Chapter 5

Discussion
Oxygen is vital for respiration and all the active processes that facilitate aerobic life. But it also leads to the formation of free-radicals, which are known to cause damage to the DNA. Respiration, oxidative burst of the macrophages in response to infection, and a variety of exogenous agents including cigarette smoke and ionising radiation give rise to an oxidative stress. This oxidative stress is either a contributing cause or an effect in a range of human diseases. Oxidation of DNA may lead to mutation and consequently, to carcinogenesis (Ames, 1983; Guyton & Kensler, 1993; Mandavilli et al., 2002; Cooke et al., 2003; Volkovová et al., 2006).

According to Caporaso (2003) the question whether the oxidative repair phenotype is associated with disease outcome can be most directly addressed in studies that include survival and treatment data, but indirectly by relating markers to tumour stage and grade, clinical data, and prognosis. Because radiation and chemotherapy influence oxidative processes and DNA repair, accounting for treatment effects in the study design is a requirement.

The inclusion of genetic biomarkers of cancer risk, such as comet assay or MN biomarkers, allows for a complete risk assessment for cancer for all the known demographic (age and sex), lifestyle exposures (smoking and alcohol), occupational and environmental exposures (heavy metals and pesticides) and medical co-morbidities (hypertension, diabetes and chronic obstructive pulmonary disease) (El-Zein et al., 2011).

Events like chromosome breakage or interference with mitotic apparatus, which are believed to be related to carcinogenesis, result in the formation of MNi (Evans, 1962; Cairns, 1975; Bishop, 1987; Nersesyan, 1996; Basu et al., 2004; Nersesyan et al., 2006; Nersesyan & Llin, 2007; Thomas et al., 2008). Since more than 90% of all human cancers are of epithelial in origin, MN assay with buccal epithelial cells is the most suitable biomonitoring approach for the detection of increased cancer risk in humans. Buccal cells have limited DNA repair capacity relative to PBLs, and therefore, may more accurately reflect age-related genomic instability event in epithelial tissue (Dillon et al., 2004). Besides, the minimal invasiveness of cell collection, low cost, ease of storage and slide preparation make the MN assay with buccal epithelial cells the ideal choice for molecular
epidemiological studies (Fenech et al., 2011). The presence of MNi and other nuclear anomalies within these cells has been shown to be associated with genetic defects in genome maintenance, accelerated ageing, exposure to genotoxic agents, oral cancer risk and neurodegenerative diseases and was also useful in chemo-preventive studies (Holland et al., 2008). Genetic damage is measured via the mean frequencies of MNC, TMN, BE, BN, KH and KL.

The comet assay is a rapid, versatile, and sensitive visual method for measuring DNA damage (SSBs and DSBs), protection, and repair at the level of individual cells (Collins et al., 1997, 2008; Tice et al., 2000; Collins, 2004; Wong et al., 2005; Møller, 2006). It is one of the highly sensitive techniques and can detect as few as 50-15,000 breaks/cell (Gedik et al., 1992; Piperakis, 2008). It documents effects at the single cell level, therefore, can reveal heterogeneity within cell populations, in addition to measuring mean responses (Ross et al., 1995; Collins et al., 1997). It provides a visual method for assessing DNA breakage quantitatively in single cells (Ross et al., 1995) and is much more accurate at estimating low levels of damage (Collins, 2009). The percentage of DNA that moves into the tail reveals quantitatively the frequency of DNA breaks, over a range from a few hundred per cell up to several thousand (Ross et al., 1995; Collins et al., 2001), therefore, any increase or decrease in the DNA damage will likewise lead to increase or decrease in the % tail DNA, tail length (µm), tail moment, Olive tail moment and tail area in the PBLs of the population under study.

5.1 COMPARISON OF NORMAL SUBJECTS AND CANCER PATIENTS

During the present investigation the mean frequencies of various nuclear anomalies were significantly (P<0.001) higher in cancer patients than in normal healthy subjects (Table 3) which signifies an association between cancer and the increased occurrence of MNi and other nuclear anomalies. The value of Pearson correlation coefficients between various nuclear anomalies was found to be positive in both normal subjects and cancer patients. This positive correlation with MN and BN suggests the possibility of a similar mechanism of formation of these nuclear anomalies.

The comparison of the mean values of different comet parameters, viz. % tail DNA, integral intensity, tail length, tail moment, Olive tail moment and tail area between cancer patients and normal subjects gave highly significant (P<0.001)
differences (Table 31). A highly significant (P<0.01) positive correlation was observed in the different comet parameters in cancer patients as well as normal subjects (Table 32, 33).

Similar results were found in other reports which showed an increase in MN frequency. In a study with exfoliated buccal mucosa cells from 59 primary cancer patients and 45 healthy volunteers, significant (P<0.001) increase of MN number in cancer patients’ cells compared to control subjects was observed (Nersesyan et al., 2002).

Shao et al. (2005), in a study with 102 subjects with previously untreated oesophageal cancer and 112 healthy controls, quantified baseline (untreated), benzo[a]pyrene diol epoxide (BPDE)-induced, and γ-radiation-induced DNA damage by the Olive tail moment. The mean tail moment was significantly higher in cancer cases than in controls at baseline after BPDE induction, and after γ-radiation-induction.

