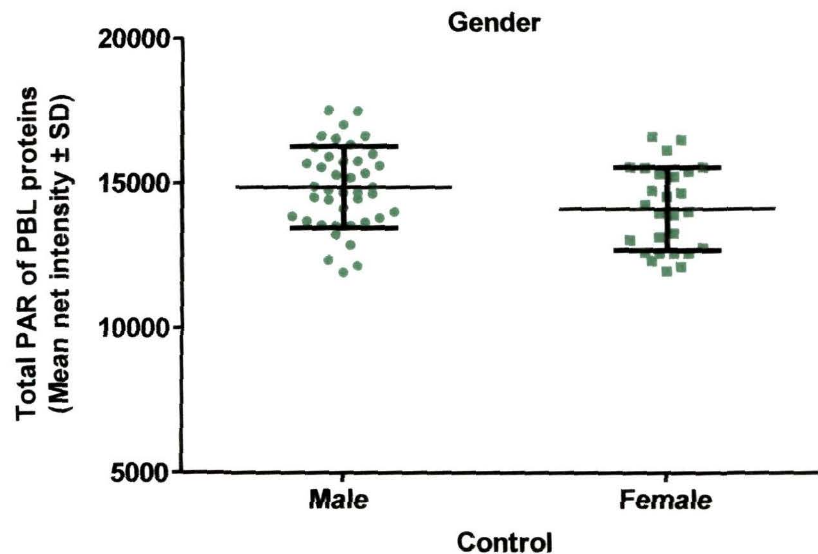


Figure 3.19: A plot showing the influence of gender on total PAR level of control (P = 0.0463). Number of controls: male - 41, female - 27. PAR level (Mean ± SD): male - 14868 ± 1405, female – 14125 ± 1431.

In this study the effect tobacco consumption on total PAR of PBL proteins was analyzed. Even though we had a relatively small number of tobacco+ subjects in control group,

tobacco consumption did not seem to exhibit any statistically significant effect on the total PAR level (P = 0.1402) of control as shown in Fig. 3.20.

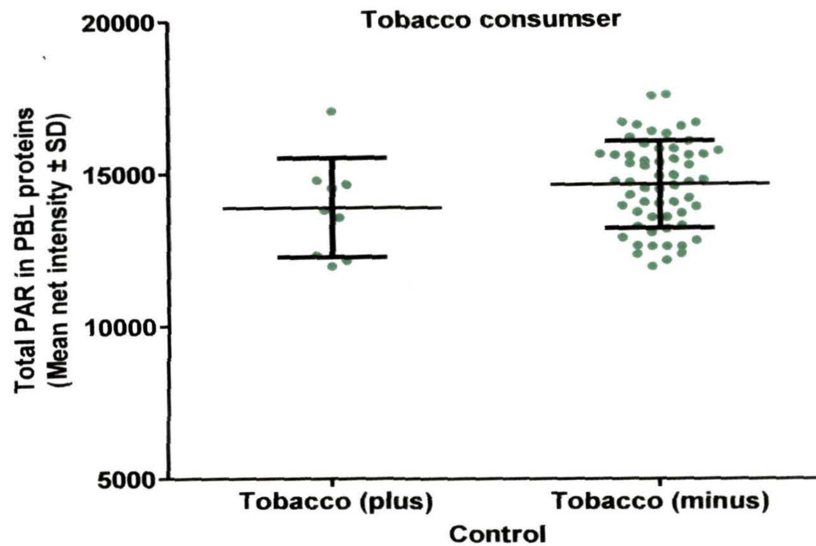


Figure 3.20: A plot showing the effect of tobacco on total PAR level of controls. Number of controls: tobacco consumer - 09, non-tobacco consumer - 59. Total PAR level (Mean ± SD): tobacco consumer - (13879 ± 1617), non-tobacco consumer (14605 ± 1418).

The effect of alcohol consumption on total PAR level of PBL proteins was also examined. Figure 3.21 shows that alcohol consumption did not have reveal any statistically significant (P = 0.9379) effect on total PAR of PBL proteins of control subjects.

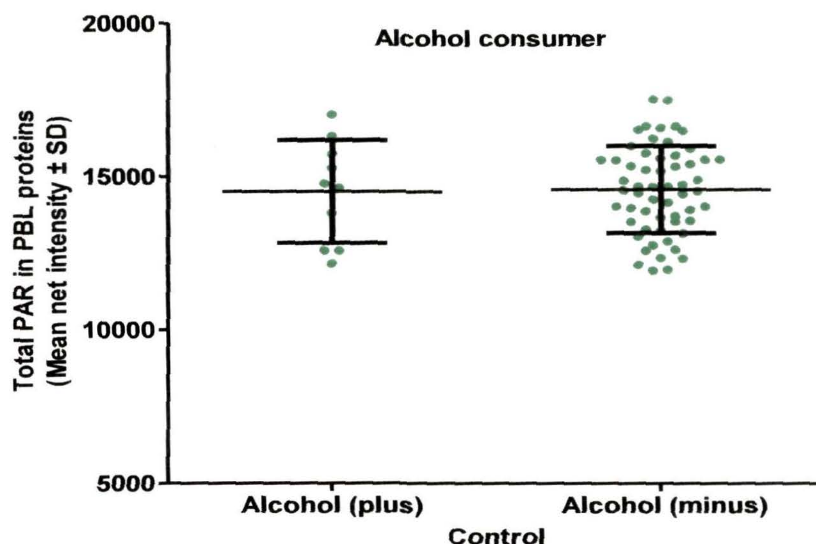


Figure 3.21: A plot showing the effect of alcohol consumption on total PAR of PBL proteins in controls. Number of control subjects: alcohol consumer - 10, non-alcohol consumer - 58. PAR level (Mean ± SD): alcohol consumer – 14510 ± 1679, non-alcohol consumer – 14584 ± 1425.

Betel nut chewing is another important etiological factor, which was analyzed for its effect on total PAR of PBL proteins. As shown in Figure 3.22, betel nut chewing did not have even slightest of statistically significant (P = 0.9839) effect on total PAR of PBL proteins in control subjects.

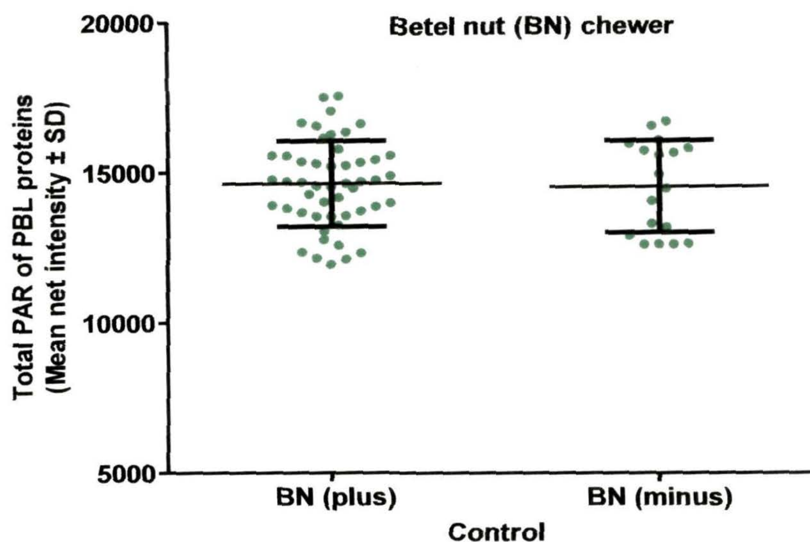


Figure 3.22: A plot showing the effect of betel nut consumption on total PAR of PBL proteins in control group. Number of control subjects: betel nut consumer - 50, non-betel nut consumer - 18. PAR level (Mean ± SD): betel nut consumer - 14613 ± 1422, non-betel nut consumer - 14496 ± 1516.

Diet, in terms of non-vegetarian and vegetarian meal, was another factor that was analyzed for its effect on PAR level within the control group. Diet also did not show any statistically significant (P = 0.6194) effect on total PAR of PBL proteins as shown in Fig 3.33.

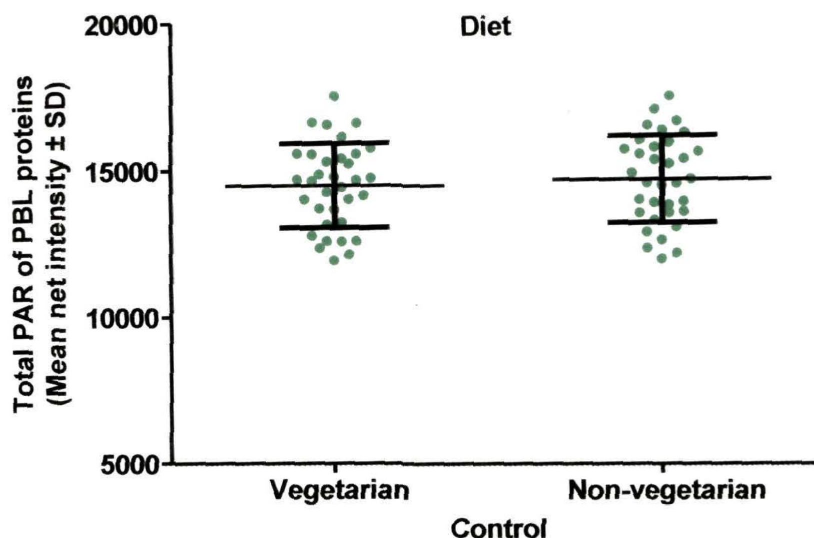


Figure 3.23: A plot showing the effect of diet on total PAR of PBL proteins in control group. Number of control subjects: vegetarian - 31, non-vegetarian - 37. PAR level (Mean ± SD): vegetarian – 14478 ± 1442, non-vegetarian – 14667 ± 1477.

A summary of the influence of the etiological factors on total PAR of PBL proteins for the control group is shown in Table XX. A Spearman rank correlation test of control age versus PAR level (P = 0.0945) did not show any significant effect of age. In male and female controls, the mean PAR level were 14870 ± 1405 and 14130 ± 1431, respectively. A Mann-Whitney rank sum test between gender and PAR level in control showed a statistically significant lower level of PAR in female (P = 0.0463).

Table XX: Table shows the effect of various factors on PAR level of control group. A Spearman rank correlation test was performed to analyze the relation between PAR level and age of control. A Mann-Whitney rank sum test was performed to analyze the effect of the other factors on PAR level of control. A significant reduction of PAR level was observed in control female when compared to control male.

