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Assessment of genotoxicity in oral epithelial cells of health professionals occupationally exposed to ionizing radiations

Universidade dos Açores

Ponta Delgada

Outubro 2014

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Dissertação apresentada ao Departamento de Biologia, no âmbito do Mestrado em Ambiente, Saúde e Segurança, para obtenção do título de Mestre.

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Outubro 2014

Agradecimentos

A Deus, por me dar força para seguir em frente nos momentos mais difíceis e desanimadores e pela bênção concedida durante toda a minha vida.

À minha mãe, por me conceder o dom da vida, uma boa educação e iluminar o meu caminho, sempre com carinho, amor e esperança de um futuro melhor. Mesmo quando o sol deixa de brilhar "no meu mundo", é contigo o meu porto de abrigo.

À minha irmã, pela pouca paciência que ainda lhe resta para me aturar nos momentos de imenso trabalho.

Ao Professor Doutor Armindo Rodrigues, orientador da minha tese de mestrado, pela imediata aceitação deste projecto, pelos ensinamentos que me transmitiu, pelo apoio e incentivo constantes, bem como pela simpatia e excelência prestada.

À Professora Doutora Patrícia Garcia, também minha orientadora da tese, por me acolher neste projecto de investigação, pelo entusiasmo demonstrado desde o início da realização deste projecto, pela disponibilidade, simpatia e ajuda prestada ao longo da realização deste trabalho, sendo incansável durante a realização do mesmo.

Ao Mestre Ricardo Camarinho pela ajuda prestada ao longo deste projecto. Sem a sua ajuda a concretização desta investigação seria muito mais difícil. Agradeço ainda o bom ambiente profissional e bom humor com que sempre me presenteou durante as longas horas de trabalho e a amizade cultivada durante esse tempo.

Ao meu colega e amigo, Filipe Bernardo, por todo o apoio prestado nestes dois anos de mestrado, pela partilha de saberes e vivências e por me incentivar a ser melhor.

À minha amiga Isabel Santos, por me ouvir e me apoiar nos momentos mais difíceis, por me incentivar a conseguir mais e melhor e a lutar pelos meus objectivos.

A todos vós, o meu muito obrigado…

"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota".

(Madre Teresa de Calcutá)

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List of Abbreviations

ADD_5 – Accumulated Deep Dose in the last five years

ADD – Annual Deep Dose

ASD – Annual Surface Dose

CI – Confidence Intervals

DNA – Deoxyribonucleic Acid

γ – Gamma

NC – Normal Cell

MDD – Monthly Deep Dose

MNC – Micronucleated cells

MSD - Monthly Surface Dose

MN- Micronucleus

ONA – Other Nuclear Anomalies

RR – Relative Risk

WHO – World Health Organization

Chapter 1

1. Introduction

1.1. General Overview

The genotoxic damage induced by ionizing radiation has been observed in DNA, chromosomes and nuclei, as in case of DNA strand breaks, chromosomal anomalies and micronucleus (MN) formation (Morgan *et al*., 2002; Chadwick & Leenhouts, 2003; Vral *et al.*, 2011).

Ionizing radiations are recognized mutagens and carcinogens to the human population. In order to detect the effects of ionizing radiation, in low doses exposure, careful and precise analysis is required and needed (Popova *et al*., 2007). Thus, the MN test works as a biological dosimeter (Muller *et al.*, 1996; Hendry & West, 1997; Vral *et al.*, 2011), widely used as a "standard test" for the evaluation of the dose in radiological biomonitoring programs (eg., Vral, *et al*., 2011; Ropolo *et al*., 2012; Saberi *et al.*, 2013) and in studies of assessment of genotoxicity resulting from acute or therapeutic exposure (eg., Norpa & Falck, 2003; Popova *et al*., 2004; Cerqueira *et al.*, 2004; Ribeiro, 2012).

Many studies have shown that the number of MN induced by ionizing radiation is strongly correlated with the dose and type of radiation (Vral *et al.*, 2011).

Micronuclei results from the exposure to various clastogenic agents, like ionizing radiation. However, taking into account that ionizing radiation is a strong clastogenic agent, thus a potential MN inductor, the MN test has proven to be quite

feasible to study the ionizing radiation genotoxic effects (Vral *et al*., 2011). Nevertheless, the evaluation of genotoxic effects resulting from exposure to ionizing radiation in humans has been widely performed on lymphocytes, which implies invasive methods. Recently, cells from the oral epithelium have been used for the evaluation of exposure to various genotoxic agents, particularly in human populations (Holland *et al*., 2008; Ceppi *et al*., 2010; Bolognesi *et al*., 2013). The easy access to oral epithelial cells through noninvasive methods, associated with its recognized sensitivity for the assessment of DNA damage (Holland *et al.*, 2008), makes these epithelial cells a biomarker with high potential for biomonitoring the effects of exposure to ionizing radiation in occupational context. The use of oral epithelial cells has more advantages over the use of lymphocytes when the target tissue of interest is epithelial tissue (Jois *et al*., 2010). In this case, it is important to study this tissue, since 90% of cancers have epithelial origin (Rosin, 1992).

1.2. Objectives

Since ionizing radiation is a well recognized mutagenic and carcinogenic agent in the human population (Popova, *et al*., 2007), it is important to study the genotoxic effects of long term occupational exposure to ionizing radiation.

Considering the case of health professionals who deal daily with ionizing radiation, including interventional cardiologists, radiographers (medical imaging technologists), orthopedic surgeons, nurses, medical auxiliaries, among others, it is necessary to perform a study that addresses the "invisible" risk of occupational hazards of exposure to ionizing radiation. Thus, the present study aims to:

i) Assess the genotoxic effects and the carcinogenicity risk of occupational exposure to ionizing radiation in health care workers of the Hospital do Divino Espírito Santo;

ii) Test nuclear anomalies in oral epithelial cells as biomarkers of effect for occupational exposure to ionizing radiation.

Chapter 2

2. Main Concepts

2.1. Matter and Energy

According to Bushong (2008), matter is every material substance of physical objects that occupies space, and energy is the "ability to do work".

It is known that are different types of energy, like potential, kinetic, chemical, electrical, thermal, nuclear and electromagnetic energy. The last one is used in radiological exams, called X-rays, which is not the only one in the electromagnetic spectrum. The radio waves, microwaves, infrared, visible light, ultraviolet and γ-rays also take part of the electromagnetic radiation spectrum (Bushong, 2008).

Ionizing radiation is any electromagnetic radiation or any radioactive particle with sufficient energy to ionize molecules and atoms (Cameron, 1991; Little, 2003). X-rays belong to the group of ionizing radiation, which are capable of removing electrons from an atom (ionizing). This happens when radiation, such X-rays and also γ-rays, passes through matter, transferring enough energy to the electron to remove it from the atom (IAEA, 2004; Bushong, 2008).

2.2. Ionizing Radiation and Health Effects

The molecules of a cell can be changed when ionizing radiation, such X-rays and γ-rays (Gamma rays), target those molecules, culminating in changes of molecules,

like DNA, and ultimately cell death (Mi-Young & Tae- Hwan, 2002; Bushong, 2008; Holland *et al*., 2008). Mi-Young and Tae- Hwan (2002) also refer that this change is related to dose, time of exposure, age, sex, and even to every single individual radiosensitivity.

Whenever a cell death or a cell alteration occurs, we are witnessing early deterministic effects or stochastic effects, respectively (Bushong *et al*., 2008). Nonetheless, most of the effects of ionizing radiation on cells are not noticed because human metabolic processes are able to recover and repair this kind of damage.

Dose and exposure time are the keys factors for tissue responses (Bushong, 2008; Lima, 2009).Different tissues respond in various different ways due to the organ-specific radiosensitivity, dose, irradiation conditions (Bushong, 2008; Lima, 2009) and cell proliferation and maturation. Skin, gonads and bone marrow are the tissues that can immediately be affected by this kind of radiation (Bushong, 2008).

Nowadays it is known that a threshold-type dose-response relation exists. In several cases is well known the minimum dose required to produce a deterministic response, and if this dose is exceeded, the severity of the response increases (Bushong, 2008). However, the existence of a threshold doesn't mean that cell anomalies can't happen at doses bellow the stipulated (Lima, 2009).

2.2.1. Deterministic Effects *vs*. Stochastic Effects

According to Linet *et al.* (2012), deterministic and stochastic effects are the two cellular types of damage produced by ionizing radiation when natural cell repair does not occur.

A deterministic effect happens when an individual is exposed to a high dose of radiation, above the threshold. The effects severity increases proportionally with the dose (ICRP, 2007; Bushong, 2008; Sgouros *et al*., 2009; Linet *et al*., 2012). According to Lima (2009), induction of cataracts, erythema (radiodermitits), radiation syndrome, sterility, epilation and death are some of the effects that appear just after irradiation.