In a meta-analysis of 37 publications with 1179 cancer patients and 1260 control subjects, Iarmarcvovai et al. (2008) found a significant increase in baseline MN frequency in cancer patients as compared to control subjects.

Our results are in substantial agreement with the previous reports on the induction of micronuclei and chromosome damage by different diseases. Pitozzi et al. (2003) used the alkaline comet assay to measure the basal levels of DNA strand breaks and purine oxidation in total leukocytes from fresh whole blood and in isolated mononuclear leukocytes of type 2 diabetic subjects and found that normal healthy subjects exhibited lower levels of both DNA breaks and FPG-sensitive oxidative DNA damage than diabetics and the difference was more relevant and statistically significant for oxidative damage.

Comet assay analysis on a total of 30 leprosy patients and 15 controls revealed about 38% of the cells to be damaged in all patients while the DNA damage in normal subjects was restricted to only 5% of their cells. In treated and untreated leprosy patients, smoking and alcohol consumption did not influence DNA damage (Gandhi & Singh, 2004).

Migliore et al. (2005) observed a significantly elevated comet response in PBLs from individuals with mild cognitive impairment (a clinical condition between normal ageing and Alzheimer’s disease) and considered a possible association between oxidative damage and the onset of the disease.
de Almeida et al. (2010) analysed the hepatocytes of hepatic parenchyma from 62 cases diagnosed with chronic hepatitis and displaying different degrees of fibrosis (F1-F4), 15 cases without fibrosis (F0) and 12 healthy liver parenchyma samples as control and showed that the rates of MN-Hepatocytes in the F4 group were statistically significant ($P < 0.05$) and higher than those in the control group, F0, F1, F2 and F3 cases.

In a study with 20 patients with oral submucous fibrosis and 20 age-sex-matched healthy individuals as control group, MNC and MNi in oral submucous fibrosis patients were statistically significantly elevated ($P < 0.05$) as compared to control group (Anila et al., 2011).

Contrary to our findings, Lockett et al. (2006) used lymphocyte samples from 158 prostate cancer cases and 128 controls and reported that basal DNA damage did not differ between cancer cases and controls.

5.2 GENETIC DAMAGE IN CANCER PATIENTS WITH RESPECT TO THE DISEASE AND TREATMENT TAKEN

5.2.1 Organ and Organ System Affected

When the mean frequencies of nuclear anomalies in cancer patients were analyzed according to the organ system affected, it was found that the mean frequencies of MNC, TMN, BN, and KH were the highest in patients with cancer of circulatory system (Table 6). This might be due to the fact that there was only one patient with the cancer of circulatory system (Figure 13).

The observation of the mean values of comet parameters in cancer patients with respect to the organ system affected revealed that the mean values of tail length, tail moment and Olive tail moment were observed to be the highest in the circulatory system while the digestive system showed the lowest mean values in % tail DNA, tail length and tail moment and Olive tail moment (Table 34; Figure 27). The high mean frequencies of the comet parameters in the circulatory system may be due to the reason that there is only one patient in this group.

Among different types of cancer, lung cancer had the least genetic damage with the lowest mean frequencies of MNC, TMN and BE while colon cancer showed the maximum genetic damage with the highest mean frequencies of MNC, TMN and KH. The difference in the mean frequencies of MNC, TMN, BE, and KH with respect to the organ affected was found to be mostly non-significant (Table 7).
On comparing the mean values of comet parameters in cancer patients with respect to the organs affected, gall bladder cancer patients had the highest mean values of integral intensity, tail length, tail moment and Olive tail moment in the group while neck cancer patients had the lowest mean values of % tail DNA, tail moment and Olive tail moment (Table 35; Figure 28).

Our results were in agreement with a number of earlier studies where an increase in the basal DNA damage had been reported in PBLs of patients suffering from a variety of different cancers, including ovarian (Baltaci et al., 2002), bladder (Schabath et al., 2004), breast (Sanchez et al., 2005), oesophageal (Olliver et al., 2005), renal (Lin et al., 2007) and lung (Lou et al., 2007).

Bloching et al. (2000) observed two-fold higher rates of buccal MNi in pharyngeal cancer patients compared to healthy subjects.

Rajeswari et al. (2000) reported increased background DNA damage in lymphocytes of breast cancer patients as compared to healthy controls using the comet assay, while, in a study involving 70 breast cancer patients and 70 controls, Smith et al. (2003) the increase in the background DNA damage was statistically. The present study too found a significant increase in the background DNA damage as well as the mean frequency of MNi in breast cancer patients as compared to normal subjects.

Ban et al. (2001) studied the spontaneous MN frequencies in eight human cancer cell lines and four human skin fibroblast cell strains and reported that MN frequencies were significantly higher in the BRCA1-defective HCC1937 and BRCA2-defective Capan-1 cell lines than in the wild-type BRCA1 and BRCA2 mammary and pancreatic tumour cell lines ($P < 0.001$).