Control	Defined groups	Number of samples	PAR level (Mean ± SD)	P
Age	All	68	14573 ± 1452	0.0945
Gender	Male	41	14870 ± 1405	0.0463*
	Female	27	14130 ± 1431	
Tobacco	Yes	09	13879 ± 1617	0.1402
	No	59	14605 ± 1418	
Alcohol	Yes	10	14528 ± 1657	0.9379
	No	58	14541 ± 1452	
Betel nut	Yes	50	14646 ± 1436	0.9839
	No	18	14286 ± 1507	
Diet	Vegetarian	34	14478 ± 1442	0.6194
	Non-vegetarian	34	14667 ± 1447	

In addition, influences of three other factors like history of cancer in the family (HCF), early sexual exposure and the late first pregnancy (LFP) among the female control subjects on total PAR of PBL proteins were also analyzed.

The history of cancer in family (HCF) did seem to influence the level of total PAR in PBL proteins. The control subjects with HCF exhibited statistically lower (P = 0.0284) total PAR than that in the non-HCF control subjects (Fig. 3.24).

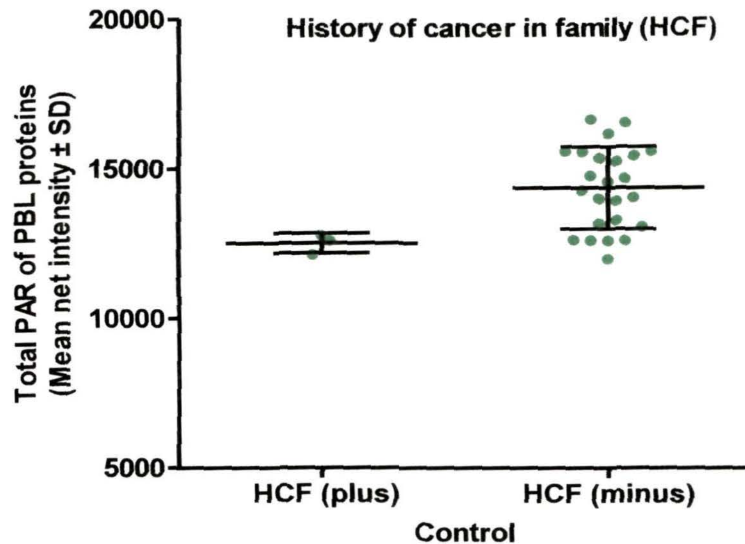


Figure 3.24: A plot of control with history of cancer in family (HCF) versus PAR level. Number of control: HCF - 3, non HCF - 24. Total PAR level (Mean ± SD): HCF – 12506 ± 333, non-HCF – 14339 ± 1371.

In the control group none of the female respondents had early sexual exposures; therefore analysis of this factor was not possible.

In contrast, the late first pregnancy (LFP) factor did not show any statistically significant effect (P = 0.8471) on the total PAR of PB proteins in female control subjects as shown in Fig. 3.25.

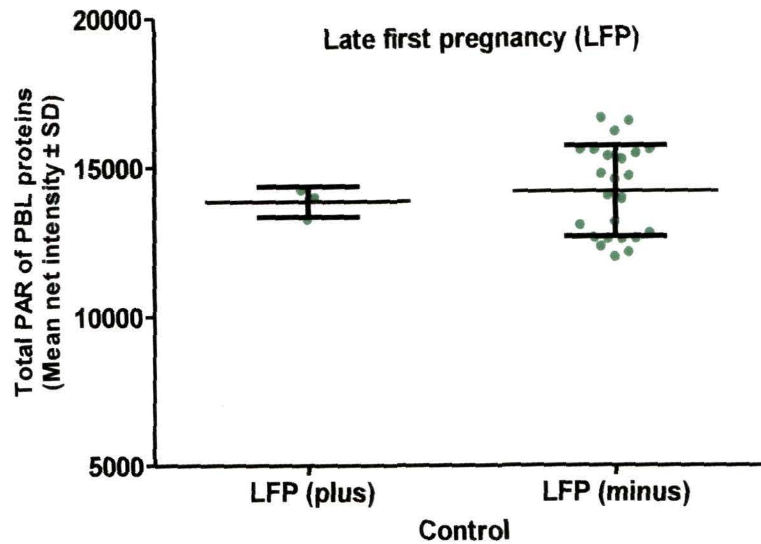


Figure 3.25: A plot of control with late first pregnancy (LFP) versus PAR level. Number of control: LFP - 3, non LFP - 24. Total PAR level (Mean ± SD): LFP – 13851 ± 509, non- LFP – 14159 ± 1511.

Table XXI shows a summary of these two special factors that were analyzed in female control subjects only.

Table XXI: Table shows Mann-Whitney rank sum test of the effect of etiological factors on total PAR level of control group. A significant reduction of PAR level in female control with history of cancer in the family was observed. NA- Not applicable.

Control	Defined groups	Number of samples	PAR level Mean ± SD	P
Family history of cancer	Yes	3	12506 ± 333	
	No	24	14414 ± 1482	0.0284*
Early sexual exposure	Yes	0	-	
	No	27	14130 ± 1431	NA-
Late first pregnancy	Yes	3	13851 ± 5 09	
	No	24	14159 ± 1511	0.8471

In summary, the female control subjects with history of cancer in the family showed a marginally significant (P = 0.0284) lowering of level of total PAR than female control subjects with no history of cancer in family. None of the female control subjects covered in this study had early sexual exposure. Therefore, the analysis was not possible.

3.7.2. In cancer group (cases)

The value of total PAR of PBL proteins among cancer patients (cases) ranged between 6324 and 10318. A Spearman rank correlation test was performed to determine the effect

of age on the PAR level. A mildly significant correlation (P = 0.0117) was observed between total PAR of PBL proteins and age with an r value = 0.2319 as shown in Fig 3.26. This is, however, opposite to the observations within the control group.

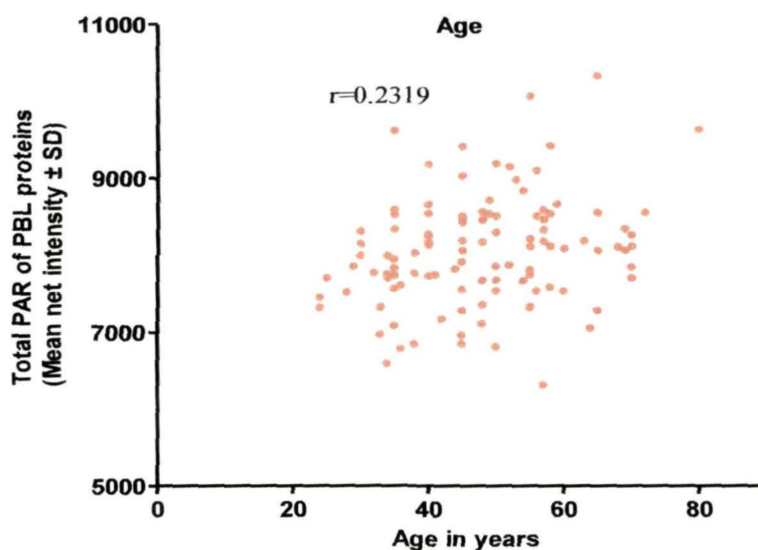


Figure 3.26: A Spearman rank correlation test of control age versus PAR level (P = 0.01168, r = 0.2376) Total number of cases - 112. Age range was 25-80 years. Total PAR in cancer group: Minimum - 6324, Maximum - 10318.

Almost as in the control group, there was very mildly statistically significant (P = 0.0673) influence of gender on PAR level among the cancer patients. Fig 3.27 shows that the total PAR of PBL proteins in cancer patients was only marginally higher in males than in females.

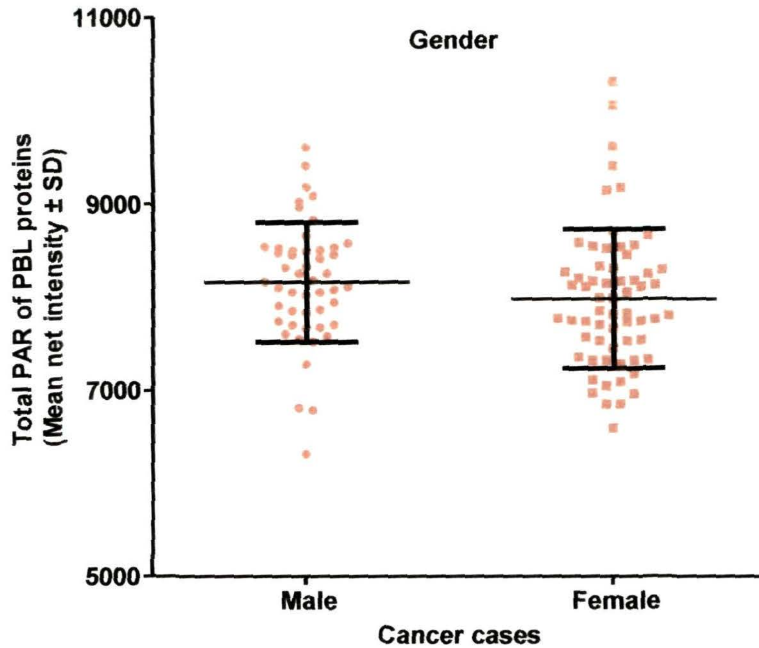


Figure 3.27: A plot showing the effect of gender on PAR level of cases. Number of cases: male - 49, female - 63. Total PAR level (Mean ± SD): male – 8161 ± 643, female - 7982 ± 748.

However, tobacco consumption had a very significant effect (P = 0.0033) on total PAR level recorded in cancer patients. Fig 3.28 shows that the total PAR of PBL proteins was statistically significantly lower in the tobacco consumers as compared to tobacco non-consumers.

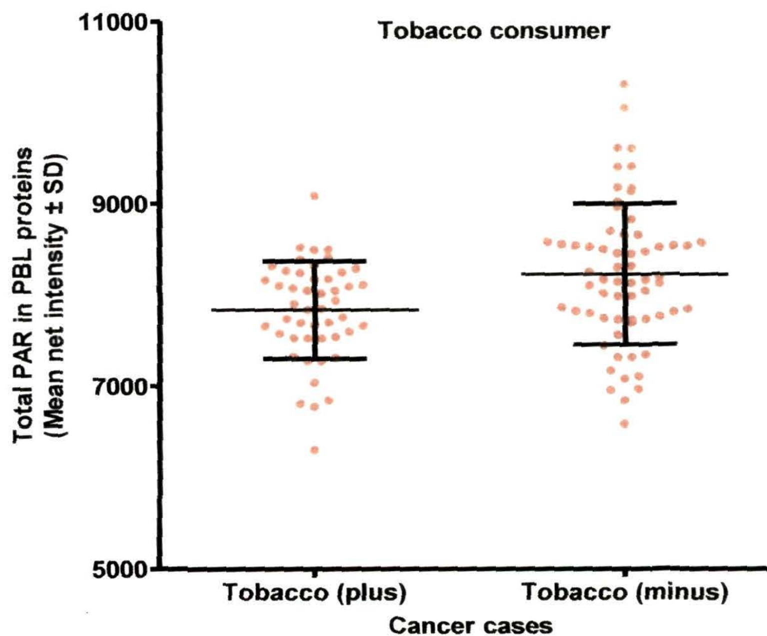


Figure 3.28: A plot showing the effect of tobacco on total PAR level of cases. Number of cases: tobacco consumer - 48, non tobacco consumer - 64. Total PAR level (Mean ± SD): tobacco consumer – 7840 ± 534, non-tobacco consumer – 8232 ± 771.