Stochastic effects occur when an individual is exposed to low levels of ionizing radiations over long periods of time (Bushong, 2008). This effect also depends on the exposure dose. There is no recognizable severity or threshold, only odds that a specific event will happen after exposure. Stochastic effects can be divided between somatic and genetic effects. Somatic effects happen when somatic cells (any cell not directly involved in reproduction) are affected, and genetic effects happen when the cells responsible for heritage transmission are affected. Cancer is a recognizable somatic effect (NAS, 2006; ICRP, 2007; Lima, 2009).

The use of X-rays has been growing at clinical and technological level, since it was discovered in the XIX century, increasingly becoming the medicine of yesterday in tomorrow's medicine (Seibert, 1995; Hall & Giaccia, 2006).

Accordingly to Frieben (1902), Rollins (1904), Scott (1911) and Von *et al*. (1911), many were the cases of radiodermitis, cataracts, leukemia, several carcinomas and other health problems that appeared, many years later, as a consequence of X-rays manipulation. Recommendations about ionizing radiation protection were implemented, such the use of lead aprons, radiological dosimeter and consequent measurement of radiation dose that the worker is exposed (Hall & Giaccia, 2006).

According to Cameron (1991), the biological effects related to ionizing radiations are carcinogenic, mutagenic and teratogenic. It is known that X-ray high doses are able to cause different alterations to human organism, from cellular structure

deterioration to a cancer development; it is well recognized the relation between low doses of X-rays and cancer (Brenner *et al*., 2003; Bushong, 2008), being therefore assigned a significant risk to interventional cardiologists (Venneri *et al*., 2009).

2.2.2. Quantification of human irradiation effects

To understand the quantification of human irradiation effects, the measurement scales and what it measures must be known.

These are the basic unit conversions (Lima, 2009):

- 1 gray (Gy) = 100 rad
- 1 rad = 10 milligray (mGy)
- 1 sievert (Sv) = 1.000 millisieverts (mSv) = 1.000.000 microsieverts (μSv)
- 1 sievert = 100 rem

The absorbed dose (D) is the indicator of the energy released in the biological environment and the fundamental quantity in radiation protection; $d\bar{E}$ is the mean energy imparted to matter of mass (d_m) by ionizing radiation. Gray (Gy) is the unit of measurement (IAEA, 2007; ICRP, 2007; Lima, 2009).

$$D = \frac{d\bar{E}}{dm}$$

The equivalent dose (H_T) is the dose of ionizing radiation absorbed by one specific part of the human body and well adjusts to the different types of energy. Equivalent dose is calculated through the product of an average absorbed dose in a specific tissue or organ due to radiation (D_{TR}) and radiation weighting factor (W_R). Millisieverts (mSv) is the unit of measurement (IAEA, 2007; ICRP, 2007; Lima, 2009).

$$H_T = D_{T,R} \cdot w_R$$

Note: The radiation weighting factor (W_R) is described on the ICRP (1991).

According to Cameron (1991), equivalent dose takes into account the Relative Biological Effectiveness (RBE), but the received dose is not uniform to the body, since this calculation "would result in the same radiation risk if it had been given to the whole body".

The effective dose (E) is a quantity of radiological protection that takes into account the different radiosensitivity of the different organs and the associated risk factors, representing the sum of the equivalent doses (H_T), where W_T is the tissue weighting factor. Millisieverts (mSv) is the unit of measurement (IAEA, 2007; ICRP, 2007; Lima, 2009).

$$E = \sum_T W_T \sum_R W_R D_{T,R}$$

or

$$E = \sum_T W_T H_T$$

Note: The tissue weighting factor (W_T) is described on the ICRP (1991).

2.2.3. Allowed doses

According to Lima (2009) the biological effects are dependent of the absorbed dose. It is also stated that doses higher than 1-2 Gy (deterministic limit) can lead to acute damage of the irradiated tissue, and tissue and burns necrosis can occur if the absorbed dose is comprised between 3 and 5 Gy.

The NCRP (1993), on the Report No. 116, published the limit dose recommendations to occupational exposures, public exposures and others. These limits have been established for levels that if any individual should be exposed to this dose, acute or in chronic form, the somatic and genetic effects observed would be acceptable. Table 1 shows the effective and equivalent dose limits for occupational exposures and public exposures.

Table 1 - Effective and equivalent dose limits for occupational exposures and public exposures (adapted from NCRP, 1993).

Exposures	Dose
Occupational exposures	
Effective dose limits	
Annual	50 mSv
Cumulative	10 mSv x age
Equivalent dose annual limits	
Lens of eye	150 mSv
Skin, hands and feet	500 mSv
Public exposures (annual)	
Effective dose limit, continuous or frequent exposure	1 mSv
Effective dose limit, infrequent exposure[a]	5 mSv
Equivalent dose limits for tissues and organs[a]	
Lens of eye	15 mSv
Skin, hands, feet	50 mSv
Education and training exposures (annual)[a]	
Effective dose limit	1 mSv
Equivalent dose limits for tissues and organs	
Lens of eye	15 mSv
Skin, hands and feet	50 mSv
Embryo-fetus exposures (monthly)	
Equivalent dose limit	0.5 mSv

a - Sum of external and internal exposures but excluding doses from natural sources.

2.3. Biomonitorization

2.3.1. Biomonitoring

According to Bocca *et al.* (2010) human biomonitoring is a technique for assessing human exposure to certain chemical compounds present in the environment, being this assessment carried out through tests made on tissues or fluids, such as blood, urine, hair, and also epithelial tissue. This assessment, if combined with the measure of

the substance and the individual exposure, can provide a straight correlation between exposed individuals and health effects (Kuno, 2010), like occupational exposures to ionizing radiations.

Human biomonitoring allows an evaluation of numerous factors, such as cumulative exposure or genetic susceptibility, to a certain chemical compound (Paustenbach & Galbraith, 2006; Al Bakheet *et al*, 2013), and may subsequently extrapolate the case studies to adverse health effects, including cancer (de la Monte *et al*., 2009). Human biomonitoring may act as periodic measure of a certain biomarker (Mutti, 1999).

When performing a study of biomonitoring it is possible to quantify the exposure and thus relate it with health outcomes, allowing an estimate of health risks in exposed individuals, as it is the case of exposures in working contexts (occupationally exposure). With this approach, it is possible to implement mitigation measures regarding the exposure to certain compounds (Angerer *et al*., 2007) and strategies to enhance health and safety conditions at work (Norppa, 2004; Boogaard, 2007), like the drawing up of occupational exposure limits.

2.3.2. Biomarkers

Biomarkers are markers of cellular and molecular alterations that occur in an organism (Kuno, 2010), being predictive of any anomaly or alteration, such as cancer.

Biomarkers are used as “agents” of measurement, that reflects interactions between a biological system and environmental compounds, assisting in decision making within the public health. These markers make possible what is not possible through assessments made by a questionnaire or environmental measurements (WHO,

1993). Also, these biomarkers contain the substance or the product resultant from the substance biotransformation, wherein the amount present in it determines the intensity of the exposure to the agent and the health risk (WHO, 1996).

According to WHO (1993) there are three classifications of biomarkers, biomarkers of exposure, effect and susceptibility.

Biomarkers of exposure are those that are used to demonstrate a relation between an external agent and the exposure of an individual to this same agent.

Biomarkers of effect are those capable of measure behaviour, chemical, physical and other type of alterations on tissues and fluids of an organism. These measurements can be associated to a probable disease.

Biomarkers of susceptibility measures the level of response/capability, innate or acquired, that an organism has when exposed to a particular substance.

Biomarkers of effect and exposure are the most commonly used.

2.4. Radiological Dosimetry

Exposure to ionizing radiations during medical procedures constitutes the majority of human population exposure to artificial radiation. Thus, it is necessary to control the dose of radiation exposure of individuals that are occupationally exposed (IAEA, 2007).

The radiological dose control in health professionals exposed to ionizing radiations is made through a radiological dosimeter (IAEA, 2004), an instrument that detects and measures the exposure of an individual to ionizing radiations (Bushong, 2008), which can be used in different parts of the body. Therefore, every hospital has a unique protocol for the use of the radiological dosimeter (Lima, 2009).

According to Bushong (2008), the use of Thermoluminescent Dosimeters (TLD) is very frequent, due to the size, sensitivity and accuracy of dose reading. These dosimeters are used for personal radiation monitoring (health professionals) and also for patients submitted to diagnosis and therapeutics that involves ionizing radiations.

TLD have crystals that have the capability to store all the energy that an individual was exposed. In order to quantify the radiation dose that the individual was exposed, the crystal emits a light when exposed to high temperatures, being the intensity measured subsequently (Cameron, 1991).

