2.1. Betel Quid Chewing and Human Health

Betel quid chewing as a habit has existed in human civilization since ages. It has been prevalent in India and South-East Asia for over 2000 years. Chewing of betel quid is mentioned in Susruta Samhita, which was written approximately around 600 BC while ancient writings refer to prevalence of betel quid in Sri Lanka as early as 504 BC. Stone inscriptions in Harappa and Mohenjodaro from the year AD 473 are historical evidence of its existence. In ancient Indian culture, paan (betel quid) chewing is referred to as one of the eight bhogas (enjoyments) of life (Gupta, 2004). Betel quid chewing was adopted even by invading kings and settlers in India and was also a part of the Mughal culture (Gupta, 2004). Offering of betel quid to guests is an ancient
custom in many South-East Asian countries which is sanctimoniously preserved
till the present time.

_Areca catechu_ belongs to Family Arecaceae (Palmae), palm family and
Subfamily Arecoideae. The “nut” (actually the seed endosperm) is chewed as a
stimulant masticatory by 5% of the world’s population, making it more popular
than chewing gum but not as popular as tobacco. The seeds of many other
palms, including at least eight species of Areca, are used as inferior substitutes
for _Areca catechu_. Use of betel nut is often culturally or socially ritualized, and
there are elaborate ceremonies attending its use in various Asian and Pacific
cultures (Staples et al., 2006).

_Areca nut catechu_ and _Piper betle_ plants are native to West Malaysia.
Betel quid chewing is believed to have been introduced to the Indian subcontinent
during the period of colonization of Malaysia and South-East Asia by Indian emperors
(Bhisey, 2000). Global estimates report upto 600 million chewers (Gupta and
Warnakulasuriya, 2002). The pattern and usage of chewing betel quid is different
in different countries and sometimes varies within the same country too with
geographical and ethnic variation. The countries which occupy prominence with
regard to prevalence of betel quid chewing comprises India, Srilanka, Pakistan,
Bangladesh, Taiwan, China, Myanmar, Thailand, Lao People’s Democratic
Republic, Cambodia, Malaysia, Singapore, Indonesia, Palau, Guam and migrant
populations from the aforesaid countries residing in South Africa, United
Kingdom, Canada and North America. In several other countries including Nepal, Vietnam, Kenya and Solomon Islands, the practice of betel quid chewing is known but no reports are available (IARC, 2004).

Traditionally betel quid consisted of betel leaf, pieces of Areca nut, a few drops of lime (calcium hydroxide), several condiments, sweetening, and flavoring agents, depending on regional practices and individual preferences. Countrywide surveys on the use of Areca nut have not yet been conducted in India. Reports from different parts indicate variations in the constituent of betel quid consumed in India. Studies from Andhra Pradesh, Bihar, Gujrat, Kerala, Maharashtra, Karnataka and West Bengal report betel quid comprising of Flakes of Areca nut (ripe), catechu, lime paste and sometimes few sweeteners and flavouring agents along with or without tobacco wrapped around in a leaf of Piper betle (Mehta et al., 1971; Mehta et al., 1972; Dayal et al., 1971; Chakrabarti et al., 1990; Gupta et al., 1996).

Betel quid chewing is widely prevalent in the North Eastern part of India which is populated by ethnic hill tribes, plain tribes and plain settlers. Fresh unripe Areca nut along with slaked lime locally quarried from limestone wrapped in a piece of betel quid is chewed by Khasis of Meghalaya (Stitch et al., 1981), which is locally known as ‘kwai’. The Assamese people in Assam consume unripe or fermented Areca nut along with slaked lime lime and leaf of Piper betle (Phukan et al., 2001). In Southern Assam, the Cacharis and Sylhetis
consume ripe Areca nut flakes with betel leaf and lime along with Sadagura, a tobacco preparation unique to this region.

A consensus workshop held in 1996 recommended that the term ‘quid’ should be defined as “a substance, or mixture of substances, placed in the mouth, usually containing at least one of the two basic ingredients, tobacco or Areca nut, in raw or any manufactured or processed form” (Zain et al., 1999). A chewing substance may primarily consist of:

- Areca nut alone, without any betel leaf, slaked lime or tobacco.
- Chewing tobacco without any Areca nut.
- Areca nut with components of betel vine and any other ingredients except tobacco (betel quid without tobacco)
- Areca nut with components of betel vine and any other ingredients including tobacco (betel quid with tobacco).

Areca nut is the 4th most commonly used psychoactive substance in the world after tobacco, alcohol and caffeine containing beverages (Sullivan and Hagen, 2002). Betel nut use as a stimulant presents significant health risks. Heavy use of betel quid humans causes serious health problems (Staples et al., 2006). Areca nut, fresh or dried, ripe or unripe, that is chewed as a stimulant narcotic. The betel quid (wad of chewable ingredients) includes the fresh or dried seed of betel nut, a fresh leaf of betel pepper (Piper betle), a dab of slaked lime, and various flavorings (cutch, cardamom, clove, tobacco, or gambier). Eight
closely related alkaloids are responsible for the stimulant effect; the alkaloid levels are highest in the unripe fruit (IARC, 2004). The Indian pan (betel quid) is a common after-dinner treat, acting against post-meal lethargy and as a digestif (Staples et al., 2006). Betel quid chewing manifests several pharmacological effects, including euphoria, central nervous system stimulation, vertigo, salivation, miosis, tremor and bradycardia (Giri et al., 2006).

Studies on betel quid chewers in different parts of the globe testify the addictive effect of betel quid chewing (Burton-Bradley, 1997; Cawte, 1985; Yang, 2001; Kuruppuarachchi, 2003). Reported effects of betel quid chewing were relaxation, lifting of mood. Withdrawal symptoms seen in habituates trying to quit the habit include mood swings, anxiety, irritability, sleep disturbance and craving for the nut. The mean severity of dependence score was 7.3 (range 1-12) which is similar to score of problematic use of amphetamines (IARC, 2004). These findings are regarded to be consistent with the existence of a dependence syndrome among regular users. In rare cases, Areca nut psychosis has been reported to occur in heavy users following abrupt cessation of the habit (IARC, 1985).

The oral cavity suffers from potentially harmful effects due to the habit of betel quid chewing. The main effects on the hard tissue of the oral cavity are on the teeth. It caused severe wearing of the enamel covering which may result in increased dentinal sensitivity. Root fractures have also been reported in long
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term chewers probably as a result of increased masticatory load and repetitive masticatory stress during chewing betel quid (Yeh, 1997; Gao et al., 2001).

Discolouration of teeth with brownish staining occurs in chronic users with poor oral hygiene (Gao et al., 2001). Report on the effect of betel quid chewing on dental caries is contradictory. Some studies have reported that prevalence of dental caries is higher in non chewers than in chewers (Nigam and Srivastava, 1990), while others have shown that there is no difference in the occurrence of dental caries in betel quid chewers and non chewers (Williams et al., 1996).

Incidence of gingivitis has been found to be higher among betel quid chewers with tobacco (Amarasena et al., 2003). Loss of periodontal attachment and calculus formation has been found to be higher in betel quid chewers (Anerud et al., 1991). Areca nut chewing activates sympathoadrenal response and increases plasma concentration of adrenaline and nor adrenaline (Chu, 1995, 2002). It also increases central sympathetic activity in humans, leading to increased heart rate and increased blood flow through the common and external carotid arteries (Chu, 2002; Lin, et al., 2002). Increase in serum homocysteine levels which is a risk factor for heart disease and betel quid chewing has been reported to be associated with homocysteine level in chewers (Alfthan et al., 1997; Obeid et al., 1998). It is a risk factor for Meige’s syndrome which is a movement disorder (Bihari et al., 2000). Betel quid chewers have an increased...
risk of developing diabetes mellitus (Tung et al., 2004; Benjamin, 2001) and liver cirrhosis (Tsai et al., 2003).

Arecoline, which is the major alkaloid in Areca nut, causes constriction of smooth bronchial muscles and severe asthma has been reported in asthmatics on betel quid chewing (Kiyingi, 1991; Kiyingi and saweri, 1994). A statistically significant association has been found between Low birth weight, pre-term birth and maternal betel quid chewing (Yang et al., 2001). Prevalence of adverse pregnancy outcome such as spontaneous abortion, premature delivery, stillbirth and fetal malformation has been reported to be 2.8 times higher among betel quid chewing women compared to non users (Yang et al., 1999).

2.2. *Smokeless Tobacco, Smoking Tobacco and Its Effect on Human Health*

King James I of England, while describing tobacco smoking, had said - "A custom loathsome to the eye, hateful to the nose, harmful to the brain, dangerous to the lungs, and in the black stinking fume thereof nearest resembling the horrible stygian smoke of the pit that is bottomless" (Gupta et al., 2002).

The word 'tobacco' is thought to be derived from the Arabic word tabaq, meaning euphoria producing herb. It is said that the word tobacco is the Carib word tabaco (the name of the pipe in which tobacco was smoked) (Christen et al., 1982). It is possible that the word tobacco comes from the island of Tobago
in the Caribbean. Some sources refer to the origin of this word from the Tabasco state in Mexico. The word ‘cigare’ is derived from the Mayan word sikar which means ‘to smoke’ (Christen et al., 1982). According to yet another theory, the word tobacco is derived from a Spanish word tobaca which is a Y-shaped instrument used by early American Indians to inhale snuff (Christen et al., 1982). Tobacco appears to be as old as human civilization. Cultivation of the tobacco plant probably dates back 8000 years (Christen et al., 1982). Tobacco seeds were discovered in archaeological excavations in Mexico and Peru, and the remains of permanent settlements built around 3500 BC showed that tobacco was an important article to the inhabitants. Tobacco belongs to the family of plants called Solanaceae which contains about 60 species. *N. rustica*, a mild-flavoured, fast-burning species, was the tobacco originally raised in Virginia, but it is now grown chiefly in Turkey, India and Russia. Both the species of tobacco are annuals. Modern commercial varieties of tobacco have descended directly from *N. tabacum* (Gupta, 2004).