Palyvoda et al. (2003) found a significant increase in both background DNA damage as well as the frequency of cells with MNi in patients as compared to controls in a study using lymphocytes of Head and neck SCC patients. Similar results were obtained in the present study where both the DNA damage and the mean frequency of MNi in the neck cancer patients were significantly higher than that in the normal subjects.

Lou et al. (2007) applied comet assay and MN assay to the blood samples of 36 untreated lung cancer patients and 30 controls and observed a significantly higher mean value of tail moment in lung cancer patients with respect to controls ($P < 0.05$). The mean values of MNC frequency and MN frequency in lung cancer patients were also significantly higher than those of controls ($P < 0.01$). Similar increase in genetic damage was also observed in lung cancer patients in the present study.
A significant increase in MN frequency was observed in PBLs of thyroid cancer patients in comparison to healthy controls by Joseph et al. (2009). Nersesyan et al. (2002) observed the exfoliated buccal mucosa cells collected from 41 primary cancer patients with different types of cancer, viz. 12 with breast cancer, 8 with Hodgkin’s disease, 21 with cancer of cervix uteri and 30 healthy women as controls. While the increase was significant (P<0.001) for all other cancer types, women with Hodgkin’s disease did not show such a significant difference from control subjects. This is in contrast to the present study, where highest frequencies of nuclear anomalies have been observed in patients with Hodgkin’s disease (circulatory system).

Yildirim et al. (2006) applied MN assay to the PBLs and buccal epithelial cells of 45 previously untreated cancer patients of which 35 were men (14 with lung cancer, 13 with stomach cancer, 8 with colorectal cancer) and 10 women with colorectal cancer and 44 control volunteers (31 men and 13 women) of corresponding ages and reported that the highest frequency of MNi was in the patients with lung cancer while patients with colorectal cancer had the lowest frequency of MNi. This is exactly opposite to our findings where patients with colorectal cancer had the highest frequency of MNi while patients with lung cancer had the lowest.

### 5.2.2 Duration and Stage of Disease

In the present study, the mean frequencies of nuclear anomalies of cancer patients increased significantly (P<0.001) with respect to the duration of disease (Figure 15). Pearson correlation coefficients were positive between duration of disease and most of the nuclear anomalies and were statistically significant (P<0.001) (Table 9), thus, indicating that the onset of cancer triggers the factors that increase level of genetic damage in an individual.

When the mean values of different comet parameters in cancer patients were compared with respect to the duration (in months) of disease, the mean values of the comet parameters decreased slightly from cancer patients with 1-12 months of disease to cancer patients with 13-24 months of disease before rising significantly (P<0.05) in the group with disease duration >24 months. A reverse phenomenon was encountered in integral intensity which had lower mean value in >24 months category as compared to 1-12 months category (Table 36; Figure 29). Pearson correlation coefficients of duration of disease were positive with all comet parameters except integral intensity
(Table 37). Probably, the breaks in the DNA accumulate over time, resulting in shorter DNA fragments. The decrease in the integral intensity with the increase in the duration of disease may be due to the excessive loss of DNA, in the form of smaller fragments, during the experimental process.

In the current investigation, the mean frequency of MNC increased continuously with the stage of cancer and the difference was highly significant (P<0.01) between patients with Stage II cancer and those with Stage IV cancer. Similar trends were observed in case of TMN and BE (Table 10; Figure 16). The stage of cancer was significantly (P<0.01) correlated with MNC, TMN and BE. A weaker correlation was also observed between the stage of cancer and BN and KH (P<0.05) (Table 11). This supports the view that the advancement of cancer results in an increased genotoxic insult on the body.

Similar results were observed for the comet parameters and the mean values of % tail DNA, tail length, tail moment, Olive tail moment and tail area showed a significant increase from Stage II to Stage IV (Table 38; Figure 30). When correlation analysis was done, a highly significant (P<0.01) Pearson correlation coefficient with the stage of cancer was observed for all the comet parameters, except for integral intensity (Table 39). This increase in the DNA damage with the advancement of the stage of the disease may be due to the increased oxidative stress caused by the rapid proliferation of the tumour.

The present findings are in agreement with previous works where a gradual increase in frequency of MNi from normal to precancerous to cancerous lesions was observed in patients with oral lesions, suggesting a link of MN frequency with neoplastic formation (Casartelli et al., 2000; Halder et al., 2004).

According to Ahuja & Rajeshwari (2003), there was a significant stepwise increase in the mean basal DNA damage in leukocytes as well as cervical epithelial cells from controls to patients with mild dysplasia, severe dysplasia and cancer of the cervix.

Halder et al. (2004) observed an increasing MN frequency from healthy subjects with no oral lesions to patients with precancerous lesions to patients with histopathologically proven oral squamous carcinoma which further decreased in preoperative cases to patients with histopathologically proven oral squamous carcinoma, post operative cases.
Hussien et al., (2005) observed significantly higher DNA damage levels in mono-nucleated cells of breast cancer patients compared to benign breast disease control patients using the comet assay.

Kopjar et al. (2006) studied 30 breast cancer patients and 30 healthy female donors with no known familial history of breast cancer and observed marked inter-individual variations in baseline DNA damage among patients, some of them related to the disease stage and status.

