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Genotoxicity Biomarkers: Application in Histopathology Laboratories

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1. Introduction

Most cancers results from man-made and natural environmental exposures (such as tobacco smoke; chemical pollutants in air, water, food, drugs; radon; and infectious agents) acting in concert with both genetic and acquired characteristics. It has been estimated that without these environmental factors, cancer incidence would be dramatically reduced, by as much as 80%-90% (Perera, 1996). The modulation of environmental factors by host susceptibility was rarely evaluated. However, within the past few years, the interaction between environmental factors and host susceptibility factors has become a very active area of research (Perera, 2000). Molecular biology as a tool for use in epidemiological studies has significant potential in strengthening the identification of cancers associated with environmental exposures related to lifestyle, occupation, or ambient pollution. In molecular epidemiology, laboratory methods are employed to document the molecular basis and preclinical effects of environmental carcinogenesis (Portier & Bell, 1998).

Molecular epidemiology has become a major field of research and considerable progress has been made in validation and application of biomarkers and its greatest contribution has been the insights provided into interindividual variation in human cancer risk and the complex interactions between environmental factors and host susceptibility factors, both inherited and acquired, in the multistage process of carcinogenesis (Perera, 2000).

The possibility to use a biomarker to substitute classical endpoints, such as disease incidence or mortality is the most promising feature and one that is most likely to affect public health. The use of events that are on the direct pathways from the initiation to the occurrence of disease to surrogate the disease incidence is a very appealing approach, which is currently investigated in different fields (Bonassi & Au, 2002).

Biological monitoring of workers has three main aims: the primary is individual or collective exposure assessment, the second is health protection and the ultimate objective is occupational health risk assessment. It consists of standardized protocols aiming to the periodic detection of early, preferably reversible, biological signs which are indicative, if compared with adequate reference values, of an actual or potential condition of exposure,

effect or susceptibility possibly resulting in health damage or disease. These signs are referred to as biomarkers (Manno et al., 2010).

There has been dramatic progress in the application of biomarkers to human studies of cancer causation. Progress has been made in the development and validation of biomarkers that are directly relevant to the carcinogenic process and that can be used in large-scale epidemiologic studies (Manno et al., 2010).

There are many important aspects to consider when a biomonitoring study is designed. For instance, there is needed a detail information on genotoxin exposure, e.g. type of toxin, duration of exposure, commencing date of exposure relative to sampling date of buccal cells, in order to achieve a meaningful interpretation of data. It will also helps to identify key variables affecting the observed frequency of biomarkers, like age, gender, vitamin B status, genotype and smoking status (Thomas et al., 2009).

Based on the impact on genotoxicity biomarkers in peripheral blood lymphocytes on the design of biomonitoring studies, Battershill et al. (2008) study have considered a strong/sufficient correlation between micronucleus (MN) frequency and increasing age. The effect is more pronounced in females than in males, with the increase more marked after 30 years of age. There are studies that also demonstrated a strong correlation between age and MN frequency and suggested that chromosome loss is a determining factor in this increase.

In what concern to gender, is also documented a gender difference in the background incidence of MN in peripheral blood lymphocytes (PBL), with the frequency being consistently higher in females. A study that assessed MN, chromosomal aberrations and sister chromatid exchange showed highly significant elevations in MN in lymphocytes of women (29% when adjusted for age and smoking) whereas chromosomal aberrations and sister chromatid exchange remained unchanged. This may reflect aneuploidy detected in MN assays (Battershill et al., 2008).

In respect to smoking, although the link between smoking and cancer is strong and exposure to genotoxic carcinogens present in tobacco smoke has been convincingly demonstrated, interestingly the same convincing association is less apparent when assessing biomonitoring studies of genotoxicity. HUMN project study about tobacco smoke, the majority of the laboratories showed no significant differences between smokers and non-smokers and the pooled analysis, interestingly, indicated an overall decrease for all smokers compared to controls (Battershill et al., 2008).

It was verified a weak/insufficient evidence for association with genotoxicity end points and alcohol consumption. Alcohol consumption has been causally associated with cancer at a number of sites (e.g. head and neck cancer). Alcoholic beverages have not been reported to induce mutagenic effects in rodents. The evidence regarding an effect of drinking alcoholic beverages on increased MN or substitute for chromosomal aberrations formation in PBL is inconclusive (Battershill et al., 2008).

2. Biomarkers – General definitions

Biomarkers have been defined by the National Academy of Sciences (USA) as an alteration in cellular or biochemical components, processes, structure or functions that is measurable

in a biological system or sample. The traditional, generally accepted classification of biomarkers into three main categories - biomarkers of exposure, effect, and susceptibility; depending on their toxicological significance (Manno et al., 2010).

A biomarker can potentially be any substance, structure or process that could be monitored in tissues or fluids and that predicts or influences health, or assesses the incidence or biological behaviour of a disease. Identification of biomarkers that are on causal pathway, have a high probability of reflecting health or the progression to clinical disease, and have the ability to account for all or most of the variation in a physiological state or the preponderance of cases of the specified clinical outcome, have largely remained elusive (Davis et al., 2007).

A biomarker of exposure is a chemical or its metabolite or the product of an interaction between a chemical and some target molecule or macromolecule that is measured in a compartment or a fluid of an organism (Manno et al., 2010).

A biomarker of effect is a measurable biochemical, structural, functional, behavioural or any other kind of alteration in an organism that, according to its magnitude, can be associated with an established or potential health impairment or disease. A sub-class of biomarkers of effect is represented by biomarkers of early disease (Manno et al., 2010).

A biomarker of susceptibility may be defined as an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a chemical (Manno et al., 2010).

Although the different types of biomarkers are considered for classification purposes, as separate and alternative, in fact it is not always possible to attribute them to a single category. The allocation of a biomarker to one type or the other sometimes depends on its toxicological significance and the specific context in which the test is being used (Manno et al., 2010).