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Chapter 3

3. Assessment of genotoxicity in oral epithelial cells of individuals occupationally exposed to ionizing radiations

3.1. Abstract

The present study was designed to assess the genotoxic effects and the carcinogenicity risk of occupational exposure to ionizing radiation in health care workers, testing the nuclear anomalies in oral epithelial cells as biomarkers of effect resulting from occupational exposure to ionizing radiation.

Buccal epithelial cells were collected from a total of 42 health professionals occupationally exposed to ionizing radiations (exposed group) and 39 non-exposed health professionals and administrative workers (non-exposed group), and examined for the frequency of MNC and ONA (pyknosis, karyolysis, and karyorrhexis). The frequency of MNC and ONA per 2000 cells was higher in the exposed group (5.26 *vs*. 146.62, respectively) than in the control group (1.33 *vs*. 88.46, respectively). Significant and positive correlations between MNC or ONA and the Annual Surface Dose were observed, showing that exposure to ionizing radiations is a risk factor for DNA damage. The consumption of alcoholic drinks was also significantly and positively correlated with the frequency of MNC, revealing that alcohol consumption is also a risk factor for DNA damage.

The risk analysis showed that the Annual Surface Dose of ionizing radiation is a significant predictor for the development of nuclear anomalies (MN and ONA), being the risk of having high frequency of MNC or of ONA increased by 1.8-fold in the individuals exposed to ionizing radiations, compared to non-exposed ones. The multivariate analysis showed that the confounding factors (age, gender, tobacco use, alcoholic drinks use, elixir use) were not significantly associated with the frequency of MNC or ONA.

The findings in this study show a significant association between occupational exposure to ionizing radiations and the occurrence of MNC in oral epithelial cells, enlightening exposure to ionizing radiations as a carcinogenic agent. It is important to highlight the significantly higher risk for DNA damage observed in the exposed group. The studied biomarkers can be used for human biomonitoring, in order to analyze the mutagenic and clastogenic effects of ionizing radiations on occupationally exposed individuals.

3.2. Resumo

O presente estudo foi elaborado com o intuito de avaliar os efeitos genotóxicos e o risco de carcinogénese da exposição ocupacional a radiações ionizantes em profissionais de saúde, testando as alterações nucleares em células do epitélio bucal como biomarcadores de efeito decorrentes da exposição ocupacional à radiação ionizante.

Foram recolhidas células do epitélio bucal de 42 profissionais de saúde ocupacionalmente expostos às radiações ionizantes (grupo exposto) e de 39 profissionais de saúde e administrativos (grupo não exposto), as quais foram analisadas para a frequência de células micronucleadas e outras anomalias nucleares (picnose, cariólise e cariorrexe). A frequência de células micronucleadas e de outras anomalias nucleares, por cada 2000 células, foi maior no grupo exposto (5.26 *vs*. 146.62, respectivamente) do que no grupo de controlo (1.33 *vs*. 88.46, respectivamente). Foi observada uma correlação significativa e positiva entre a frequência de células micronucleadas ou de outras anomalias nucleares e a dose anual de superfície, demonstrando que a exposição a radiações ionizantes é um factor de risco para a ocorrência de danos no ADN. Verificou-se também que o consumo de bebidas alcoólicas apresentava uma correlação significativa e positiva com a frequência de células micronucleadas, revelando que o consumo de álcool é também um factor de risco para a ocorrência de danos no ADN.

A análise de risco demonstrou que a dose anual de superfície de radiações ionizantes é um preditor significativo para o desenvolvimento de anomalias nucleares (micronúcleos, picnose, cariólise e cariorrexe), sendo o risco da ocorrência de células micronucleadas e outras anomalias nucleares 1.8 vezes maior nos indivíduos expostos a

radiações ionizantes, comparativamente aos não expostos. Na análise multivariada, os fatores de confundimento (idade, sexo, consumo de tabaco, consumo de bebidas alcoólicas e uso de elixir) não revelaram qualquer associação significativa com a frequência de células micronucleadas ou outras anomalias nucleares.

Os resultados deste estudo mostram uma associação significativa entre a exposição ocupacional a radiações ionizantes e a ocorrência de células micronucleadas no epitélio bucal, revelando que a exposição a radiações ionizantes é um fator de risco de carcinogénese. É importante destacar a existência de um risco significativamente maior no grupo exposto, no que diz respeito aos danos no ADN observados. Os estudos de biomarcadores podem ser utilizados para efeitos de biomonitorização humana, de forma a analisar os efeitos mutagénicos e clastogénicos decorrentes da exposição ocupacional a radiações ionizantes.

3.3. Introduction

Ionizing radiations are any electromagnetic waves or particles able to ionize a molecule or an atom. The process of ionization involves the act of removing electrons from the medium which it propagates, if there is sufficient energy for that (Cameron, 1991; Little, 2003). All this process can culminate in cellular alterations, even cellular death, if the body cannot repair the damage caused by the radiation to which the individual was exposed (Mi-Young & Tae- Hwan, 2002; Little, 2003; Bushong, 2008; Holland *et al*., 2008).

X-ray presents itself as an asset in medical diagnosis, even being a major inducer of genetic damage and a cumulative genotoxic agent (Cerqueira *et al*., 2004). This kind of radiation represents the major "slice" of radiations made by man received by the general population (UNSCEAR, 1982; Ropolo *et al*., 2012). Health professionals are the majority of individuals that are exposed to low doses of ionizing radiations, being subjected to several adverse biological effects during the performance of medical diagnostic procedures (Mettler & Upton, 2008; Ropolo *et al*., 2012), as cardiac catheterizations, support in the operating room, radiographies, CT scans, among others.

Ionizing radiations are known as mutagenic agents able to cause chromosomal damage (Ropolo *et al*., 2012; Saberi *et al*., 2013) and also as carcinogenic agents. In order to detect the effects of ionizing radiation, in low doses of exposure, careful and precise analysis is required and needed (Popova *et al*., 2007). Thus, the micronucleus (MN) test works as a biological dosimeter (Muller *et al.*, 1996; Hendry & West, 1997; Vral *et al.*, 2011), widely used as a "standard test" for the evaluation of the dose in radiological biomonitoring programs (eg., Vral, *et al*., 2011; Rapolo *et al*., 2012; Saberi *et al.*, 2013) and in studies of assessment of genotoxicity resulting from acute or

therapeutic exposure (eg., Norpa & Falck, 2003; Popova *et al*., 2004; Cerqueira *et al.*, 2004; Ribeiro, 2012).

According to Norppa (2004), it is important to perform the biomonitoring (human biomonitoring) of the effects caused by genotoxic agents in individuals occupationally exposed to ionizing radiations, in order to implement mitigating measures to improve the health quality and the work conditions of these professionals. The micronucleus assay has been used in the last 15-20 years as a biomarker of effect in individuals exposed to several mutagenic and carcinogenic agents, in order to evaluate chromosomal damage (Holland *et al*., 2008). Micronuclei are originated from acentric chromosomes, chromatid fragments or whole chromosomes that have failed to be incorporated in the daughter nuclei during mitosis (Fenech & Bonassi, 2011; Bolognesi *et al*., 2013).

In a study conducted by Bonassi *et al*. (2007) it was confirmed that a high frequency of micronuclei in human peripheral blood lymphocytes is a predictive of increased cancer risk, making MN a biomarker of great importance in the planning and validation of cancer surveillance. Besides the carcinogenic risk, Thomas *et al*. (2009) reported that MN are also associated with the increased risk of accelerated ageing and neurodegenerative diseases.

Stich *et al*. (1983) used, for the first time, cells from the oral epithelium to analyse micronuclei. Since then, this method has been increasingly applied (Holland *et al*., 2008) for the biomonitoring of the effects of carcinogenic substances. The MN assay with epithelial cells may be done with exfoliated cells from the oral mucosa, urothelial or nasal epithelium, allowing the analysis of genetic damage induced *in vivo* (Holland *et al*., 2008; Ceppi *et al*., 2010).

The use of cells from the oral epithelium is an asset in the analysis of MNC, because the oral epithelium is in an area of easy access, resulting in a minimally invasive technique to collect cells (Holland *et al.*, 2008; Thomas *et al*., 2009; Bolognesi *et al*., 2013), enabling the direct study of a particular tissue (Nerseysyan *et al*., 2002). Furthermore, the use of these cells is of major importance, since 90% of cancers have epithelial origin (Rosin, 1992).

The oral epithelium consists of four layers, the *stratum germinativum* (which is closely linked to connective tissue and also called basal layer), the *stratum spinosum*, the *stratum granusolum* and the *stratum corneum* (Thomas *et al*., 2009). This epithelium is constantly being renewed by successive mitosis that occurs in the basal layer of the epithelium, which cells later migrate to the surface (Holland *et al*. 2008).

The presence of MN in oral epithelial cells reflect genotoxic events that occurred between one to three weeks earlier, when these cells were yet in the basal layer (Stich & Rosin, 1983); these events are only observed later in the exfoliated oral mucosa, after their differentiation (Cerqueira *et al*., 2004).