Guido et al. (2008) observed a progressively increasing number of MNi/BE in cirrhotic nodules (CN) to large regenerative nodules (LRN), to dysplastic nodules (DN) and hepatocellular carcinoma (HCC) (P<0.001)

Saran et al. (2008) observed a significant stepwise increase in the MN frequency in buccal epithelial cells and PBLs from controls to pre-cancer patients and from pre-cancer patients to cancer patients.

Samanta et al. (2011) reported a gradual increase in MNi frequencies from normal to inflammatory, abnormal squamous cell of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL), and to high-grade squamous intraepithelial lesion (HSIL) and invasive cancer (IC).

Verma & Dey (2013) studied a total of 55 cases, out of which 9 were grade I, 9 grade II and 37 grade III carcinomas. They observed a significant correlation between the mean number of chromatin bridges, multipolar mitoses and the mean number of MNi per 1000 cells and increasing grade of carcinoma (P < 0.001).

Wang et al. (2013) studied the PBLs from 50 patients with acute leukaemia, 49 patients with myelodysplastic syndrome, 54 patients with benign blood diseases, 45 patients with malignant solid tumour, and 50 healthy controls and observed that the MN frequency of MNi showed a highly significant (P<0.001) decrease from the patients with acute leukaemia or solid tumour to myelodysplastic syndrome patients to benign blood diseases to healthy controls, where it was the lowest.

On the other hand, a study by Chang et al. (2010) showed that elevated level of MNi in PBLs was not associated with the stage of disease or resection status.

5.2.3 Treatment Taken

When the mean frequencies of nuclear anomalies in cancer patients were compared with respect to the treatment taken by them, the lowest values were observed in patients who had received no treatment so far. Among the treatment
groups, the lowest mean frequencies of MNC, TMN, BE and BN were observed in patients who had undergone surgery alone, while the highest mean frequencies were observed in treatment groups with radiation therapy as an integral part of the treatment. This may be due to the genotoxic (clastogenic) effects of the radiations used in radiation therapy (Table 12; Figure 17).

The comparison of different comet parameters in cancer patients with respect to the treatment taken by them resulted in a non-significant difference in the mean values of these parameters between different treatment groups although the values were invariably high in treatments having both chemotherapy and radiation therapy as a component (Table 40; Figure 31).

The present results are in agreement with Halder et al. (2004) who observed a decrease in the MN frequency in patients with histopathologically proven oral squamous carcinoma from preoperative cases to post operative cases.

Jianlin et al. (2004) used the MN assay and the comet assay to study the increase in genetic damage with treatment with radiotherapy in 24 cancer patients and found a significant increase in MNI rate (MNR), mean micronucleated cells rate (MCR), mean tail length (MTL) and the mean tail moment (MTM) with cumulative radiation dose (0, 10, 30 and 50 Gy) for each patient.

Kopjar et al. (2006) studied 30 breast cancer patients and 30 healthy female donors with no known familial history of breast cancer and observed a significantly elevated DNA migration in blood cells of patients just one hour after completion of the first cycle of chemotherapy.

In a study by Gamulin et al. (2007) the assessment of two comet parameters, i.e., tail length and tail moment in blood samples collected after administration of the first fraction of radiotherapy confirmed a strong positive response to the therapy (P<0.05) in almost all patients. The values of both comet parameters recorded in most of the blood samples collected after the administration of the last fraction of radiotherapy show a slight decrease, indicating the possibility of an adaptive response.

5.3 COMPARISON WITH RESPECT TO INHERENT FACTORS

5.3.1 Sex

Comparison of the nuclear anomalies was also made on the basis of the sex of the subjects under the study (Table 13). In both normal subjects and cancer patients, the mean frequencies of different nuclear anomalies were higher in women than men.
This may be due to the preferential loss of the inactive X chromosome in women (Hando et al., 1994; Surralles et al., 1996, 1999; Norppa and Falck 2003). On the other hand, when the mean frequencies of all the nuclear anomalies in men and women in the normal subjects group were compared to their counterparts in the cancer patients group, the differences were significantly high (P<0.001). These results confirm the genotoxic effect of cancer in both men and women (Table 14; Figure 18).

The mean values of different comet parameters of normal subjects and cancer patients were compared with respect to their sex and slight but statistically non-significant increase was observed in women as compared to men in both normal subjects and cancer patients (Table 41; Figure 32). There may be two possible explanations for this behaviour. It may either be the protective effect of the genes on the Y chromosome in men or the preferential loss of the inactive X chromosome in women. When the mean values of different comet parameters in men and women in the cancer patients group were compared to their counterparts in the normal subjects group, the differences were found to be significantly high (P<0.001) for all the comet parameters (Table 42) suggesting a clear effect of oxidative stress induced by the disease on both the sexes.

The present results are in agreement with earlier studies by Hando et al. (1994) and Surralles et al. (1996, 1999) who described an increase in MN frequency with respect to sex, with elevated loss of X chromosome by micro-nucleation being prevalent in women (Norppa and Falck 2003). Piyathilake et al. (1995) too reported 2.8 fold higher frequencies of micronucleated cells in women as compared to men.