2.1 Genotoxicity biomarkers

As a subtype of biomarkers of effect there are biomarkers of genotoxicity, generally used to measure specific occupational and environmental exposures or to predict the risk of disease or to monitor the effectiveness of exposure control procedures in subjects to genotoxic chemicals (Manno et al., 2010).

Cytogenetic biomarkers are the most frequently used endpoints in human biomonitoring studies and are used extensively to assess the impact of environmental, occupational and medical factors on genomic stability (Barrett et al., 1997; Battershill et al., 2008) and lymphocytes are used as a surrogate for the actual target tissues of genotoxic carcinogens (Barrett et al., 1997). The evaluation of MN in PBL is the most commonly used technique, although cells such as buccal epithelium are also utilized (Battershill et al., 2008).

MN assay is one of the most sensitive markers for detecting DNA damage, and has been used to investigate genotoxicity of a variety of chemicals. MN testing with interphase cells is more suited as a cytogenetic marker because it is not limited to metaphases, and has the advantage of allowing rapid screening of a larger numbers of cells than in studies with sister chromatid exchanges or chromosomic aberrations (Ishikawa et al., 2003).

MN analysis, therefore, appears to be a good tool for investigating the effects of clastogens and aneuploidogens in occupational and environmental exposure in human epidemiological studies (Ishikawa et al., 2003) and are described as a promising approach with regard to assessing health risks (Battershill et al., 2008).

2.1.1 Cytokinesis-Block micronucleus assay

The scope and the application of cytokinesis-block MN assay (CBMN) in biomonitoring has also been expanded in recent years so that in addition to scoring MN in binucleate cells, there are proposals to evaluate MN in mononucleate cells (to provide a more comprehensive assessment of DNA damage), nucleoplasmic bridges (indicative of DNA misrepair, chromosome rearrangement or telomere endfusions) and nuclear buds (a measure of gene amplification or acentric fragments). Fenech (2007), has proposed that CBMN assay can be used to measure chromosomal instability, mitotic dysfunction and cell death (necrosis and apoptosis) and has suggested the term CBMN assay. Identification of the contents of MN (e.g. presence and absence of centromeres) is now considered important in the evaluation of MN in biomonitoring studies, providing insight into mechanisms underpinning the positive results reported, i.e. to differentiate between clastogens and aneugenic responses (Battershill, et al., 2008).

The CBMN assay is a comprehensive system for measuring DNA damage; cytostasis and cytotoxicity-DNA damage events are scored specifically in once-divided binucleated cells and include: micronucleus (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUDs). Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios (Fenech, 2002a, 2006, 2007).

MN originate from chromosome fragments or whole chromosomes that lag behind anaphase during nuclear division. The CBMN assay is the preferred method for measuring MN in cultured human and/or mammalian cells because scoring is specifically restricted to once-divided binucleated cells, which are the cells that can express MN. In the CBMN assay, once-divided cells are recognized by their binucleated appearance after blocking cytokinesis with cytochalasin-B (Cyt-B), an inhibitor of microfilament ring assembly required for the completion of cytokinesis.

The CBMN assay allows measuring chromosome breakage, DNA misrepair, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis. Also measure NPB, a biomarker of dicentric chromosomes resulting from telomere end-fusions or DNA misrepair, and to measure NBUDs, a biomarker of gene amplification.

Because of its reliability and good reproducibility, the CBMN assay has become one of the standard cytogenetic tests for genetic toxicology testing in human and mammalian cells (Fenech, 2002b, 2007).

NPB occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. There are various mechanisms that could lead to NPB formation following DNA misrepair of strand breaks in DNA. Typically, a dicentric chromosome and an acentric chromosome fragment are formed that result in the formation of an NPB and an MN, respectively. Misrepair of DNA strand breaks could also lead to the formation of dicentric ring chromosomes and concatenated ring chromosomes which could also result in the

formation of NPB. An alternative mechanism for dicentric chromosome and NPB formation is telomere end fusion caused by telomere shortening, loss of telomere capping proteins or defects in telomere cohesion. The importance of scoring NPB should not be underestimated because it provides direct evidence of genome damage resulting for misrepaired DNA breaks or telomere end fusions, which is otherwise not possible to deduce by scoring MN only (Fenech, 2007 ; Thomas et al., 2003).

NBUD are biomarkers of elimination of amplified DNA and/or DNA repair complexes. The nuclear budding process has been observed in cultures grown under strong selective conditions that induce gene amplification as well as under moderate folic acid deficiency. Amplified DNA may be eliminated through recombination between homologous regions within amplified sequences forming mini-circles of acentric and atelomeric DNA (double minutes), which localized to distinct regions within the nucleus, or through the excision of amplified sequences after segregation to distinct regions of the nucleus. The process of nuclear budding occurs during S phase and the NBUD are characterized by having the same morphology as an MN with the exception that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process. The duration of the nuclear budding process and the extrusion of the resulting MN from the cell remain largely unknown (Fenech, 2007; Serrano-García & Montero-Montoya, 2001; Utani et al., 2007).

Most chemical agents and different types of radiation have multiple effects at the molecular, cellular and chromosomal level, which may occur simultaneously and to varying extents depending on the dose. Interpretation of genotoxic events in the absence of data on effects in nuclear division rate and necrosis or apoptosis can be confounding because observed increases in genome damage may be due to indirect factors such as inhibition of apoptosis or defective/permissive cell-cycle checkpoints leading to shorter cell-cycle times and higher rates of chromosome malsegregation. Furthermore, determining nuclear division index (NDI) and proportion of cells undergoing necrosis and apoptosis provides important information on cytostatic and cytotoxic properties of the agent being examined that is relevant to the toxicity assessment. In human lymphocytes, the NDI also provides a measure of mitogen response, which is a useful biomarker of immune response in nutrition studies and may also be related to genotoxic exposure. The cytome approach in the CBMN cytome assay is important because it allows genotoxic (MN, NPB and NBUD in binucleated cells), cytotoxic (proportion of necrotic and apoptotic cells) and cytostatic (proportion and ratios of mono-, bi- and multinucleated cells, NDI) events to be captured within one assay (Fenech, 2005, 2007; Umegaki & Fenech, 2000).

