Genotoxicity of Acrylic Resin

In Vitro Study on Gingival Fibroblasts

Mariana do Souto Lopes

Porto

2012

U.PORTO

FACULDADE DE MEDICINA DENTÁRIA
UNIVERSIDADE DO PORTO

Genotoxicity of Acrylic Resin

In Vitro Study on Gingival Fibroblasts

Mariana do Souto Lopes

Porto

2012

U.PORTO

FACULDADE DE MEDICINA DENTÁRIA
UNIVERSIDADE DO PORTO

Thesis presented to the

Faculty of Dental Medicine of the University of Porto

as a candidature to obtain the Master Degree in Oral Rehabilitation

Supervisor: Professor Doutor Daniel Pérez Mongiovi

Assistant Professor – Faculty of Dental Medicine of the University of Porto

Co-Supervisor: Professor Doutor José Albertino da Cruz Lordelo

Associate Professor – Faculty of Dental Medicine of the University of Porto

Faculty of Dental Medicine of the University of Porto

Scientific Council

Prof. Doutor Afonso Manuel Pinhão Ferreira (Prof. Catedrático)

Prof. Doutor Américo dos Santos Afonso (Prof. Associado c/ Agregação)

Prof. Doutor António Cabral Campos Felino (Prof. Catedrático)

Prof. Doutor César Fernando Coelho Leal Silva (Prof. Associado c/ Agregação)

Prof. Doutor Germano Neves Pinto Rocha (Prof. Associado)

Prof. Doutora Irene Graça Azevedo Pina Vaz (Prof. Associado)

Prof. Doutora Inês Alexandra Costa Morais Caldas (Prof. Auxiliar)

Prof. Doutor João Carlos Antunes Sampaio Fernandes (Prof. Catedrático)

Prof. Doutor João Carlos Gonçalves Ferreira de Pinho (Prof. Associado c/ Agregação)

Prof. Doutor João Fernando Costa Carvalho (Prof. Catedrático)

Prof. Doutor Jorge Manuel Carvalho Dias Lopes (Prof. Catedrático)

Prof. Doutor José António Macedo Carvalho Capelas (Prof. Associado c/ Agregação)

Prof. Doutor José Carlos Reis Campos (Prof. Auxiliar c/ Agregação)

Prof. Doutor José Mário Castro Rocha (Prof. Auxiliar)

Prof. Doutor Manuel José Fontes de Carvalho (Prof. Associado)

Prof. Doutora Maria Cristina Pinto Coelho Mendonça de Figueiredo Pollmann (Prof. Associado)

Prof. Doutora Maria Helena Guimarães Figueiral da Silva (Prof. Associada c/ Agregação)

Prof. Doutora Maria Helena Raposo Fernandes (Prof. Catedrático)

Prof. Doutora Maria Lurdes Ferreira Lobo Pereira (Prof. Auxiliar)

Prof. Doutor Mário Augusto Pires Vaz (Prof. Associado da FEUP - personalidade convidada)

Prof. Doutor Mário Jorge Rebolho Fernandes Silva (Prof. Catedrático)

Prof. Doutor Mário Ramalho Vasconcelos (Prof. Associado c/ Agregação)

Prof. Doutor Miguel Fernando Silva Gonçalves Pinto (Prof. Catedrático)

Prof. Doutor Paulo Rui Galrão Ribeiro Melo (Prof. Associado c/ Agregação)

Prof. Doutor Ricardo Manuel Lobo Faria Almeida (Prof. Associado c/ Agregação)

Jubilated Professors

Prof. Doutor Adão Fernando Pereira (Prof. Catedrático)

Prof. Doutor Amílcar Almeida Oliveira (Prof. Associado)

Prof. Doutor António Manuel Machado Capelas (Prof. Associado - falecido)

Dr. António Ulisses Matos dos Santos (Assistente Convidado)

Prof. Doutor Durval Manuel Belo Moreira (Prof. Associado c/ Agregação)

Prof. Doutor Francisco António Rebelo Morais Caldas (Prof. Catedrático)

Dr. José Maria Vaz Osório (Assistente Convidado)

Prof. Doutor José Serra Silva Campos Neves (Prof. Catedrático)

Prof. Doutor Manuel Desport Marques (Prof. Associado Convidado - falecido)

Prof. Doutor Manuel Guedes de Figueiredo (Prof. Associado)

to Pedro

to my parents Paula and Alexandre,

to my sister Sara,

to my dogs Lai and Tim

“If I have seen further it is by standing on ye sholders of Giants.”

Sir Issac Newton, 1676

AKNOWLEDGEMENTS

If I have successfully reached the end of this journey, it was, to a great extent, due to the lessons and support I have received from several people.

Firstly, I would like to thank to my supervisor, Professor Daniel Pérez Mongiovi, for the most dedicated guidance of this research, for teaching me what I wanted to know and for sharing with me the difficulties and the joys of this journey. His friendship, encouragement and patience were fundamental for the successful conclusion of this thesis.

I would also like to thank to Professor José Lordelo, not only for co-supervising and encouraging me in this research, but also for the clinical teaching during the Master course.

I would like to thank to Professor Alexandra Teixeira for the friendship, care and close following of the research developments, as well as the opportune observations and corrections.

I am also very grateful to Professor Álvaro Azevedo for the care showed for this research, the precious help in the statistical analysis and for the wise scientific advices.

I would like to thank to Professor Helena Fernandes and all the collaborators at the Laboratory of Pharmacology and Biocompatibility of the Faculty of Dental Medicine of the University of Porto, for providing all the equipments and materials necessary for the research. I am also very grateful for the wise and experienced scientific advices and for establishing the connection with the Faculty of Pharmacy of the University of Porto.

I am very grateful to Professor Conceição Branco and the co-workers at the Laboratory of Applied Chemistry of the Faculty of Pharmacy of the University of Porto, for the precious collaboration in this research. In particular, I would like to thank to

Professor Célia Amorim for her friendship, care and patience to complete and revise the analytical part of this thesis.

I would also like to thank to Professor Paula Sampaio from the Laboratory of Advanced Light Microscopy of the Institute for Molecular and Cell Biology of the University of Porto, for the availability and wise explanations on how to use the confocal fluorescent microscope.

I would like to thank Professor Helena Figueiral for authorizing the use of the facilities of the Laboratory of Removable Prosthodontics of the Faculty of Dental Medicine of the University of Porto. In particular, I would like to express my gratitude to the dental technicians Sr. Fernando Faria and Sr. Carlos Costa for kindly and patiently teaching me the laboratory techniques necessary for the fabrication of the acrylic samples.

I would also like to thank Professors Maria de Lurdes Pereira, Benedita Sampaio and Inês Caldas for the scientific and moral support along all steps of this research.

I am very grateful to all Professors of the Master in Oral Rehabilitation for the scientific and clinical knowledge I was taught.

I would like to thank the friendship and kindness of the assistants at the clinic.

Finally, I would like to thank my fellow colleagues of the Master of Oral Rehabilitation for the companionship during these two years, especially to my partner and friend Marcelo Lopes.