The HUMN project provided strong data based on a large number of subjects and laboratories and clearly showed a gender difference, with women demonstrating a higher incidence of MN compared to men (Bonassi et al., 2001).

A gender difference in the background incidence of MN in PBLs was reported by Battershill et al. (2008), with the frequency being consistently higher in women.

On the other hand, in some studies contradicting results were also observed. In a study by Nishioka et al. (1981) higher values of MNi were reported in men as compared to women. The present finding was also in contrast to reports of Benites et al. (2006) who studied Brazilian population and found significantly higher number of MNi in men.

However, in the significantly more comprehensive analysis by Neri et al. (2003), a gender difference was not observed. Yildirim et al. (2006) too, observed no
significant difference between men and women in MN frequencies of PBLs and buccal cells in colorectal cancer patient and control groups \((P>0.05)\).

Similarly, in exfoliated buccal cells, the role of gender as a determinant of MN frequency was traced in a review of 15 studies by Holland et al. (2008) and conflicting results were obtained, with only three studies reporting significant differences.

5.3.2 Blood Group

When the mean frequencies of nuclear anomalies of cancer patients and normal subjects having different blood groups were compared, it was found that the mean frequencies of all the nuclear anomalies except KL were significantly higher in cancer patients with blood groups A, B and O as compared to normal subjects with the same blood groups, though none of the nuclear anomalies showed a significant difference between cancer patients and normal subjects having blood group type AB (Table 15; Figure 19).

A comparison of the mean values of comet parameters in normal subjects and cancer patients was made with respect to their blood groups. All parameters showed a statistically significant difference in the two groups for the blood group types A, B and O except for integral intensity which showed a statistically significant difference only for the blood group type AB (Table 43; Figure 33). The blood group AB showed the lowest mean values for all the comet parameters except for integral intensity where it was the highest. This suggests that cancer patients with this blood type had suffered a lower level of genetic damage in comparison to other blood type. This may be due to the protective effect of this blood type against oxidative stress.

Perusal of literature revealed that no previous work was available on the correlation of genetic damage with ABO blood group system.

5.3.3 Family History of Cancer

During the present investigation, cancer patients having a family history of cancer had statistically higher \((P<0.01)\) mean frequencies of MNC and KH than those who did not have a family history of cancer (Table 16). Similar trend was observed in the mean frequencies of the other nuclear anomalies although the differences were not statistically significant (Figure 20).
Among cancer patients, those who had a family history of cancer had higher mean values for all the comet parameters as compared to those who did not have a family history of cancer while the differences in the mean values of % tail DNA, tail moment and Olive tail moment in cancer patients with a family history of cancer and those without it were found to be significant (P<0.05) (Table 44; Figure 34).

This suggests that the people having a family history of cancer exhibit greater genetic instability and they are genetically more susceptible than people without a family history of cancer and that the onset of cancer is accompanied by an increased level of genetic damage.

The present findings are supported by earlier studies by Rothfuß et al. (2000) who compared 12 women with a BRCA1 mutation and 10 relatives without the familial mutation to 17 healthy women without family history of breast or ovarian cancer as controls and observed a close relationship between the presence of a BRCA1 mutation and sensitivity for the induction of MNi. The carriers of a familial BRCA1 mutation exhibited strongly increased MN frequencies, whereas FDRs without the mutation had induced MN frequencies in the range of the controls.

In a study with 121 controls with no family history of any type of cancer, 188 FDRs of breast cancer patients, and 88 breast cancer patients, Ahuja & Rajeshwari (2003) observed a stepwise increase in DNA damage and micronucleus frequency from controls to FDRs, and from FDRs to breast cancer patients using MN assay and comet assay.

Similarly, Burgaz et al. (2011) observed a statistically significant (P<0.01) increase in the mean frequencies of MNi in 59 patients having HNC and their 34 healthy FDRs (P<0.05) with respect to 31 healthy control volunteers who did not have any HNC history in their family.

Tozan-Becerent et al. (2011) found significantly higher DNA damage in colorectal cancer patients and their FDRs as compared to the control group.

Çelik et al. (2013) too, analyzed the frequency of SCE and MNi in the PBLs of 30 women with breast cancer, 22 of their FDRs, and 20 age-matched healthy female volunteers and observed that both SCE and MNi occurred at a significantly higher rate in breast cancer patients, and in their FDRs, as compared to controls (P<0.001 and P=0.001, respectively).
5.4 COMPARISON WITH RESPECT TO LIFESTYLE FACTORS

5.4.1 Dietary Habit

In the present study, comparisons were also made between the nuclear anomalies of cancer patients and normal subjects with respect to their dietary habits. The mean frequencies of all the nuclear anomalies in normal subjects group were found to be lower in vegetarians as compared to omnivores (Table 17). This may be because vegetarian food contains antioxidants, which reduce the genetic damage (Duthie et al., 1996; Pool-Zobel et al., 1997). The mean frequencies of all the nuclear anomalies of vegetarians and omnivores in both cancer patients and normal subjects were not significantly different.