In conclusion, the CBMN method has evolved into an efficient "cytome" assay of DNA damage and misrepair, chromosomal instability, mitotic abnormalities, cell death and cytostasis, enabling direct and/or indirect measurement of various aspects of cellular and nuclear dysfunction such as: unrepaired chromosome breaks fragments and asymmetrical chromosome rearrangement (MN or NPB accompanied by MN originating from acentric chromosomal fragments); telomere end fusions (NPB with telomere signals in the middle of the bridge and possibly without accompanying MN); malsegregation of chromosomes due to spindle or kinetochore defects or cell-cycle checkpoint malfunction (MN containing whole chromosomes or asymmetrical distribution of chromosome-specific centromere signals in the nuclei of BN cells); nuclear elimination of amplified DNA and/or DNA repair

complexes (NBUD); chromosomal instability phenotype and breakage-fusion-bridge cycles (simultaneous expression of MN, NPB and NBUD); altered mitotic activity and/or cytostasis (NDI) and cell death by necrosis or apoptosis (ratios of necrotic and apoptotic cells) (Fenech, 2007).

2.1.2 Micronucleus in exfoliated buccal cells

Regeneration is dependent on the number and division rate of the proliferating (basal) cells, their genomic stability and their propensity for cell death. These events can be studied in the buccal mucosa (BM), which is an easily accessible tissue for sampling cells in a minimally invasive manner and does not cause undue stress to study subjects. This method is increasingly used in molecular epidemiology studies for investigating the impact of nutrition, lifestyle factors, genotoxin exposure and genotype on DNA damage, chromosome malsegregation and cell death (Thomas et al., 2009).

The assay has been successfully to study DNA damage as measured by MN or by the use of fluorescent probes to detect in BM is an indication of the regenerative capacity of this tissue. The BM provides a barrier to potential carcinogens that can be metabolized to generate potential reactive products. As up to 90% of all cancers appear to be epithelial in origin, the BM could be used to monitor early genotoxic events as a result of potential carcinogens entering the body through ingestion or inhalation. Exfoliated buccal cells have been used non-invasively to successfully show the genotoxic effects of lifestyle factors such as tobacco smoking, chewing of betel nuts and/or quids, medical treatments, such as radiotherapy as well as occupational exposure, exposure to potentially mutagenic and/or carcinogenic chemicals, and for studies of chemoprevention of cancer.

In this assay cells derived from the BM are harvested from the inside of a patient's mouth using a small-headed toothbrush. The cells are washed to remove the debris and bacteria, and a single-cell suspension is prepared and applied to a clean slide using a cytocentrifuge. The cells are stained with Feulgen and Light Green stain allowing both bright field and permanent fluorescent analysis that can be undertaken microscopically (Thomas et al., 2009).

The Buccal Mucosa Cytome (BMCyt) assay has been used to measure biomarkers of DNA damage (MN and/or nuclear buds), cytokinetic defects (binucleated cells) and proliferative potential (basal cell frequency) and/or cell death (condensed chromatin, karyorrhexis, pyknotic and karyolitic cells). The protocol can also make use of molecular probes for DNA adduct, aneuploidy and chromosome break measures within the nuclei of buccal cells. Furthermore, chromosome-specific centromeric probes have been used to measure aneuploidy by determining the frequency of nuclei with abnormal chromosome number. Tandem probes have been successfully applied to measure chromosome breaks in specific important regions of the genome (Thomas et al., 2009).

The methodology and concepts described in this protocol may be applied to other types of exfoliated cells such as those of the bladder, nose and cervix but the morphological characteristics, sampling and scoring methods are neither properly described nor standardized for cells from these tissues (Thomas et al., 2009).

The time of sampling is also an important variable to consider. As the buccal cells turn over every 7-21 days, it is theoretically possible to observe the genotoxic effects of an acute exposure approximately 7-21 days later.

Ideally, repeat sampling, at least once every 7 days after acute exposure, should be performed for 28 days or more so that the kinetics and extent of biomarker induction can be thoroughly investigated. In the case of chronic exposure due to habitual diet or alcohol consumption or smoking it is recommend that multiple samples are taken at least once every 3 months to take into account seasonal variation (Thomas et al., 2009).

The uniformity of sampling is one of the many aspects to consider; therefore a circular expanding motion is used with toothbrush sampling to enhance sampling over a greater area and to avoid continual erosion in a single region of the BM. This is performed on the inside of both cheeks using a different brush for sampling left and right areas of the mouth to maximize cell sampling and to eliminate any unknown biases that may be caused by sampling one cheek only. It is important to note that repeated vigorous brushing of the same area can lead to increased collection of cells from the less differentiated basal layer. About transportation, in some investigations buccal cells may have to be collected from a distant site which may cause sample deterioration. About cell fixation, there are many possible alternatives of fixatives such as methanol: glacial acetic acid (3:1), 80% methanol or ethanol: glacial acetic (3:1). The staining technique recommend is Feulgen because is a DNA-specific stain and because permanent slides can be obtained that can be viewed under both transmitted and/or fluorescent light conditions. There are many false-positive results in MN frequency as a result of using Romanowsky-type stains such as Giemsa, May-Grunwald Giemsa and/or Leishmann's which leads to inaccurate assessment of DNA damage. Romanowsky stains have been shown to increase the number of false positives as they positively stain keratin bodies that are often mistaken for MN and are therefore not appropriate for this type of analysis. For these reasons, it is advisable to avoid Romanowsky stains in favour of DNA-specific fluorescent-based stains such as propidium iodide, DAPI, Feulgen, Hoechst 33258 or Acridine Orange (Thomas et al., 2009).