Historians believe that Native Americans began using tobacco for medicinal and ceremonial purposes before 1 BC. First pictorial records of tobacco being smoked have been found on Guatemalan pottery. The documentation of the practice of inhaling the smoke of dried tobacco plants is available from the Mayan culture as early as the sixth century (Christen and Glover, 1987; Christen et al., 1982). In 1493, Ramon Pane, who accompanied Columbus on his second voyage, described the habit of Indians taking snuff through a Y-
shaped tube. Pane is credited for being the first person to introduce tobacco seeds into Europe (Christen and Glover, 1987; Christen et al., 1982).

Tobacco was introduced into India by Portuguese traders during 1600 AD. Its use and production proliferated to such a great extent that today India is the second largest producer of tobacco in the world. Soon after its introduction, it became a valuable commodity of barter trade in India.

### 2.2.1. Smokeless Tobacco

Perhaps one of the fastest growing detrimental health habits over the past few years has been the use of smokeless tobacco. As a result there is an urgent need to alert the populace about health problems associated with the use of smokeless tobacco (Glover et al., 1988). Smokeless tobacco use may be of two kinds: oral use and nasal use. In India and South-East Asia, nasal use is uncommon whereas in European countries and North America, sniffing (inhaling) dry snuff through the nostrils is more common. Snuff is a finely ground tobacco of which the user places a pinch (called a dip or rub) in the gingival groove. Snuff can be dry, moist, or in sachets (tea bag-like pouches). The most common position to place snuff is in the mandibular labial mucosa (cuspid to cuspid). In European countries, sniffing (inhaling) dry snuff through the nostrils is more common than in North America (Christen and Glover, 1987). In India, the neighboring countries, and some other countries of the southeast region,
smokeless tobacco use is very common. Reliable prevalence data from some selected parts of India when large cross-sectional, house-to-house surveys of tobacco habits were conducted in populations in widely dispersed areas which do represent a large part of the country revealed that tobacco use is very common in India. However, the prevalence of smokeless tobacco use varied markedly in different regions, although in general it was comparable between smokers and nonsmokers. In most places, smokeless tobacco use was found to be more common among women but among women there is great variability with geographical variation (Mehta et al., 1969, 1972). The most common methods of smokeless tobacco use in India are with betel quid chewing and its variants. Although the betel quid itself, as well as various combinations of its ingredients, can be chewed alone, most habitual chewers include tobacco in their quid (Mehta et al., 1969, 1972). The tobacco and lime mixture is probably the most common variant of chewing tobacco without betel nut. The mixture is known as ‘khaini’ in the northern part of India, and it is popular in other parts as well. To prepare the quid, the user places a small amount of tobacco in the palm; a dash of lime is flicked by a thumb or forefinger, and it is mixed and rubbed vigorously with the tobacco in the hand. The mixture is then ready for use and is placed in the mouth. The exact placement of the tobacco and lime mixture in the mouth varies among the people of different regions. The most common sites of oral cancers and precancers also vary correspondingly in those regions. Mawa is another variant of betel quid that contains Areca nut,
tobacco, and lime. Mawa is popular in Bhavnagar district and nearby areas. By weight, more than 90 percent of mawa is Areca nut. One quid may be chewed for 10 to 20 min. Some users may chew only half of the quid at one time (Sinor et al., 1990). There are also several methods of smokeless tobacco use. One of them is use of manufactured snuff, which is common in the Western Region. The finer snuff is used for nasal use and coarser snuff for oral use. Mishri is a powdered form of roasted tobacco. It is common in Maharashtra and central regions of India, especially among women. People begin using mishri as a dentifrice, but it soon turns into an addiction. A typical user applies mishri to the teeth and gums several times a day.

Tobacco is also used in the form of ‘gudakhu’, a paste made of tobacco and molasses. This is common in the eastern region. Creamy snuff, common in Goa, is a manufactured item marketed in toothpaste-like tubes. Its marketing technique exploits the prevailing misconception that tobacco is good for the teeth and gums. There are several herbal and medicinal tooth powders that contain tobacco (Gupta et al., 2002).

The most extensively studied and best documented health consequence of smokeless tobacco use in India is oral cancer. Numerous case-control and some cohort studies have clearly demonstrated the causal role of smokeless tobacco in oral cancer (IARC, 1984). Oral mucosal lesions are common in smokeless tobacco users.
In surveys of adolescents, lesions ranging from small local mucosal changes involving slight colour and texture modifications to more significant color changes and deep furrowing have been observed in between 23 and 63 percent of smokeless tobacco users (Centers for Disease Control, 1988; Greer and Poulson, 1983; Offenbacher and Weathers, 1985; Poulson et al., 1984), far exceeding those among non-users (Offenbacher and Weathers, 1985).

Smokeless tobacco users have increased risk of ischaemic heart disease (Bolinder, 1994). However, the actual scenario is controversial as two Scandinavian case-control studies found that snuff dippers had no increased risk of myocardial infarction compared with non-tobacco users (Huhtasaari, 1992, 1999). A recent analysis of adults suggested that chewing tobacco may be a risk factor in the development of root surface caries and possibly coronal caries. Interestingly, the decayed or filled root surfaces tended to match the side of the mouth on which the smokeless tobacco was used although this did not reach statistical significance (Tomar, 1999). Experimental evidence also suggests that chewing tobacco may be cariogenic (Going, 1980). Numerous studies have shown a strong association between smokeless tobacco and adverse pregnancy outcomes, particularly low birth weight (Krishnamurthy, 1993).

The population attributable risk (PAR) is a measure of the proportion of the disease that could theoretically be prevented in the population if the use of smokeless tobacco was eradicated. PAR value for European countries like Sweden
was lower (between 0 and 60); but in India it is very high with over 10,000 deaths possibly attributable to smokeless tobacco use alone (IARC, 2000; WHO, 1997).

The adverse health effects of smoking tobacco have been investigated in great details. However, besides positive correlation between smokeless tobacco consumption and oral cancer, other effects of smokeless tobacco still remains an under explored area. Generalisation of these studies is also difficult because of differences in smokeless tobacco types, which vary in their constitution across the globe (Critchley and Unal, 2003).

### 2.2.2. Smoking Tobacco

Smoking is known to be a major source of Cd exposure. The Cd content of cigarettes may vary over a wide range from 0.29–3.38 mg/g and smokers are subjected to ill effect of slow Cd poisoning (Watanabe et al., 1987). The quantity of Cd inhaled from smoking one cigarette is about 10% of the total Cd content in the cigarette (Elinder et al., 1983). A two-fold higher level in milk Cd concentration in mothers who are current smokers has been reported by Radisch et al. (1987). Maternal Cd exposure was found to be associated with pre-term birth and a lower birth weight (Nishijo et al., 2002). Exposure to environmental tobacco smoke (ETS) has been reported to endanger the foetus and has adverse pregnancy outcomes such as diminished birth weight, preterm
delivery, term-low birth weight as well infant length (National Cancer Institute, 1999; WHO, 1999; Windham, 1999).

Cigarette smoke contains many oxidants and free radicals and may therefore cause considerable oxidative stress, resulting in higher levels of lipid peroxidation products (Rust et al., 2001). Lower levels of vitamin C, folate, and provitamin A carotenoids (Alberg, 2002; Kim et al., 2003), commonly found in smokers, are thought to be caused by oxidative stress as well as by diverse diet habits: smokers differ from non-smokers with respect to several lifestyle behaviors, including eating less healthful diets, such as less fruits or vegetables that are major sources of vitamin C and carotenoids. Irrespective of the reasons for a poor vitamin status, it may be assumed that smokers need larger quantities of supplemental antioxidants in order to compensate their a priori lower body depots (compared to non-smokers). Cardiovascular disease and asthma demonstrate a strong etiologic association with smoking (Benovitz, 1991).

2.3. Betel Quid Chewing and Genotoxicity

The DNA is the critical target for lethal, carcinogenic, teratogenic and mutagenic effects of various toxins and environmental mutagens (Chaubey, 2001). In humans, approximately 107 cells divide per second. Estimates suggest that spontaneous mutations arise in about a third of those cells. The chemical events that lead to DNA damage include hydrolysis, oxidation and electrophilic attack.
These reactions are triggered by exposure of cells to exogenous chemicals (e.g. environmental agents, food constituents, etc.), or they can result from endogenous metabolic processes (Marnett, 2001). The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. Mutagenesis is involved in the pathogenesis of many neoplasias and often occurs through mechanisms that involve chromosome damage (Roth 2003). A requisite for the survival of living forms is the ability to continually adapt and change. Since the beginning of the industrial revolution, organisms, including humans, have been required to adapt to a large number of new man-made chemicals in their environments. We are challenged to cope, not with discreet pure chemicals, but with various mixtures of chemicals and the products of their interactions. Our ability to respond and change is far from perfect, and even the very process of adaptation may lead, in some individuals, to adverse health outcomes (Legator et al., 1994).

There is an increasing effort worldwide to determine the impact of environmental, genetic and life-style factors on genomic stability in human populations (Doina, 2004). The cytological alterations found in the exposed groups consisted not only in evidences of ferruginous bodies and inflammatory processes, but also in nuclear atypias, mainly micronuclei in respiratory cells, and other abnormalities as karyolysis, karyorrhexis, broken-egg type or binucleated cells. Various guidelines and strategies have been established over the past two decades for evaluating chemicals that may be mutagenic and/or carcinogenic in
humans. These procedures have been applied to pharmaceuticals, food additives, chewable products such as betel quid, pesticides, and industrial and environmental chemicals (Waters et al., 1999).