INDEX

Abbreviations List

α-MEM – α-minimal essential medium

APC – anaphase promoting complex

bis-GMA – bisphenol A-glycidyl methacrylate

BA – *n*-butyl acrylate

BMA – *n*-butyl methacrylate

BPO – benzoyl peroxide

CA – chromosomal aberrations

CBMN – cytokinesis-block micronucleus assay

CHO – chinese hamster ovarian cell line

CPC – bovine dental papilla primary cells

CytoB – cytochalasin B

DBP – dibutyl-phthalate

DIN – Deutsches Institut für Normung (German Institute for Standardization)

DMA – *n*-dodecyl methacrylate

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

EGDMA – ethyleneglycol dimethacrylate

EDTA – ethylenediamine tetraacetic acid

EMS – ethyl methanesulfonate

FBS – fetal bovine serum

FDA – US Food and Drug Administration

FISH – fluorescence *in situ* hybridization

FTIR – Fourier transform infrared spectroscopy

GC – gas chromatography

GMA – glycidyl methacrylate

HeLa – human epithelial cell line

HEMA – 2-hydroxyethyl methacrylate

HGF – human gingival fibroblastos (primary or cell line)

HMA – *n*-hexyl methacrylate

HPLC – high performance liquid chromatography

HQ – hydroquinone

HSG – human submandibular gland adenocarcinoma cell line

IBMA – isobutyl methacrylate

ISO – International Organization for Standardization

LC- liquid chromatography

LOD – limit of detection

LOQ – limit of quantification

log *P* – logarithm of the octanol/water partition coefficient

L929 – mouse fibroblast cell line

MA – methacrylic acid

MDL – method detection limit

MeOH – methanol

MMA – methyl methacrylate

MN – micronucleus/micronuclei

MNvit – i*n vitro* mammalian cell micronucleus test

MP – mobile phase

MQL – method quantification limit

MTA – mineral trioxide aggregate

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromid assay

NAC – *N*-acetylcystein

NBUD – nuclear bud

NPB – nucleoplasmic bridge

NRU – neutral red uptake

OECD – Organization for Economic Co-operation and Development

PAA – polyacrylic acid

PBS – phosphate buffered saline

PDL – periodontal ligament

PI – propidium iodide

PDMS – poly(dimethylsiloxane)

PMMA – poly(methyl methacrylate)

ROS – reactive oxygen species

RPC-C2A – pulp cell line

SAC – spindle assembly checkpoint

SCE – sister chromatid exchange

SD – standard deviation

SFC – supercritical fluid chromatography

SP – stationary phase

TEGDMA – triethylene glycol dimethacrylate

TMPTMA – trimethylolpropane trimethacrylate

UDMA – urethane dimethacrylate

V79; V79-379A; V79B; V79-4 – chinese hamster lung fibroblast cell line (and subcultures)

XTT – (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl] -2H-tetrazolium hydroxide) assay

WI-38 – human fetal lung fibroblast cell line

1,6-HDMA – 1,6-hexanediol dimethacrylate

3T3 – mouse fibroblast cell line

Tables List

ABSTRACTS

Abstract

Genotoxicity of Acrylic Resin: *In Vitro* Study on Gingival Fibroblasts

Poly-methyl methacrylate based materials have a wide range of applications in prosthodontics and orthodontics. However, complete polymerization of the methyl methacrylate monomer is never achieved and may therefore leach into the oral environment in physiological conditions. It has been often associated with adverse biological effects such as allergic reactions in the patients' oral mucosa and contact sensitization/irritation in dental professionals.

In the present study we hypothesized whether the residual monomer leached in approximate clinical conditions is capable of inducing *in vitro* cytotoxic and/or genotoxic hazards to a human gingival fibroblast cell line.

All methodologies in this study followed ISO standards and international guidelines. At first, heat polymerizable acrylic resin samples were fabricated and then submersed independently in artificial saliva for 24 or 72 hours at 37ºC, in order to simulate the mouth clinical conditions. The leached residual monomer was quantified by means of high performance liquid chromatography.

Moreover, a thoroughly characterization of the methyl methacrylate cytotoxic profile on the viability of human gingival fibroblasts was performed and compared to that on human fetal lung fibroblasts and chinese hamster lung fibroblasts. Ethyl methanesulfonate and formaldehyde were tested as well as positive controls. Methyl methacrylate showed to be cytotoxic to human gingival fibroblasts, for concentrations ranging 40 to 160 mM. However, the determined values for the leached residual monomer from polymerized pieces were 10^5- fold less concentrated, which does not cytotoxically affect the cells. Subsequently, the genotoxicity effect of these three

chemicals was also determined using human gingival fibroblasts and chinese hamster lung fibroblasts, by means of the micronuclei *in vitro* test.

Within the limitations of this *in vitro* research, the outcomes show that the residual monomer leached by the polymer tested does not affect cyto and genotoxically the cell lines tested. Moreover, it provided some evidence that the human gingival fibroblast cell line is a good model for cytotoxicity assays. Still, further studies must be developed so as to draw definite conclusions on the suitability of these cells as oral models for the micronuclei *in vitro* test.

Resumo

Genotoxicidade das Resinas Acrílicas: Estudo *In Vitro* em Fibroblastos Gengivais

Os materiais baseados no poli-metil metacrilato têm uma ampla aplicabilidade em prótese dentária e ortodontia. No entanto, a reação de polimerização do monómero de metil metacrilato nunca é completa, podendo ocorrer lixiviação para o meio oral em condições fisiológicas. Tem sido associado recorrentemente a efeitos biológicos adversos como reações alérgicas na mucosa oral de pacientes e a sensibilização/irritação de contacto em profissionais dentários.

No presente estudo formulou-se a hipótese de que o monómero residual lixiviado em condições clínicas aproximadas poderia induzir danos citotóxicos e/ou genotóxicos *in vitro* numa linha celular de fibroblastos gengivais humanos.

Todos os métodos deste estudo seguiram normas ISO e diretivas internacionais. Em primeiro lugar, produziram-se amostras de resina acrílica termo-polimerizável, que foram submersas independentemente em saliva artificial durante 24 ou 72 horas a 37ºC, de modo a simular as condições clínicas da cavidade oral. O monómero residual lixiviado foi quantificado por cromatografia líquida de alta eficiência.

Além disso, realizou-se uma caracterização meticulosa do perfil citotóxico do metil metacrilato na viabilidade dos fibroblastos gengivais humanos e comparou-se com o de fibroblastos fetais de pulmão humano e fibroblastos de pulmão de hamster chinês. O etil metanossulfonato e formaldeído foram também testados como controlos positivos. O metil metacrilato mostrou ser citotóxico para os fibroblastos gengivais humanos, para concentrações a variar entre 40 e 160 mM. No entanto, os valores determinados para o monómero residual lixiviado das peças polimerizadas foram

cerca de 10^5 vezes menos concentrados, o que não afeta as células ao nível citotóxico. Subsequentemente, o efeito genotóxico destas três substâncias químicas também foi determinado para os fibroblastos gengivais humanos e fibroblastos de pulmão de hamster chinês, através do teste de micronúcleos *in vitro*.

Dentro das limitações desta pesquisa *in vitro*, os resultados mostram que o monómero residual lixiviado pelo polímero testado não afeta cito nem genotoxicamente as linhas celulares testadas. Além disso, forneceu alguma evidência de que a linha celular de fibroblastos humanos gengivais são um bom modelo para ensaios de citotoxicidade. No entanto, é necessário desenvolver estudos adicionais para que se possam retirar conclusões definitivas sobre a adequação destas células como modelos orais para o teste de micronúcleos *in vitro*.

A. INTRODUCTION

Dental acrylic resins are biomaterials that are commonly used for the fabrication of oral devices in the prosthodontic field. The present research project intends to develop methodologies to evaluate the amount of leachable molecules released by a conventional heat-polymerizable denture base polymer and their potential harmful effects. The biological risk assessment will include cytotoxic and genotoxic *in vitro* assays performed on an oral cell model.