The criterion of scoring is originally based in the described by Tolbert et al. that are intended for classifying buccal cells into categories that distinguish between "normal" cells and cells that are considered "abnormal" on the basis of cytological and nuclear features, which are indicative of DNA damage, cytokinetic failure or cell death. Therefore, some definitions of the cytological findings are (Thomas et al., 2009):

Normal "differentiated" cells have a uniformly stained nucleus, which is oval or round in shape. They are distinguished from basal cells by their larger size and by their smaller nucleus-to-cytoplasm ratio. No other DNA-containing structures apart from the nucleus are observed in these cells. These cells are considered to be terminally differentiated relative to basal cells, as no mitotic cells are observed in this population.

Cells with MN are characterized by the presence of both a main nucleus and one more smaller nuclear structures called MN. The MN are round or oval in shape and their diameter should range between 1/3 and 1/16 of the main nucleus. MN has the same staining intensity and texture as the main nucleus. Most cells with MN will ontain only one MN but it is possible to find cells with two or more MN. Baseline frequencies for micronucleated cells in the BM are usually within the 0.5-2.5 MN/1000 cells range. Cells with multiple MN are rare in healthy subjects but become more common in individuals exposed to radiation or other genotoxic events.

Cells with nuclear buds contain nuclei with an apparent sharp constriction at one end of the nucleus suggestive of a budding process, i.e. elimination of nuclear material by budding.

The NBUD and the nucleus are usually in very close proximity and appear to be attached to each other. The NBUD has the same morphology and staining properties as the nucleus; however, its diameter may range from a half to a quarter of that of the main nucleus. The mechanism leading to NBUD formation is not known but it may be related to the elimination of amplified DNA or DNA repair (Thomas et al., 2009).

The scoring method should include coded slides by a person not involved in the study in order to be a blind study. The best magnification to the observation is 1000X. An automated procedure of scoring, by image cytometry have to be developed and validated. The authors suggested first determine the frequency of all the various cell types in a minimum of 1000 cells, following this step, the frequency of DNA damage biomarkers (MN and NBUD) is scored in a minimum of 2000 differentiated cells (Thomas et al., 2009).

At the end the results with the BMCyt are dependent on the level of exposure and potency of genotoxic or cytotoxic agents, genetic background and the age and gender of the donor cells being tested (Thomas et al., 2009).

Is important to define the role of BMCyt in human biomonitoring as a new tool, less invasive in comparison with the CBMN assay, and with many potentialities in molecular epidemiology (Thomas et al., 2009).

Genotoxicity biomonitoring endpoints such as micronucleus, chromosome aberrations and 8-OHdG and DNA repair measured by comet assay are the most commonly used biomarkers in studies evaluating environmental or occupational risks associated with exposure to potential genotoxins. A review made by Knudsen and Hansen (2007) about the application of biomarkers of intermediate end points in environmental and occupational health concluded that MN in lymphocytes provided a promising approach with regard to assessing health risks but concluded that the use of chromosome aberrations in future studies was likely to be limited by the laborious and sensitive procedure of the test and lack of trained cytogeneticists. Methodologies like comet assay in lymphocytes, urine and tissues are increasingly being used as markers of oxidative DNA damage (Battershill et al., 2008).

Studies investigating correlations between endpoints used in genotoxicity biomonitoring studies have yielded inconsistent results, where we can find studies that correlate cytogenetic and comet and studies there do not achieve a correlation between micronucleus, chromosome aberrations and comet. The relative sensitivities of the different endpoints discussed, together with the importance of other factors which influence the persistence of the biomarkers such as DNA repair, may plausibly impact on background levels in the studies considered and would need to be considered before the relationship regarding increases in genotoxicity endpoints with exposure to environmental chemicals or endogenous factors is explored (Battershill et al., 2008).

2.2 Application of genotoxicity biomarkers in an occupational setting – Histopathology laboratories

A biomonitoring study was conducted in 7 histopathology laboratories in Portugal in order to assess the genotoxicity effects in occupational exposure to formaldehyde (FA).

FA is a reactive, flammable and colourless gas with a strong and very characteristic pungent odour that, when combined with air, can lead to explosive mixtures. FA occurs as an

endogenous metabolic product of N-, O- and S-demethylation reactions in most living systems. It is used mainly in the production of resins and their applications, such as adhesives and binders in wood product, pulp and paper, synthetic vitreous fibre industries, production of plastics, coatings, textile finishing and also as an intermediate in the synthesis of other industrial chemical compounds. Common non-occupational sources of exposure to FA include vehicle emissions, particle boards and similar building materials, carpets, paints and varnishes, food and cooking, tobacco smoke and its use as a disinfectant (Conaway et al., 1996; Franks, 2005; IARC, 2006; Pala et al., 2008; Viegas & Prista, 2007).

Commercially, FA is manufactured as an aqueous solution called formalin, usually containing 37 to 40% by weight of dissolved FA (Zhang et al., 2009), which is commonly used in histopathology laboratories as a cytological fixative to preserve the integrity of cellular architecture for diagnosis.

Exogenous FA can be absorbed following inhalation, dermal or oral exposure, being the level of absorption dependent on the route of exposure. The International Agency for Research on Cancer (IARC) reclassified FA as a human carcinogen (group 1) in June 2004 based on *"sufficient epidemiological evidence that FA causes nasopharyngeal cancer in humans"* (IARC, 2006; Zhang et al., 2009). In their review, IARC also concluded that there was *''strong but not sufficient evidence for a causal association between leukaemia and occupational exposure to FA"* (Zhang et al., 2009, 2010). However, some studies have also led to mixed results and inconclusive evidence (Franks, 2005; Speit et al., 2010).