Similarly, alcohol consumption exhibited a highly significant (P = 0.0001) effect on total PAR. Fig 3.29 shows that total PAR level of PBL proteins in alcohol consuming cancer patients was statistically highly significantly lower than the alcohol non-consuming cancer patients.

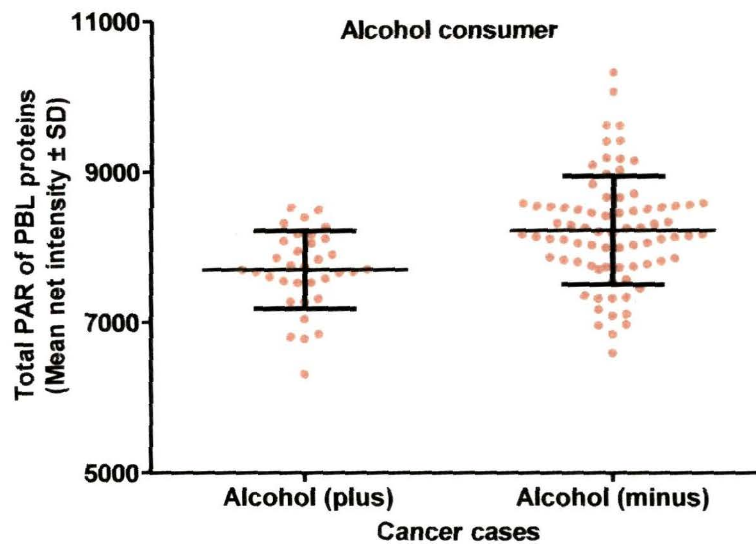


Figure 3.29: A plot showing the effect of alcohol on total PAR level of cases (P = 0.0001***). Number of cases: tobacco consumer-34, non tobacco consumer - 78. Total PAR level (Mean ± SD): tobacco consumer - 7699 ± 518, non-tobacco consumer – 8223 ± 718.

Interestingly, a major factor associated with cancer, betel nut chewing, seems not to influence (P = 0.0935) the level of total PAR of PBL proteins in cancer patients. In Fig. 3.30, total PAR level of PBL proteins was statistically almost similar among the betel nut chewers and non-chewers.

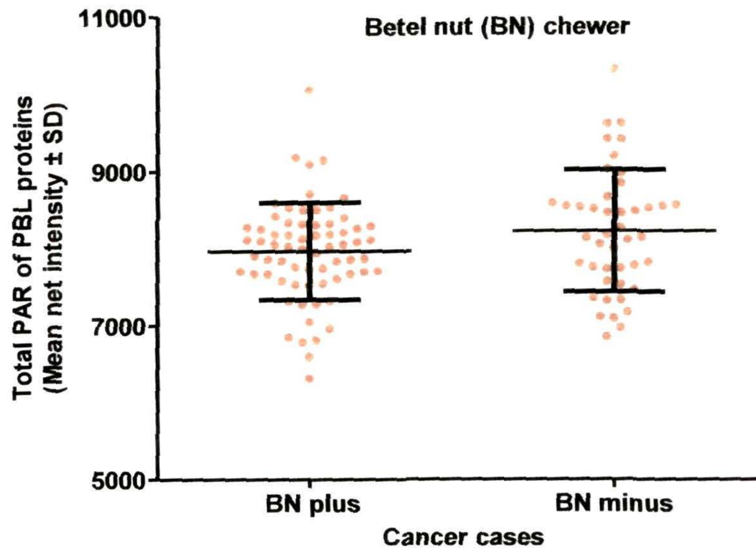


Figure 3.30: A plot showing the effect of betel nut mastication on total PAR of PBL proteins among cancer patients (cases). Number of cases: betel nut consumer - 68, non-consumer - 44. Total PAR level (Mean ± SD): betel nut consumer – 7963 ± 628, non-consumer – 8220 ± 791.

When diet was examined for its influence on the total PAR of PBL proteins, it did not seem to exhibit any significant effect (P = 0.6902). Fig 3.31 shows that vegetarian or non-vegetarian dietary habit did not alter total PAR level among cancer patients.

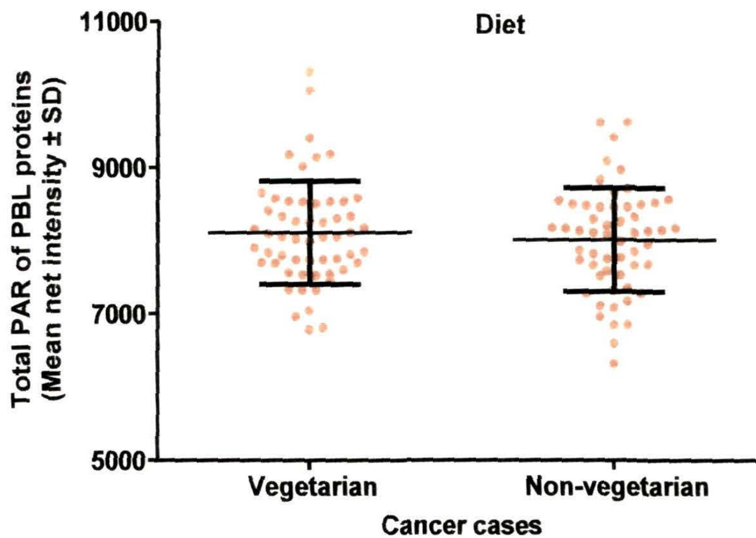


Figure 3.31: A plot showing the effect of diet on PAR level of cancer patients (cases). Number of cases: vegetarian - 56, non-vegetarian - 56. Total PAR level (Mean ± SD): vegetarian – 8110 ± 707, non-vegetarian – 8010 ± 709.

Statistical analysis of inter-individual factors such as age, gender, tobacco consumption, alcohol consumption and betel nut mastication for the cases group, i.e. cancer patients, is summarized in Table XXII.

Table XXII: Table shows the effect of various factors on total PAR level of among cancer patients. A Spearman rank correlation test showed a mildly significant correlation between age and total PAR level of cases. A Mann-Whitney rank sum test was performed to analyze the effect of other factors on total PAR level. A significant reduction of total PAR level was observed in tobacco consumers while alcohol consumption seemed to have lowered the total PAR level very significantly.

Cases	Defined groups	n	PAR level (Mean ± SD	P
Age	All	112	8060 ± 707	0.0116*
Gender	Male	49	8161 ± 643.6	0.0673
	Female	63	7982 ± 748.2	
Tobacco	Yes	48	7840 ± 543	0.0033**
	No	64	8232 ± 771	
Alcohol	Yes	34	7699 ± 518.1	0.0001***
	No	78	8223 ± 718.9	
Betel nut	Yes	68	7963 ± 628.3	0.0935
	No	44	8220 ± 791.9	
Diet	Yes	56	8110 ± 707.8	0.6902
	No	56	8010 ± 709.2	

Similarly, other etiological factors like, history of cancer in the family (HCF), early sexual exposure (ESE) and late first pregnancy (LFP) among the female cancer patients were also analyzed to find out their influence on the level of total PAR of PBL proteins.

Among cancer patients, history of cancer in the family (HCF) did not show statistically significant effect ($P = 0.7111$) on the total PAR of PBL proteins in female cancer patients (Fig 3.32). It is to be noted that we had only 6 female cancer patients in the HCF+ group.

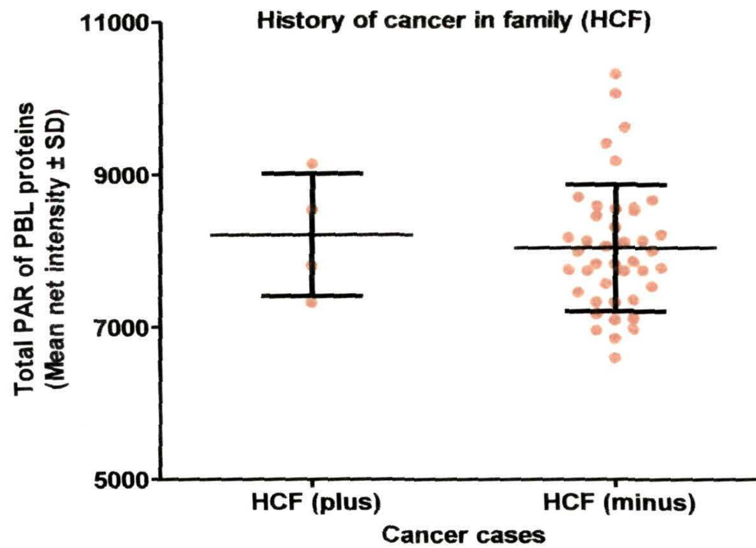


Figure 3.32: A plot showing the effect of history of cancer in family (HCF) on total PAR level among female cancer patients. Number of female subjects: HCF - 06, non-HCF - 57. Total PAR level (Mean ± SD): HCF - 8209 ± 803, non-HCF - 8035 ± 830.