A.1. Oral Rehabilitation and Dental Polymers

Edentulism can be defined as a state of loss of all natural permanent teeth[1] and is considered by the World Health Organization as a physical impairment.[2] It is a global health problem, affecting the populations of both developed and developing countries, with an estimated international prevalence between 7 and 69%.[2] Despite the recent improvements in oral health care, there are still barriers to public preventive oral treatments and too many people are still being affected by oral chronic diseases like caries and periodontitis, which lead to the partial or total loss of teeth.[2,3] Other biological conditions like pulpal pathology, trauma and oral cancer, or the socio-economical circumstances (gender, income, education level, access to health care, culture) also account for tooth loss.[2] It has also been related to several systemic diseases like osteoporosis, hypertension and coronary artery disease, respiratory illnesses, diabetes, neuropathies, rheumatoid arthritis and cancer, as well as oral conditions such as alveolar ridge resorption, impairment of the oro-facial function (mastication, speech) and esthetics.[2] Moreover, edentulism has been associated with increasing age, which nowadays is becoming a global issue, with the aging of the populations, especially in developed countries.[4] As a consequence, the peoples' needs in terms of prosthodontic partial and total rehabilitation are growing as well.[4]

Generally speaking, a prosthesis is an artificial therapeutic device which substitutes a missing part of the body, improving or changing its function.[1] More specifically, a dental prosthesis (denture) is an artificial device that substitutes the missing teeth (partial or total edentulism) and associated dental/alveolar structures,[1] which may as well include any additions needed for optimum function.[5] Therefore, it can be considered a medical device since it is an *apparatus* (…)/ *appliance (…) intended by the manufacturer to be used, alone or in combination, for human beings (…)* with the *purpose of (…) replacement, modification or support of the anatomy or of a physiological process*.[6] Dental prostheses (as well as orthodontic appliances) are permanent surface-contacting devices (with mucosal membranes), since the cumulative single, multiple or repeated long-term use frequently exceeds 30 days.[6] Many times, this close contact is promoted by the denture base that rests on the soft tissue foundations and to which the artificial teeth are attached.[1,5] One of the most fundamental requirements of the denture base is its adaptation, which is its degree of fit to the supporting area, including the surface of the palate, maxillary and mandibular edentulous ridges, best suited to carry the chewing forces during the denture's function.[1] Base adaptation is one of the factors that influence the retention of a denture, avoiding the vertical dislodging forces along the path of placement, away from the supporting tissues.[1,7] For that reason, it may be stated that the base adaptation directly contributes to the patients' acceptance and daily functional using of the denture, thus the treatment success.[7]

Acrylic resin-based dental materials became popular in the decade of 1950 and since then the range of applications and products has increased and evolved considerably. Thus, the poly(methyl methacrylate) (PMMA) polymer is ubiquitous in the dental profession and not limited only to the fabrication of conventional removable prosthesis, but also in implant supported prosthodontics, orthodontic devices, denture liners, individual impression trays and temporary crowns.[8,9] In addition, it is widespread in non-dental human use products like the orthopedic bone cements, intraocular lens,

prosthesis for plastic and reconstructive surgery or artificial fingernails and nail varnishes.[9,10]

A.2. Physico-Chemical Features of Acrylic Resins

A polymer is a high molecular weight chemical compound, which, by means of a repeated intermolecular chemical reaction (polymerization) becomes a long-chain and/or cross-linked macromolecule composed of several repetitive united molecules with a lower molecular weight (monomers).[1,11,12] A polymer may be a fiber, a rigid or rubberlike material, depending on the form and morphology of the monomer. Although some polymers may be inorganic, in the dental field most of them are organic molecules, particularly derived from methacrylates (denture and orthodontic bases, restorative composite resins).[12] Other types of polymers based on polyacrylic acid (PAA) (glass ionomers, adhesives) or poly(dimethylsiloxane) (PDMS) (soft denture liners) are also widely employed in dental practice.[13]

In general, polymers may consist of only one type of molecule (homopolymer) or two or more kinds of monomers (copolymers).[12] The monomers usually are mono- (e.g. methyl methacrylate – MMA) or di-methacrylates (e.g. 1,6-hexanediol dimethacrylate – 1,6-HDMA) and may even have more functional groups (multifunctional monomers), though the latter are more common in restorative composite resins (e.g. bisphenol A-glycidyl methacrylate – bis-GMA, triethylene glycol dimethacrylate – TEGDMA, urethane dimethacrylate – UDMA).[12,13] UDMA is also very common in light and microwave cured denture and orthodontic base polymers.[9] Multifunctional monomers have been developed in order to reduce the materials' viscosity and improve the polymerization efficiency.[12] Individual monomer types have other different chemical features in terms of their hydrophilicity/lipophilicity. In a study

cited by Lai *et al.* (2004) it was reported that the logarithm of the octanol/water partition coefficient (log *P*) directly relates to lipophilicity.[14] The hydroxyl groups on acrylates and methacrylates seem to contribute to the lipophilicity; also the longer the oxyethylene chains of the dimethacrylates like 1,6-HDMA and the longer the alkyl chains, the more hydrophobic.[14] Moreover, as it will be further explored bellow, lipophilicity (and a higher log *P*) is related to longer retention times in high performance liquid chromatography (HPLC) runs in reversed phase columns.[14] Though these monomers are not considered to be hydrophilic, they have some affinity to water and tend to absorb it or being dissolved.[13] Additionally, the size of the monomers' molecules is important, because smaller ones shall leach more easily than larger molecules.[15-17]

Polymers may be characterized by their network structure: the length, the branching, the cross linking and organization of the chains. Cross linking consists of permanent connections between different chains.[12] A more dense cross-linking results in a closed structure (composite resins), whereas a lower density arrangement generates a more open network (denture and orthodontic bases).[13] Di-functional monomers such as ethyleneglycol dimethacrylate (EGDMA), trimethylolpropane trimethacrylate (TMPTMA) or 1,6-HDMA are usually employed to promote cross-linking.[8,9] As the polymer chain becomes longer, more branched and with more cross linking, the molecular weight increases, which is favorable to the mechanical and physical features of the material. Hence, it becomes more resistant to distortion, more rigid and the fusion temperature rises. Besides, materials with cross linking have a higher glass-transition temperature (softening temperature). Additionally, these features contribute to a polymer with a higher proportion of a random amorphous organization, which is preferable to a highly ordered structure, since crystallinity increases brittleness.[12]