It was Herman Druckrey, at a conference in Sweden who first used the word genotoxic for chemicals that can react with DNA, and thus have the potential of being mutagenic, cell transforming, and carcinogenic (Weisberger, 1994). The term genotoxic is popularized as agents that were DNA reactive, directly or after biochemical activation, with appropriate fractions from liver or other tissue of rodents or humans. In sharp contrast, there are other chemicals and agents that are clearly not mutagenic, but that have the ability of increasing the effectiveness or efficiency of a genotoxic carcinogen. The classic promoters of carcinogenesis fall into that class. The fidelity of DNA replication is decreased during carcinogenesis (Weisberger, 1994). A major goal for genetic toxicologist is to provide precise information on exposure and health risk assessment for effective prevention of health problems (Salama et al., 1999). DNA repair can operate effectively and restore the integrity of DNA but only if this has a chance of operating prior to the synthesis of new DNA and mitosis, underwriting the importance of cell division (Weisberger, 1994). Several studies in the past decade have implicated a probable role of betel quid chewing in causing genotoxic damage in humans. In order to understand the genotoxicity of betel quid it is necessary to have a brief overview of its constituents as given below.
2.3.1. Chemical Constituents of Betel Quid

Comprehensive analyses of the chemical composition of Areca nut have been reported and reviewed (Raghavan & Baruah, 1958; Shivashankar et al., 1969; Arjungi, 1976; Jayalakshmi & Mathew, 1982). The major constituents of the nut are carbohydrates, fats, proteins, crude fibre, polyphenols (flavonols and tannins), alkaloids and mineral matter. Among the chemical ingredients, tannins, alkaloids and some minerals that may have biological activity and adverse effects on tissues have been subjected to detailed study. Polyphenols (flavonols, tannins) constitute a large proportion of the dry weight of the nut. The polyphenol content of a nut may vary depending on the region where Areca catechu is grown, its degree of maturity and its processing method. The tannin content is highest in unripe Areca nuts and decreases substantially with increasing maturity (Raghavan & Baruah, 1958). The roasted nut possesses the highest average content of tannins, ranging from 5 to 41% (mean, 21.4%); the average tannin content of sun-dried nuts is 25%; and the lowest levels are seen in boiled nuts, which contain 17% (Awang, 1987). Among the chemical constituents, alkaloids are the most important biologically. The nut has been shown to contain at least six related alkaloids, of which four (arecoline, Arecaidine, guvacine and guvacoline) (Figure 1) have been conclusively identified in biochemical studies (Raghavan and Baruah, 1958; Huang and McLeish, 1989; Lord et al., 2002). The relative proportion of these four alkaloids in Areca nut is given in Table 1.
2.3. Betel quid chewing and genotoxicity

Arecoline is generally the main alkaloid. Holdsworth et al. (1998) and Self et al. (1999) reported the presence of at least six other related alkaloids in addition to arecoline and guvacoline which were identified as nicotine (~0.02%), methyl nicotinate, ethyl nicotinate, methyland ethyl-N-methyl piperidine-3-carboxylate and ethyl-N-methyl-1,2,5,6-tetrahydro-pyridine-3-carboxylate.

Table 1: Alkaloid content of fresh Areca nuts from Darwin, Australia.

<table>
<thead>
<tr>
<th>Name of Alkaloid</th>
<th>% of Total Alkaloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arecoline</td>
<td>0.30–0.63</td>
</tr>
<tr>
<td>Arecaidine</td>
<td>0.31–0.66</td>
</tr>
<tr>
<td>Guvacoline</td>
<td>0.03–0.06</td>
</tr>
<tr>
<td>Guvaccine</td>
<td>0.19–0.72</td>
</tr>
</tbody>
</table>

* Adopted from Huang & McLeish (1989)
2.3. Betel quid chewing and genotoxicity

2.3.1. Elemental composition

Concentrations of sodium, magnesium, chlorine calcium, vanadium, copper, manganese and bromine were measured in Areca nut (Ridge et al., 2001). There is about 36 other trace elements have been reported to be present in Areca nut (Zaidi et al., 2002). The copper content in samples of raw and processed Areca nut was analysed and reported to be much higher than that found most frequently in other nuts consumed by humans and probably plays a significant role in fibrogenic, mutagenic and toxic effects of Areca nut (Trivedy et al., 1997). The concentration of copper in samples of commercially available Areca nut was found to be 18 ± 8.7 μg/g, which was higher than raw betelnut (Trivedy et al., 1999).

2.3.1.2. Betel leaf

The major accompaniment for chewing Areca nut is the leaf of Piper betle. Betel leaves contain betel oil, a volatile liquid, which contains several phenols including hydroxychavicol, eugenol, betel phenol and chavicol. Vitamin C (1.9 mg/g) and a large amount of carotenes (80.5 mg/g) have also been reported (Wang & Wu, 1996). Mean concentrations of 36 trace elements are present in betel leaf of which Manganese content is highest (Mn 380 ± 38 μg/g) (Zaidi et al., 2002).

2.3.1.3. Slaked lime

Slaked lime (calcium hydroxide) is often combined with Areca nut. In coastal areas, it is obtained by heating the covering of shell fish (sea shells) or
is harvested from corals. In central parts of a country, it is quarried from limestone (Gupta & Warnakulasuriya 2002). Mean concentrations of 35 trace elements (Zaidi et al., 2002). Free calcium hydroxide, iron(II) and magnesium(II) are the major components (Nair et al., 1990).

2.3.2. Genotoxicity and Mutagenicity

Elevated micronucleus formation and chromosome breaks have been reported in oral exfoliated cells in chewers of betel quid with or without tobacco (Stitch et al., 1981). Studies on betel quid chewers revealed that betel quid chewers in Orissa (India) who regularly chewed dried Areca nut, slaked lime, betel leaf, tobacco and catechu had the highest frequencies of micronucleated cells (6.1%), followed by those in Khasis (4.7%) of Meghalaya (India), who chewed fresh Areca nut, slaked lime and betel leaf; the Philippines (3.9%) chewing fresh Areca nut, betel leaf, slaked lime and tobacco; Guam (1.8%) chewing fresh green Areca nut with husk, slaked lime and betel leaf; and Hualien (1.7%) (in Taiwan, China), with fresh Areca nut with husk, slaked lime and betel leaf chewing habits (Stich et al., 1986).

The frequencies of micronuclei in exfoliated mucosal cells were shown to be higher in chewers (with or without oral submucous fibrosis) of 'tamol (raw fermented Areca nut, betel leaf and slaked lime) and Areca nut in different regions of India compared with healthy individuals with no habit (n = 10–36 for

29
each group) (Kayal et al., 1993). Nair et al. (1991) reported that the frequency of micronuclei in exfoliated human oral mucosa cells was $4.83 \pm 0.7$ per 1000 cells in chewers of betel quid with tobacco ($n = 35$; tobacco, Areca nut, betel leaf, slaked lime and catechu) and $5.2 \pm 0.66$ per 1000 cells in chewers of tobacco and slaked lime ($n = 35$), whereas the frequency in the control group ($n = 27$) was $2.59 \pm 0.37$ per 1000 cells. No correlation between the frequencies of micronucleated cells and the duration or frequency of the chewing habit was noted. In another study cytogenetic effects in controls ($n = 15$), healthy Areca nut chewers ($n = 10$), Areca nut chewers with oral submucous fibrosis ($n = 10$) and Areca nut chewers with oral cancer ($n = 8$) were evaluated. All three groups of Areca nut chewers showed significantly large numbers of chromosomal aberrations and sister chromatid exchange in peripheral blood mononuclear cells and the frequencies of micronucleated exfoliated buccal cells increased by approximately 3.8-fold (Dave et al., 1992a).

The secretion of TIMP-1 in response to increases in circulatory MMP-9 in humans varies with the Taq 1 polymorphism of the vitamin D receptor (VDR) gene (Taq 1 VDR genotypes) has been reported to increase in relation to Areca nut consumption (Timms et al., 2002a).

Aqueous betel quid extract induced sister chromatid exchange in Chinese hamster ovary-K1 cells in vitro, (Wang et al., 1999). Aqueous betel
2.3. Betel quid chewing and genotoxicity

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Betel quid extract has been reported to induce micronucleus formation in polychromatic erythrocytes in Swiss mice *in vivo* (Shirname et al., 1984) and chromosomal aberrations in Chinese hamster ovary-K1 cells *in vitro* (Wang et al. (1999). Aqueous Areca nut extract induced DNA strand breaks and DNA–protein crosslinks in human primary buccal epithelial cells *in vitro* (Sundqvist et al., 1989). Exposure of Hep 2 human laryngeal carcinoma cells to aqueous acetic acid, hydrochloric acid or ethanol extracts of Areca nut induced unscheduled DNA synthesis (Sharan and Wary 1992). It also resulted in elevated sister chromatid exchange (Panigrahi & Rao, 1984), micronucleus formation and chromosomal aberrations in the bone marrow cells of Swiss mice *in vivo* (Deb & Chatterjee, 1998; Deb & Chatterjee, 1998; Bhisey et al., 2004).