The inhalation of vapours can produce irritation to eyes, nose and the upper respiratory system. Whilst occupational exposure to high FA concentrations may result in respiratory irritation and asthmatic reactions, it may also aggravate a pre-existing asthma condition. Skin reactions, following exposure to FA are very common, because the chemical is both irritating and allergenic (Pala et al., 2008). FA induces genotoxic and cytotoxic effects in bacteria and mammals cells (Ye et al., 2005) and its genotoxicity and carcinogenicity has been proved in experimental and epidemiological studies that used proliferating cultured mammalian cell lines and human lymphocytes (Pala et al., 2008; Speit et al., 2007) by DNA-protein cross-links, chromosome aberrations, sister exchange chromatides, and MN (Zhang et al., 2009).

The goal of this study was to compare the frequency of genotoxicity biomarkers, provided by CBMN assay in peripheral lymphocytes and MN test in buccal cells between workers of histopathology laboratories exposed to FA and individuals non-exposed to FA and other environmental factors, namely tobacco and alcohol consumption.

The study population consisted of 56 workers occupationally exposed to FA from 7 hospital histopathology laboratories located in Portugal (Lisbon and Tagus Valley region), and 85 administrative staff without occupational exposure to FA. The characteristics of both groups are described in Table 1.

Ethical approval for this study was obtained from the institutional Ethical Board and Director of the participating hospitals, and all subjects gave informed consent to participate in this study. Every person filled a questionnaire aimed at identifying exclusion criteria like history of cancer, radio or chemotherapy, use of therapeutic drugs, exposure to diagnostic X-rays in the past six months, intake of vitamins or other supplements like folic acid as well

as information related to working practices (such as years of employment and the use of protective measures). In this study, none of the participants were excluded.

	Control group	Exposed group
Number of subjects	85	56
Gender		
Females	54 (64%)	37 (66%)
Masculine	31 (36%)	19 (34%)
Age (mean ±standard deviation, in years)	32.42±8.1	39.45±11.5
Range	20-53	20-61
Years of employment (mean ± sandard deviation, in years)	-	14.5
Range		1-33
Tobacco consumption		
Non-smokers	60 (70,6%)	45 (80,4%)
Smokers	25(29, 4%)	11 (19,6%)
Alcohol consumption		
Non-drinkers	19 (22,4%)	19 (33,9%)
Drinkers	66 (77,6%)	37 (66,1%)

Table 1. Characteristics of the studied sample.

2.2.1 Environmental monitoring of FA exposure

Exposure assessment was based on two techniques of air monitoring conducted simultaneously. First, environmental samples were obtained by air sampling with low flow pumps for 6 to 8 hours, during a typical working day. FA levels were measured by Gas Chromatography analysis and time-weighted average (TWA_{8h}) was estimated according to the National Institute of Occupational Safety and Health method NIOSH 2541 (NIOSH, 1994).

The second method was aimed at measuring ceiling values of FA using Photo Ionization Detection (PID) equipment (11.7 eV lamps) with simultaneous video recording. Instantaneous values for FA concentration were obtained on a per second basis. This method allows establishing a relation between workers activities and FA concentration values, as well to reveal the main exposure sources (McGlothlin et al., 2005; Viegas et al., 2010).

Measurements and sampling were performed in a macroscopic room, provided with fume hoods, always near workers breath.

2.2.2 Biological monitoring

Evaluation of genotoxic effects was performed by applying the CBMN assay in peripheral blood lymphocytes and exfoliated cells from the buccal mucosa.

Whole blood and exfoliated cells from the buccal mucosa were collected between 10 a.m. and 12 p.m., from every subject and were processed for testing. All samples were coded and analyzed under blind conditions. The criteria for scoring the nuclear abnormalities in lymphocytes and MN in the buccal cells were the ones described by, respectively, Fenech et al. (1999) and Tolbert et al. (1991).

Heparinized blood samples were obtained by venipuncture from all subjects and freshly collected blood was directly used for the micronucleus test. Lymphocytes were isolated using Ficoll-Paque gradient and placed in RPMI 1640 culture medium with L-glutamine and red phenol added with 10% inactivated fetal calf serum, 50 ug/ml streptomycin + 50U/mL penicillin, and 10 ug/mL phytohaemagglutinin. Duplicate cultures from each subject were incubated at 37ºC in a humidified 5% CO_2 incubator for 44h, and cytochalasin-b 6 ug/mL was added to the cultures in order to prevent cytokinesis. After 28h incubation, cells were spun onto microscope slides using a cytocentrifuge. Smears were air-dried and double stained with May-Grünwald-Giemsa and mounted with Entellan®. One thousand cells were scored from each individual by two independent observers in a total of two slides. Each observer visualized 500 cells per individual. Cells from the buccal mucosa were sampled by endobrushing. Exfoliated cells were smeared onto the slides and fixed with Mercofix®. The standard protocol used was Feulgen staining technique without counterstain. Two thousand cells were scored from each individual by two independent observers in a total of two slides. Each observer visualized 1000 cells per individual. Only cells containing intact nuclei that were neither clumped nor overlapped were included in the analysis.

2.2.3 Statistical analysis

The deviation of variables from the normal distribution was evaluated by the Shapiro-Wilk goodness-of-fit test. The association between each of the genotoxicity biomarkers and occupational exposure to FA was evaluated by binary logistic regression. The biomarkers were dichotomized (absent/present) and considered the dependent variable in regression models where exposure was an independent variable. Odds ratios were computed to evaluate the risk of biomarkers presence and their significance was assessed. The non-parametric Kuskal-Wallis and Mann-Whitney U-tests, were also used to evaluate interactions involving confounding factors. All statistical analysis was performed using the SPSS package for windows, version 15.0.