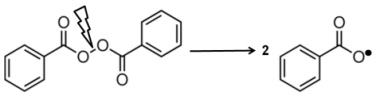
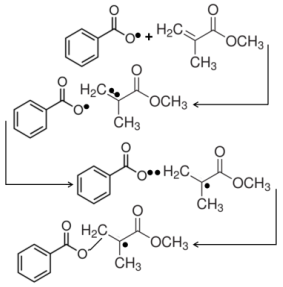
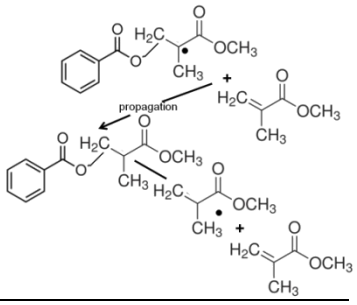
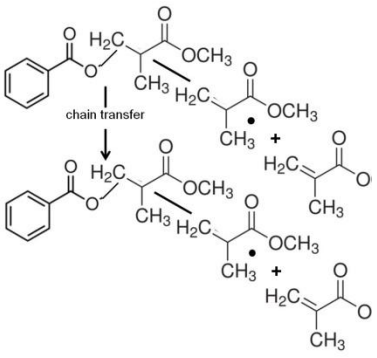
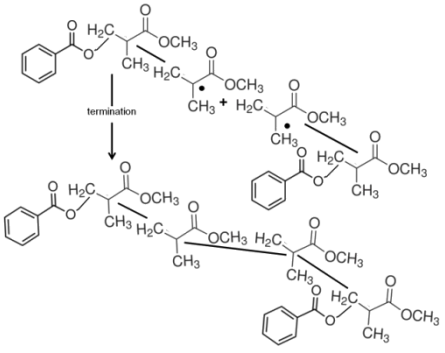
In general, dental polymers are considered insoluble in water, though imbibition may occur, resulting in undesirable dimensional alterations. The water/solvent molecules are adsorbed through the porosities and inter-chain spaces and expand the

matrix network (plastification).[12,13,18,19] As a consequence, the polymer softens and swells, but does not dissolve, compromising the material's clinical performance.[12] Moreover, it may also suffer oxidation and hydrolysis.[13] If the tridimensional polymer network is more complex (high molecular weight, cross linking, crystalline regions and chain ramifications), it is does not absorb as much water and its solubility decreases.[12]. Water and other solvents may act as external plasticizers,[12] while other molecules, including monomers (e.g. butyl methacrylate – BMA), work as internal plasticizers, which soften and reduce the resistance of materials, widely used in denture reliners.[12,20] Since that PMMA is a linear polymer it is soluble in a variety of organic solvents such as acetone or chloroform.[12]

According to the 8th Edition of the Glossary of Prosthodontic Terms PMMA is a stable, hard transparent resin of marked clarity with a Knoop hardness number ranging from 18-20, a tensile strength of approximately 60 MPa, a density of 1.19 g/ml and a modulus of elasticity of approximately 2.4 GPa.[1]

Chemically speaking, polymerization may occur by two distinct processes: addition or condensation. The condensation (growth-step) reaction is typical of elastomers and it is characterized by a simultaneous reaction of the bifunctional monomers that gradually connect each other and many times, as a consequence, may produce low molecular weight byproducts. Conversely, during the addition polymerization, which is the most usual in the dental field, the monomers are activated one by one, but are rapidly added to the main chain without changing the composition and, theoretically, can produce almost unlimited giant molecules if monomer is available.[12] Addition polymerization is divided in 4 main stages, as generally summarized in Table 1 and Figure 1. The addition polymerization reaction is exothermal, reaching considerably high temperatures. It has been reported that e.g. autopolimerizable PMMA acrylic bone cements peak temperature may range 50-120ºC.[21]

Table 1. Main stages of the addition polymerization (adapted from [12])

Stage		General description	Fig. 1 – Reaction diagram
Induction	Activation	An activator agent breaks the –O–O– connection of the initiator molecule, creating two free radical sites with an unpaired electron (•) each.	
	Initiation	The unpaired electron interacts with another electron from the monomer's double bond, creating a covalent bond. Consequently, a new electron becomes unpaired and available to establish new bonds with other monomers.	
Propagation		The unpaired electron of the complex free radical-monomer interacts with the double bond of a new monomer, forming a dimer. The process is subsequently repeated, rapidly creating large macromolecules.	
Chain Transfer		When a hydrogen atom is donated by an active free radical to another monomer, the former chain becomes non-reactive and the free radical site is transferred to the monomer, which continues the reaction. It may also occur between two separate chains, where one passive chain becomes active and vice-versa.	
Termination		It may result from a chain transfer where a previously active chain becomes inactive. More often, the terminal free radical sites of two separate chains establish a direct coupling or exchange a hydrogen atom and both become inactive.	

The agents that promote the beginning of the addition polymerization will be discussed in the next section. In addition, the reaction may be inhibited or delayed by the presence of impurities and the contact with oxygen, which react with the free radical sites of either the activator agent or a growing polymer chain.[12,22] Besides, hydroquinone (HQ) (<0.006%) may be added to the monomer composition as an inhibitory agent, avoiding its spontaneous polymerization.[12,22]

A.3. Polymerization Methods and Classification of Dental Base Polymers

One of the most popular and accepted criteria for the classification of dental base polymers is the polymerization method. As mentioned in the previous section, the beginning of the reaction requires an activation agent, which may vary in terms of the source and/or type, determining the nature of the polymerization method. The activation agent may be a secondary molecule, UV light, visible light, heat or transferred energy from another free radical site. The most common polymer general types used in dentistry and respective activator and initiator are listed in Table 2.[9,12]

Table 2. Main polymerization methods used in dentistry [9,12]

Polymerization	Activation agent	Initiator molecule
Thermo	Heat (>65ºC) from water bath or microwave	e.g. benzoyl peroxide
Chemical/auto	Tertiary amine (e.g. N,N-dimethyl p-toluidine)	
Light	Visible light (~470 nm)	e.g. camphorquinone

The most commonly used initiator in dental acrylic resins is benzoyl peroxide (BPO), which is activated between 50º and 100ºC. The higher the polymerization temperature, the faster will the formation of free radical sites occur and the shorter will the induction stage be.[12] Therefore, a material is classified as a heat-polymerizable if it requires temperatures above 65ºC in order to polymerize.[5] Conversely, an auto or chemical-polymerizable material does not require temperatures higher than 65ºC,[5] since the tertiary amine, which is packed separately, reacts with BPO during manipulation and together form a complex that does not require as much energy to break the –O–O– connection.[9,12] For that reason, autopolymerizable materials can be activated and polymerized at room or the mouth temperature.[12]

On the other hand, in light cured materials the energy required for the induction of the reaction comes from an external source of radiation.[23] UV light has been discarded because of its hazardous biological effects, limited penetration into the materials and decrease of the light source intensity with time. As a result, visible light in the spectrum of blue/violet (usually around 470 nm) is used to activate initiator molecules like camphorquinone.[12]

A more general classification takes into account the thermal properties of polymers. Most of them are thermo-rigid, since they are permanently hard and inflexible upon polymerization. Even if the material is re-heated until its polymerization temperature, its dimensional and structural features do not change. Besides, their mechanical properties are better and are virtually insoluble.[12] Conversely, thermoplastic polymers are softened after the application of heat above its glass transition temperature and become moldable because the distance between the molecular chains increases. They return to their hardened state upon cooling and may be re-heated and re-molded several times, though their physical characteristics are not so advantageous.[5,12]

The International Organization for Standardization (ISO) has also established a classification for both denture and orthodontic base polymers, which is presented in

Table 3. Besides, according to the type of polymer considered, it should comply with certain characteristics, namely the maximum content of unpolymerized residual monomer,[5,23] which will be further discussed in the next section.