Areca nut extract with lime induced increased frequency of micronucleus formation in hamster cheek pouch buccal epithelial cells *in vivo* (Nair et al., 1992). Cultured CHO cells were exposed to urine concentrates (10 μl/ml) collected from 20 Areca nut chewers (without smoking or alcohol drinking) for 3 h. Marked elevation of chromosomal aberrations and sister chromatid exchange was noted compared with exposure to urine concentrates from control subjects, even without metabolic activation (Trivedi et al., 1995). Eugenol and dihydroxychavicol, metabolites of safrole, are found in the urine of betel quid chewers (Krol & Bolton, 1997; Nikolic et al., 1999; Thompson et al., 1993).
2.3.3. Formation of Nitrosamines from Arecoline and Betel Quid

Betel quid or Areca nut chewing results in exposure to Areca nut alkaloids, N-nitroso-compounds formed from these compounds during chewing, polyphenols and trace elements which are probably responsible for the DNA damaging properties of betel quid (IARC, 1985). Three Areca nut derived nitrosamines, N-nitrosoguvacoline (NGL), N-nitrosoguvacine (NGC) and 3-methylNitrosamino-propionitrile (MNPN), a rodent carcinogen, were detected in the saliva of chewers of betel quid with or without tobacco and are most probably produced in situ during betel quid chewing (Wenke et al., 1984; Nair, et al., 1987; Prokopczyk et al., 1987). The highest levels of Areca nut derived nitrosamines (NGL) were found in the sediment of saliva collected from Taiwanese betel quid chewers (Stich et al., 1986).

2.3.4. Formation of N-nitroso Compounds in Oral Cavity

Areca nut and tobacco contain secondary and tertiary amines that can be nitrosated in the saliva during the chewing of betel quid when they react with available nitrite in the presence of nitrosation catalysts such as thiocyanate. Micrograms per millilitre levels of nitrite and thiocyanate have been reported in the saliva of chewers of betel quid (Nair et al., 1985; Nair, et al., 1987). Endogenous nitrosation has been demonstrated in chewers of betel quid mixed with proline, by measuring N-nitrosopropline in saliva and urine (IARC, 1985).
There is more extensive formation of nitrosamines in subjects with poor oral hygiene if they chew tobacco. The enhanced endogenous nitrosation in subjects with poor oral hygiene may be due to the increased conversion of nitrate to nitrite or bacterial enzyme mediated formation of nitrosamines or both (Calmels et al., 1988). Increased formation of nitrite and nitric oxide (NO) in the mouth has been reported during the formation of dental plaque (Carossa et al., 2001).

2.3.5. Formation of Reactive Oxygen Species in Oral Cavity

Reactive oxygen species such as the hydroxyl radical (HO•) are generated in the oral cavity during betel quid chewing which probably contribute to the genetic damage observed in the oral epithelial cells of chewers (Chen et al., 2002). This reaction is enhanced by alkaline pH (from the slaked lime that is consumed with the quid) and by the presence of the transition metals like copper and iron (present in Areca nut) (Nair et al., 1990). Alkaline pH is important for the formation of reactive oxygen species that is likely to occur via autoxidation of polyphenols, redox cycling via quinone semiquinone radicals and iron catalysed Haber-Weiss and Fenton reactions (Nair et al., 1996). Thus, micronuclei and other markers of DNA damage have been demonstrated in exfoliated cells obtained from chewers of betel quid. Animal studies and in-vitro test systems support the suggestion that extracts of Areca nut can exert genotoxic effects.
However, most of the studies on betel quid genotoxicity have been done in Far East South Asian countries. Reports are scarce on the genotoxic effects of betel quid in the North Eastern part of India.

2.4. Smokeless Tobacco, Smoking Tobacco and Genotoxicity

As Christofides has noted, there is a need for reliable data on trends in tobacco use, disaggregated by sex, age, social class, ethnicity and other social factors (Christofides, 2001). Tobacco use also must be widely defined to include smokeless forms popular in south-east Asia (Christofides, 2001; Morrow, 2003).

2.4.1. Chemical Constituents of Tobacco

Tobacco contains 28 carcinogens, including tobacco specific nitrosamines (TSNAs) (NIDCR-NCI, 2003). The genotoxic effect from long term smoking tobacco use can probably be attributed to the presence of TSNAs. There are four principal compounds: N-nitrosonomicotine (NNN), 4-methyl-N-nitrosamino-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB).

Only two TSNAs such as NNN and NNK are considered to be potential carcinogens (IARC, 1985a), based on the fact that there is sufficient evidence of their carcinogenicity in experimental animals but no data in humans. On the other hand, both NAT and NAB are designated by IARC as not classifiable with
regard to carcinogenicity (IARC, 1985b). There are inadequate data in experimental animals and no data in humans. Benzo(a)pyrene, which has been classified by IARC in Group 2A (IARC, 1983) has been reported to be present in tobacco largely as a result of fire-curing. The burning of tobacco produces numerous genotoxic compounds, including polynuclear aromatic hydrocarbons, heterocyclic amines, nitrosamines, and aromatic amines (IARC, 1986). Benzo[a]pyrene, considered to be the bullet of the 'smoking gun', is the most extensively studied carcinogen in cigarette smoke (Osborn, 1987). Tobacco smoke contains many thousands of chemicals including a plethora of carcinogens (Hoffmann, 1990). Many carcinogens undergo metabolic activation in mammalian tissues to reactive intermediates that interact with and modify informational macromolecules such as DNA (Miller, 1981; Phillips, 2002) with potentially mutagenic consequences.

### 2.4.2. Formation of Nitrosamines from Tobacco

When tobacco is smoked, nicotine enters the bloodstream through the lungs. When it is sniffed or chewed, nicotine passes through the mucous membranes of the mouth or nose to enter the bloodstream. The chemical structure of nicotine is similar to that of acetylcholine and it is also able to activate cholinergic receptors. But, unlike acetylcholine, when nicotine enters the brain and activates cholinergic receptors, it can disrupt the normal functioning of the brain. NNN and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), tobacco-specific nitrosamines, formed from nicotine by nitrosation during the processing and storage of
tobacco and in the mammalian organism (Hecht and Hoffmann, 1990). The alkaloid nicotine is a tertiary amine consisting of a pyridine and a pyrrolidine ring. NNN closely resembles nicotine in structure whereas the formation of NNK from nicotine results in ring opening of the pyrrolidine ring (Fischer et al., 1990). NNN and NNK are powerful carcinogens. This effect is thought to be initiated by the formation of pro mutagenic DNA-adducts from reactive metabolites (Hecht, 1993). In addition, the volatile nitrosamines like N-nitrosodimethylamine and N-nitrosodiethylamine (Bhide et al., 1986) and the TNSAs like N-nitrosonomicotine, 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanone and N'-nitrosoanabasine are present in the saliva of chewers of betel quid with tobacco (Bhide et al., 1986; 1984; Wenke et al., 1984; Nair, et al., 1987). The highest levels of tobacco-specific nitrosamines were detected in samples collected from India (Bhide et al., 1986). Volatile nitrosamines and tobacco specific nitrosamines found in the saliva of chewers could result from the leaching of those present in tobacco or could be formed endogenously during chewing from abundant precursors.

2.4.3. Genotoxicity and Mutagenicity

Different forms of smokeless tobacco have been reported to induce genotoxicity. Adhvaryu et al. (1991) analysed sister chromatid exchange and chromosomal aberrations in peripheral lymphocytes and micronuclei in exfoliated mucosal cells in healthy mava (tobacco, Areca nut and lime) chewers, mava chewers with oral submucous fibrosis and mava chewers with oral cancer (n =
2.4. Smokeless tobacco, smoking tobacco and genotoxicity

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15 for all groups). Chromosomal aberrations and sister chromatid exchange were significantly higher for all three groups of chewers than for controls. Adhvaryu et al. (1986) compared the frequency of sister chromatid exchange in cultured lymphocytes isolated from controls \((n = 15)\), tobacco chewers \((n = 10)\) and oral submucous fibrosis subjects \((n = 10)\) who chewed a combination of tobacco, Areca nut and slaked lime. The frequencies of micronuclei were higher in healthy chewers and oral submucous fibrosis subjects. Similar results were reported in Gudakhu (Das et al., 1992) and Masheri users (Mahimkar et al., 2000).\(32^\text{P}\)-postlabelling technique in Indian Khaini tobacco chewers \((n = 22)\) revealed differential amounts of five aromatic DNA adducts (Dunn & Stich, 1986). Dave et al. (1991) compared 30 healthy controls and 15 ‘pan masala’ consumers with respect to cytogenetic effects in peripheral blood lymphocytes and exfoliated cells from buccal mucosa. Sister chromatid exchange and chromosomal aberrations of peripheral blood lymphocytes were more frequent in cells of ‘pan masala’ chewers than in those isolated from control subjects. The percentage of micronucleated cells in exfoliated buccal mucosal cells was also higher in ‘pan masala’ consumers than in healthy controls. Nair et al. (1991) reported that the frequency of micronuclei in exfoliated human oral mucosa cells was significantly different in chewers of betel quid with tobacco compared to chewers of tobacco and salked lime. No correlation between the frequencies of micronucleated cells and the duration or frequency of the chewing habit was noted. Similar results were reported in other studies outside India on other
forms of smoking tobacco use (nass, naswar, maras, snuff, Qat, miraa, cathine) (Aleksandrova et al., 1970; Merchant et al., 2000; Ozkul et al., 1997; Celik et al., 2006; Gupta et al., 1984; Ahmed et al., 2003). Elevated micronucleus frequency along with other nuclear abnormalities was also reported in exfoliated buccal epithelial cells in female snuff users (Tolbert, 1991).