It is possible to visualize other nuclear anomalies besides MN, like karyorrhexis, karyolysis and pyknosis, by the analysis of the exfoliated oral mucosa cells. These abnormalities are due to cytotoxic (necrosis and keratinization) and genotoxic (apoptosis) events, acting as effective biomarkers of individuals exposed to mutagenic and carcinogenic agents (Garcia *et al*., 2012; Rodrigues *et al*., 2012; Holland *et al*. 2008).

3.4. Material and Methods

3.4.1. Subjects and sample collection

The samples for the study were collected from workers of the Hospital do Divino Espirito Santo and consisted of two separate groups. These were divided by health professionals occupationally exposed to ionizing radiations (exposed group) and health professionals and administrative workers non-exposed to ionizing radiations (non-exposed group). The exposed group comprised 42 (51.9%) health professionals exposed to ionizing radiations (technicians, physicians, nurses and auxiliary health care personnel); the non-exposed group consisted of 39 (48.1%) individuals working at several services of the Hospital do Divino Espirito Santo, such administrative services, infirmaries and technical laboratories.

All individuals in the exposed group were routinely monitored for exposure to ionizing radiation by use of personal film badge dosimeters. The personal film badge dosimeter is fixed to the hospital uniform, near the chest, and all radiological workers are trained to use it correctly. The dosimeters are read every 90 days. Every three months a report is given to the Radiology Service of the Divino Espirito Santo Hospital, with Monthly Surface Dose (MSD), Monthly Deep Dose (MDD), Annual Surface Dose (ASD), Annual Deep Dose (ADD) and Accumulated Deep Dose in the last five years (ADD_5). The effective dose to an individual was found by calculating a weighted average of the equivalent dose to different body tissues, with the weighing factors designed to reflect the different radio-sensitivity of the tissues. No dosimeter was available for unexposed subjects, because they were not occupationally exposed to X-rays.

The Ethics Board of Hospital do Divino Espirito Santo (Ponta Delgada, Azores, Portugal) approved the study. All individuals gave written informed consent in compliance with the Helsinki Declaration and Oviedo Convention, to participate in this study. The informed consent contained information about the procedures for collecting the necessary data and biological samples, and also data confidentiality (Appendix 1). It was also explained to each individual that they could abandon the study at any time without any consequences. It was also insured that the individual samples would be destroyed after the study.

A questionnaire (Appendix 2), structured accordingly to Ferris (1978) and Cerqueira *et al*. (2004), was used to interview each person about their age, gender, smoking habits (consumption of smoking and/or smokeless tobacco), alcohol consumption, frequent use of mouthwash, performance of X-rays in the previous week and general health status. Individuals who have done radiographies to the head, in a time window of fifteen days, were excluded from this study. This window of time was calculated because the oral epithelium cells migration from the basal layer up to the *stratum corneum* varies from 7 to 21 days (Bolognesi, 2013; Fenech *et al*., 1999; Cerqueira *et al*., 2004 and Ribeiro, 2012). If the individual had performed an X-ray to the head during this time line, at the time of the interview, it would not be possible to distinguish any anomalies caused by the dose that the individual was exposed during the X-ray exam and the exposure during labor time.

After responding the questionnaire, all individuals in the study were sampled by collecting exfoliated epithelial cells of their oral epithelium. The samples from the individual's oral mucosa were collected through a sterilized cervical brush, obtained from both sides of the cheeks. Each individual brushed the cheeks vigorously, in order to maximize the number of collected cells to eliminate any unknown deviations that

could be caused by sampling one side only (Cerqueira *et al*., 2004; Thomas *et al*., 2009). It is important to note that vigorous brushing repeated in the same area may lead to an increase of collected cells from a deeper and less differentiated layer (Thomas *et al*., 2009).

After the sampling, the researcher insured a correct cell spreading on two microscopic slides through a circular and dispersive movement. The collected cells were then stained by the Feulgen method (Annex 1) for the observation of micronuclei and other nuclear anomalies. The Fuelgen method is commonly used due to its DNA specificity and is indicated for situations of DNA quantification (Carrard *et. al*, 2007). Besides that, the nuclear anomalies found with this method present the lowest values of confound factors compared to other methods, such as May-Grunwald/Giemsa (Carrard *et al*., 2007), Papanicolaou and Hemotoxylin & Eosin (Grover, *et al*., 2012), due to the possibility of miss interpretation of the nuclear anomalies in study.

All the slides were examined with the use of an optical microscope (LEICA DM1000, Leica Microsystems®), with a magnification of 400X. For each individual 2000 cells were analysed for the observation of MN, karyorrhexis, karyolysis and pyknosis. A similar methodological approach was used by Sellappa *et al*. (2011), Jyoti *et al*. (2012) and Ceretti *et al*. (2014) for 2000 analysed cells.

3.4.2. Nuclear anomalies analysis criteria

To consider the occurrence of nuclear anomalies, several criteria were considered, such as the fact that the cells had to be intact and in a relatively flat position on the slide, with almost none or none overlap with adjacent cells, almost none or none residue in the cytoplasm and intact nucleus, smooth and distinct nuclear perimeter.

These criteria were also used by Tolbert *et al*. (1992) when analysing cells with these types of nuclear anomalies.

To identify each type of nuclear anomaly in this study, specific criteria (according to Thomas *et al*., 2009) were used.

Micronucleated cells are characterized by the presence of a main nucleus and one or more small nucleus (micronucleus). The presence of only one MN is more common in general, and the presence of two or more MN is more frequent in subjects exposed to radiation and other genotoxic agents. The normal range of MNC frequency for human oral epithelia varies from 0.5 to 2.5 per 1000 analysed cells (Holland *et al*., 2008; Ceppi *et al*., 2010).

The parameters to be taken into account for the analysis of MN (Figure 1A) is the texture and tonality which should be equal to the main nucleus, round or oval shape, a diameter between 1/3 and 1/16 of the diameter of the main nucleus with an evident edge – which suggests a nuclear membrane- as well as being present in the cytoplasm (Tolbert *et al*., 1992; Thomas *et al*. 2009.).

Regarding karyorrhexis (Figure 1B), the nucleus is characterized by a speckled pattern, indicative of nuclear fragmentation, which naturally leads to a nuclear disintegration associated with loss of nuclear membrane integrity. This pattern is due to the aggregation of nuclear chromatin (Thomas et al., 2009). This is a step that is part of the cell death (Carrard *et al*. 2007).

For the analysis of karyorrhexis it must be taken into account the existence of a fragmented nucleus, within the intact cytoplasm (Carrard *et al*., 2007).

Karyolysis (Figure 1C) is the name given to cells devoid of DNA, characterized by the absence of nucleus – ghost nucleus-, representing the last stage of cell death (Thomas *et al*., 2009). The analysis of this cell abnormality is relatively simple, being

based on just the complete absence of a nucleus, which is not stained by the Fuelgen method (Carrard *et al*., 2007; Thomas *et al*., 2009).

Pyknotic cells (Figure 1D) are characterized by extremely small nucleus, but with a high density of cellular material, resulting in a uniform and intense staining, compared with normal cells. Moreover, these cells have a nucleus with a diameter between 1/3 to 2/3 in nucleus in normal differentiated cells (Thomas *et al*., 2009).