Table 3. Classification of base polymers and maximum residual monomer [5,23]

ISO Reference	Classification	Material	Residual MMA monomer (% mass fraction max.)*
ISO 20795-1:2008 Denture base polymers [5]	Type 1	Heat-polymerizable	2.2
	Class 1	Powder/liquid	
	Class 2	Plastic cake	
	Type 2	Autopolymerizable	4.5
	Class 1	Powder/liquid	
	Class 2	Powder/liquid (pour-type)	
	Type 3	Thermoplastic	2.2
	Type 4	Light-activated	2.2
	Type 5	Microwave cured	2.2
ISO 20795-2:2010 Orthodontic base polymers [23]	Type 1	Autopolymerizable	5
	Type 2	Light-activated	5
	Type 3	Thermoplastic	5

* Residual monomer content shall not be more than 0.2% higher than that claimed by the manufacturer

A.4. Residual Monomer Content and Leaching

It has been broadly described in the literature that total polymerization of the monomer is never achieved.[9,24] According to ISO 10993 Part 13 (2010), residual monomer is defined as *unreacted chemical compound(s) used to build the polymeric chains, which is still present in the final polymeric material*.[11] Three main parameters may influence the residual monomer content of a polymer piece: the polymerization method, the polymerization cycle and the post-polymerization treatment.[9,25]

In terms of the polymerization method, studies have shown that thermo polymerizing materials, including heat and microwave cured, present the lowest values of unpolymerized residual monomer.[9,20,26,27] Koruglu *et al*. (2012) found lower residual monomer values in microwaved resins than in heat polymerizable,[28] with the additional advantages that the curing cycles are very short and the physical properties are comparable to those of conventional heat polymerizable materials.[9,29] Conversely, autopolymerizable polymers are undoubtedly the materials with the most incomplete polymerization reaction, since they present recurrently the highest values of residual monomer,[20,26] even when compared with light curing materials.[27,30]

Ideally, during the thermo-polymerization cycle the increase of the resin internal temperature should be controlled in order to avoid the monomer boiling (100,8ºC) and the consequent formation of internal porosity, which weakens the polymer structure.[12] A study by Harrison and Huget (1992) showed that the most effective polymerization cycle to minimize the residual monomer content was 7 hour incubation in water at 70ºC followed by a post polymerization treatment at 1 h at 100ºC.[9] Though many authors support that longer curing cycles are preferable, Bayraktar *et al*. (2003) found that long curing cycles (9 hours at 70ºC) without post polymerization terminal boil had a higher residual monomer content than a shorter cycle followed by a short terminal boil (20 min. at 70ºC, followed by 22 min. at 100ºC).[26] This and other studies show that in order

to reduce the final content of residual monomer the most effective procedure is to perform a final post polymerization treatment, either a terminal boil, immersion in water for at least 24 hours or microwaving for a few minutes.[9,20,26,31,32]

Moreover, it has been demonstrated that the reinforcement of acrylic resins with fibers of any type contributes to an incomplete polymerization, independent on the method or cycle employed.[26,28]

Besides, the most appropriate powder/liquid proportion in commercially available dental PMMA is 3:1, which limits the volumetric contraction during polymerization caused by the monomer, as well as the excess of unpolymerized monomer.[12] It has been reported that when a material is prepared with a higher proportion of polymer (higher ratio), the levels of residual monomer are lower, although it may result in a resin too difficult to work with.[25] Though as a rule most manufacturers respect this proportion, some of them, especially in auto-polymerizable denture and orthodontic materials, do not comply with it, increasing the amount of monomer in the recommended proportion (about 2.5:1). To illustrate this situation, some examples are presented in Table 4, based on the respective manufacturers' instructions of use. As a consequence, this is another factor contributing to the increase in the content of residual monomer.[25]

One must, however, distinguish between the total residual monomer content, which did not polymerize, and the fraction of that monomer which may leach to a greater or lesser extent in physiological conditions.[33] Therefore, ISO 10993 Part 17 (2002) considers a leachable substance as any *chemical removed from a medical device by the action of water or other liquids related to the use of the device*.[34] Though most dental polymers are chemically stable and insoluble, they undergo biodegradation, due to the aggressive and complex oral environment, mainly because of the permanent contact with water.[8,9] As water penetrates the matrix, an expansion occurs inside the polymer network, leaching its trapped toxic components such as residual monomer and byproducts.[8,13,18,19]

Table 4. Powder/liquid ratios of commercial autopolymerizable acrylic resins		
Commercial Product	Manufacturer	Powder/Liquid Ratio
Duraliner II (denture)	Reliance Dental Manufacturing Co., Worth, IL, USA	10 ml : 7 ml
JET Clássico (denture)	JET, Clássico, São Paulo, Brazil	2.5 : 1
Kooliner (denture)	GC America Inc., Alsip, Chicago, IL, USA	15 ml : 6 ml
Poly Seal (denture)	Kamemizu Chemical Ind. Co. Ltd., Osaka, Japan	2 g : 1 ml
Vertex Self-Curing (denture)	Vertex-Dental B.V., Zeist, The Netherlands	1.7 g : 1 ml (~0.95 g)
Orthocryl (orthodontic)	Dentaurum GmbH & Co. KG, Ispringen, Germany	2.5 : 1
Steady Resin M (orthodontic)	Scheu Dental GmbH, Iserlohn, Germany	10 : 5

Furthermore, a study by Danesh *et al*. showed that there is not a mandatory correlation between the monomer leaching characteristics and the residual monomer content in a polymer piece.[30] This phenomenon may be explained by the nature (polarity) and concentration of the solvent, the polymer structure, the size and chemical (hydrophilic or hydrophobic) features of the leachable molecules.[19,35,36] Moreover, some studies have also been pointing out that apart from monomer, other leached toxic

substances, such as initiators (BPO, tertiary amines, camphorquinone) and additives (HQ, pigments) are released, increasing potential exposure to these potentially harmful substances.[9,37,38] Many plasticizers derived from phthalate esters (like dibutyl-phthalate – DBP) are present in tissue conditioners, hard and soft relining materials and have been considered toxic, mutagenic, carcinogenic, teratogenic and xenoestrogenic, affecting the reproductive organs and fertility.[9,39,40] Moreover, MMA toxic byproducts are also released such as methacrylic acid and formaldehyde, which results from MMA oxidation.[9,18,38,41,42]

Since the residual monomer acts as a plasticizer, many studies have established a relation between the degree of conversion and the physical and mechanical properties of acrylic resins, like the flexural strength, the hardness, water sorption, solubility, dimensional stability, relaxation modulus, transverse strength and cubical expansion and specific volume with the temperature.[36,43-51] Hence, acrylic devices show better mechanical performances if polymerized by methods that increase the degree of conversion and reduce the residual monomer content.

A.5. Analytical Methods for Residual Monomer Quantification

ISO has been publishing updated international standards that specify which test methods and conditions should be employed to appropriately analyze the denture [5] and orthodontic [23] polymers' physical and chemical characteristics. Among other tests, it is specified which test methods and conditions shall be used in order to accurately measure the total residual monomer content. In general, it is recommended that the MMA shall be extracted from the solid polymer with an appropriated organic solvent and quantified by means of a chromatographic method, which may be gas chromatography (GC), HPLC or another similar chromatographic technique.[5,23] The

same chromatographic methods are recommended by ISO 10993-13:2010 in order to identify and quantify the degradation products from polymeric medical devices, including residual monomers, additives and leachables.[11]

However, other techniques such as chemical detection, UV and infrared-spectrophotometry have also been employed to quantify the residual monomer content,[9,44,45,52,53] and the degree of conversion of carbon-carbon double bonds has been measured by the Fourier transform infrared spectroscopy (FTIR).[36,54-56]

An accurate residual monomer measurement requires an analytical method sufficiently capable of separating, identifying and quantifying the different, but closely related, molecules from the multicomponent and complex samples that many times are to be analyzed.[57,58]

Generally speaking, in chromatographic techniques the phase that contains the sample to be analyzed (mobile phase – MP) is forced through or upon an immiscible fixed phase (stationary phase – SP), compelling the sample components to distribute themselves distinctly between the two phases. Therefore, if a molecule has more affinity to the MP, it will travel faster than one which has more affinity to the SP. Consequently, since the migration rates of the substances are different, it becomes possible to separate, identify and quantify them.[57,58]