Comparisons were also made between the mean values of different comet parameters of normal subjects and cancer patients with respect to their dietary habits. While in the normal subjects group, the mean values of all the comet parameters, except integral intensity, in omnivores were found to be higher than vegetarians. Surprisingly, in case of cancer patients, most of the comet parameters showed increased (but statistically non-significant) mean values in vegetarians as opposed to omnivores (Table 45; Figure 35).

Our results are in agreement with earlier studies comparing vegetarians with non-vegetarians and did not provide any indication that diet alters MN or chromosomal aberrations frequency in PBLs (Fenech & Rinaldi, 1995; Kazimirova et al., 2004, 2006).

Various studies carried out to investigate the effect of specific food types, viz. vegetables and fruit, on oxidative DNA damage have been found to be inconsistent too, with only small effects reported (Giovannelli et al., 2002; Staruchova et al., 2006).

Raimondi et al. (2007) used a detailed dietary questionnaire during the examination of polycyclic aromatic hydrocarbon-exposed workers in the Czech Republic and reported no effects of diet on a number of indicators of DNA damage including chromosomal aberrations.

It was only when Bonassi et al. (2011) reanalyzed a total of 30 databases, containing 5424 subjects that some evidence in support of the effect of dietary habits was found. They observed that the subjects with a daily consumption of fruits or green vegetables had lower mean MN frequencies than those who reported no consumption at all of these foods with subjects reporting daily consumption of fruits
versus those never eating fruits having a 32% lower frequency of MN in their exfoliated buccal cells.

5.4.2 Smoking Habit

On comparing the smoking habits of the subjects, the mean frequencies of MNC, TMN and BE were found to be lower in non-smokers than in smokers, while an opposite trend was observed among cancer patients, where non-smokers had higher mean frequencies of nuclear anomalies. No significant differences were observed in the mean frequencies of nuclear anomalies in patients consuming >20 cigarettes/day when compared to patients consuming 0 cigarette/day (Table 22; Figure 23). While a positive correlation was observed between number of cigarettes smoked and nuclear anomalies under observation in normal subjects (Table 23), the correlation was negative in case of cancer patients (Table 24).

Comparison of mean values of different comet parameters of normal subjects and cancer patients were also made with respect to their smoking habits. Oddly, among both normal subjects and cancer patients, the mean values of most of the parameters were higher but statistically non-significant in case of non-smokers than smokers (Table 47; Figure 36).

No significant difference was observed in the mean values of different comet parameters in both normal subjects group (Table 49) and cancer patients group (Table 50; Figure 37), when comparisons were made on the basis of the number of cigarettes smoked daily. Thus, an inconclusive response was obtained for the effect of smoking on genetic damage in cancer patients. Positive but non-significant Pearson correlation coefficients for the number of cigarettes smoked (daily) were observed with respect tail moment and Olive tail moment in both normal subjects (Table 51) and cancer patients (Table 52).

The increased damage in non-smokers, observed in the present study may be due to the exposure to passive smoking in case of non-smokers. Also, many smokers who are the cancer patients in the current study may have actually stopped or reduced smoking and adopted healthy lifestyles because of respiratory symptoms directly or indirectly related to the diagnosis or after being diagnosed with cancer. This may result in the consideration of a former heavy smoker as a non-smoker and hence, mask the results.
The findings of the present study are supported by a pooled reanalysis of 24 databases (3501 non-smokers, 1409 current smokers and 800 former smokers) from the HUMN international collaborative project performed by Bonassi et al. (2003) to understand the influence of smoking habit on MN frequency. The results showed a small decrease in MN frequencies in current and former smokers (all smoking levels combined) as compared to non-smokers, among non-exposed subjects. In contrast, MN frequency was not influenced by the number of cigarettes smoked per day among occupationally exposed subjects. This analysis confirmed that smokers generally do not experience an overall increase in MN frequency.

Speit et al. (2003) too reported no significant changes in DNA migration in the comet assay of PBLs from heavy smokers (>20 cigarettes/day).

In a study assessing DNA damage in the PBLs of smokers by Hoffmann & Speit (2005), neither MN assay nor comet assay gave significant differences in any of the nuclear anomalies or the comet parameters between smokers and non-smokers.

In contrast, Stich & Rosin (1983) observed an elevated frequency of MNi of buccal cells from the cheek and tongue only for subjects who both smoked and drank alcohol. Neither smoking alone nor drinking alone was associated with an elevated frequency of MNi, thus, indicating a synergistic effect of both smoking and alcohol drinking habits.

Fenech (1993) showed that, after adjustment for age and sex, individuals with high cigarette usage (>30 cigarettes/day) had statistically greater MNi frequency as compared to non-smokers.

Rojas et al. (1996) used comet assay for detection of DNA strand breaks and observed a significantly greater extent of DNA damage in smokers than non-smokers while in a study by Oßwald et al. (2003) comet assay failed to detect any changes in buccal cells of smokers.

Barrera et al. (1998) reported higher chromosomal instability in smokers than in non-smokers, in a study using FISH while another small study, with 12 smokers and 12 non-smokers as controls, also established the association between smoking and significantly higher MN levels in circulating lymphocytes (Schneider et al., 2001).