Among the female cancer patients, early sexual exposure (ESE) appeared to exert a statistically very mild (P = 0.0103) effect on total PAR level. Females with early sexual exposure had a marginally lower level of total PAR of PBL proteins (7460 ± 396), when compared to female cancer patients who did not have an early sexual exposure (8175 ± 835), as shown in Fig 3.33

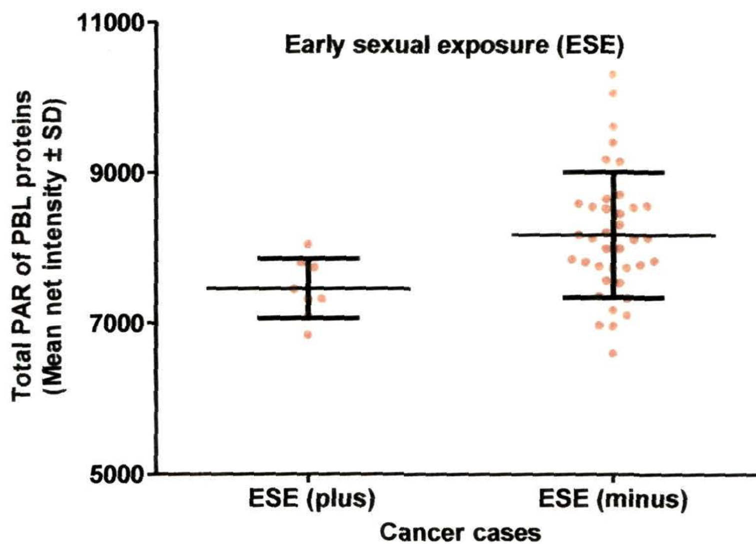
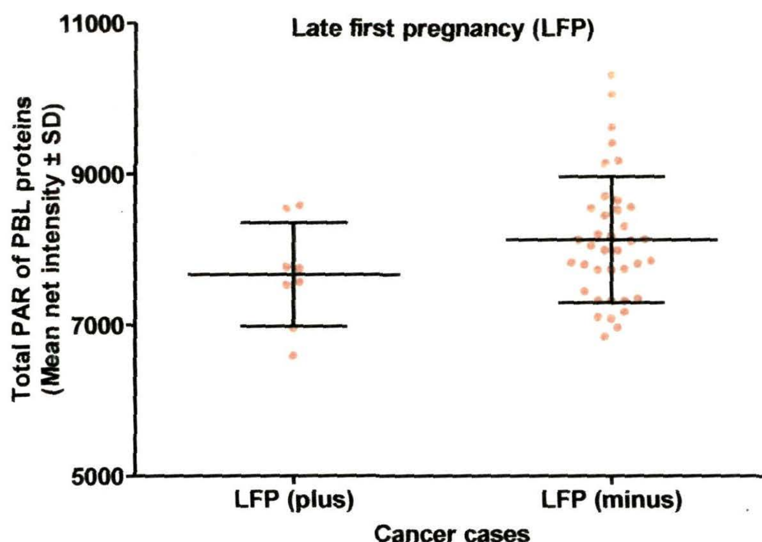


Figure 3.33: A plot showing the effect of early sexual exposure (ESE) on PAR level of female cancer patients. Number of female cases: ESE - 8, non-ESE - 55. Total PAR level (Mean ± SD): ESE – 7460 ± 396, non-ESE – 8175 ± 835.

On the other hand, late first pregnancy (LFP) did not seem to influence the total PAR of PBL proteins among female cases as shown on Fig 3.34. The levels of total PAR among the two groups were statistically not different (P = 0.2785).



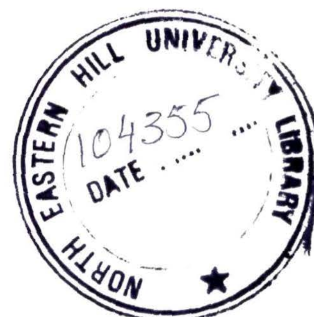


Figure 3.34: A plot showing the effect of late first pregnancy (LFP) on total PAR level of cases. Number of control: LFP - 8, non-LFP - 38. Total PAR level (Mean ± SD): LFP – 7669 ± 686, non-LFP – 8131 ± 832.

The results of influence of these etiological factors that were analyzed in female cancer patients only have been summarized in Table XXIII.

Table XXIII: Mann-Whitney rank sum test of the effect of etiological factors on total PAR level of female cases group. A mildly significant reduction of total PAR level in female cases with early sexual exposure was observed.

Cases	Defined groups	Number of samples	PAR level Mean ± SD	P
Family history of cancer	Yes	06	7983 ± 725	0.8883
	No	57	7982 ± 757	
Early sexual exposure	Yes	08	7469 ± 397	0.0149*
	No	55	8063 ± 756	
Late first pregnancy	Yes	08	7669 ± 686	0.2785
	No	55	8027 ± 751	

3.8. LEVEL OF TOTAL PAR IN PBL PROTEINS IN AGE AND GENDER MATCHED SUBJECTS

To complete the analysis it was necessary to compare total PAR of PBL proteins in age and gender matched subjects. Applying this criterion, we matched subjects among control and cancer groups for age and gender. We obtained 17 male subjects in the control group and 23 male subjects among case group who were aged between 30 and 56 years. Likewise, we had 11 female control subjects and 33 female cases with an age range of 24 – 65 years.

Fig. 3.35 shows a plot of total PAR of PBL proteins for each male subject from controls and cases, who matched for age.

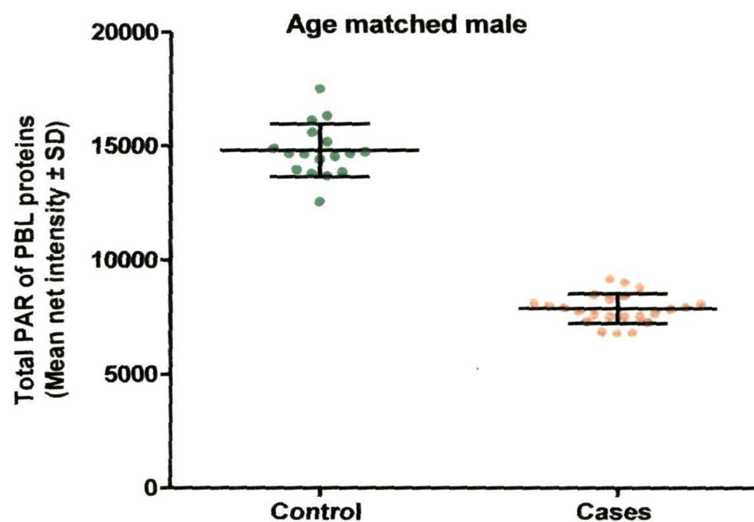


Figure 3.35: A plot showing total PAR level in control and cases matched for age and gender [male] (P <0.0001). Number of male subjects ~ Control: 17, Cases: 23. Total PAR level (Mean ±SD): Control – 14803 ± 1154, Cases – 7874 ± 657.

Fig. 3.36 shows a plot of total PAR of PBL proteins for each female subject from controls and cases, who matched for age.

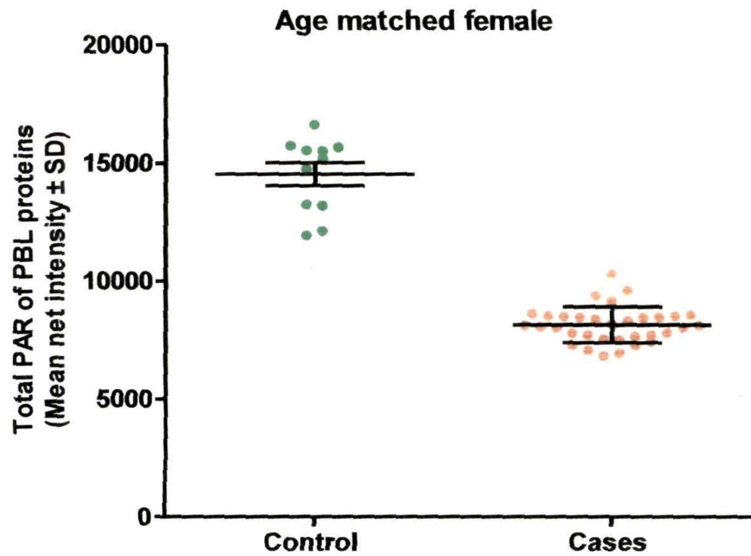


Figure 3.36: A plot showing total PAR level in control and cases matched for age and gender [female] (P <0.0001). Number of female subjects ~ Control: 11, Cases: 33. Age range: 24 – 65 years. Total PAR level (Mean ±SD): Control – 14543 ± 1595, Cases – 8159 ± 760.

Thus, after adjusting for age and gender (male), the level of total PAR of PBL proteins in cancer group (cases) was 7874 ± 657 and in the control group 14803 ± 1154. A Mann-Whitney test compares this difference as statistically highly significant (P <0.0001). In age matched female subjects, the level of total PAR of PBL proteins in cancer group (cases) was 8159 ± 760 and in the control group 14543 ± 1595. A statistically highly significant difference was observed (P <0.0001). The results have been summarized in Table XXIV.

Table XXIV: A Mann-Whitney test comparing total PAR level in age and gender matched control and cases groups. A highly significant reduction of total PAR was observed in cases when compared to control.

Gender	Controls	Cases	P
Male	17	23	
PAR level (Mean ± SD)	14803 ± 1154	7874 ± 657	P <0.0001
Female	11	33	
PAR level (Mean ± SD)	14543 ± 1595	8159 ± 760	P <0.0001

DISCUSSION

Poly-ADP-ribosylation (PAR) of proteins was first discovered in the 1960s and since then we have come a long way in understanding this enigmatic metabolic process and its highly spread out biological involvement. PAR is a unique post translational modification of cellular proteins. It involves a reversible enzymatic reaction catalyzed by two major enzymes, PARP (mainly PARP-1) and PARG. Although 18 isoforms of PARPs have been identified till date, PARP-1 is the main and most understood enzyme of this metabolic pathway (Ame *et al.*, 2004; Rouleau *et al.* 2004; Sharan, 2009). The reaction starts with PARPs transferring ADP-ribose units from the NAD^+ unto the acceptor or target proteins. This adds a highly heterogeneous, linear or branched polymeric adducts of variable lengths, sizes and architecture on the acceptor or target proteins. The other enzyme PARG acts in the opposite direction by catalysing the sequential removal of these polymers from the target proteins. The two enzymes acting in opposite direction, therefore, regulate the level of PAR *in vivo*. Over the years the involvement of PAR with several cellular and molecular processes has been reported. It is now established that PAR has strong influence on the structural organization of chromatin. Since the structural state of the chromatin is intricately associated with the functionality of the chromatin, all chromatin functions can potentially be strongly influenced by the status of PAR of chromosomal proteins. We know that normally the chromatin is structurally organized in positively highly supercoiled state in the nucleus. This positively supercoiled state is maintained by strong electrostatic and other weak interactions of chromosomal proteins, particularly histone core octamer and linker proteins, which are highly basic proteins, and DNA, which is highly acidic, in such a way that very ordered structural levels of organization (nuclesomes, polynucleosomes, primary, secondary and tertiary levels of supercoiling, etc.) are created. Ultimately, approximately 50000 loops/domain of DNA in conjuncture with nuclear matrix proteins make the chromatin. It is obvious that this state of highly organized structure is maintained by sustained histone-DNA interaction. However, in order to perform DNA repair, replication, transcription, gene expression, etc., the most fundamental biological functions of the chromatin, the chromatin needs to fully negatively supercoil, or totally uncoil to the extent that even the double helical structure of DNA collapses to become single stranded structure. Only in this state of DNA structural organization can the fundamental function of the genome listed above can be performed. It is understandable that to convert the highly positively supercoiled chromatin to fully negatively supercoiled or totally uncoiled state, the electrostatic and weak interactions between the

chromosomal (mainly histone) proteins and DNA, should be abolished. Here the critical role of PAR is envisioned. As the presence or absence of the highly variable and heterogeneous PAR adduct on target proteins greatly influence the net charge of the protein, the extent as well as the strength of electrostatic interactions of these proteins with DNA will be determined by the status of PAR of chromosomal (mainly histone) proteins. Therefore, any change in the net charge of the chromosomal and histone proteins due to change in the status of their PAR modification, would influence both structural organization of chromatin as well as its functionality. Only when the chromatin is transiently negatively supercoiled can the enzymes, proteins, cofactors, and other associated factors access strands of DNA to perfom DNA repair, replication or transcription. Unlike all other known post-translational modifications of chromosomal proteins, PAR modification is the most suitable avenue for efficiently, quickly and reversibly winding-unwinding the DNA superstructure because of two reasons. The first is its capacity to drastically alter the charge state of chromosomal proteins due to the polymeric state of this modification – unique to PAR modification. Secondly, the changing (growing or shrinking) bulk of the PAR adduct would act as a physical force to break or remake electrostatic interaction between the chromosomal proteins and the DNA.