Depending on the physical presentation of the SP, chromatography may be classified as planar or columnar. The latter is the most used and consists of a narrow tube with an inert porous solid that supports the SP (solid or liquid). Depending on the MP physical state, three types are possible: gas (GC), liquid (LC) and supercritical fluid chromatography (SFC).[58]

Most analytical separations are currently made by LC, due to its sensitivity and adaptability, simple automation and wide applicability in science and industry.[58] LC has the advantages of not destroying the sample and being suitable for nonvolatile substances, which cannot be analyzed by GC. Nowadays, the latest LC technology is HPLC and is performed using pumping pressures that reach hundreds of atmospheres

inside a thin stainless steel column. Hence, this analytical technique requires sophisticated and quite expensive equipment.[58]

If the nature of the equilibrium and the separation mechanism are considered, chromatography may be classified as: adsorption (liquid-solid), partition (liquid-liquid), ion exchange, molecular/size exclusion, affinity and chiral.[57,58] Partition chromatography (most widely employed in HPLC) varies according to the relative polarities of the MP and SP. The first used type was normal-phase chromatography (highly polar SP, relatively nonpolar MP), but has more recently been substituted by reversed-phase chromatography, where the SP is nonpolar (e.g. hydrocarbon) and the MP is polar (water, methanol, acetronitrile, tetrahydrofuran).[58] Consequently, the polar MP increases the elution time and the most polar molecules eluate first. It has the advantage of being possible to use water as a mobile phase (inexpensive, non toxic, UV-transparent and compatible with biological solutes) [58] and it is not as sensitive to polar impurities.[57]

An HPLC system comprises many devices controlled by a computer which also records, analyses and retrieves the results.[57] The signal detected by the device at the end of the analytical column is processed and the data is plotted as function of time (or eluent volume) in a chromatogram. Having in mind that two molecules in a sample have different retention rates in the SP and MP (have different distribution constants) they are detected at different times, originating two distinct bands or peaks, which resemble a Gaussian curve. Thus, the chromatogram gives a qualitative (the time position of the peak is characteristic for each molecule at those conditions) and quantitative information (the area under the peak) about the sample composition.[58]

When performing a HPLC quantitative analysis it is necessary to compare the integrated areas under the peaks (preferable to the peak heights) with that of previously prepared standards.[58] The most direct method is to analyze at least four samples of known concentrations of the analyte in the same conditions of the subsequent test (the range should cover the concentrations to be determined in the

test samples, for higher accuracy). A calibration plot is built using the standards peak areas as a function of the known concentrations. The linear zone of the curve (the signal given by the detector is proportional to the concentration [57] is used in order to interpolate the concentration of the analyte in the test sample from the peak area.[58,59]

Nevertheless, if the molecules in a sample are unknown, a HPLC chromatogram does not give their identification, but only their retention time in certain MP, SP and temperature conditions. Still, if a peak for a determined molecule does not appear in the chromatogram it is either absent or its concentration is so low that it is bellow the limit of detection (LOD).[58] Theoretically, the LOD is the lower analyte concentration that produces a signal that is significantly different from the blank/background signal. Moreover, the limit of quantification (LOQ) is defined as the lower analyte concentration which is possible to determine with precision. Yet, the LOD is not the same as the sensitivity of the method, which is numerically equal to the slope of the linear zone of the calibration curve. The LOD depends on both the slope and the standard deviations (SD) of the points in the calibration curve (blank signal plus three SDs of the blank).[59]

A.6. Residual Monomers as Biological Hazards

Stomatitis is a multifactorial oral condition that has been extensively associated with PMMA oral devices, particularly dentures, which may be caused or related to poor oral hygiene, mechanical trauma, wearing during the night, smoking, systemic and nutritional conditions, bacterial and fungal infections, as well as reactions to chemical aggressions such as mucosal irritation or allergy by MMA.[60-63] Its prevalence has been reported between 15 and 70% of denture wearers, with significant variations dependent on the sample population, and it is more frequent in elderly people and women.[62]

In patients, diverse systemic reactions to dental acrylic resin have been registered such as contact dermatitis and asthma, and local inflammation like lichen planus, gingivitis, ulcerations, eczema, erythema, blisters and erosions, papilloma, fibroma, and burning mouth sensation, especially on the surface of the mucosal prosthetic support and oral adjacent tissues.[25,64-68] Despite the cases described in the literature,[33,63,69-71] genuine acrylic resin contact allergy in patients is a rare condition, since the polymerized PMMA is non-sensitizing.[33,63,70] Given that polymers are macromolecules (molecular weights 5000–1 million), there is not the risk of gastrointestinal or dermal absorption and the respiratory tract contact is considered negligible.[72]. However, unpolymerized acrylic monomers in general are capable of inducing sensitization and/or irritation and it is widely recognized that professionals in the dental area (dentists, dental assistants and technicians and methacrylate manufacturing personnel) are commonly affected by allergic contact dermatitis in the hands or face, occupational respiratory hypersensitivity and local neurological injuries.[9,10,49,63,64,70,73] Moreover, clinical gloves only provide limited protection from MMA contact.[49]

Moreover, some authors have been investigating whether a relationship exists between wearing a conventional removable prosthesis and cancer. It has been reported that oral lesions caused by chronic trauma and irritation due to ill-fitting dentures may lead to an increased risk of cancer in association with other factors such as age, smoking, alcohol consumption, poor oral hygiene, defective or missing teeth.[74-76] However, no studies were found trying to establish a relationship between oral cancer and MMA aggression. On the other hand, Tomenson *et al*. (2000, 2005) investigated whether an increased prevalence of death caused by respiratory, stomach or colo-rectal cancers was related to the occupational exposure to MMA in the manufacture of PMMA products.[77,78] They came to the conclusion that the cancer cases were probably more related to life style habits and so there was little evidence that MMA is a human carcinogen.[78]

As formerly mentioned, part of the trapped residual monomer may leach from the polymer in clinical conditions and consequently cause biological hazards, such as cytotoxicity and genotoxicity.[9,18] The smaller molecular monomers are also more cytotoxic and there is a direct linear relationship between cytotoxicity and molecular hydrophobicity.[14,35,79] As referred before, since the lipophilicity relates to the log *P*, it has been hypothesized that the mechanism of the action of monomers is membrane-mediated and relatively non-specific.[14] The hydrophobic molecules interact with the phospholipid bilayer of the biologic membranes: cell membrane, endoplasmatic reticulum, mitochondrial membrane and nuclear membrane.[35,79]

However, the ISO 20795 standard does not cover biocompatibility tests and refers to ISO 10993 in order to specify the most appropriate methods to assess possible biological or toxicological hazards in medical devices.[5,23] Therefore, part 12 of ISO 10993 specifies the procedures to select and prepare a test sample and to prepare extracts from those samples.[80] It states that factors like period of extraction, temperature, the nature of the vehicle solvent, the type, shape and the phase equilibrium of the material and the ratio of surface-area-of-material to volume-of-extraction solvent should be considered when planning extraction procedures.[80] The extraction conditions recommended by ISO 10993-12:2007 are summarily presented in Table 5. Other parts of ISO 10993 detail how to perform the biocompatibility assessment tests, as will be discussed in the next chapter.