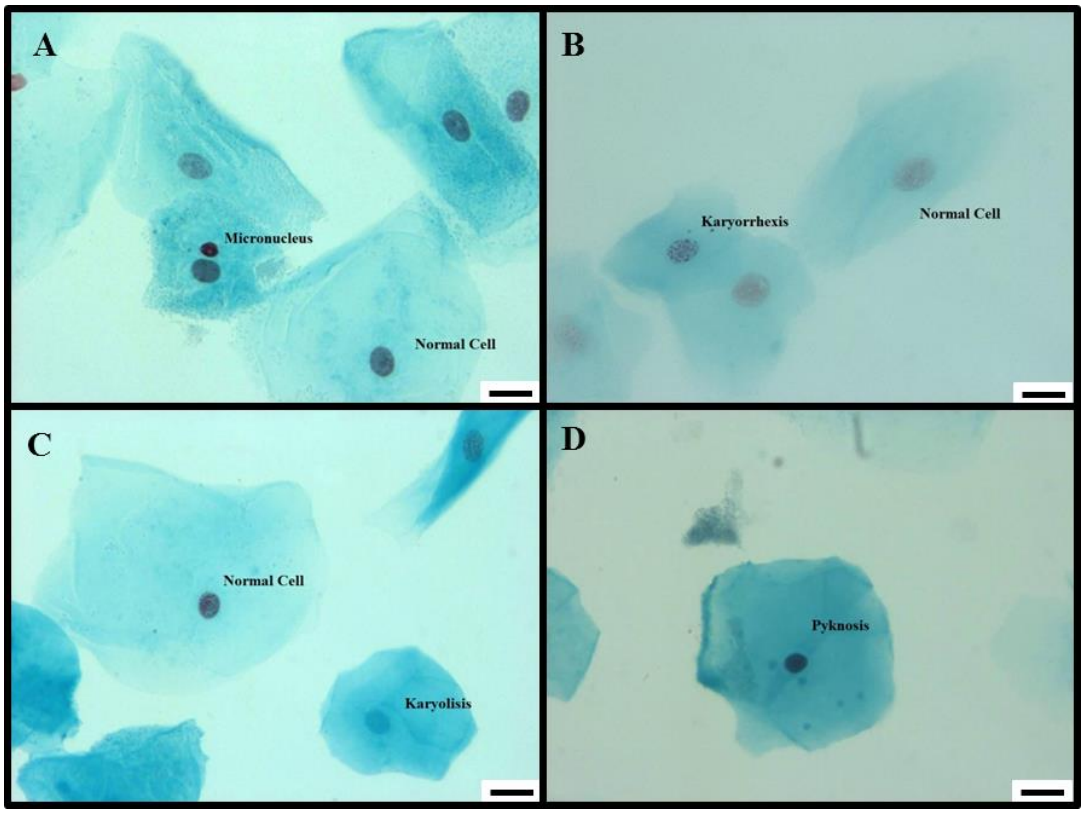

Figure 1 - Photomicrograph of exfoliated oral mucosa cells stained with the Feulgen method. Micronucleus (A) karyorrhexis (B), karyolysis (C) and pyknosis (D) at optical microscopy (scale bar=15µm).

3.4.3. Statistical analysis

The Mann-Whitney U test or *t*- student test were used to compare the frequencies of MNC and of ONA between individuals exposed to ionizing radiations and non-exposed individuals (exposed group *vs*. control group, respectively). To

estimate the association between occupational exposure to ionizing radiations and the frequency of MNC, relative risks (RRs) and 95% confidence intervals (95% CIs) were calculated using a negative binomial regression model (Cameron & Trivedi, 1998; Ceppi *et al*., 2010), adjusting for age, gender, annual deep dose, tobacco use (yes *vs*. no), alcoholic drinks use (yes *vs*. no) and elixir use (yes *vs*. no). To estimate the association between occupational exposure to ionizing radiations and frequency of ONA, relative risks (RRs) and 95% confidence intervals (95% CIs) were also calculated using a negative binomial regression model, adjusting for age, gender, annual deep dose, tobacco use (yes *vs*. no), alcoholic drinks use (yes *vs*. no), elixir use (yes *vs*. no) and number of cigarettes smoked per day.

Qui-Square tests were performed to compare gender, tobacco use (yes *vs*. no), alcoholic drinks use (yes *vs*. no) and elixir use (yes *vs*. no) distributions between exposed and non-exposed groups, while age distribution was compared by *t*-test. Spearman's rank or Pearson's correlations were performed between cell nuclear anomalies (MN and ONA), confounding factors and dose of exposure.

All statistical analyses were performed using SPSS 21.0 for Windows (SPSS Inc., 2012), at the level of statistical significance of $P \leq 0.05$.

3.5. Results

3.5.1. Subjects

A total of 81 subjects, which included 42 exposed workers and 39 controls, were recruited in the Hospital do Divino Espírito Santo for our study. Characteristics of exposed workers and controls are presented in Table 2.

The exposed group and the control group were compared relatively to the binary variables, gender, tobacco use, alcoholic drinks use and elixir use (Table 2). The Qui-Square test, for a confidence level of 95%, revealed that only sex and alcoholic drinks showed significant differences between both groups (X^2= 4.391, *P*= 0.036; X^2= 12.825, *P*<0.001, respectively).

Table 2 - Characteristics and main habits of the group exposed to ionizing radiation and control group and dosimeter readings (average ± S.E.).

Parameters	**Exposed Subjects, n (%)**	**Controls, n (%)**	***P*-value**
Number of subjects	42	39	
[1]Mean age (years) ± S.E.	44.81 ± 1.347	44.51 ± 1.844	0.896
[2]Gender			0.036
Female	23 (54.8%)	30 (76.9%)	
Male	19 (45.2%)	9 (23.1%)	
Job category			
Physicians	11 (26.2%)	-	
Technicians	18 (42.8%)	-	
Nurses	6 (14.3%)	-	
Auxiliary health care	7 (16.7)	-	
[2]Tobacco use			0.745
No	31 (73.8%)	30 (76.9%)	
Yes	11 (26.2%)	9 (23.1%)	
[2]Alcoholic drinks use			<0.001
No	21 (50%)	34 (87.2%)	
Yes	21 (50%)	5 (12.8%)	
[2]Elixir use			0.223
No	18 (42.9%)	22 (56.4%)	
Yes	24 (57.1%)	17 (43.6%)	
Dosimeter			
ASD (mSv)	0.633 ± 0.132	-	
ADD (mSv)	0.715 ± 0.134	-	

ASD – Annual Surface Dose; ADD – Annual Deep Dose
[1]*t*- test; [2]Pearson Chi-Square

The exposed group and the control group were also compared relatively to the continuous variable age (Table 2). The *t*-Test test, for a confidence level of 95%, revealed that there is no significant difference between both groups (t=-0.131; *P*=0.896).

3.5.2. Nuclear Anomalies (MN and ONA): Comparative Analysis

The frequency (mean ± SE) of MNC per 2000 cells was higher in the exposed group than in the control group (5.26 ± 0.653 *vs*. 1.33 ± 0.215 respectively). Similarly, the frequency of ONA per 2000 cells was higher in the exposed group than in the control group (146.62 ± 10.984 *vs*. 88.46 ± 6.943, respectively). All nuclear anomalies in study show a higher frequency in the exposed group (Table 3, Figure 2 and 3).

Table 3 - Frequency (mean ± SE) of cells (per 2000 cells) with micronuclei, karyorrhexis, pyknosis, karyolysis and the total of these last 3 anomalies (ONA) in epithelial buccal cells of individuals exposed (Exposed group) and non-exposed (Control group) to ionizing radiation.

Nuclear anomalies / Groups	Mean ± Std. Error	Range	*P*-value
[1]**Micronucleus**			<0.001
Exposed	5.26 ± 0.653	0 - 17	
Control	1.33 ± 0.215	0 - 6	
[1]**Karyorrhexis**			0.004
Exposed	110.81 ± 11.195	17 - 333	
Control	66.64 ± 5.885	1 - 154	
[1]**Karyolysis**			0.002
Exposed	16.69 ± 0.378	0 - 47	
Control	10.41 ± 1.720	0 - 43	
[1]**Pyknosis**			0.001
Exposed	19.12 ± 1.791	2 – 47	
Control	11.41 ± 1.527	0 - 35	
[2]**ONA**			<0.001
Exposed	146.62 ± 10.984	35 - 359	
Control	88.46 ± 6.943	7 - 202	

[1]Mann-Whitney test; [2]*t*-test

It is possible to verify that 75% of the control group sample presents values of MNC below the observed in the 1st quartile for the exposed group. Significant differences between both groups were observed, where the median of MNC in the exposed and in the control group are equal to 4 and 1, respectively (Figure 2A).

Regarding to ONA, 75% of the sample of the exposed group presents ONA values above the median of the control group. Also significant differences were found between the exposed and the control group, where the median of ONA is equal to 92 and 130.50 in the control and the exposed group, respectively (Figure 2B).

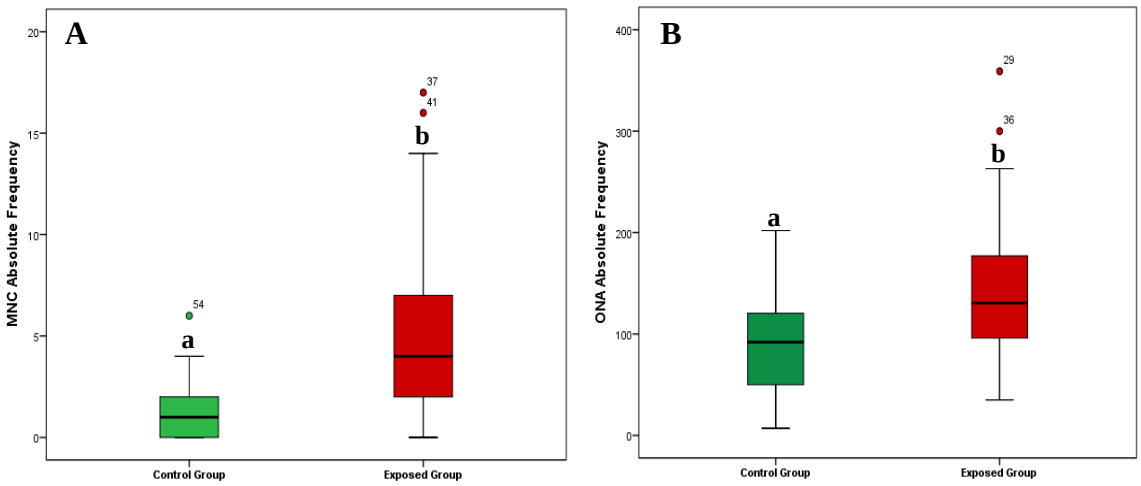

Figure 2 - Box-plots diagrams showing frequency distribution of cells with MN (A) or with ONA (B) per 2000 cells in the exposed group to ionizing radiation and control group: line within the box, median; thin horizontal lines represent minimum and maximum values and outliers (O); different letters over the bars indicate significant differences at *P*=0.05 (A- Mann-Whitney test; B- *t*-test).