Therefore, all metabolic and physiological processes, which involve alteration in chromatin organization and functionality, shall be influenced by the PAR of cellular proteins. Carcinogenesis is a complex and multistep process, which incorporates multiple genetic alterations. Various kinds of mutations, chromosomal aberrations, gene rearrangements, gene silencing and neogene expression have been observed in different cancers. All these processes are, in many ways, dependent on chromatin organizational and functional state. Therefore, the involvement of PAR of chromosomal proteins in carcinogenesis is obvious. Besides, the role of PARP-1 in DNA strand breaks (Burkle *et al.*, 2000; Rolli *et al.*, 2000) and base excision repair (Dantzer *et al.*, 1999) have also been reported. In addition, the role of PARP-1 in the induction of necrotic cell death through cellular NAD depletion after excessive DNA damage has been described (Affar *et al.*, 2000). PARP-1 is also involved in transcriptional control (Ha *et al.*, 2002) and cellular differentiation (Masutani *et al.*, 2001). PARP-1 deficiency has been shown to be a risk factor for carcinogenesis in mice after treatment with alkylating agents (Tsutsumi *et al.*, 2001) or in the combination of a

deficiency of DNA-PK (Morrison *et al.*, 1997) or p53 (Tong *et al.*, 2001) PAR also plays a major role in maintaining genomic stability of cells exposed to genotoxic stress (Herceg and Wang, 2001). In general, it appears scientifically reasonable to say that PAR of cellular proteins are involved with global cellular metabolisms, including genome maintenance, cell cycle control, proliferation, differentiation, and cell death. Since PAR has been implicated in these processes, its role in neoplastic transformation cannot be ruled out.

Different approaches have been adopted in the attempt to understand the role of PAR of cellular proteins in carcinogenesis. Several studies concentrated on the biosynthetic enzyme of PAR metabolism, that is, PARP. In this either the level of gene expression of PARP was monitored or the enzyme activity was measured. The earliest observation of the involvement of PAR in carcinogenesis was the up regulation of PARP in malignant or transformed cell *in vitro* when compared to control (Miwa *et al.*, 1977), and in leukemia and ovarian cancers (Singh, 1991). In some cancers an increased expression of the gene has been reported (Masutani *et al.,* 2005) while others have reported a decreased expression (Bieche *et al.,* 1996). These studies suggested that PARP gene expression was not consistent and, therefore, could not be used to interpret carcinogenesis. In the other approach involving monitoring of PARP activity, for example, low PARP level in PBL was reported in laryngeal cancer patients (Rajaee-Behbahani *et al.,* 2002). In both these approaches, there seems to be a serious flaw. Up- or down-regulation of neither PARP gene nor its activity could alone shed much light on the status of actual PAR of cellular or chromosomal proteins. That is because, the actual extent of PAR of cellular proteins *in vivo* is the net outcome of biosynthetic activity of PARP and bioderivative activity of PARG, simultaneously acting in opposite directions (Sharan *et al.*, 1996; Sharan, 2009). Therefore, the enzymatic activity of PARP, which may be enhanced or reduced during carcinogenesis, cannot be inferred as the true representative of the metabolic level of PAR of cellular proteins. Only simultaneous measure of the expressions or activities of PARP as well as PARG will be able to reliably tell us about the extent of PAR of cellular proteins, which actually influences the structural and functional status of chromatin. However, it has to be kept in mind that even then, measures of PARP and PARG genes or their products would only give an indirect measure of the level of PAR adduct on the target proteins.

Keeping this in mind, we took a direct approach in this study. We envisaged to measure the PAR adducts directly and quantitatively. Obviously, our assay had to ensure that we measured true metabolic level of PAR adducts on cellular proteins. A variety of methods have been developed and employed for the quantification of ADP-ribose polymers on cellular proteins *in vivo*. One of the most widely used methods is the radioisotopic assay, which utilized radiolabeled NAD^+. This method, however, had its own share of problems, the major being that it measured only part of the ADP-ribose polymer adduct(s) that has been added since start of the assay (with radiolabelled ADP-ribose(s)) and did not measure the already existing ADP-ribose adduct(s), which was without the radiolabel. Therefore, the quantification of PAR by radioisotopic method did not revealed the actual metabolic level of the polymer (Schneeweiss *et al.* 1995, Sharan, 2009). Therefore, to measure the true PAR level *in vivo* a highly specific and sensitive method was designed, developed and standardised in the laboratory. This assay utilized the highly immunogenic property of the ADP-ribose polymer. This novel ELISA based slot- and Western blot immunoprobe assay has been deployed in this study to quantitatively measure the true metabolic level of poly-ADP-ribosylation of cellular proteins in order to correctly determine the correlation between PAR and carcinogenesis. The method has been described in details elsewhere (Sharan *et al.*, 1998; Sharan *et al.*, 2005). Briefly, the ELISA based slot- and Western blot immunoprobe assay employed polyclonal antibody (PAb) raised specifically against natural, heterogeneous ADP-ribose polymers of mouse spleen cells. The PAb was used to immunoprobe the ADP-ribose polymer adducts attached to cellular proteins on the nitrocellulose membrane. Using an alkaline phosphatase conjugated second Ab and BCIP/NBT based color developer, the poly-ADP-ribosylated proteins were quantified. This method provides us with the measure of true metabolic level of ADP-ribose polymers, since it detected entire heterogeneous poly-ADP-ribose polymer populations of all sizes and architecture (Sharan *et al.*, 1998; Sharan *et al.*, 2005).

Previous work done in our laboratory employing this novel method has shown a strong negative correlation between PAR of cellular proteins and initiation, promotion and progression of cancer in mouse model in both target organ as well as other organs (Devi, 2001; Kma, 2003; Kma and Sharan 2003; Devi *et al.*, 2005; Devi and Sharan, 2005; Sharan *et al.*, 2005; Kma and Sharan, 2006; Kma and Sharan, 2008). PAR of total cellular proteins as well as histone proteins of liver and spleen cells were

significantly lowered during initiation of carcinogenesis in mice exposed to dimethlynitrosamine (DMN) (Devi *et al.,* 2005; Kma and Sharan, 2006; Kma and Sharan, 2008). Aqueous extract of betel nut was also found to lower PAR of different proteins in various tissues of mice (Saikia *et al.,* 1998; Pariat and Sharan, 2002). In Dalton's lymphoma ascites tumorigenesis, PAR of histone proteins was progressively lowered during the later stage of cancer development (Devi and Sharan, 2006). Based on these observations we had suggested that lowering of PAR is a hallmark of carcinogenesis as well as tumorigenesis (Kma and Sharan 2003; Devi *et al.*, 2005; Kma and Sharan, 2005; Sharan *et al.*, 2005; Kma and Sharan, 2006; Devi and Sharan, 2006; Kma and Sharan, 2008 and Sharan, 2009). Since both carcinogen target tissues as well as non-target tissues showed the negative correlation, albeit at different levels, it was logical to hypothesize that measurement of the level of PAR of blood lymphocyte proteins in mice would also mirror the status of measured PAR of cellular proteins in target tissue. Furthermore, blood, being a circulating tissue, would be sentinel of the whole body as has also been contented by Satyanarayana (2006). If this hypothesis were true, blood would become an ideal target tissue for early cancer detection program because it is quite easy to access blood for any assay. Therefore, this hypothesis was tested in mouse model rigorously. Indeed, the hypothesis was found to be correct. Blood lymphocyte proteins of mouse exposed to DMN also mirrored statistically significant lowering of total PAR of lymphocyte proteins mirroring the trend that was noted in target and non-target tissues (Kma, 2003, Kma and Sharan, 2003, Kma and Sharan, 2006).

Having proved the hypothesis in a mouse model, it was important to test it in human cancers as well. Therefore, using the novel immunoprobe assay we set out to investigate the correlation between the total cellular PAR of PBL proteins in human cancer. PBL was chosen since a blood based assay of PAR level would be easy to perform. Moreover, drawing of blood for such an assay was also most non-invasive in nature. The need of tissue samples or biopsies poses a problem as it requires surgical intervention. We focused this study on three classes of human cancers i.e. cancers of (a) head and neck (H&N) region, (b) breast and (c) cervix. These three types of cancers were chosen due to their high rates of prevalence in the north-eastern part of India. The hospital registry of the Dr. B. Barooah Cancer Institute, Guwahati, the collaborating institute, shows that while the largest number of male patients came to the institute with

one or the other cancer of H&N region, female patients mostly came with cancers of cervix and breast. The H&N cancers differ in their sites of origin. We attempted to cover all possible types of cancers, totalling to fourteen different types of cancers in this class (Table - VI). The number of individuals covered in the study widely varied in cancers of different sites. Therefore, a total of sixteen different types of cancers have been studied in this investigation in a total of 112 patients. The control subjects consisted of 68 volunteers with no known history of cancer. Attempt was made to spread out the controls covering, as best as possible, both genders, all possible age groups and relations of cancer patients. To assess the level of total PAR of PBL proteins among the control as well as experimental (cancer patients or cases) subjects, blood samples were collected and slot blot immunoprobe assay performed as described (§ 2.15). The quantification of PAR of PBL proteins was done by densitometric analysis (§ 2.18). The concentration corrected mean net intensity of each control sample was plotted (Figs. 3.1 & 3.2). Even though a large inter-individual variability could be observed in PAR level of PBL among the control subjects, it was obvious that they statistically belonged to one group (Fig. 3.2), which is distinctly different from the other cancer groups (cases).