A.7. *In Vitro* Biological Assessment Tests

According to ISO 10993 Part 1 (2009) it is important to assess the potential biological risks arising from the use of medical devices, within a risk management process that includes a review and evaluation of existing data, as well as the selection and application of additional tests when necessary.[6] Having in mind that denture

Table 5. Summary of ISO 10993-12 recommended criteria (conditions and procedures) for extraction of leaching residual monomer [80]	
Criteria	Recommendation
Thickness	>1 mm (larger moulded items)
Sample dimensions (mm)	Indifferent (e.g. T: 10; D: 50/T: 5; D: 25)
Standard surface area	Indifferent
Solvent volume	Indifferent
Extraction ratio	3 cm^2/ml
Temperature and extraction period: 37ºC/50ºC – 72h	Solvent*: polar, non polar, additional
Temperature and extraction period: 37ºC – 24h	Solvent: cell culture media with serum
Quantification method [11]	GC, HPLC, other chromatographic method

D – Diameter; GC – Gas chromatography; HPLC – High performance liquid chromatography; T – Thickness; * More than one option acceptable. Examples of solvents – Polar: water, physiological saline, culture media without serum; Non-polar: freshly refined vegetable oil; Additional: ethanol/water; ethanol/saline, diluted polyethylene glycol 400, dimethyl sulfoxide, culture media with serum

base polymers may be one of the components of prosthodontic medical devices, it is important to perform biological risk assessment tests on them, in order to cautiously foresee their behavior during clinical usage. Therefore, the priority of these tests is to ensure not only the protection the humans' health, but also to guarantee animal welfare and the minimum of animal testing.[6]

Hence, whenever possible, preference should be given to methodologies that simulate and gather likewise relevant information as *in vivo* models, by performing *in vitro*, *ex vivo* and analytical chemistry tests. Also, *in vitro* screening tests should always be carried out prior to *in vivo* assays.[6] The major advantages of *in vitro* biological tests is their simplicity, repeatability, reproducibility, controlled environment and are cost-effective,[6,25,81,82] when compared to *in vivo* tests.

ISO 10993 describes the guidelines for a wide range of tests including acute toxicity, irritation to the skin, eye and mucosal surfaces, haemolysis and thrombogenicity, subchronic and chronic toxic effects, sensitization, allergy, genotoxicity, carcinogenicity and teratogenicity.[6]

More specifically, cytotoxicity tests use cell cultures to assess several endpoints, including qualitative evaluation of cell damage by morphological means (apoptosis, membrane and cytoplasm markers), quantify the cell damage and death, determine the degree of cell growth and measure the function and cellular metabolism.[38,83] These tests may be performed using extracts, by direct or indirect contact with medical devices or representative parts of them.[83] ISO 10993-5:2009 considers that a test sample has a cytotoxic effect if a 30% reduction in viability occurs.[83]

Some cytotoxicity test protocols are recommended by ISO, such as neutral red uptake (NRU) test, which measures the cells growth inhibition; colony formation test, to quantify the cells plating efficiency; XTT ((2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl] -2H-tetrazolium hydroxide)) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromid) tests measure the cells function and metabolism.[83] In particular, MTT is a yellow water soluble substance metabolically reduced by viable cells into a blue-violet insoluble formazan. The cells viability is photometrically determined by measuring the color intensity of dissolved formazan.[83] Other tests that contribute to the direct or indirect assessment of cytotoxicity have also been employed, such as ^{3}H-thymidine [68,84] and crystal violet.[24]

The aim of genotoxicity *in vitro* tests is to determine to what extent substances are capable of inducing genetic damage to mammalian and non-mammalian somatic cells, bacteria and yeasts that may be transmitted to the descendent cells.[85] Knowing that DNA (deoxyribonucleic acid) damage events may trigger or contribute in some way to the initiation of a cancer process, it implies that a genotoxic substance may also have a potential carcinogenic effect [68,85] that should be further scrutinized in *in vivo* models.[85] Since that genotoxicity includes a wide variety of endpoints, more than one should be investigated for each candidate test sample. These endpoints include: gene or point mutations, small deletions, mitotic recombination, microscopically visible changes in the chromosome (or other DNA) structure (clastogenicity) and number (aneugenicity).[85] Therefore, a battery of *in vitro* tests should be performed, in order to cover separately gene mutations and chromosomal damages.[85] ISO 10993-3:2003 recommends that the OECD (Organization for Economic Co-operation and Development) Guideline for the Testing of Chemicals should be followed, in order to test for gene mutations in bacteria and gene mutations and clastogenicity in mammalian cells.[85] Widely published genotoxicity assays for dental materials include Ames test on prokaryotic cells such as *Salmonella* strains [86-88] and the bacterial *umu*-test;[89] for mammalian cells: the single cell microgel electrophoresis (Comet assay),[90,91] hprt gene mutation,[92,93] chromosome aberration (CA) [68] and sister-chromatid exchange (SCE) assays [68] and the *in vitro* micronuclei test (MNvit).[24,94-97]

A.8. Cell Cultures for *In Vitro* Biological Testing

One of the most employed and profitable methods of running *in vitro* biological assays is to grow eukaryotic cells outside the living organism or tissue in the laboratory environment – cell culture [82]–, providing a more convenient and homogeneous

population of cells to test and manipulate.[98] Moreover, a large amount of cells can be obtained; it is possible to isolate a single cell type from a complex tissue or to explore the interactions between two cell types; cells are able to multiply and differentiate; since that many cells' properties and functions are maintained in culture; the cellular activities may be deeply studied and it is possible to test the cells response when challenged by drugs, hormones, growth factors and other molecules.[82,98]

Because eukaryotic cells cannot live in fluid suspension like bacteria and yeasts, they are seeded in appropriated culture vessels, including glass petri dishes, plastic culture flasks or plastic multiwells and microtitre plates, with a surface treatment (polylysine or extracellular matrix components), to which they can attach, forming a monolayer, in order to grow and divide.[83,98,99] Besides, the culture dish must contain a rich nutrient media, implying that the manipulation must occur in sterile conditions in order to avoid contamination by bacteria and fungi.[82,99] Besides, in order to prevent contamination, cell culture medium includes large spectrum antibiotic and antifungal.[82,83] In 1955, Eagle revolutionized the animal cell culture science, by determining the necessary ingredients of culture medium, which included salts, glucose, amino acids, vitamins, and natural animal serum with polypeptide growth factors that are important for the stimulation and regulation of the cell cycle.[99,100] Nowadays, it is possible to grow cells using entirely synthetic medium, containing variable proportions of nutrients, vitamins, proteins such as insulin and epidermal growth factor, and transferring.[82]

In relation to the source of the culture cells, if they are collected directly from a tissue or organ (generally embryonic, but also adult) they are called primary cultures.[82,98,99] In average, animal cells take approximately 20 hours to complete a cell cycle and divide in optimal conditions.[99] When they are almost covering the surface of the culture plate (80% subconfluent, corresponding to the end of the logarithmic phase of growth) they are treated with trypsin (proteolitic enzyme) to digest the extracellular adhesions to the dish and a Ca^{2+} chelanting substance, such as EDTA

(ethylenediamine tetraacetic acid).[82,83,98] Cells are replated on new dishes with lower density, and this process is consecutively repeated (passages) originating secondary cultures.[98,99] Many times, secondary culture cells are acquired in frozen vials (for conservation during months or years in liquid nitrogen at -196ºC), which recover their activity upon thawing.[82,83,98]