Wu et al. (2004) reported a positive trend between micronuclei frequency and either smoking intensity (e.g., daily cigarette consumption) or cumulative smoking (e.g., packyears). All confounders studied were negatively associated with the frequency of micronuclei in buccal cells. In a study of 120 healthy subjects, Konopacka (2003) reported that the frequency of micronuclei of oral epithelial cells was three times greater in smokers \( (n = 50) \) than nonsmokers \( (n = 70) \). Reports to the contrary have also been found (Nerseyan, 2006). Tobacco associated oral changes have been summarized by Greer (1986).

Urine isolated from controls and chewers of masheri \( (n = 23) \) and betel quid with tobacco in addition to masheri \( (n = 34) \) induced little or weak mutation in S. typhimurium TA98 and TA100. In the presence of metabolic activation and nitrite, the mutagenicity of urine samples collected from controls and chewers showed greater mutagenicity in S. typhimurium TA98 but not in TA100 compared with urine samples from control subjects (Govekar & Bhisey, 1993). Aqueous extracts of pan masala with tobacco induced chromosomal aberrations, sister chromatid exchange and micronucleated cells in Chinese hamster ovary (CHO)
cells in the presence and absence of an exogenous metabolic system, although metabolic activation markedly inhibited the chromosomal damaging effect, implicating the presence of direct acting mutagens and clastogens (Jaju et al., 1992). Ozkul et al. (1995) reported the SCE inducing effect of Maras powder (a smokeless tobacco preparation used in south eastern Turkey) on T-lymphocytes. Tobacco smoking has been reported to increase risks to oral and lung cancer and to the formation of micronucleated cells in the buccal mucosal cells (Celik et al., 2006). Animals have been studied as potential surrogates to define human health risks associated with smoking. Studies of lung macrophages and blood lymphocytes a significant increase in micronuclei frequencies was observed for exposed animals compared with nonexposed animals exposed to tobacco smoke (D’Agosini, 2001; Balansky, 2000) and smokeless tobacco (Stich, 1987; Chen, 1989).

2.4.4. Precancerous Lesions and Condition

Studies on the natural history of oral cancer suggest that several potentially malignant lesions and conditions precede the development of cancer of the oral cavity (IARC, 2004). In a World Health Organization (WHO) Workshop, held in 2005, the terminology, definitions and classification of oral lesions with a predisposition to malignant transformation have been discussed. The term “potentially malignant” was preferred above “premalignant” or “precancerous” (Warnakulasuriya et al., 2007).
Barnes et al. (2005) proposed various criteria for classification of oral dysplasia according to various criteria as summarized below.

- **Architecture**
- Irregular epithelial stratification
- Loss of polarity of basal cells
- Drop-shaped rete ridges
- Increased number of mitotic figures
- Abnormal superficial mitoses
- Premature keratinization in single cells (dyskeratosis)
- Keratin pearls within rete pegs
- **Cytology**
- Abnormal variation in nuclear size (anisonucleosis)
- Abnormal variation in nuclear shape (nuclear pleomorphism)
- Abnormal variation in cell size (anisocytosis)
- Abnormal variation in cell shape (cellular pleomorphism)
- Increased nuclear-cytoplasmic ratio
- Increased nuclear size
- Atypical mitotic figures
- Increased number and size of nucleoli
- Hyperchromasia

The histological changes of oral epithelia under different pathological conditions as proposed by Barnes et al. (2005) are summarized in Table 2.
Table 2: Histopathological staging of oral epithelial precancerous lesions.

<table>
<thead>
<tr>
<th>Sl.</th>
<th>Type of Lesion</th>
<th>Histological Descriptions</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Squamous hyperplasia</td>
<td>This may be in the spinous layer (acanthosis) and/or in the basal/parabasal cell layers (basal cell hyperplasia); the architecture shows regular stratification without cellular atypia.</td>
</tr>
<tr>
<td>2.</td>
<td>Mild dysplasia</td>
<td>The architectural disturbance is limited to the lower third of the epithelium accompanied by cytological atypia.</td>
</tr>
<tr>
<td>3.</td>
<td>Moderate dysplasia</td>
<td>The architectural disturbance extends into the middle third of the epithelium; consideration of the degree of cytological atypia may require upgrading.</td>
</tr>
<tr>
<td>4.</td>
<td>Severe dysplasia</td>
<td>The architectural disturbance involves more than two thirds of the epithelium; architectural disturbance into the middle third of the epithelium with sufficient cytologic atypia is upgraded from moderate to severe dysplasia</td>
</tr>
</tbody>
</table>

2.4.4.1. Leukoplakia

Leukoplakia is at present defined as “A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer” (Warnakulasuriya et al., 2007). The estimated reported prevalence of oral leukoplakia, worldwide, is approximately 2% (Petti, 2003). The prevalence of oral leukoplakia has to be set at a more realistic figure of less than 0.5%. There are some geographical differences with regard to the gender distribution.
Leukoplakia is 6 times more common among tobacco users than among nonusers. Dose response relationship between the development of leukoplakia and chewing betel quid with or without tobacco has been reported. The age adjusted prevalence of leukoplakia was higher among men than women and the prevalence increased with the number of quids chewed per day (Gupta et al., 1984). Chewers of lao-hwa quid had the highest risk for oral leukoplakia (Lee et al., 2003). There are conflicting results of studies related to the possible role of human papillomavirus infection leukoplakia (Bagan et al., 2007; Campisi et al., 2004).

Leukoplakia may affect any site of the oral and oropharyngeal cavity. Clinically, leukoplakia can be subdivided in a homogeneous type (flat, thin, uniform white in colour) and a non-homogeneous type. The non-homogeneous type has been defined as a ‘white and red lesion’ ("erythroleukoplakia"), that may be either irregularly flat (speckled) or nodular. Verrucous leukoplakia is yet another type of non-homogeneous leukoplakia. Although verrucous leukoplakia usually has a uniform white appearance, its verrucous texture is the distinguishing feature from homogeneous (flat) leukoplakia. Verrucous leukoplakia is clinically indistinguishable from the clinical aspect of verrucous carcinoma. Proliferative verrucous leukoplakia (PVL) is a subtype of verrucous leukoplakia, (Hansen et al., 1985) being characterized by multifocal presentation, resistance to treatment and a high rate of malignant transformation.
Histopathologically, a distinction can be made between dysplastic and non-dysplastic leukoplakia. The assessment and severity of dysplasia is based on architectural disturbance accompanied by cytological atypia. The WHO 2005 classification recognizes five histopathological stages in epithelial precursor lesions (Barnes et al., 2005). Dysplasia is a spectrum and no specific criteria exist to precisely divide this spectrum into mild, moderate and severe categories. Sometimes, a diagnosis of verrucous carcinoma, carcinoma in situ or invasive squamous cell carcinoma is made in the clinical presentation of leukoplakia. In such cases the histopathological diagnosis replaces the clinical diagnosis of leukoplakia. Proliferative verrucous leukoplakia may show a spectrum of histopathological changes, ranging from hyperkeratosis with or without dysplasia to verrucous hyperplasia and verrucous carcinoma. Some authors consider verrucous hyperplasia an early stage of verrucous carcinoma, (Murrah et al., 2004) while others regard to be distinct from verrucous carcinoma (Shear and Pindborg, 1980).

Annual malignant transformation rate of 0.3% - 1% have been reported (Gupta et al., 1980). This figure is much higher for non-homogeneous types (Holmstrup et al., 2006) including proliferative verrucous leukoplakia. The latter probably nearly always transforms into verrucous carcinoma or squamous cell carcinoma and may do so in a protracted course of over 10–15 years. Malignancies may develop within the site of pre-existing leukoplakia, but may also occur elsewhere in the oral cavity or the upper aerodigestive tract.
The commonly recognized factors that statistically carry an increased risk of malignant transformation into a squamous cell carcinoma are the presence of epithelial dysplasia – often correlating with a clinical non-homogeneous, erythroleukoplakic subtype – is in general regarded the most important indicator of malignant potential. Nevertheless, it should be recognized that some dysplastic lesions may remain clinically unchanged or may even show complete regression (Gupta et al., 1980). Furthermore, carcinomatous transformation may also take place in non-dysplastic leukoplakia (Holmstrup et al., 2006). Genetic changes, particularly at chromosomes 3, have been demonstrated in the majority of keratotic, non-dysplastic lesions (Schwarz et al., 2007).

2.4.4.2. Erythroplakia

Erythroplakia is defined as “A fiery red patch that cannot be characterized clinically or pathologically as any other definable disease” (WHO, 1978; Reichart and Philipsen, 2005). The clinical appearance may be flat or even depressed with a smooth or granular surface. In case of a mixture of red and white changes such lesion is categorized as non-homogeneous leukoplakia (erythroleukoplakia).

Tobacco and alcohol use are considered important etiologic factors. An association was observed between chewing betel quid with tobacco and the risk for erythroplakia, after adjustment for age, sex, education, body mass index, pack-years of smoking and years of alcohol drinking (Hashibe et al., 2000;). An increase in the risk for erythroplakia was observed with an increase in the
frequency and duration of betel quid chewing, as well as for swallowing the juice and keeping the quid in mouth overnight (Hashibe et al., 2000a; Shiu et al., 2000). The possible role of *C. albicans* is at present still unclear.

Prevalence figures of erythroplakia are only available from studies performed in South and South-East Asia and vary between 0.02% and 0.83% (Reichart and Philipsen, 2005). Erythroplakia mainly occurs in the middle aged and the elderly. There is no distinct gender preference. Any site of the oral and oropharyngeal cavity may become involved, usually in a solitary fashion. This solitary presentation is often helpful in clinically distinguishing erythroplakia from erosive lichen planus, lupus erythematosus and erythematous candidiasis, since these lesions occur almost always in a bilateral, more or less symmetrical pattern.