Fifty percent of the samples of the control group show values of karyorrhexis below the median of the exposed group. Significant differences were observed between the exposed and the control group, both with a median equal to 93 and 71, respectively (Figure 3A).

Similarly to the previous nuclear anomaly, 50% of the control group samples show values of karyolysis below the median of the control group. Also, significant differences were found between the two groups, where the control and exposed group presented a median equal to 6 and 14 karyolysis, respectively (Figure 3B).

Regarding to pyknosis, 75% of the exposed group sample shows values of pyknosis above the observed in the 1st quartile of the control group. It is possible to verify that a significant difference exists between both groups and that the median of pyknosis is equal to 7 and 17, in the control and exposed group, respectively (Figure 3C).

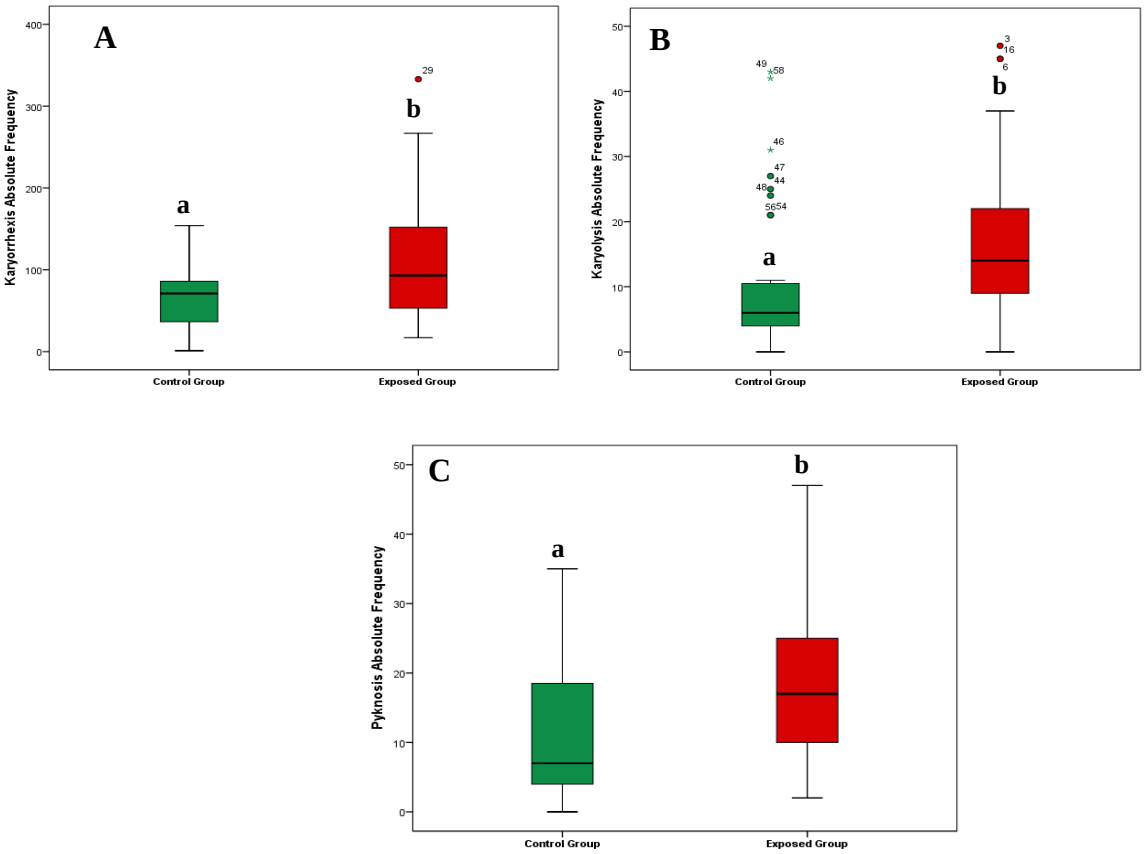

Figure 3 - Box-plots diagrams showing frequency distribution of cells with karyorrhexis (A), karyolysis (B) or pyknosis (C) per 2000 cells in the exposed group to ionizing radiation and control group: line within the box, median; thin horizontal lines represent minimum and maximum values, extreme values (*) and outliers (O); different letters over the bars indicate significant differences at *P*=0.05 (Mann-Whitney test).

3.5.3. Association between studied variables

Correlation between exposure to ionizing radiation (Annual Surface Dose) and the studied variables

A significant, strong and positive correlation between MNC and ASD (Rho= 0.652; *P*<0.001; Spearman Correlation) was observed. It was also observed a significant, moderate and positive correlation between karyorrhexis and ASD (Rho= 0.394; *P*<0.001; Spearman Correlation). No significant correlations were observed between karyolysis or pyknosis with ASD. However, when considering the last 3

nuclear anomalies in total (ONA), a significant, positive and moderate correlation was observed between this variable and the ASD (Rho= 0.425; *P*<0.001; Pearson Correlation).

Finally, Spearman's Rank correlations revealed a significant, positive and moderate correlation between alcoholic drinks use and MNC (Rho= 0.400; *P*<0.001), and also a significant, positive and moderate correlation between gender and MNC (Rho= 0.389; *P*<0.001). For all the other variables, no significant correlations were observed (all *P*> 0.05).

Association between exposure to ionizing radiation and the frequency of MNC or ONA: Relative Risk

In order to assess the association between the frequency of MNC and the annual surface dose of exposure to ionizing radiation, a negative binomial regression adjusted for age, gender, tobacco use, alcoholic drinks use, elixir use was carried out (Table 4). Results showed that ASD of ionizing radiation is a significant predictor of MNC in the multivariate analysis (RR= 1.8; 95% CI, 1.1-2.8; *P*= 0.018). These results show that the risk of having high frequency of MNC is increased by 1.8-fold in the individuals exposed to ionizing radiations, compared to non-exposed individuals.

Table 4 - Association between the frequency of MNC and age, gender, tobacco use, alcoholic drinks use, elixir use and annual surface dose, expressed as Relative Risk (RR) at 95% confidence intervals (95%CI).

Factor	RR (95% CI)	*P*-value
Age	1.01 (0.983-1.038)	0.480
Gender		
Female	1	-
Male	1.691 (0.816-3.504)	0.158
Tobacco use		
No	1	-
Yes	0.673 (0.357-1.268)	0.220
Alcoholic drinks use		
No	1	-
Yes	1.063 (0.505-2.238)	0.872
Elixir Use		
No	1	-
Yes	1.158 (0.689-1.947)	0.580
Annual Surface Dose	1.763 (1.101-2.824)	0.018

A similar approach was carried out to assess the association between the frequency of ONA and the ASD of exposure to ionizing radiation (Table 5). Similarly to MNC, the ASD of ionizing radiation is a significant predictor of ONA in the multivariate analysis (RR= 1.8; 95% CI, 1.1-2.8; P= 0.020). These results show that the risk of having high frequency of ONA is increased by 1.8-fold in the individuals exposed to ionizing radiations, compared to non-exposed individuals.

Table 5 - Association between the frequency of ONA and age, gender, tobacco use, alcoholic drinks use, elixir use, annual surface dose and cigarettes per day, expressed as Relative Risk (RR) at 95% confidence intervals (95%CI).

Factor	RR (95% CI)	*P*-value
Age	1.011 (0.983-1.039)	0.457
Gender		
Female	1	-
Male	1.685 (0.810-3.504)	0.163
Tobacco use		
No	1	-
Yes	0.842 (0.289-2.457)	0.753
Alcoholic drinks use		
No	1	-
Yes	1.106 (0.514-2.379)	0.796
Elixir Use		
No	1	-
Yes	1.193 (0.700-2.032)	0.517
Cigarettes per day	0.976 (0.890-1.070)	0.605
Annual Surface Dose	1.745 (1.090-2.792)	0.020

3.6. Discussion

Ionizing radiations are recognized mutagens and carcinogens to the human population (Popova *et al*., 2007; Vellingiri *et al*., 2014), capable of inducing several forms of DNA damage, such as micronuclei formation, strand breaks and chromosomal anomalies (Morgan *et al*., 2002; Chadwick & Leenhouts, 2003; Vral *et al.*, 2011; Vellingiri *et al*., 2014). Our results show that occupational exposure to ionizing radiations can cause DNA damage, resulting in an increase of MNC and of cells with other nuclear anomalies.