2.2.4 Results

FA exposure levels

Results of FA exposure values were determined using the two methods described – the NIOSH 2541 method for average concentrations (TWA_{8h}) and the PID method for ceiling concentrations. For the first exposure metric, FA mean level of the 56 individuals studied was 0.16 ppm (0.04 – 0.51 ppm), a value that lies below the OSHA reference value of 0.75 ppm. The mean ceiling concentration found in the laboratories was 1.14 ppm (0.18 – 2.93 ppm), a value well above the reference of the American Conference of Governmental Industrial Hygienists (ACGIH) for ceiling concentrations (0.3 ppm). As for the different tasks developed in histopathology laboratories, the highest FA concentration was identified during macroscopic specimens' exam. This task involves a careful observation and grossing of the specimen preserved in FA, therefore has direct and prolonged contact with its vapors (Table 2).

Tasks	Ceiling values (ppm)
Macroscopic specimen's exam	2.93
Disposal of specimen and used solutions	0.95
Jar filling	2.51
Specimen wash	2.28
Biopsy exam	1.91

Table 2. FA ceiling values (ppm) by tasks in the macroscopy room.

Genotoxicity biomarkers

For all genotoxicity biomarkers under study, workers exposed to FA had significantly higher mean values than the controls (Table 3).

In peripheral blood lymphocytes, significant differences (Mann-Whitney test, $p<0.001$) were observed between subjects exposed and non-exposed to FA, namely in mean MN (respectively, 3.96±0.525 *vs* 0.81±0.172), NPB (3.04±0.523 *vs* 0.18±0.056), and NBUD (0.98±0.273 *vs* 0.07±0.028). In buccal mucosa cells, the MN mean was also significantly higher ($p=0.002$) in exposed subjects (0.96±0.277) than in controls (0.16±0.058).

	Mean, MN lymphocytes ±S.E. (range)	Mean, NPB±S.E. (range)	Mean, NBUD±S.E. (range)	Mean, MN buccal cells ±S.E. (range)
Controls	0.81±0.172 (0-7)	0.18±0.056 (0-3)	0.07±0.028 (0-1)	0.16±0.058 (0-2)
Exposed	3.96±0.525 (0-14)	3.04±0.523 (0-15)	0.98±0.273 (0-13)	0.96±0.277 (0-9)
p-value	<0.001	<0.001	<0.001	0.002

Table 3. Descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means in the studied population (mean ± mean standard error, range and p-value of Mann-Whitney test)

Discriminating by occupation, technologists group mean of MN in lymphocytes was 3.76±0.647; in NBP was 2.62±0.629; in NBUD was 1.09±0.401 and in MN in BM was 1.18±0.406. In pathologists, the means were 5.00±1.243; 3.75±1.467; 0.33±0.188 and in MN in BM was 0.58±0.434, respectively.

The odds ratios indicate an increased risk for the presence of biomarkers in those exposed to FA, compared to non-exposed (Table 4) and they were all significant ($p<0.001$).

	OR	CI 95%	p-value
MN lymphocytes	9.665	3.81-24.52	<0.001
NPB	11.97	4.59-31.20	<0.001
NBUD	9.631	3.12-29.70	<0.001
MN buccal cells	3.990	1.38-11.58	0.011

Table 4. Results of binary logistic regression concerning the association between FA and genotoxicity biomarkers, as evaluated by the odds ratio (OR).

Regarding the impact of the duration of exposure to FA, the mean values of MN in lymphocytes and in buccal cells tended to increase with years of exposure (Table 5) but the association was not statistically significant.

Group	Years of exposure	N	Mean MN lymphocytes ± S.E. (range)	Mean NPB± S.E. (range)	Mean NBUD± S.E. (range)	Mean MN buccal cells ± S.E. (range)
Exposed	> 5	8	2.75±0.940 (0-8)	5.13±1.381 (0-10)	1.38±0.498 (0-3)	0.63±0.625 (0-5)
	6-10	19	3.05±0.775 (0-12)	2.42±0.668 (0-9)	1.53±0.731 (0-13)	0.63±0.326 (0-6)
	11 - 20	12	5.50±1.317 (0-14)	3.33±1.443 (0-14)	0.33±0.188 (0-2)	0.83±0.458 (0-5)
	>21	15	5.00±1.151 (0-13)	2.33±1.036 (0-15)	0.73±0.248 (0-2)	1.20±0.8 (0-9)

Table 5. Descriptive statistics according to years of exposure to formaldehyde of MN in lymphocytes and buccal cells, NPB, and NBUD means in the two groups (mean ± mean standard error, range)

Age and gender are considered the most important demographic variables affecting the MN index. However, Table 6 shows that the mean of all the genotoxicity biomarkers did not differ between men and women within the exposed and the controls ($p > 0.05$).

Groups	Gender	N	Mean MN lymphocytes ± S.E. (range)	Mean NPB ± S.E. (range)	Mean NBUD ± S.E. (range)	Mean MN buccal cells± S.E. (range)
Exposed	Females	37	4.43±0.676 (0-14)	3.03±0.699 (0-15)	1.34±0.418 (0-13)	1.14±0.353 (0-8)
	Males	19	3.47±0.883 (0-13)	2.95±0.818 (0-14)	0.42±0.158 (0-2)	0.74±0.495 (0-9)
Controls	Females	54	0.87±0.229 (0-7)	0.22±0.078 (0-3)	0.11±0.043 (0-1)	0.11±0.057 (0-2)
	Males	31	0.71±0.255 (0-6)	0.10±0.071 (0-2)	0.00	0.26±0.122 (0-2)

Table 6. Descriptive statistics by gender of MN in lymphocytes and buccal cells, NPB, and NBUD means in the two groups (mean ± mean standard error, range)

In order to examine the effect of age, exposed and non-exposed individuals were stratified by age groups: 20-30, 31-40, and ≥ 41 years old (Table 7). There was no consistent trend regarding the variation of biomarkers with age, the only exception being the MN in lymphocytes in the exposed group (Kruskal-Wallis, $p = 0.006$), where the higher means where found in the older group. According to Mann-Whitney test, there is a statistical significant result between the elder and the older group (20–30 and > 41 years old, $p = 0.02$), however the comparison between 20-30 and 31–40 groups ($p = 0.262$) and 30–40 and > 41 groups ($p = 0.065$) did not reach statistical significance.