Histopathologically, erythroplakia commonly shows at least some degree of dysplasia and often even carcinoma in situ or invasive carcinoma (Shafer and Waldron, 1975). Erythroplakia has high risk of malignant transformation. Probably by far erythroplakias undergoes malignant transformation. There are not enough documented series that would allow calculating a reliable annual malignant transformation rate.

### 2.4.4.3. Erythroleukoplakia

A mixture of red and white changes such lesion is usually categorized as non-homogeneous leukoplakia or ‘erythroleukoplakia’. It is a more aggressive form of leukoplakia. Erythroleukoplakia can be speckled, nodular or verruciform.
Nodular lesions have irregular blunt or sharp projections (Schepman et al., 1998).

Tobacco and betel quid chewing is believed to be a major etiological factor for erythroleukoplakia and often associated with the site of tobacco quid usage (Warnakulasuriya et al., 2007). The other known predisposing factors are exposure to alcohol, UV radiation, microbial infection, trauma and candidiasis (Shiu et al., 2000; Reichart and Philipsen, 2005).

This type of lesion can occur in all age group and in both gender. However, it is seen affecting a higher percentage of males older than 40 years. The common sites of occurrence are lip commissure, lip vermilion, buccal mucosa and gingival. Most cases of erythroleukoplakia show a hyperkeratotic response to an irritant (Van der Waal et al., 1997). Histologically, erythroleukoplakic lesions shows severe dysplasia. Erythroleukoplakia is recognized as having a higher risk status for malignant transformation (Warnakulasuriya et al., 2007).

2.4.4.4. Oral submucous fibrosis

Oral submucous fibrosis (OSF) is a chronic disorder characterized by fibrosis of the lining mucosa of the upper digestive tract involving the oral cavity, oropharynx and frequently the upper third of the oesophagus. The occurrence of oral submucous fibrosis is more or less restricted to South-East Asia, although a number of cases have been reported in other parts of the world, such as South Africa, Greece and the United Kingdom.
The disease is most likely caused by the habit of chewing Areca and betel quid or substitute (Tilakaratne et al., 2006). Taking into account atrophy as the underlying factor, an alternative pathway related to undernutrition has been proposed (Rajendran et al., 1989). A number of epidemiological surveys, case series reports, large sized cross sectional surveys, case control studies, cohort and intervention studies provide overwhelming evidence that Areca nut is the main aetiological factor for oral submucosal fibrosis. Most convincing evidence is derived from case control studies that estimate the odds ratios for Areca nut use among oral submucous fibrosis cases and a definite dose dependent relationship between Areca nut and causation of the disease (IARC, 2004; Yang et al., 2001; Jacob et al., 2004).

Clinically, oral submucosal fibrosis is characterized by a burning sensation, blanching and stiffening of the oral mucosa and oropharynx, and trismus. In advanced stages vertical fibrous bands appear in the cheeks, faucial pillars, and encircle the lips with an increasing loss of tissue mobility (Meghji et al., 1997). The hallmark of the disease is that it affects most parts of the oral cavity, pharynx and upper third of the oesophagus (Tilakaratne et al., 2006). Through an as yet unknown process, fibrosis and hyalinization occur in the lamina propria, which results in atrophy of the overlying epithelium (Trivedy et al., 2000). The atrophic epithelium apparently predisposes to the development of squamous cell carcinoma in the presence of carcinogens.
Oral submucosal fibrosis is well recognized as a potentially malignant disorder (Zain et al., 1999). In a long-term follow-up study the annual malignant transformation rate was approximately 0.5% (Murti et al., 1985). Besides oral precancerous lesions (oral leukoplakia and erythroplakia) and oral precancerous conditions (oral submucous fibrosis), some other betel quid associated lesions of the oral mucosa occur in habitual betel quid chewers prominent among which is betel chewer’s mucosa (Reichart et al., 2002). Betel chewer’s mucosa was first described by Mehta et al., 1971) and is characterized by a brownish-red discoloration of the oral mucosa. This discoloration is often accompanied by encrustation of the affected mucosa with quid particles, which are not easily removed, and a tendency for desquamation and peeling. The lesion is usually localized in and associated with the site of quid placement in the buccal cavity, and is strongly associated with the habit of betel quid chewing, particularly in elderly women (Reichart et al., 1996). Several epidemiological studies have shown that the prevalence of betel chewer’s mucosa may vary between 0.2 and 60.8% in different South-East Asian populations. At present, betel chewer’s mucosa is not considered to be potentially malignant (Rahman et al., 1997).

2.5. Chewing Habit and Carcinogenesis

Cancer of the oral cavity comprises approximately 30% of all malignant tumours of the head and neck. Nearly 95% of these are squamous cell carcinoma (OSCC). Worldwide, head and neck cancer is the sixth most common human
cancer. In South-East Asia and particularly in India cancer of the oral cavity is the most common cancer comprising 35% of all cancers in men and 18% in women. Numerous risk factors contribute in the genesis of squamous cell carcinoma in the oral cavity. Social factors and lifestyle habits have a major impact on the development of oral cancer (Shah and Zelefsky, 1998).

It has become clear that the induction of malignancies is much more complicated than originally thought, involving a series of genetic and epigenetic changes. Current evidence suggests that the mutagenic events involved in carcinogenesis are themselves produced by one of two broad modes of action. The first may be mediated by the covalent binding of a chemical or its metabolites to DNA or chromatin, or by their interference with DNA related processes, such as spindle function or transcription, thereby directly affecting the integrity of the genome (i.e., the structure or content of DNA). The second mechanistic process involves chemical alterations in homeostasis that may be mediated via tissue necrosis, apoptosis, or cellular turnover leading indirectly to the expression of mutations in DNA. From the point of view of risk assessment, being able to distinguish between these two modes of action (i.e., DNA reactive and DNA nonreactive) is important since chemicals that express mutagenicity via the second mode of action may provisionally be considered nongenotoxic. With evidence that gene mutations, gene amplifications, chromosomal rearrangements, and aneuploidy are associated with numerous types of tumors (Barrett, 1992), it remains essential to identify chemicals and other agents that are capable of
inducing the types of genetic alterations that could damage the genes involved in carcinogenesis. Short-term tests that detect these types of genetic damage, specifically in mammalian cells in vivo, continue to provide vital information needed for evaluating carcinogenic risks of chemicals to humans. The expression of mutagenic activity per se (the ability of a chemical to produce alterations in DNA structure or content) is clearly a critical mechanistic consideration when assessing carcinogenic potential. Most of the human carcinogens, with the exception of certain hormonal and immunosuppressive agents, have been demonstrated to be mutagenic in animal or human somatic cells (IARC, 1994). Various guidelines and strategies have been established over the past two decades for evaluating chemicals that may be mutagenic and/or carcinogenic in humans. These procedures have been applied to pharmaceuticals, food additives, pesticides, and industrial and environmental chemicals (Waters et al 1999).

A fundamental observation in cancer epidemiology during the last century was that cancer incidence and mortality rates vary dramatically across the globe (Armstrong, 1975). In addition, rates of cancer among populations migrating from low to high incidence countries change markedly; in most cases, they approximate the rates in the new region within one to three generations. These lines of evidence indicate that the primary determinants of cancer rates are not genetic factors, but rather environmental and lifestyle factors that could, in principle, be modified to reduce cancer rates in high-risk areas (Doll and Peto, 1981). While carcinogens can interact directly with DNA, more often carcinogens
are metabolized into reactive intermediates. Reactive intermediates that are not
detoxified cause damage to DNA by binding to DNA, forming carcinogen-DNA
adducts. Most frequently, DNA damage occurs at the C8 and N2 atoms of
guanine leading to transversions and frameshift mutations. Betel quid chewing
significantly enhanced the accumulation of mt DNA deletions in non-tumorous
oral tissues. Frequent loses in chromosome arms, high frequency and breakage
at the 1cen-1q12 region and allelic imbalance in short tandem repeat markers
were observed in analysis of cytogenetic changes betel quid and tobacco
associated oral squamous carcinomas (Mahale and Saranath, 2000; Lin et. al.
2002; Pai et al, 2002). These observations are consistent with the mutagenic
effects of betel quid and tobacco.

There is a substantial risk of oral cancers associated with the types of ST
used in India (chewing betel quid with tobacco). Studies from different regions
with varying chewing practices have consistently found statistically significant
and clinically important ORs associated with betel and tobacco chewing.(Sankaranarayanan, 1990; Dikshit, 2000). Many also found clear trends with
increased consumption, that is, dose-response relationships (Sankaranarayanan,

Tobacco smoking is the greatest single cause of preventable illness and
premature death, killing half of all people who continue to smoke for most of
their lives (Doll, 1994). Half of these will die before the age of 69. Thirty per cent
of all cancer deaths, including nearly 90% of deaths from lung cancer are caused by smoking. The cancers for which epidemiological studies have established a causal association with tobacco smoking are lung, urinary tract, kidney, oral cavity, sinonasal, nasopharynx, hypopharynx and oropharynx, oesophagus, larynx, pancreas, stomach, liver, colorectal, cervix and myeloid leukaemia (IARC, 2003). The exposure of human tissues and organs to these carcinogens, and their metabolic activation therein, is the mechanism by which smoking-related cancer is initiated (Alexandrov, 2002).