As expected, the obtained results in this study are consistent with the hypothesis that the frequency of MNC and ONA is higher in the group exposed to ionizing

radiations than in non-exposed group, which is highlighted by the significant differences observed between the two groups for the frequency of MNC and all the other studied nuclear anomalies (karyorrhexis, karyolysis and pyknosis).

The frequency of MNC (mean ±SE) in the control group was 1.3 ± 0.26 per 2000 cells, which is within the normal range for human oral epithelia (0.5-2.5 MNC/ 1000 cells), reported by Holland *et al*. (2008) and Ceppi *et al*. (2010). However, even though only slightly above the normal range, the mean (±SE) frequency of MNC in the exposed group (5.3 ± 0.67 MNC/2000 cells) was much higher than in the control group. These results help in explaining the wide application of the MN test (although in lymphocytes) in populations exposed to ionizing radiations (Bao *et al*. 1997; Bonassi *et al*., 2007; Dias *et a*l, 2007; Ropolo *et al*, 2012), since MN are recognized as reliable biomarkers of effect, acting as a predictors of carcinogenic risk (Bloching *et al*., 2000; Saran *et al*., 2008; Mahimkar *et al*., 2010,). Furthermore, the high frequency of MNC observed in this study is consistent with results obtained in studies that analyze the lymphocytes of individuals exposed to ionizing radiations (Cardoso *et al*., 2001; Zakeri & Hirobe, 2010) and the oral epithelial cells (Cerqueira *et al*., 2008). Likewise, all the other studied nuclear anomalies were always more frequent in the group of individuals occupationally exposed than in unexposed individuals. Our results are consistent the study by Ribeiro (2012) that also observed higher frequencies of these nuclear anomalies in healthy adults who had been submitted for panoramic dental radiography (acute exposure). All these results show that chromosomal damage in epithelial cells induced by ionizing radiations can also be karyorrhexis, karyolysis and pyknosis, and not only MN.

Moreover, in this study 2000 cells were analyzed, which gives a greater accuracy to the obtained results, even though there are several other studies which only

used 1000 cells epithelial cells (eg. Cerqueira *et al*., 2004; Sahin *et al*., 2009; Waingade & Medikeri, 2012; Garcia *et al*., 2012; Ropolo *et al*., 2012; Lorenzoni *et al*., 2013; Rodrigues *et al*., 2013; Aurora *et al*., 2014). Nevertheless, it has not yet been clearly defined the number of cells that should be counted/analysed (Fenech *et al*., 1999; Majer *et al*., 2001), but the importance of 2000 cells or more (>4000 counted cells) is made clear by Ceppi *et al*. (2010), by saying that "increasing the number of cells scored results in a smaller confidence interval odd estimates".

One of the reasons why this Feulgen method was chosen is related to the fact that the nuclear anomalies observed using the MN test present the lowest values of confounding factors, compared to other methods for evaluation of genetic damage, such as May-Grunwald/Giemsa (Carrard *et al*., 2007), Papanicolaou and Hemotoxylin & Eosin (Grover, *et al*., 2012), mainly due to the possibility of miss interpretation of the nuclear anomalies in study. Another reason is related to the easy access to oral epithelial cells through noninvasive methods, associated with its recognized sensitivity for the assessment of DNA damage (Holland *et al.*, 2008). The use of cells from the oral epithelium in an asset in the analysing of MNC (Holland *et al.*, 2008; Thomas *et al*., 2009; Bolognesi *et al*., 2013), enabling the direct study of a particular tissue (Nerseysyan *et al*., 2002). The accomplishment of the present study is important since there are not many studies on occupational exposure to ionizing radiation using this biomarker in epithelial cells. Most of the studies for occupational exposure to ionizing radiation use lymphocytes, which is a more invasive method.

Our results show that consumption of alcoholic drinks is positively and moderately correlated with the frequency of MNC. An association between alcohol consumption and risk of cancer has been found in several studies, not being explained yet the biological mechanism of this relationship (Williams & Horm, 1977; Maffei *et*

al., 2000; Ellison *et al*., 2001). Maluf & Erdtmann (2000) consider alcohol a genotoxic substance, and there are some studies evidencing that ethanol is capable of producing chromosome breakage (Maffei *et al*., 2002; Ristow *et al*., 1995; Blasiak *et al*., 2000), which meets the results obtained in this study. This association was also found in other studies (Stich & Rosin, 1983; Greenrod *et al*., 2005; Ishikawa *et al*., 2006; Benassi-Evans & Fenech, 2011), although counteracted in many other (Corrêa *et al*. 2009; Cheng *et al*., 2012; Garcia *et al*., 2012; Rodrigues *et al*., 2013). In an *in vitro* study, carried out by Greenrod and Fenech (2003), it was found that ethanol promotes an increase of MNC formation, when compared with other components of the wine; furthermore, according to Greenrod *et al*. (2005) it increased the susceptibility of the individual to DNA damage induced by radiation. Nevertheless, no association between ONA and alcohol consumption was observed.

Our results show a positive and moderate correlation between the frequency of MNC in cells of the oral epithelium and gender, in accordance with studies conducted by Gonsebatt *et al*. (1997), Bonassi *et al*. (2001) e Pastor *et al*. (2001). The effects of gender on MN was first recognized in studies, with lymphocytes, performed by Fenech and Morley (1985), Fenech and Morley (1986) and Fenech *et al*. (1994). Rodrigues *et al*. (2012) mentions that gender has been associated with DNA damage in different literature. However, it is important to understand this factor when performing a biodosimetry study (Fenech & Bonassi, 2011).

Our results show no significant association between tobacco consumption (yes *vs*. no) and both groups and MNC and ONA, although tobacco is known to contain various genotoxic chemicals (Speit *et al*., 2003). However, Martino-Roth *et al*. (2002) and Bolognesi *et al*. (2013) even referred a small decrease of MNC frequency in smokers, and Benites *et al*. (2006) found no association between MNC frequency and

smoking status, similarly to our results. On the other hand, authors like Burgaz *et al*. (1995) found a significant increase of MNC in smokers, when compared to non-smokers.

In this study it was not found an association between the use of elixir and MNC or ONA, as Garcia *et al*. (2012) have observed. Contrarily, the study of Erdemir *et al*. (2007) showed an increase of MNC and ONA frequencies in individuals that used elixir.

It is known that high doses of X-ray are able to cause different alterations to the human organism (Brenner *et al*., 2003; Bushong, 2008), and many studies have pointed in this direction, particularly regarding the association between the occurrence of MN in lymphocytes and the dose received by individuals exposed to these same radiation (Vral *et al.*, 2011). However, these associations were made based on the evaluation of lymphocytes and not cells from the oral epithelial, since there are not many studies for occupational exposure to ionizing radiation using oral epithelial cells.

In our study, the physical dosimeters used by health professionals recorded values between 0 and 5.05 mSv, being these values within the limits established by the NCRP (1993). The highest dose values were recorded for the physicians of interventional cardiology, as expected, since these are, in addition to the technicians and nurses who work in rooms of fluoroscopy, the highly exposed group to radiation during fluoroscopic examination (Zakeri & Hirobe, 2010). However, even with these values situated within the parameters of normality, it was found, in this study, a strong and positive correlation between the frequency of MNC (Rho= 0.652; $P<0.001$) or of ONA (Rho= 0.425; $P<0.001$) with the annual surface dose, having this same association being observed in other studies (Hadjidekova *et al*., 2003).

Regarding the association between ASD and the frequency of MNC and ONA, and considering the confounding factors age, gender, tobacco use, alcoholic drinks use, elixir use and number of cigarettes smoked, we observed that the risk of developing MNC or cells with ONA was almost the double in the group exposed to ionizing radiations. These results show that the frequency of MNC increases with the dose of radiation, in an occupational context, as it has been suggested by Kumari *et al*. (2005).

Our results indicate that the MNC of the oral epithelia are good biomarkers for occupational exposure to ionizing radiation, confirming the relevant role of micronuclei as biological dosimeters, as previously mentioned by several authors for lymphocytes (Muller *et al.*, 1996; Hendry & West, 1997; Vral *et al.*, 2011). Salama *et al*. (1999) mentioned the fact that karyorrhexis, karyolysis and pyknosis are effective biomarkers to assess the exposure of individuals to mutagenic and carcinogenic agents. Our study highlights, for the first time, the relevance of the using altogether these 3 nuclear anomalies as biomarkers of exposure/effect to ionizing radiations.