Thorne et al. (2008) demonstrated that human pulmonary carcinoma cells exposed to mainstream cigarette smoke from Kentucky reference-cigarettes showed considerable DNA damage in terms of strand-breaks, ALSs and oxidative DNA lesions.
In a reanalysis of total 30 databases, containing 5424 subjects Bonassi et al. (2011) found that only subjects who smoked the most (i.e., ≥40 cigarettes/day) had a significant increase in MN over non-smokers.

In order to evaluate genetic instability and risk of lung cancer associated with exposure to smoking, McHugh et al. (2013) studied the genetic damage in 500 lung cancer cases and 500 controls, using the CBMN assay and observed a difference in lung cancer risk between non-exposed men and women heavy smokers, although it was not statistically significant (P=0.09). This suggested that heavy smoking may have an effect on DNA repair capacity and in turn modulate the risk of lung cancer.

5.4.3 Alcohol Drinking Habit

As for the alcohol drinking habit, all the nuclear anomalies showed higher mean frequencies for abstainers when compared to alcoholics in the normal subjects group. Similar trend was observed in case of MNC, BN, and KL in the cancer patients group. But the differences were non-significant in both cancer patients group and normal subjects group (Table 25; Figure 24). While the mean frequencies of nuclear anomalies in normal subjects first decreased from the abstainers to the light drinkers and then again increased for the heavy drinkers, an opposite trend was observed in case of cancer patients where the mean frequencies were the highest for light drinkers. No significant difference was observed in the mean frequencies of nuclear anomalies in either of the groups (Table 27, 28). When correlation between the nuclear anomalies and amount of alcohol was calculated, a negative but non-significant correlation for most of the nuclear anomalies was observed in case of normal subjects, while in cancer patients most of the nuclear anomalies had a positive but non-significant correlation (Table 29, 30).

During the current study, the mean values of all the comet parameters viz. % tail DNA, integral intensity, tail length, tail moment, Olive tail moment and tail area were higher in abstainers as compared to alcoholics in both normal subjects and cancer patients (Table 53; Figure 38). When compared with respect to the amount of alcohol consumed (per month), the mean values of comet parameters in both normal subjects (Table 55) and cancer patients (Table 56; Figure 39) decreased from abstainers to light drinkers and then increased again in heavy drinkers, although the differences in the mean values were not statistically significant. This suggests that consumption of low amount of alcohol may not have a damaging effect on the DNA.
The present study is in accordance with the reports of Bloching et al. (2000) who did not find any relationship between daily alcohol intake and MN rate (P>0.05) in normal mucosa in 55 patients with SCC and 16 patients with leucoplakias, and 99 healthy, age-matched controls.

Similar results were obtained in a reanalysis of total 30 databases, containing 5424 subjects by Bonassi et al. (2011) when no association was found between daily alcohol consumption and increased MN frequency.

Although drinking alcoholic beverages has been causally associated with cancer at a number of sites, viz. head and neck cancer, yet there has been no report on induction of mutagenic effects in rodents (Committee on Carcinogenicity, 1995; Committee on Mutagenicity, 2000).

On the contrary, Stich & Rosin (1983) reported a pronounced combined effect of alcohol in combination with smoking. Both Castelli et al. (1999) and Maffei et al. (2002) reported an increase in MN and chromosomal aberrations in PBLs that have been observed in subjects consuming alcoholic beverages but not in abstainers of a year or more.

5.5 DISTRIBUTION OF ABO AND Rh (D) ALLELES

5.5.1 ABO Alleles

When the phenotypes and gene frequencies of ABO blood group in both normal subjects and cancer patients were compared, the difference was found to be non-significant.

Perusal of literature revealed that no previous work was available on the correlation of cancer risk with ABO and Rh (D) allele frequencies in the population of Haryana. But the distribution of ABO and Rh (D) allele frequencies has been studied in different populations of India.

Previous studies on the North West Indian populations have reported that the frequency of allele A varied from 0.068 to 0.667 (Kushwaha et al., 1990; Ravikiran, 2004). The frequency of allele B has been reported to range from 0.185 to 0.566 (Kushwaha et al., 1990; Sidhu, 1999) and the frequency of allele O has been found to vary from 0.170 to 0.689 (Sidhu, 1999; Karve, 1948). The frequencies of all the allele A, B and O, in both normal subjects (0.151, 0.316, and 0.533, respectively) and cancer patients (0.196, 0.257, and 0.546, respectively) observed in the present study fit well within the range of the previous studies. Therefore, suggesting a homogeneous distribution.
Similar results were reported by Anderson & Haas (1984) and Tursen et al. (2005), who found no significant relationship between ABO blood group and cancer risk while Wolpin et al. (2009, 2010) showed that people with blood group A, AB, or B were more likely to develop pancreatic cancer than those with blood group O and the risk increased with the addition of each non-O allele. No such relation was observed in the present study.