2.5.1. Experimental Studies

Desai et al. (1996) analysed the frequencies of micronuclei in exfoliated oral mucosa cells and sister chromatid exchange in lymphocytes isolated from healthy volunteers, and patients with oral submucous fibrosis, oral leukoplakia and oral lichen planus. These patients chewed pan masala, Areca nut, Areca nut with tobacco, or had mixed habits. The number of micronuclei in exfoliated cells was elevated in patients with oral submucous fibrosis, oral leukoplakia and oral lichen planus, respectively. A large number of micronucleated cells were also seen in circulating lymphocytes obtained from these three groups. Hsieh et al. (2003) reported that polymorphism in the DNA repair gene XRCCI, 399 Gln/Gln phenotype showed an independent association with the frequency of TP53 mutations (after adjustment for smoking, Areca quid chewing and alcohol drinking) in oral cancer patients in Taiwan, China. Comparison of p53 protein
expression in 22 baseline biopsies of oral precancerous lesions that transformed to cancer 4–25 years later with 68 other similar lesions that did not transform over the same period, no significant relationship between p53 protein expression and malignant transformation could be observed (Murti et al., 1998). Several studies reported a high incidence of p53 protein expression in oral premalignant lesions and squamous cell carcinomas from betel quid and/or tobacco consumers (Kerdpon et al., 2001; Chiang et al., 2000a; Chiang et al., 1999; Baral et al., 1998; Kaur et al., 1998a; Yan et al., 1996). High prevalence of H-ras mutations (codons 12, 13 or 61) and loss of allelic heterozygosity were reported in oral squamous cell carcinomas in Indian populations as compared to populations in the West (Saranath et al., 1991a, b; Munirajan et al., 1998). Betel quid chewing with or without tobacco is a common habit in this population. However, detailed data on chewing habits were not given in these studies. Ki-ras codon 12 mutations or p21 ras protein accumulation have been observed in Areca quid chewing and tobacco smoking related oral squamous cell carcinomas in a population in Taiwan, China (Kuo et al., 1994, 1995b). Mutations and/or alterations in the expression of cancer related genes (MTSl/p16, pRb, FHIT, APC, H-ras, Ki-ras, cyclin D1, MDM2, c-myc, p21WAF1, Stat-3, p27kip1, Bcl-2, Ets-1, RAR and HSP70) associated with betel quid chewing with or without tobacco have been observed in oral squamous cell carcinomas (Lin et al., 2000a; Chang et al., 2002; Nagpal et al., 2002; Pande et al., 2001; Kuo et al., 2002). Betel quid has potential tumour promoting activity and has been found to enhance
skin hyperplasia and inflammation. These promoting effects on mouse skin were associated with the induction of the expression of PKC and NF-κB (Lee et al., 2002). TP53 mutations were infrequent or absent in oral premalignant lesions and squamous cell carcinomas in subjects chewing betel quid without tobacco, but accumulation of p53 protein was observed of chewers of betel quid with tobacco (Hsieh et al., 2001; Ralhan et al., 2001).

A high prevalence of HPV-16 infection in 13/17 (76.4%) oral squamous cell carcinomas was reported in betel quid chewers and smokers in a Taiwanese population (Chang et al., 1989). A high prevalence of HPV-18 in addition to HPV-16 was also reported in 67/91 (74%) oral squamous cell carcinomas in betel quid chewers and smokers in an Indian population (Balaram et al., 1995). A lower prevalence of HPV infection (37/110; 33.6%) was observed in oral squamous cell carcinomas associated with reduced frequency of Pro/Pro allele frequency at codon 72 of TP53 in an eastern Indian population consuming betel quid and chewing tobacco (Nagpal et al., 2002).

2.5.2. Oncogenes and Tumour Suppressor Genes

Treatment of oral mucosal fibroblasts with Areca nut extract or arecoline induced an approximately threefold increase in c-jun mRNA levels. This increase was transient and c-jun mRNA returned rapidly to control levels thereafter. Furthermore, preincubation of cells with either N-acetyl-cysteine, a
GSH precursor, or BSO, a specific inhibitor of GSH biosynthesis, had a minimal effect on arecoline-induced c-jun expression, suggesting that this effect is independent of GSH status (Ho et al., 2000).

Lin et al. (2000) established a cell line, HCDB-1, from tumours induced by the application of 7,12-dimethylbenz[a]anthracene (DMBA)/betel quid extract from Taiwan, China, to hamster buccal pouch. Mutational analysis of TP53 revealed a C→T transition at codon 141 (Ala→Val) in these cells. The HCDB-1 cells were tumorigenic in nude mice. The APC gene of cultured cells from oral submucous fibrosis patients (8/8) had a CGA→GGA missense mutation at codon 498 (Arg→Gly) and 7/8 cell cultures from these patients had an adenine deletion at nucleotide 1494 that created a stop codon (TGA) at codon 504, while all (8/8) normal human gingival fibroblast cultures expressed the wildtype APC protein (Liao et al., 2001).

O6-Methylguanine-DNA methyltransferase (MGMT) repairs premutagenic O6-methylguanine lesions induced in DNA by alkylating agents (Pegg, 1990, 1995; Mitra, 1993). This reaction leads to the restoration of intact DNA concomitant with the functional inactivation of MGMT. The level of MGMT expression has a decisive role in the protection against toxic, mutagenic and carcinogenic effects of alkylating agents as demonstrated by clinical and experimental studies. Areca nut contains various mutagenic and carcinogenic N-nitroso compounds, some of which such as the tobacco specific carcinogen
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, can be metabolized by the oral mucosal epithelium to alkylating intermediates and aldehydes. The unrepaired O6-alkylguanine lesions are important in human oral carcinogenesis. Habitual use of the betel quid is likely to involve a significant formation of O6-alkylguanine in the oral mucosa (Liu, 1997). Alterations of DNA repair genes which are critical for maintaining DNA integrity can increase cancer susceptibility by increasing genomic instability.

2.5.3. Polymorphism in Carcinogen Metabolizing Enzymes

Several isozymes of cytochrome P450 (CYP) are involved in the metabolic activation of polycyclic aromatic hydrocarbons and nitrosamines and the phase II enzymes such as GST are predominantly involved in detoxification. The underlying molecular mechanisms of a number of these genetic polymorphisms in chewers in different populations have been found to be important for the onset to carcinogenesis (Vineis et al., 1999; Nair & Bartsch, 2001).

2.5.4. Genetic Susceptibility

Genetically determined host factors may thus modify the extent of DNA damage (Alexandrov, 2002). The inherited differences in the effectiveness of detoxification/activation of carcinogens play a crucial role in host susceptibility. The impact of GSTM1-null genotype on oral cancer risk was also analysed in
separate groups of individuals from India with different tobacco habits (297 cancer patients and 450 healthy controls). The odds ratios associated with GSTM1-null genotype was significantly high in chewers of tobacco with lime or with betel quid, bidi smokers and cigarette smokers. Furthermore, increased lifetime exposure to tobacco chewing appeared to be associated with a twofold increase in oral cancer risk in GSTM1-null individuals (Buch et al., 2002).

The effect of genetic variants of GSTM1 and GSTT1 on modifying the risk for oral leukoplakia was ascertained in genomic DNA from biopsies taken from 98 oral leukoplakia patients and exfoliated cells from 82 healthy controls from India (Nair et al., 1999). Most leukoplakia cases were heavy chewers (betel quid with or without tobacco; 15–20 quids per day), whereas the chewers among controls were regular but not heavy chewers (betel quid with or without tobacco; 1–2 quids per day). An extremely strong association was found between null genotypes of GSTM1 and GSTT1 singly or in combination with high risk for oral leukoplakia (Kietthubthew et al., 2001). GSTM1-null genotype predisposes towards oral cancer in individuals exposed to cigarette smoke, alcohol and betel quid (Hung et al., 1997).

2.5.5. Other susceptibilities and polymorphisms

Genotypes associated with the highest risk for oral submucous fibrosis for COL1A1, COL1A2, COLase, TGF-β1, LYOXase and CST3 were CC, AA, TT, CC, AA and AA, respectively, for the low exposure group, and TT, BB, AA,
CC, GG and AA, respectively, for the high exposure group. A trend was noted for an increased risk for oral submucous fibrosis with increasing number of high risk alleles for those with both high and low exposures to betel quid. The results imply that susceptibility to oral submucous fibrosis could involve multigenic mechanisms modified by the dose of exposure to betel quid (Chiu et al., 2002).

These pertinent health risks highlight the need for cessation of betel quid chewing. Indeed, betel quid chewing is a recognized public health problem. Study of the relationship among carcinogenic exposures and mutation studies have revealed that specific carcinogens can leave specific mutation spectra ‘fingerprints’ (Veneis et al., 1999).

2.6. Occupational Exposure and Health

The usage of biomarkers to study the association between exposure and early biological genotoxics effects is very relevant in individuals at high risk due to occupational exposure. Biomarkers of exposure and of early biological effects may help overcome the severe limitations of environmental exposure assessment in very complex occupational or environmental settings (Zabadi et al., 2008).

Several studies investigated population with different occupational exposure (Karahalil et al., 1998; Burgaz et al., 1998; Bolognesi et al., 1997;
Sarto et al., 1990; Zabadi et al., 2008; Zabadi et al., 2008). There are several studies on occupational exposure using micronucleus in exfoliated cells and lymphocytes as an endpoint. Studies on cytogenetic damage in road-paving workers (Burgaz et al., 1999), traffic police exposed to air pollutants (Bolognesi et al., 1997), automobile workshop workers (Karalahil et al., 1998) chromium platers and workers exposed to ethylene oxide (Sarto et al., 1990), found statistically significant increase in exposed groups compared to control groups.

Pesticides are the most important method in self poisoning in the developing world. Three million cases of pesticide poisoning, nearly 220,000 fatal, occur world wide every year (Eddleston et al., 2002). A total of about 890 active ingredients are registered as pesticides and currently marketed in some 20,700 pesticide products (US EPA, 1998).