In the H&N cancer group we covered 65 patients with cancers of 14 different types (Table VIII). When total PAR of PBL proteins of different sites of cancer of H&N region were compared to control, an average 55 % reduction in PAR level was seen (range 7681 - 8558) in the cancers of the H&N (Fig. 3.3). Among the 22 patients with cancer of the breast examined in this study, an average reduction of 53 % of total PAR level was found when compared to female controls, while a reduction of 54 % of total PAR level was observed when compared to all the controls, that is males + females (Fig. 3.4). Similarly, there was a highly significant reduction of total PAR of PBL proteins among all 24 patients with cancer of the cervix. An average 55 % and 57 % reduction of total PAR of PBL proteins was observed among the cervical cancer patients when compared to female controls and all the controls, respectively (Fig. 3.5). The observed reductions of total PAR of PBL proteins in all types of cancers studied in this investigation were statistically significant at different levels of confidence as shown in Figs. 3.3, 3.4 and 3.5 and Tables X, XI and XII. Appropriate tests of confidence have been applied to different results in line with current medical practices.

These results are in line with previous studies done on mice, which showed a statistically significant reduction of PAR level during carcinogenesis (Pariat, 1997; Sharan *et al.*, 1998; Devi, 2001; Kma, 2003; Kma and Sharan, 2003; Devi *et al.,* 2005; Devi and Sharan, 2005; Sharan *et al.*, 2005; Kma and Sharan, 2006; Kma and Sharan, 2008). It has to be noted that we have directly measured the PAR of PBL protein in 16 different cancers of humans using the novel immunoprobe assay in this study. We find statistically highly significant downregulation of the level of PAR of the PBL proteins in all types of cancers covered in this study. This information is very important in our quest to establish PAR of PBL proteins as a reliable biomarker of human cancers. As has been hypothesized earlier based on mouse model research (Sharan et al., 1998; Devi, 2001; Kma, 2003; Kma and Sharan, 2003; Devi *et al.,* 2005; Devi and Sharan, 2005; Sharan *et al.*, 2005; Kma and Sharan, 2006 and Kma and Sharan, 2008), PAR of PBL proteins could potentially become a convenient blood based cancer screening tool. The results of this investigation is an important step in that direction wherein we find that PAR of PBL proteins is strongly downregulated in all 16 types of cancers of humans covered in this study.

In this study, the subjects under investigation belonged to different backgrounds exposed to a variety of etiological, nutritional and other factors that are known to strongly influence course and extent of human cancers. These factors might also influence level of PAR that was measured as PAR of cellular proteins have been shown to be influenced by a variety of intra- and extracellular factors (Schneeweiss *et al.*, 1995; Sharan *et al*, 2005; Sharan 2009). Therefore, in our considered opinion, it was necessary to also analyze the influences of these factors on (I) incidence of cancers and (II) total PAR of PBL proteins in control as well as experimental (cancer) subjects.

(I) Etiological factors and incidence of cancer:

As the study was performed on the basis of consent from both the cancer patients and control subjects, factors such as number, age and gender were not well matched among the controls and cases. The age of the control and case subjects varied between 25 and 80 years (Fig. 3.6). There was a significant difference in the mean age of the two groups, with the cases (mean age 47.7 years) being slightly older than the controls (mean age 39.9 years) (Fig. 3.6 and Table XIII). The subjects were then grouped according to their gender and their mean age was analyzed. The male cancer patients

(mean age 51.33 years) were much more (highly significant) older than male controls (mean age 40.39 years) with the number of male subjects in both the groups being approximately the same (Fig. 3.7 and Table XIII). Among the female subjects, the mean ages (control mean age 42.77 years; cases mean age 45.16 years) were similar although the number of subjects was more in the cancer group (n = 63 subjects) as compared to the control group (n = 27 subjects) (Fig. 3.8 and Table XIII). As incidence of cancer is strongly influenced by age of the subject, we divided our subjects into four groups according to their age (Table XIV and Fig. 3.9). The groups were analyzed in terms of the number of subjects in each group. With the exception of Group I (25 – 34 years), the numbers of subjects were more in the case group than the control group (Fig. 3.9). The frequency of occurrence of cancer expressed as percentage among the case subjects was also analyzed. Group IV (55 + years) had the highest percentage in terms of occurrence of cancer (Table XIV). As cancer is considered a disease of aging, the frequency of incidence of cancer is likely to higher among older subjects. Similarly, we looked at our data to analyse if gender of subjects influenced the cancer incidence. When the distribution of gender among the subjects of controls and cases was examined, a significant difference was observed (Fig. 3.10). Our investigation had more females than males among cancer patients (cases), while males dominated the control group (Fig. 3.10). The difference in the distribution of gender and the cancer incidence can be accounted by the fact that two out of three classes of cancer (cancer of breast and cervix) covered in this study are exclusive to females.

We chose to examine some other etiological factors known to be associated with cancer to assess their influence on incidence of cancer. Tobacco consumption, an important etiological factor believed to be strongly associated with cancer (IARC, 1987; Sharan, 1996 and Choudhury and Sharan, 2009), was analyzed for its influence on the incidence of cancer. A statistically significant difference was observed in the distribution of tobacco consumption pattern among the subjects in control and case groups (Fig. 3.11). The incidence of cancer in terms of absolute number was more among the non-tobacco consumers when compared to the tobacco consumers. However the relative occurrence of cancer in tobacco consuming subjects was several fold higher (Fig. 3.11). Alcohol consumption is another factor that is strongly associated with human cancer. In our study, the control and case subjects exhibited different patterns of alcohol consumption (Fig. 3.12). In our study group, most of the control subjects as

well as cancer patients (about 70 %) did not report the habit of alcohol consumption. As the observed cancer frequency was about similar in both alcohol consuming and non-consuming groups, it appears that alcohol did not show any influence on the occurrence of cancer. A third, very relevant factor was betel nut chewing habit, which is very prevalent in the north eastern part of the country and has been shown to be strongly associated with human cancer (reviewed in IARC, 1987; Sharan, 1996 and Choudhury and Sharan, 2009). Our analysis shows that betel nut chewing was highly prevalent among the subjects of our study in both control and cancer groups. Though betel nut consumers exhibited higher percent of cancer incidence, the relative frequency of cancer incidence in betel consuming and non-consuming population was statistically the same (Fig. 3.13 and Table XVI). Diet is another factor that is known to influence incidence of cancer. We analysed the influence of this factor in our study. The diet of the subjects, which was broadly classified as vegetarian and non-vegetarian, was found to be equally distributed in controls and cases (Fig. 3.14) and it did not show any influence on the incidence of cancer (Table XVI).

Cervical and breast cancer among females are known to be influenced by some additional factors. Prominent among them are the duration of estrogen exposure, early menarche, late menopause, age at first childbirth, sexual intercourse at an early age, multiple sexual partners, multiparity and long-term oral contraceptive use. It has to be kept in mind that due to sensitive nature of these factors, many of our respondents preferred not to respond to these questions. Therefore, we have relatively small number of response in this category. Due to such limitations, in this study we have analyzed influences of three such factors, namely (a) history of cancer in family (HCF), (b) early sexual exposure (ESE) and (c) late first pregnancy (LFP), on the rate of incidence of cervical and breast cancers among female subjects. Based on the limited data generated in this investigation (see Table XVI), the history of cancer in the family (HCF) among the female subjects was apparently equally present among controls and cases (Fig. 3.15). Therefore, in terms of cancer occurrence, HCF among the female cases did not exert significant influence on occurrence of cancer. Based on the limited data (Fig. 3.16), ESE as well as LFP also did not have any influence on the cancer incidence among the female cases.

(II) Etiological factors and total PAR of PBL proteins

The accumulation of macromolecular damage, especially damage to genomic DNA, underlies the aging process. Given their roles in cellular responses to various types of genomic insults, PARP-1 and other PARPs have been implicated in aging (Beneke *et al.*, 2000). PARP activity has been shown to be affected by factors such as age, smoking and inflammation (Roush *et al.*, 1990). In our investigation we observed that age of control subjects had no strong effect on the total PAR of PBL proteins (Fig. 3.18). The value of PAR was reasonably uniformly spread out. This is in line with two reports the first on an association between high poly-ADP-ribosylation capacity and longevity (Muiras *et al.*, 1998) and the other poly-ADP-ribosylation in lymphocytes of cancer patient (Rajaee-Behbahani *et al.*, 2002) have shown no effect of age on PARP activity. However, among cancer patients a weak correlation was observed between the total PAR of PBL proteins and age (Fig. 3.26). In the control group total PAR level of controls was influenced by gender, with the male subjects exhibiting a slightly higher PAR level than their female counterparts (Fig. 3.19). The influence of gender on the total PAR of PBL proteins in cancer patients was insignificant (Fig. 3.27).

Among the etiological factors, tobacco consumption did not have any effect on total PAR of PBL proteins. In the control group the total PAR of PBL proteins was almost similar between the tobacco consumers and non-consumers (Fig. 3.20). However, tobacco consumption had an opposite effect on the level of total PAR among the cancer patients with the non-consumers of tobacco showing a higher PAR of PBL proteins than the consumers (Fig. 3.28). In a previous study alcohol was shown to affect PARP activity in rats (Nomura *et al.*, 2001). The effect of alcohol on PAR of PBL proteins of controls and cases was therefore, also analyzed. Alcohol did not have a pronounced effect on the total PAR of PBL proteins among control subjects (Fig. 3.21) while among the cases a statistically significant effect was observed (Fig. 3.29). Total PAR of PBL proteins among the cancer patients was higher in the alcohol non-consumers than the alcohol consumers. Finally, betel nut chewing a common habit in this particular region and a known etiological factor was analyzed for its effect on total PAR of PBL proteins. In the controls betel nut chewing did not show any effect on level of total PAR of PBL proteins (Fig. 3.22). A similar trend was also observed in the cancer patients covered in this study (Fig. 3.29). The north eastern region is known for above

average consumption of red meat and processed food. It is known that these factors may be associated with some cancers. In relation to these facts, we also examined the effect of diet on total PAR of PBL proteins. Our results reveal that diet did not have any influence on the total PAR of PBL proteins in controls (Fig. 3.23) as well as in cancer patients (Fig. 3.30).