In several other studies, the analysis of ABO blood group frequencies within different types of cancer has been done. Many of these studies found an elevated frequency of the blood group A in gastric carcinoma (Arid et al., 1953; White & Eisenberg, 1959), ovarian cancer (Osborne & de George, 1963; Bjorkholm, 1993), breast cancer (Guleria et al., 2005; Stamatakos et al., 2009), while some of them reported that the frequency of blood group B was higher in oesophageal cancer (Guleria et al., 2005), breast cancer (Sharma et al., 2007), malignant tumours of the digestive system (Jovanović-Ćupić et al., 2008) and ovarian cancer (Gates et al., 2011). In the present study, the frequencies of the blood group A and O have remained fairly constant, whereas, that of the blood group B has decreased from 45% in normal subjects to 38% in cancer patients.

5.5.2 Rh (D) Alleles

In case of Rh (D) blood group too, only a slight difference was observed, in the allele frequencies of allele d, between normal subjects and cancer patients. The gene frequency for d was slightly higher in cancer patients (0.398) than normal subjects (0.354) but both fit in the range reported in the previous studies, i.e., 0.000 (Kushwaha et al., 1990) to 0.420 (Yadav et al., 1997), again representing a homogeneous distribution.

Jovanović-Ćupić et al. (2008) and Stamatakos et al. (2009) observed an increased frequency of Rh (D) among the cancer patients with worst prognosis.

5.6 CONCLUSION

In the current investigation, a significantly high level of genetic damage was observed in cancer patients as compared to normal subjects. The positive correlation observed between duration/stage of cancer and the genetic damage was highly significant too, suggesting that the progress of cancer results in great levels of oxidative stress, which causes genetic damage in the whole body. Interestingly, there
was an increase in genetic damage from untreated patients to the ones taking treatment, with highest damages being observed in the patients receiving radiation therapy. This means that non-cancerous tissues may also get damaged during the radiation therapy process which may lead to the development of secondary tumours. A correlation was also observed between MN, BE and BN, suggesting a similar mechanism of their formation.

Higher levels of genetic damage were also observed in women as compared to men. This may be due to the preferential loss of the inactive X chromosome in women. Among different blood groups, cancer patients with the blood group type AB showed the lowest genetic damage which was not significantly different from the normal subjects with the same blood group. This indicates possibility of an unknown resistant behaviour of the AB blood type towards genetic damage. Significantly higher genetic damage was also observed in cancer patients with a family history of cancer as compared to those without it. No significant difference in genetic damage was observed with respect to dietary or alcohol drinking habits of the subjects, while in smokers, only heavy smokers (>20 cigarettes/day) showed an increased genetic damage.

Cancer is one of the biggest medical challenges faced by the man in the recent times. Due to the asymptomatic nature of cancers in the initial stage, an early clinical diagnosis becomes difficult, which results in identification of the tumours in the advanced stages, when it has metastasized to such an extent that very little can be done to save the patient from imminent death. Therefore, emphasis should be laid upon the early detection of the cancer and more robust methods should be developed for this. Certain people have been found to be more susceptible to developing cancers and screening the whole population for individuals in the high risk group followed by regular monitoring may help in reducing the cancer mortality to a great extent.

In the present study, about 10% of the clinically healthy donors had genetic damage equivalent to that in cancer patients. These normal subjects represent the high cancer risk group which can further be subjected to advanced tests, like Pap smear test and mammography; thus, reducing the cost of mass screening to 1/10\textsuperscript{th} of the total cost otherwise. Both the tests employed in the current investigation are least invasive and low cost. Therefore, these methods are readily accepted by the patient. They can also be used for repeated examinations for follow up or evaluation of treatment response of malignant cells. The screening facility can be provided readily throughout
the country as only basic infrastructure is needed for these tests and the technicians can easily be trained. Mass screening of the whole population for individuals at high risk of developing cancer will not only help in the diagnosis of cancer in the sub-clinical stages but also allow for the preventive therapy of the high risk individuals; therefore, reducing the rate of both mortality and incidences of cancer. The development and validation of rapid, reliable, and cost effective screening tools is the need of the hour. The present work may be viewed as a small step in this direction.

5.7 FUTURE PERSPECTIVE

With the rapid rise in the incidence rate all over the world, cancer has become the cause of concern for everyone and a lot of work has to be done to find an effective solution to this problem. Therefore, further studies should be conducted with the following objectives:

1. To develop an understanding about the various underlying factors (genetic and environmental), which may affect an individual’s susceptibility to cancer, and on the basis of which individuals at high risk could be screened and monitored for disease development.
2. To validate rapid, reliable, and cost effective mass screening tools for the screening of the whole population for high risk groups.
3. To develop tests for the demonstration of mutated tumour genes in body secretions and excretions for the early diagnosis of tumours.
4. To measure the genotoxic effect of different treatments (radiation therapy, chemotherapy, etc.) on the non-cancerous tissue and its correlation with the chances of development of secondary tumours.
5. To analyze individual treatment response for the development of person specific treatment.
6. To develop a better understanding of the various protective factors (diet, antioxidants, etc.), which may enhance an individual’s chances of developing cancer.
7. To develop a molecular therapy approach using substances those counteract the molecular processes which in turn promote the clonal development of tumours by activating/inactivating different pathways in the tumour cells.