Groups	Age	N	Mean MN lymphocytes ± S.E. (range)	Mean NPB± S.E. (range)	Mean NBUD± S.E. (range)	Mean MN buccal cells ± S.E. (range)
Exposed	20-30	18	2.19±0.526 (0-8)	3.56±0.926 (0-10)	1.63±0.816 (0-13)	0.75±0.470 (0-6)
	31-40	11	3.00±0.775 (0-8)	1.20±0.467 (0-4)	0.50±0.224 (880-2)	0.40±0.221 (0-2)
	>41	27	5.54±0.876 (0-15)	3.00±0.879 (0-15)	0.69±0.234 (0-5)	1.46±0.503 (0-9)
Controls	20-30	36	0.47±0.157 (0-3)	0.14±0.071 (0-2)	0.08±0.047 (0-1)	0.19±0.96 (0-2)
	31-40	35	1.14±0.326 (0-7)	0.20±0.099 (0-3)	0.06±0.040 (0-1)	0.14±0.83 (0-2)
	>41	14	0.86±0.501 (0-6)	0.21±0.155 (0-2)	0.07±0.71 (0-1)	0.14±0.143 (0-2)

Table 7. Age effects on descriptive statistics of MN in lymphocytes and buccal cells, NPB and NBUD means in the studied population (mean ± mean standard error, range).

The interaction between age and gender in determining the frequencies of genotoxicity biomarkers was investigated and found to be significant only for MN in lymphocytes in exposed subjects (Kruskal-Wallis, $p=0.04$). In general the MN tended to be more frequent in the > 41 years old category in both genders; however women had the higher means.

Regarding smoking habits, a non-parametric analysis rejected the null hypothesis that biomarkers are the same for the four categories (control smokers and non-smokers, exposed smokers and non-smokers) (Kruskall-Wallis, $p<0.001$). However, the analysis of the interactions between FA exposure and tobacco smoke between exposed and controls (Mann-Whitney test) showed that FA exposure, rather than tobacco, has a preponderant effect upon the determination of biomarker frequencies. In the control group, non-smokers had slightly higher MN means in buccal cells in comparison with smokers; although the result did not reach statistical significance (Mann-Whitney, $p> 0.05$).

As for alcohol consumption, because uptake reported in enquires may differ considerably from real consumption, all consumers were gathered into a single entity, in contrast with non-consumers. Nevertheless, no one acknowledged having "heavy drink habits" in the questionnaires.
Overall, biomarkers in controls exhibited higher mean frequencies among alcohol consumers than among non-consumers. Among those exposed, however, mean frequencies were slightly lower among drinkers, suggesting that exposure was the major predominant factor in determining the high biomarker frequencies of those who are exposed. Differences between drinkers and non-drinkers were not statistically significant, to the exception of MN in lymphocytes in controls (Mann-Whitney, $p=0.011$), where drinkers have higher means. The interaction between alcohol consumption and smoking habits was statistically significant (Kruskal-Wallis, $p=0.043$), as subjects that do not smoke and do not drink tend to have lower frequencies of MN in buccal cells than those who drink and smoke, with a gradient of frequencies in between.

2.2.5 Discussion

Long exposures to FA, as those to which some workers are subjected for occupational reasons, are suspected to be associated with genotoxic effects that can be evaluated by biomarkers (Conaway et al., 1996; IARC, 2006; Viegas & Prista, 2007; Zhang et al., 2009). In this study the results suggest that workers in histopathology laboratories are exposed to FA levels that exceed recommended exposure limits. Macroscopic specimens' exam, in particular, is the task that involves higher exposure, because it requires a greater proximity to anatomical species impregnated with FA, as supported by the studies of Goyer et al. (2004) and Orsière et al. (2006).

A statistically significant association was found between FA exposure and biomarkers of genotoxicity, namely MN in lymphocytes, NPB, NBUD and MN in buccal cells. Chromosome damage and effects upon lymphocytes arise because FA escapes from sites of direct contact, such as the mouth, originating nuclear alterations in the lymphocytes of those exposed (He & Jin, 1998; IARC, 2006; Orsière et al. 2006; Ye et al., 2005). Our results thus corroborate previous reports (Ye et al., 2005) that lymphocytes can be damaged by long term FA exposure. Moreover, the changes in peripheral lymphocytes indicate that the cytogenetic effects triggered by FA can reach tissues faraway from the site of initial contact (Suruda et al., 1993). Long term exposures to high concentrations of FA indeed appear to have a potential for DNA damage; these effects were well demonstrated in experimental studies with animals, local genotoxic effects following FA exposure, namely DNA-protein cross links and chromosome damage (IARC, 2006).

In humans, FA exposure is associated with an increase in the frequency of MN in buccal epithelium cells (Burgaz et al., 2002; Speit et al., 2006, 2007b), as corroborated by the results presented here.

Suruda el al. (1993) claim that although changes in oral and nasal epithelial cells and peripheral blood cells do not indicate a direct mechanism leading to carcinogenesis, they present evidence that DNA alteration took place. It thus appears reasonable to conclude that FA is a cancer risk factor for those who are occupationally exposed in histopathology laboratories (IARC, 2006).

MN and NPB measured in lymphocytes had higher means in pathologists compared with technologists. This result can be explained by the exposure to higher concentrations of pathologists that perform macroscopic exam. Also this chemical mode of action is more related with the concentration than with time of exposure expressed by TWA results.