In females a number of inherited traits are related to causation of cancer. For example, a woman carrying a mutated brca1 gene has about a 70 % - 80 % lifetime risk for developing breast cancer (Easton *et al.*, 1995). Therefore, history of cancer in the family (HCF) might play a role in carcinogenesis. In controls and cases, the effect of HCF on total PAR of PBL proteins was also analyzed. Among the controls total PAR of PBL proteins was affected. Control subjects with a history of cancer in the family had lower PAR of PBL proteins than the subjects with no such history (Fig. 3.24). However, among the cancer patients, the HCF did not exhibit any influence on total PAR of PBL proteins (Fig. 3.32). Likewise and as stated earlier that due to lack of data among the female subjects only two additional etiological factors were analyzed for their influence on total PAR level. The analysis of the effect of early sexual exposure (ESE) on total PAR of PBL proteins of female controls was not possible since none of them had ESE. In the case group, however, a significant effect was observed as females with ESE had lower PAR of PBL proteins when compared to the females who did not have ESE (Fig. 3.33).On the other, late first pregnancy (LFP) did not exhibit any influence on total PAR of PBL proteins of either control (Fig. 3.25) or cancer patients (Fig. 3.34).

To sum these observations up we find that the various etiological factors did not seem to have a great deal of influence on total PAR of PBL proteins in the control group with the exception of gender and history of cancer in the family (HCF). However, among the cancer patients, total PAR of PBL proteins was highly influenced by age, tobacco, alcohol and early sexual exposure (ESE). The etiological factors examined in this study seem to exert their effects more on the cancer patients than the controls. One of the main reasons for this difference may be altered state of the cellular and molecular processes in a cancerous cell.

Finally, to systematically complete our analysis we compared the total PAR of PBL proteins in age and gender matched subjects from both the groups i.e. controls and cases. With this criterion we selected age-matched males from both controls and cases. The same was done for female subjects. When age matched males from controls and cases were compared for total PAR of PBL proteins, a statistically significant lower level of PAR (53 %) was observed in the male cases (Fig. 3.35 and Table XXIV). In the female subjects matched for age, a similar trend was observed, with female cases exhibiting a statistically significant lower level of PAR of PBL proteins (56 %) (Fig. 3.36 and Table XXIV).

In conclusion, our findings reveal a statistically significant negative correlation between PAR of PBL proteins in advance human cancers of head and neck region, breast and cervix in comparison to the control group using the novel immunoprobe assay of PAR. Thus, suggesting that the assay for PAR of PBL proteins may be a useful biomarker of cancer. This assay has a lot of potential to become a tool for cancer screening as it a simple, cost effective, non invasive and specific. However, a study in patients with early stages of cancer would be a useful to consolidate this fact. The study on the influences of etiological factors on PAR in control and cases groups should be confirmed using a larger study. Such a study would provide a stronger foundation to authenticate the method for an early detection of cancer in humans.

CONCLUSION

From this study the following conclusions can thus be drawn:

- The total PAR of PBL proteins of head & neck cancer patients showed statistically significant ($P < 0.0001$) reduction in PAR when compared to controls.

- Similarly, there were significant reduction ($P < 0.0001$) in the total PAR of PBL proteins in breast and cervical cancer patients in comparison to combined controls (males + female control values) or female controls only.

- In controls, total PAR of PBL proteins was not affected by age but gender had a mildly significant effect.

- Similarly, other etiological factors such as, betel nut chewing, tobacco consumption, alcohol consumption and diet did not show much influence on total PAR of PBL proteins of controls.

- In contrast, history of cancer in the family showed a significant effect on PAR level of female controls.

- In cancer patients, age showed a significant effect on total PAR of PBL proteins.

- However, gender, betel nut chewing and diet did not exhibit any influence on total PAR of PBL proteins among cancer patients.

- Other etiological factors, such as tobacco consumption ($P < 0.0033$), alcohol consumption ($P < 0.0001$) and early sexual exposure among the female cancer patients ($P = 0.0103$) significantly lowered total PAR of PBL proteins in cancer patients.

- Overall, a statistically highly significant lowering ($P < 0.0001$) in total PAR of PBL proteins was recorded in age and gender adjusted cancer patients in comparison to the controls.

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ANNEXURE I

Research project: **POLY-ADP-RIBOSYLATION (PAR) OF BLOOD LYMPHOCYTE PROTEINS – A BIOMARKER FOR CANCER DETECTION AND SCREENING.**

Implementing organizations:

(1) **Dr. B. Borooah Cancer Institute, Regional Institute for Treatment and Research, Guwahati – 781016, Assam**

(2) **Radiation and Molecular Biology Unit, Department of Biochemistry, North Eastern Hill University (NEHU), Shillong – 793022, Meghalaya.**

Consent Form

I.. am exercising my free power of choice here by giving my consent to be included as a subject in the project titled " **Poly- ADP-ribosylation (PAR) of Blood Lymphocyte Proteins – a biomarker for cancer detection and screening**"taken up jointly by Dr. B. Borooah Cancer Institute (BBCI), Guwahati and Radiation and Molecular Biology Unit, Department of Biochemistry, North Eastern Hill University (NEHU), Shillong. I understand that I will undergo relevant clinical examination and laboratory investigations. I may need to give required samples (blood, tissue etc.) for testing. I have been informed to my satisfaction by the attending laboratory investigator about the purpose, follow up and nature of the study including laboratory investigations. I also came to know that I might be called or examine by the investigators when necessary during the study period. I am also aware of my right to opt out of the study at any time during the course of study without having to give any reason for doing so.

This consent form has been readout to give me in my own language and I clearly understand the consent.

Signature of the investigator | **Signature of the subject / Guardian**

Date: | Date:

ANNEXURES II

Questionnaire for Epidemiological Study of Cancer

[Prepared and developed for collaborative research by Radiation & Molecular Biology Unit, Department of Biochemistry, NEHU, Shillong (R. N. Sharan *et al.*) in consultation with Dr. B. Barooah Cancer Institute, Guwahati (A. C. Kataki *et al.*)]

1. Background information

1. 1. Personal data

1.1.1. Name (optional) :
1.1.2. Contact Address with telephone no. and email: ....
....
....

1.1.3. Age : .... years   Sex : ....
1.1.4. Date of birth: ....   Place of birth : ....
1.1.5. Height : .... feet   Weight : ....kg

1.2 Demographic information

1.2.1. Marital status : ☐ Unmarried ☐ Married ☐ Divorced ☐ Widow/Widower
1.2.2. Current income of the family per month :
☐ less than Rs.500 ☐ Rs.501-Rs.2500 ☐ Rs.2501+ ☐ Not known
1.2.3. Education: ☐ Literate ☐ Illiterate. If literate, level of literacy/highest degree:
☐ Middle School ☐ High School ☐ Graduate ☐ Post-graduate
1.2.4. Highest level of education of: Father ....; Mother.....................
1.2.5. Religion (if parents are of different religion please mention both / if your religion is different than your parents, please mention all three)
Self: .... Father: .... Mother: ....

1.3.Ethnic Background

1.3.1. Which state do you originally belong to? ....
1.3.2.. What is your current state of residence? ....
1.3.3. If you belong a tribal state, please specify your tribe or clan ....
1.3.4. If you belong to a non-tribal state, please specify your caste ....
1.3.5. If father and mother are of different ethnic origins, please mention their ethnic origin too: Father: .... Mother: ....

2. Family History of Cancer

2.1. Do you or any members of your family have a history of cancer?

	Yes/ No	Type of cancer (if known)	Age at Diagnosis (if known)	Living / Deceased	Treatment given
Yourself					
Your mother					
Your father					
Your sisters					
Your brothers					
Your children					
Your half sisters and brothers					
Your mother's sisters and brothers					
Your father's sisters and brothers					
Your nieces and nephews					
Your mother's parents					
Your father's parents					

For female subjects only:

2.2.Reproductive history :

2.2.1. Age of first sexual exposure: ....years

2.2.2. Age at menarche (what age did your periods begin): .... years

2.2.3. Have you ever used birth control pills? ☐ yes ☐ no

2.2.4. If yes, how many years have you used them? ....years

2.2.5. Type of pill used .... Age at menapouse ....years

2.2.6. Have you used any other form of contraceptive? ☐ yes ☐ no

If yes, which form? ....

2.3.Pregnancy history:

2.3.1. Have you ever been pregnant - ☐ yes ☐ no . If yes how many times ?..........
2.3.2. Age of first pregnancy.........
2.3.3. Did you breast feed you children? ☐ yes ☐ no
2.3.4. If yes, whether it was exclusive breast feeding? ☐ yes ☐ no
Check whichever applicable

Pregnancy	Age	Live birth	Miscarriage	Ectopic	Abortion	Still birth	Other
Pregnancy 1							
Pregnancy 2							
Pregnancy 3							
Pregnancy 4							
Pregnancy 5							

3. Social / Lifestyle Habits & Addictions

History of Tobacco Use

3.1. **Smoking**

3.1.1. Do you or have you ever smoked cigarettes/bidis/hukka? ☐ yes ☐ no
3.1.2. Age when started smoking...........
3.1.3. Are you a : ☐ former smoker . How many years did you smoke?............years
: ☐ current smoker. How many years have you smoked?.......... years
3.1.4. Number of cigarettes smoked per day: ☐ less than 5 ☐ 5-10 ☐ 10-15 ☐ 15 +

3.2. **Chewing Tobacco** (*Khaini* with lime)

3.2.1. Have you ever chewed tobacco ? ☐ yes ☐ no Age when started ....years
3.2.2. Have you been chewing tobacco on a regular basis i.e. 5-7 times/day ☐ yes ☐ no
3.2.3. How much tobacco you chew in a day?☐less than 1 pack ☐ 2-5 packs ☐ 6 + packs
3.2.4. Did you ever stop chewing tobacco, if yes, when and for how long?
3.2.5. How many years altogether you have chewed tobacco?
☐1- 5 years ☐ 6-10 years ☐ 10 + years

3.3. History of Mastication and other Chewing Habits

Question	Pan Masala	Zarda	Betel Nuts - dried (*Supari*)	Betel Nut – Wet (*Kwai*)	Betel leaf (all types)
You have/had a regular habit of this/these (tick whichever applicable)					
As a regular habit, amount you consume everyday is approx. (use most comprehensible unit)					
Age when you started chewing/eating it regularly					
If you have ever left this habit, tell us when and for how long if you resumed later on (e.g. Left : MMYY to MMYY & For: 3 years)					

3.4. History of alcohol and other alcoholic beverages

3.4.1. Mark the type of user - ☐ Non-user/never used ☐ past user ☐ Current user

3.4.2. Whether : ☐ Indian made foreign liquor (IMFL)

☐ Country liqour

☐ Very occasional : once every three months

☐ Occasional : once or twice in a week or fortnight

☐ Moderate : one peg daily

☐ Heavy : two pegs or more daily (1 peg = 30 ml)

3.4.3. Have you ever been hospitalized due your drinking problems – ☐Yes ☐ No

3.4.4. If yes, how many times? …….. What was the diagnosis/prognosis? …….

3.4.History of substance drug abuse/recreational drug abuse

Substance Drugs	Method of Intake (Eat/drink/inhale/ snort/inject/smoke)	Frequency of Intake per week	
		Past habit	Present habit
Marijuana (*Ganja*)			
Cocaine			
Heroin			
Brown Sugar			
Opium			
Hashish			
LSD			
Amphetamines			
Any other substance			

5.1.Have you ever been hospitalized due to your habit of drug-addiction – ☐Yes ☐ No
5.2.If yes, how many times have you been admitted to hospital and for how long?