Farmers are indirectly exposed to pesticides during agricultural practices. Cytogenetic studies on agricultural workers are contradictory. Study carried out on population of agricultural workers employed in an agronomic institute in Brazil, showed a significant increase of chromosome aberration frequencies despite the adoption of protective/preventive measures (Antonucci and Syllos Colus, 2000). Banana plantation workers in Costa Rica revealed a substantial increase in chromosomal abnormalities measured by the standard chromosome aberration assay and an abnormal DNA repair response using the challenge assay (Au et al., 1999). The lack of genetic damage was also observed in a
number of studies concerning farm workers (Gomez-Arroyo et al., 1992, Pastor et al., 2002). Chronic exposure to low doses of complex mixtures of pesticides has been reported to induce cumulative cytogenetic effects (Bolognesi, 2003). It was observed that individuals working exclusively in greenhouses showed higher levels of chromosomal damage as MN than subjects working in open-fields (Joksic, et al., 1997; Bolognesi et al., 1993).

Long term exposure to low as well as chronic pesticide exposure in agriculturists is associated with a broad range of nonspecific symptoms, including headache, dizziness, fatigue, weakness, nausea, chest tightness, difficulty in breathing, insomnia, confusion, and difficulty concentrating. Many of the studies indicate that pesticide exposure is associated with deficits in cognitive function (Kamel and Hoppin, 2004). However, the degree of these manifestations varies to a great extent in farmers and plantation workers (Kamel and Hoppin, 2004).

Although tea garden is an important industry, there is scarce, if any, report on cytogenetic studies on the tea plantation workers (Medhi et al. 2006). There are few published reports on the effect of tea on respiratory function in workers occupationally exposed to tea dust (Zuskin et al 1984). It has been reported that exposure to dust during the processing of different types of tea may cause acute or chronic respiratory symptoms to develop (Zuskin et al 1984). The tea garden workers are directly or indirectly exposed to pesticides at least twice in a year during pesticide application in the garden. The pesticides
most often used are chlororganics, and more recently, carbamates, organophosphates and pyrethroids, which have been reported to be positive for genotoxic effects in bacterial and mammalian test systems (Dearfield et al. 2002; Dearfield et al. 1999). A number of studies on pesticide exposure has reported increased levels of cytogenetic damage in the exposed individuals during the spraying season but no report exist on the damage caused due to low dose of exposure to female workers of tea gardens. Farmers and tea workers consume Betel quid along with "sadagura" (a roasted formulation of homemade tobacco with flavoured agents) (Kausar et al. 2009).

2.7. Importance of Biomarkers in Risk Assessment

A major goal for genetic toxicologist is to provide precise information on exposure and health risk assessment for effective prevention of health problems. Recent advances in genetic toxicity (mutagenicity) testing methods and in approaches to performing risk assessment are prompting a renewed effort to harmonize genotoxicity risk assessment across the world. Many approaches and techniques have been developed for monitoring human populations that have been exposed to environmental mutagens (Hulka et al., 1990).

The number of studies evaluating the effect of environmental exposure to genotoxic agents has rapidly increased in the last few years (Neri et al., 2005).
The genotoxicological biomonitoring in human populations is a useful tool to estimate the genetic risk from an integrated exposure to complex mixtures of chemicals. The traditional approach has been to use the readily available blood cells, e.g., lymphocytes and red blood cells, to document biomarker of effects as well as expression of biomarker of effects e.g., DNA adducts, gene mutations and chromosome aberrations in sentinel cells (Hagmar et al., 1994). Using traditional criteria, biomarkers can be divided into biomarkers of exposure, effect, and in some cases, susceptibility (Albertini et al., 1997). Exposure biomarkers are usually more chemical-specific than effect biomarkers. DNA and protein adducts and urine metabolites can be identified with a specific chemical and hence are examples of biomarkers of exposure. Effect biomarkers, such as chromosomal aberrations or gene mutations are not chemical specific, so the association with an exposure must be established by an independent measure. Inter individual biological variability affects both biomarkers of exposure and effect. A biomarker of susceptibility is one which detects subjects who have a higher probability of an adverse effect due to individual variation (Albertini et al., 1997). A number of biomarkers are available to assess transient and permanent genotoxic responses, biomonitoring studies on human populations have essentially focused on cytogenetic end-points, namely chromosomal aberrations, micronuclei frequency and sister-chromatid exchanges (Bonassi et al., 1995).

The biomarkers using non-blood cells for population monitoring include exfoliated cell micronucleus assay, DNA adducts in buccal mucosal cells and
nasal cells, cells in hair follicles, sputum cells, exfoliated colon cells, cervical epithelial cells and exfoliated urothelial cells (Salama et al., 1999). PCR-based technique for biomonitoring mutation in exfoliated cells, Protein adducts, DNA strand breaks by the single cell gel electrophoresis assay, Bronchoalveolar cells is obtained through a mildly invasive technique known as bronchoscopy, Single cell gel electrophoresis (COMET) assay are also used as biomarkers (Salama et al., 1999; Albertini et al., 1997). Male germ cell abnormalities are also an important endpoint for biomonitoring studies (Bonde et al., 1996).

The development of 'molecular epidemiology' reflects a growing interest in the study of the relationship among genotoxic exposures and mutation studies (Vineis et al., 1999). Molecular biomarkers that have been extensively used include p53, p73, cathepsin L mRNA, matrix metalloproteinase mRNA (MMP1, 2, 9), surviving and markers of genetic abnormalities include loss of heterozygosity (LOH), allelic imbalance (AI) and DNA content (Diploid and non-diploid lesion) (Rosin et al., 2002; Rich et al., 1999; Shahnazav et al., 2000; Regezi et al., 1995; Cruz et al., 1998; Chen et al., 2004; Partridge et al., 1998; Zhou et al., 2005). The field of genomics and proteomics hold great promise in molecular epidemiology. However, requirement of sophisticated equipments and high cost is a hindrance for its wide scale application.

Risk characterization is an integral part of the genotoxicity risk assessment. The characterization is the final, integrative step of the assessment of a genotoxic
agent. The National Academy of Sciences' (NAS), National Research Council (NRC) health risk assessments and US Environmental Protection Agency (USEPA), Guidelines for Mutagenicity Risk Assessment proposed a four step paradigm within a genotoxicity context for biomonitoring studies (USEPA, 1986). The steps are:

- Hazard identification — the qualitative assessment dealing with the inherent toxicity of an agent/stressor. This qualitative assessment addresses the question of whether there is any potential for human genotoxicity.
- Dose-response assessment — the relationship between the dose of an agent/stressor and the induction of an adverse (genotoxic) effect is defined.
- Exposure assessment — the determination of the extent of human exposure.
- Risk characterization — the description of the nature and likelihood of genotoxicity risk to humans including attendant uncertainty.

Precision in intensity and specificity of the exposure should be accomplished in selecting biomarker, through adequate measures (Dolk et al., 1998). The use of non-blood cells for biomonitoring should be encouraged. Studies with the aim of providing a better understanding of the usefulness in these cells should be emphasized. Human cancer causation and development involves complex carcinogen–promoter interactions associated with tobacco use, or with nutritional
habits and traditions (Weisburger and Williams, 1995). It is utmost important to identify relevant genotoxic carcinogens and risk factors for other major chronic diseases, including hypertension and stroke, cholesterol metabolism and heart disease (Weisburger, 1998; Williams et al., 1999). Current research is active in identifying chemopreventive substances and lowering the risk of both genotoxic carcinogens or of promoters by fostering more effective identification of population at high risk (Weisburger, 1994) and this is where the role and significance of biomarkers and biomonitoring studies come into play.

2.8. Importance of Baseline Micronucleus Frequency

Population biomonitoring is becoming an extremely powerful approach to determine the effect of environmental mutagens on human populations, mainly because of the improvement of methods in molecular epidemiology combined with reliable biomarkers of exposure. The number of studies evaluating the effect of environmental exposure to genotoxic agents has rapidly grown in the recent years (Neri et al. 2005b; Suk et al. 2003). By applying conventional methods, early effects may be highlighted in accessible cell types, such as blood cells, exfoliated buccal or urothelial cells; thus, genetic biomonitoring allows to detect adverse effects of mutagenic chemicals in human somatic cells (Migliore et al., 2006). Micronuclei are considered a direct evidence of a true structural or numerical mutation. Micronuclei are consistently considered biomarkers of early
2.8. Importance of baseline micronucleus frequency

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effect (Migliore et al., 2006). The growing interest in this test, mostly due to the easy use of MN in monitoring exposure to genotoxic agents, is fueled also by the accumulating evidence that the frequency of MN in healthy subjects may be considered a marker of risk for cancer (Tucker and Preston 1996) and other diseases (Andreassi and Botto 2003; Thomas et al., 2007, 2008).

The main purpose of studying baseline micronucleus frequency is to provide reference values for researchers planning studies on genomic damage in a population. The availability of reference values is important for research teams and laboratories that need to validate protocols and analytical procedures as well as to estimate the statistical power of field studies and check the quality of data. Studies based on cytogenetic biomarkers suffer from a certain degree of heterogeneity among laboratories. It is important to establish baseline micronucleus frequencies to bring consistency and minimise such variations (Neri et al., 2005). Baseline micronucleus frequency helps to re-analyse and draw additional inferences. A pooled re-analysis of 24 databases from the HUMN international collaborative project was performed with the aim to understand the impact of smoking habits on micronucleus frequency (Bonassi et al., 2003). A case control study design was applied to the baseline micronucleus data for a population screened by oral visual inspections for cases of oral leukoplakia and interviewed with structured questionnaires by health workers on a cross section study in Kerala, India (Hashibe et al., 2000a).