In epidemiological studies, it is important to evaluate the role played by common confounding factors, such as gender, age, smoking and alcohol consumption, upon the association between disease and exposure (Bonassi et al., 2001; Fenech at al., 1999). Concerning gender, studies realized by Fenech et al. (1999) and Wojda et al. (2007) reported that biomarker frequencies were greater in females than in males by a factor of 1.2 to 1.6 depending on the age group. With the exception of MN in the buccal cells of controls, the results presented here point to females having higher frequencies than males in all genotoxicity biomarkers, although the differences usually lacked statistical significance. Such trend is concordant with previous studies that reported higher MN frequency in lymphocytes in females and a slightly higher MN frequency in buccal cells in males (Holland et al., 2008) and that can be explained by preferential aneugenic events involving

the X-chromosome. A possible explanation is the micronucleation of the X chromosome, which has been shown to occur in lymphocytes in females, both *in vitro* and *in vivo*, and that can be accounted for by the presence of two X chromosomes. This finding might explain the preferential micronucleation of the inactive X (Catalán et al., 1998, 2000a, 2000b).

Aging in humans appears to be associated with genomic instability. Cytogenetically, ageing is associated with a number of gross cellular changes, including altered size and morphology, genomic instability and changes in expression and proliferation (Bolognesi et al., 1999; Zietkiewicz et al., 2009). It has been shown that a higher MN frequency is directly associated with decreased efficiency of DNA repair and increased genome instability (Kirsch-Volders et al., 2006; Orsière et al., 2006). The data has shown a significant increase of MN in lymphocytes in the exposed group. This can be explained in light of genomic instability, understood as an increased amount of mutations and/or chromosomal aberrations that cytogenetically translate into a greater frequency of changes in chromosome number and/or structure and in the formation of micronuclei (Zietkiewicz et al., 2009). The involvement of micronucleation in age-related chromosome loss has been supported by several studies showing that the rate of MN formation increases with age, especially in women (Catalán et al., 1998). This study provides evidence that age and gender interact to determine the frequency of MN in the lymphocytes of exposed subjects. The higher incidence of MN in both genders is more manifest in older age groups and the effect of gender becomes more pronounced as age increases. Several reports link this observation to an elevated loss of X chromosomes (Battershill et al., 2008).

Tobacco smoke has been epidemiologically associated to a higher risk of cancer development, especially in the oral cavity, larynx, and lungs, as these are places of direct contact with the carcinogenic tobacco's compounds. In this study, smoking habits did not influence the frequency of the genotoxicity biomarkers; moreover, the frequencies of MN in buccal cells were unexpectedly higher in exposed non-smokers than in exposed smokers, though the difference was not statistically significant. In most reports, the results about the effect of tobacco upon the frequency of MN in human lymphocytes were negative as in many instances smokers had lower MN frequencies than non-smokers (Bonassi et al., 2003). In the current study, the analysis of the interaction between FA exposure and smoking habits indicates that exposure is preponderant in determining the frequency of biomarkers. Nevertheless, the effect of smoking upon biomarkers remains controversial. Some studies reported an increased frequency of MN in lymphocytes, NPB, and NBUD as a consequence of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Still in this study no associations were observed between tobacco and nuclear abnormalities (El-Zein et al., 2006, 2008).

As for alcohol consumption, it did not appear to influence the frequency of genotoxicity biomarkers in study, to the exception of MN in lymphocytes in controls (Mann-Whitney, $p=0.011$), with drinkers having higher means. Alcohol is definitely a recognized genotoxic agent, being cited as able to potentiate the development of carcinogenic lesions (Ramirez & Saldanha, 2002). In our study, drinkers in the control group had higher mean frequencies of all biomarkers than non-drinkers, but the differences were only significant for MN in lymphocytes. Stich and Rosin (1983) study of alcoholic individuals, reported absence of significant differences concerning MN frequencies in buccal cells. That is important to corroborate our result, because of the lack of "heavy drinkers" in our study. The same study

concluded that neither alcohol nor smoking, alone, increase MN frequency in buccal cells, but a combination of both resulted in a significant elevation in micronucleated cells in the buccal mucosa. However, the synergism between alcohol consumption and tobacco has not been observed to act upon all biomarkers and, in several studies of lifestyle factors, it was difficult to differentiate the effect of alcohol from that of smoking (Holland et al., 2008).

The CBMN assay is a simple, practical, low cost screening technique that can be used for clinical prevention and management of workers subjected to occupational carcinogenic risks, namely exposure to a genotoxic agent such as FA. The results obtained in this study provide unequivocal evidence of association between occupational exposure to formaldehyde in histopathology laboratory workers and the presence of nuclear changes.

Given these results, preventive actions must prioritize safety conditions for those who perform macroscopic exams. In general, exposure reduction to FA in this occupational setting may be achieved by the use of adequate local exhaust ventilation and by keeping biological specimen containers closed during the macroscopic exam.

3. Conclusion

Another important application of biological monitoring, besides exposure assessment, is the use of biomarkers, at either individual or group level, for the correct interpretation of doubtful clinical tests. These are usually performed as part of occupational health surveillance program when exposure assessment data are unavailable or are deemed unreliable. Health surveillance is the periodical assessment of the workers' health status by clinical, biochemical, imaging or instrumental testing to detect any clinically relevant, occupation-dependent change of the single worker's health. Biomarkers are usually more specific and sensitive than most clinical tests and may be more effective, therefore, for assessing a causal relationship between health impairment and chemical exposure when a change is first detected in exposed workers (Manno et al., 2010).

Experience in biological monitoring gained in the occupational setting has often been applied to assess (the effects of) human exposure to chemicals in the general environment. The use of biological fluids/tissues for the assessment of human exposure, effect or susceptibility to chemicals in the workplace represents, together with the underlying data (e.g. personal exposure and biological monitoring measurements, media-specific residue measurements, product use and time-activity information), a critical component of the occupational risk assessment process, a rapidly advancing science (Manno et al., 2010).

Au et al. (1998), advise to put more emphasis on monitoring populations which are known to be exposed to hazardous environmental contaminant and on providing reliable health risk evaluation. The information can also be used to support regulations on protection of the environment.

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