4. Nutritional History

4.1 Dietary History

4.1.1. Has your dietary pattern changed significantly over the last three years ☐Yes ☐No
4.1.2. If yes, in what ways did it change?
4.1.3. What reasons led you to change your dietary pattern?................................
....
4.1.4. How many <u>meals</u> do you normally take in a day – Present/ Past? (tick whichever applicable)

Meal	Present	Past
Breakfast		
Morning meal		
Noon Meal/Lunch		
Afternoon meal		
Dinner		

4.1.5.Whether : ☐ vegetarian
: ☐ non vegetarian . If so, most frequent form of meat taken
☐ freshly cooked ☐ smoked ☐ dried ☐ fermented
4.1.6. Whether spices are : ☐ not used ☐ occasionally used ☐ regularly used

5. Medical History

5.1 Use of Prescription & Non-prescription medicines

5.1.1.Did you ever take following drugs, with or without doctor's prescription regularly i.e. once per week at least for one year?

Type of drug	Age when started	Age when stopped (mark if never stopped)	Age when resumed, if stopped earlier	Average doses e.g. no. of pills per day/week
Sleeping pills (e.g. Alprazolam, Diazepam etc.)				
Cholesterol Lowering agent				
Laxatives e.g. Isabgol				
Anti - hypertensives				
Anti-diabetics				
Anti-ulcer				
Others, please specify				

5.2. History of Surgery, Conditions, and Diseases

Surgery

5.2.1. Did you ever have any surgery involving removal, either partial or total, of one or both of your ovaries, uterus (womb), or tubes (Please include any surgery to remove cysts from ovaries, uterus, or tubes) – □Yes □ No ?

5.2.2. If yes, give details: ....

5.2.3. Details of surgeries :

No. of surgery	Age at surgery	Nature of surgery (What exactly was done in the surgery)	Reason for surgery
1st surgery			
2nd surgery			
3rd surgery			

5.2.4. Did a doctor or a trained professional ever examine your breasts – □Yes □ No

5.2.5. If yes, how many times was it examined and what were the results/diagnosis?

....

....

5.2.6. Did you undergo any surgery involving breasts – □Yes □ No

5.2.7. If yes, then give the nature and the reason for surgery. ....

....

....

5.2.8. Any other surgery e.g. gall stones, appendix, colon, thyroid etc.?

....

....

....

....

6. Radiation exposure history

6.1. Personal History

6.1.1 Please indicate how frequently you have undergone any of the following diagnostic procedures during your lifetime:

Procedure performed on you	Never been performed	How many times		
		Before the age of 20	Between the age of 20-40	After the age of 40>
Chest X-ray				
Dental X-ray				
Skull X-ray				
Cervical X-ray				
Vertebral X-ray				
Any other				

6.2 Professional exposures (X-ray & other radiation exposure)

For X-ray technicians

6.2.1 Have many years have you been trained as a medical radiation technician?.............years

6.2.2 How many years have you worked as a technician in medical radiation procedures? ....years

6.2.3 Did you ever stop being a technician – ☐Yes ☐ No If yes, for how many years did you stop and when did you resume again/never resumed? ....

6.2.4 Has your WBC count ever been depressed below normal as a result of working in the field of medical radiation – ☐Yes ☐ No

6.2.5 If yes, how many times has that happened? ....

7. Occupational History

We are interested in knowing about every job – full-time, part-time, paid, unpaid, voluntary – that you may have done for six months.

7.1 Have you ever worked?

☐ Yes ; ☐ housewife ☐ never worked

7.2 If yes, at what age did you start working, even as a child labour? ....years

7.3 Please give details about all your jobs till date:

Job No.	Description of the job and nature of the job work (name of the company, if possible)	Description of the place/site/location/ surrounding where you worked	Your working hours and how many years you have spent in this job
1			
2			
3			

8. Other Information

Questions can be added to collect some other relevant information not covered above.

Name of person collecting data:---
Date: --------------------------; Place: ---

(Signature)

CURRICULUM VITAE

1. Name: Rennie Orson Lakadong

2. Date of birth: 23rd February, 1978

3. Permanent residential address: Pansy Cottage,
Nongshiliang, Malki,
Shillong – 793001

4. Telephone number +919862035368

5. Email: reonlak@yahoo.co.in

6. EDUCATIONAL QUALIFICATION

- Master of Science, **M.Sc.** (Biochemistry), 2002 — North Eastern Hill University, Shillong **(First Class with 60.39 percent)**

- Bachelor of Science, **B.Sc** (Honours in Biochemistry), 2000 — North Eastern Hill University, Shillong **(First Class with 61.12 percent)**

- Higher Secondary School Leaving Certificate, **HSSLC,** 1997 — Meghalaya Board Of School Education, Shillong **(First Division with 64.88 percent)**

- Indian Certificate of Secondary Education, **ICSE,** 1995 — Council for the Indian School Certificate Examinations **(First Division with 75.66 percent)**

7. ADDITIONAL QUALIFICATIONS:

- National Eligibility Test conducted by Council of Scientific Research and University Grants Commission, India **(CSIR-UGCNET)** in Life Sciences, June, 2002.

8. SCHOLARSHIPS/ FELLOWSHIPS:

- **CSIR-NET Senior Research Fellowship,** April, 2007-March, 2008.
- **CSIR-NET Senior Research Fellowship,** April, 2005-March, 2007.
- **CSIR-NET Junior Research Fellowship,** April, 2003-March, 2005.

9. SEMINAR/ CONFERENCES ATTENDED:

a. **96th Indian Science Congress**, held at North-Eastern Hill University, Shillong, from 3rd to 7th January, 2009.

b. **International Conference on Radiation Biology and Translational Research in Radiation Oncology**, held at Jaipur, India, on 10th to 12th November, 2008.

c. National Seminar on "**Advances in Medical and Microbial Biochemistry**"held at Department of Biochemistry, North Eastern Hill University, Shillong on 13th to 14th March, 2008.

d. National Seminar on "**Adaptation Biochemistry**" held at Department of Biochemistry, North-Eastern Hill University, Shillong, on 22nd and 23rd March, 2007.

e. Seminar on "**Trends in Biochemical research**" held at Department of Biochemistry, North-Eastern Hill University, Shillong, on 31st March, 2006.

f. Seminar on "**Advances in Biochemical Education and Research**" held at Department of Biochemistry, North-Eastern Hill University, Shillong, on 25th and 26th February, 2005.

10. WORKSHOPS ATTENDED:

a. Training course on **"Applications of Bioinformatics"** conducted by the Bioinformatics Centre, North-Eastern Hill University, at North-Eastern Hill University, from 13th to 15th, February, 2007.

b. CME programme on **"Cancer challenges in India with particular reference to North-East"** organized by Department of Radiation Oncology, NEIGHRIHMS, Shillong, on 13th and 14th July, 2006.

c. Workshop entitled **Workshop on RDBMS, Theory and Practice,** organized by St. Anthony's College, Shillong on March 1st - 19th, 2005.

11. PUBLICATIONS:

Seminar/ Conference proceedings (abstracts)

1. Lakadong, R.O., Kma, L. and Sharan, R. N. (2009) **Poly-ADP-ribosylation (PAR) of blood lymphocyte proteins in humans: A potential biomarker,** In: *Proceedings of the 96th Indian Science Congress, New Biology Section.*

2. Lakadong, R.O., Kataki, A.C., Kma, L. and Sharan, R. N. (2008) **Poly-ADP-ribosylation(PAR) of blood lymphocyte proteins in human cancers and factors influencing it,** In: *Indian Journal of Radiation Research,* **5,** page 204.

3. Lakadong, R.O., Kma, L. and Sharan, R. N. (2008) **Study of influence of different physiological, medicaland other lifestyle factors on poly-ADP-ribosylation (PAR) of blood lymphocyte proteins in humans,** In: *Proceedings of National Seminar on Advances in Medical and Microbial Biochemistry.*

4. Lakadong, R.O., Kma, L. and Sharan, R. N. (2007) **Poly-ADP-ribosylation (PAR) of blood lymphocyte protein: A potential biomarker of cancer,** In: *Proceedings of National Seminar on Adaptation Biochemistry.*

5. Lakadong, R.O., Kma, L., Kataki, A.C. and Sharan, R. N. (2006) **A novel biomarker of cancer for population detection and screening programme: Poly-ADP-ribosylation (PAR) of blood lymphocyte proteins,** In: *Proceedings of National Seminar on Biochemical Research.*

6. Lakadong, R.O., Kma, L., Kataki, A.C. and Sharan, R. N. (2006) **Poly-ADP-ribosylation (PAR) of blood lymphocyte proteins - a biomarker for cancer detection and screening: Preliminary results for human study,** In: *BBCI Newsletter*, Vol 3, No. 1, pg 7.

7. Lakadong, R.O., Kma, L. and Sharan, R. N (2005) **Poly-ADP-ribosylation (PAR) of blood lymphocyte proteins- a biomarker for cancer detection and screening: Preliminary results for human study,** In: *Proceedings of National Seminar on Advances on Biochemical Education and Research,* page 100.

12. RESEARCH EXPERIENCE:

- Ph. D scholar in Radiation and Molecular Biology Unit, Department of Biochemistry, NEHU, since March 2003.

13. EXPERTISE FOR SCIENTIFIC RESEARCH:

- **Skills developed/ techniques known:** Standard molecular biology procedures such as isolation of protein and DNA, slot-blotting, SDS-Polyacrylamide Gel Electrophoresis,Western Blotting, Gel filtration chromatography. Isolation of human blood lymphocytes. Raising of polyclonal antisera in rabbits and purification of antibodies.
- **Computer skills:** MS Office (Word, Excel, PowerPoint), Graph Pad Prism, Digital Image Processing, Documentation and Analysis (Kodak Digital Science)

15. TEACHING EXPERIENCE:

- Lecturer , Department of Allied Health Sciences, Martin Luther Christian University, Shillong, August 2007 till date.

  Instructing undergraduate students of Surgical Technology, Optometry, Physician Assistant, Medical Laboratory Technology, and Medical Imaging Technology in Theoretical courses Basic Biochemistry, Molecular biology, Medical Biochemistry.

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