3.7. Conclusion

Globally, our results suggest a significant association between occupational exposure to ionizing radiations and the occurrence of MNC and ONA in oral epithelial cells, enlightening exposure to ionizing radiations as a carcinogenic agent. With this consideration it is important to highlight the significantly higher risk for DNA damage observed in the exposed group. Therefore, healthcare professionals who deal daily with ionizing radiations are advised to not neglect radioprotection procedures in force, always using all personal and collective protective equipment available in the health unit.

It is of a great importance to further investigate the genetic damage induced by radiation and estimate the radiation effectively received by occupationally exposed healthcare professionals. Results show that the studied biomarkers can be used for human biomonitoring, in order to analyze the mutagenic and clastogenic effects of ionizing radiations on individuals occupationally exposed to ionizing radiations.

3.8. References

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Appendix

Appendix 1 – Informed Consent

Consentimento informado, esclarecido e livre para participação em estudo de investigação nos termos da Norma n.º 015/2013 da Direcção-Geral da Saúde

Por favor, leia com atenção a seguinte informação. Se achar que algo está incorrecto ou que não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe foi feita, queira assinar este documento.

Título: **Avaliação de genotoxicidade em células do epitélio bucal de sujeitos expostos ocupacionalmente a radiações ionizantes**

Este estudo enquadra-se no âmbito da realização da Tese de Mestrado em Ambiente, Saúde e Segurança da mestranda Laura Aguiar Torres, ministrado pela Universidade dos Açores, orientada pelo Doutor Armindo dos Santos Rodrigues, Professor com Agregação na Universidade dos Açores, e pela Doutora Patrícia Ventura Garcia, Professora Auxiliar na Universidade dos Açores.
Para a realização deste estudo será recolhida uma amostra das células do seu epitélio bucal, bem como de todos os indivíduos em estudo, os quais estão divididos por dois grupos, o grupo de estudo, composto por profissionais de saúde que lidam diariamente com radiações ionizantes, e o grupo de controlo, composto por profissionais de saúde que não lidam diariamente com radiações ionizantes. Será ainda necessário proceder à recolha dos valores das doses de radiação recebida por si (se aplicável), as quais encontram-se descritas na folha de controlo dosimétrico, assim como responder a um breve questionário.
A participação neste estudo é de caracter voluntário, ausente de prejuízos, anónimo e confidencial, sendo a recolha de dados exclusiva para uso no presente estudo, não havendo qualquer possibilidade de posteriormente saber o resultado da análise efectuada às suas células.
Em nome da investigadora e Técnica de Radiologia, Laura Aguiar Torres, agradecemos a sua participação. Caso tenha alguma dúvida, não hesite em contactar a investigadora através do telemóvel n.º 919026826/927660530, ou através do e-mail: lauratorresradio@gmail.com.

Assinatura e número de cédula profissional de quem pede o consentimento:

Laura Aguiar Torres C-040 334148

Declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela pessoa que acima assina. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito a utilização dos dados que de forma voluntária forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pela investigadora.

Nome: _____

Assinatura: _____

Data: ____/____/____

ESTE DOCUMENTO É COMPOSTO POR UMA PÁGINA E FEITO EM DUPLICADO: UMA VIA PARA A INVESTIGADORA, OUTRA PARA A PESSOA QUE CONSENTE.

Appendix 2 - Questionnaire

QUESTIONÁRIO

IDENTIFICAÇÃO:_____ NOME:_____ _____
(Apelido) (1º Nome)

Freguesia _____ Área _____

Morada _____ Permanência (anos) _____

ENTREVISTADOR: _____ DATA DA ENTREVISTA: ____-_____-_____
MÊS DIA ANO

==========

1. IDADE: _____ **2.** Data nascimento: _____ **3.** Local Nasc. _____

4. Sexo: 1.(M)___ 2.(F)___ **5.** Raça: 1. Branco (B)___ 2. Negro (N)___ 3.Asiático (A)___ 4. Outro (O)____

6. Estado Civil: Solteiro(a)___ Casado(a)____ Viúvo(a) ____ Separado(a)/Divorciado(a)____

7. Peso (kg):_____ **8.** Altura (m):_____ **9.** Qual é o seu grau escolar completo mais elevado? _____

==========

CONSUMO DE ÁLCOOL

1.1. Consome bebidas alcoólicas? 1.S___ 2.N___

1.2. Que bebida alcoólica consome mais?
1.Cerveja___ 2.Vinho___ 3.Destiladas____

1.3. Quantos copos bebe por dia? _____

==========

CONSUMO DE TABACO (cigarro = cigarrilha = charuto = cachimbo)

2.1. Fuma? 1.S___ 2.N___

Se respondeu "**Não**" à anterior:

2.2. Já fumou mais de 1 cigarro por dia, por mais de 1 ano, ou mais de 20 maços, por mais de 1 ano? 1.S___ 2.N___

2.3. Com que idade parou de fumar? _____

Se respondeu "**Sim**" à 2.1.:

2.4. Quantos cigarros fuma por dia? _____

2.5. Quantos anos tinha quando começou a fumar? _____

2.6. Em sua casa há fumadores? 1.S___ 2.N___

==========

OUTROS

3.1. Usa elixir bucal (p/ dentes)? 1.S___ 2.N___

3.2. Usa 'spray' para mau hálito? 1.S___ 2.N___

3.3. Fez radiografias à zona da boca nas últimas duas semanas? 1.S___ 2.N___

3.4. Toma alguma medicação? S___ 2.N___

Se respondeu "**Sim**" à 3.4.:

3.5. Qual?_____

==========

OCUPAÇÃO PROFISSIONAL

4.1. Qual a sua profissão? _____

4.2. Qual o local de trabalho?_____

4.3. Quantas horas trabalha por semana? _____

Se aplicável, responda às seguintes questões:

4.4. Em que valências da Radiologia labora? Seleccione todas aquelas que se aplicam a si. 1.Radiologia Convencional___ 2. Hemodinâmica___ 3. Tomografia Computorizada___ 4. Bloco Operatório___

4.5. Quantas horas, por semana, julga estar exposto às radiações ionizantes aquando do exercício das suas funções nas salas de hemodinâmica e bloco operatório? ___

4.6. Há quantos anos exerce funções que estejam relacionadas com a exposição às radiações ionizantes? ___

==========

USO DO DOSÍMETRO (se aplicável)

5.1. Com que frequência utiliza o dosímetro? 1.Sempre___ 2. Às vezes___ 3. Nunca___

Se respondeu "**Nunca**" à 5.1:

5.2. Quantas horas, por semana, julga estar exposto às radiações ionizantes?_____

Algumas questões baseadas em:
1. Ferris BG, 1978. Epidemiology standardization project II. *Am. Rev. Resp.Dis.* 118(6):7-53.
2. Cerqueira EM *et al.*, 2004. Genetic damage in exfoliated cells from oral mucosa of individuals exposed to X-rays during panoramic dental radiographies. Mutat. Res. 562 (1-2): 111-7.

Annexes

Annex 1 - Authorization of the Council of Ethics and the Board of Directors of HDES to conduct the study

PCA 28/4/2014

CES/HDES CHKS

O Conselho de Administração do H.D.E.S. reunido em 30/4/14, apreciou este assunto, tendo deliberado:

- [X] Aprovar/Autorizar
- [ ] Dar parecer favorável
- [ ] Tomar conhecimento
- [ ] Solicitar Informação
- [ ] Outro: .....

Enviar a .....

Exmo. Sr.
Presidente do Conselho de Administração
do HDES
Dr. Fernando Mesquita

Ponta Delgada, 28 de Abril de 2014

ASSUNTO: Apreciação do Projeto de Investigação *"Avaliação de Genotoxicidade em Células do Epitélio Bucal de Sujeitos Expostos Ocupacionalmente a Radiações Ionizantes*

Relativamente ao assunto em epígrafe do qual é Investigadora Principal a Drª Laura Aguiar Torres (a aluna do mestrado em Ambiente, Saúde e Segurança da Universidade dos Açores) cumpre informar V. Exc.ª que a Comissão de Ética para a Saúde, reunida a 10 de Abril do corrente ano, deliberou por unanimidade emitir parecer favorável à sua efetivação no HDES.

Com os melhores Cumprimentos,

O Presidente da Comissão de Ética para a Saúde

Dr. Dionísio Faria e Maia

Annex 2 - Feulgen method protocol

Feulgen method protocol

1 – Air dry the slides: maximum of 24 hours;

2 – Fix in methanol. 0°C for 20 minutes;

3 – Wash in distilled water for 1 minute;

4 – Place the slides in HCL5N for 20 minutes;

5 – Place in Schiff reagent for 20 minutes in the dark;

6 – Rinse with water for 5 minutes;

7 – Put the slides in Light Green for 1 minute;

8 – Proceed to dehydration with 70% ethanol (1 minute), 96% ethanol (1 minute) and 100% ethanol (2 minutes);

9 – Lighten with xylene;

10 – Assemble small slides with DPX.