However, secondary cells from normal tissue have a limited life span and cannot be passaged infinitely, because eventually they enter in a process of replicative cell senescence and stop dividing.[98,101] These culture populations can only be doubled typically 50 to 100 times, depending on the cell type.[82,99] Moreover, their phenotype begins to change with increasing number of passages [101] and factors such as age, metabolic and hormonal conditions of the donor may influence primary cell cultures.[68]

In order to solve these problems, genetic modifications are induced, allowing the cells indefinite division, becoming immortal/permanent cell lines.[82,99] The so-called transformed cell lines, derived from malignant tumors, grow rapidly and proliferate to a high density,[98] have good reproducibility and are genetically and metabolically stable.[14] Embryonic stem cells also share these properties and are capable of differentiating into any type of adult cell.[99] Still, cell lines usually show significant differences in their activity from their respective progenitor tissues [98] and there is a certain concern that many cell lines present mutant genes capable of disrupting the process of apoptosis, influencing the cell death.[14]

Cell types derived from malignant tumors are the most widely used in laboratory experiments such as fibroblasts (e.g. mouse – 3T3), epithelial cells (e.g. human – HeLa), ovary cells (e.g. chinese hamster – CHO), myoblasts, chromaffin cells, plasma cells, kidney and macrophages.[98] ISO 10993 Part 5 (2009) recommends (for cytotoxicity testing) the employment of established cell lines from recognized repositories, though primary cell cultures shall be used for specific sensitivity requirements, if their accurate and repeatable response is demonstrated.[83] Some of the recommended cell lines are L929 (mouse fibroblasts), 3T3, WI-38 (human fetal lung

fibroblast) and V79-379A (chinese hamster lung fibroblast).[83] When it comes to testing the biological activity of monomers such as MMA, the cell types that have been employed are immortalized cell lines like V79-B,[24,93] L929,[32,102] CHO [68] and HSG (human submandibular gland adenocarcinoma),[35,79] primary cells from human biopsies or tooth extractions like HGF (human gingival fibroblasts),[14,35,79] PDL fibroblasts (human periodontal ligament),[14] CPC (bovine dental papilla).[101]

A.9. Cell Cycle and Mitosis Overview

The cell cycle comprises a series of complex, coordinated and controlled processes that are fundamental for the continuation of life, through the growth and division of both prokaryotic and eukaryotic cells.[99] Cells' self-reproduction is essential for the proliferation of unicellular species and for the maintenance of multi-cellular organisms.[103]

The major stages of the eukaryotic cell cycle are the interphase and M phase, which are subsequently divided in substages. Once the cell decides to go forward with the cell cycle, there is no turning back to the previous stage.[103]

The interphase is the period between the cells divisions and may last for days, weeks or years, depending on the cell type and on the environment conditions.[82] During the first substage of interphase, G1, the cell has the opportunity to grow, to exert its metabolic functions, to interact with and evaluate the surrounding environment, to differentiate and to duplicate its organelles.[82,103] If the cell is not stimulated, it may enter a G0 period (quiescence), continuing with its metabolic functions, but not growing. If the cell receives an external stimulus such as growth factors, it is induced to begin proliferation, thus surpassing the restriction point, which commits the cell to enter and finish the division process.[99,103]

Next, the S (synthesis) phase is a critical moment, when the cell carefully replicates its DNA, duplicating its genome.[82] The cell possesses several specific mechanisms (checkpoints) to regulate and correct the DNA replication. Protein kinases ATM and ATR become activated with DNA incorrect replication and activate other enzymes such as Chk1, Chk2 and p53 (very often mutated in cancer cells) that stop the cell cycle progression, in order to gain time for DNA reparation.[99,103] Mechanisms such as proofreading (DNA polymerase) and mismatch repair correct the mistakes resulting from an erroneous base pairing and therefore avoid that incorrect genomic information (e.g. mutations, DNA double stranded breaks) is transmitted to the daughter cells.[103] The next step is called G2, when the cell is preparing to actually start the division if everything is under control. A G2 checkpoint arrests the cell cycle if unreplicated or damaged DNA is detected.[99]

The M phase is subdivided in mitosis, which in turn has its own substages, and cytokinesis, comprising the cell division itself. During the first mitosis substage, prophase, DNA condensates and the chromosomes become more compact. The two equal chromosomes that result from replication are paired (sister chromatids), united by a specific DNA sequence, the centromere, with bonded proteins, forming the kinetochore.[82,99] The cytoskeleton disassembles and the centrosomes travel in opposite directions towards the cell poles to start forming the mitotic spindle.[99,103]

When the dissolution of nuclear envelope begins, the cell enters the promethaphase stage. Chromosomal condensation continues and the actin microtubules attach to the paired chromosomes kinetochore.[82,99,103] At this moment, the spindle assembly checkpoint (SAC) verify if all chromosomes were correctly connected to the respective microtubule.[103] If not, the chromosomes risk not being segregated to the daughter cells and are left behind (lagging chromosome).[82]

During the next step, metaphase, the dichromatids finish condensation and align their kinotochores in the equatorial zone of the cell.[103] In this stage, the microtubules of the mitotic spindle are functionally differentiated into three types: astral

microtubules (help with the correct positioning of the spindle apparatus), chromosomal/kinetochore microtubules (connect the centrosome to the kinetochores and exert a pulling force toward the poles) and polar/interpolar microtubules (extend from one centrosome to the other past the chromosomes and maintain the integrity of the whole apparatus).[82]

During the transition from metaphase to anaphase, an anaphase promoting complex (APC) is activated and an enzymatic complex destroys the proteins (securins) responsible for the maintenance of the kinetocore.[82] Consequently, at the anaphase, segregation occurs with the dissolution of the centromere, separating the sister chromatids (anaphase A), which migrate in opposite directions pulled by the microtubules (anaphase B). The cell becomes longer because of this movement, increasing the distance between the centrosomes.[103]

The last stage of mitosis, telophase, is characterized by the decondensation of the chromosomes that become indistinguishable again. The nuclear envelope is reorganized, involving the genetic material of each potential daughter cell. The mitotic spindle disassembles and the organelles are equally distributed through the two daughter cells.[103]

In the last part of the M phase of the cell cycle, cytokinesis, the cytoplasm is divided by the formation of a contractile ring (actin and myosine filaments) that cleavages the cytoplasmic membrane.[82,103] It has been reported that the place where the contractile ring is positioned matches the zone once occupied by the aligned chromosomes during methaphase.[82,103]

Ideally, two similar daughter cells, with the same genetic material, should form upon the end of cytokinesis. However, despite the numerous checkpoints and repairing mechanisms, failure in the mitotic process may occur for various reasons. Some of these factors will be further discussed in the next chapter.

A.10. *In Vitro* Mammalian Cell Micronucleus Test

Ever since Boveri a hundred years ago, it has been described that disturbances during the chromosome segregation in mitosis may cause cancer and that many cancer cells present several nuclear morphological abnormalities.[104,105] Later, haematologists described the Howel-Jolly bodies, firstly identified in bone marrow dividing erythrocytes, associated with the lack of vitamin B12 and folate.[105,106] Since the 1980s that these cellular bodies are known as micronuclei (MN), which represent the damage that has been transmitted by an affected mother cell through a genotoxic agent or event to the daughter cells.[107,108] In 1997 the International Human Micronucleus (HUMN) Project was founded to coordinate investigation groups all around the world that dedicated to research on micronuclei with human lymphocytes as a tool for studying the DNA damage in human populations.[108]

Most MN are originated by an acentric chromosome or chromatid fragment or even a whole chromosome that is left behind (lagging chromossome) during the anaphase and can be observed in interphasic cells as a smaller additional nucleus.[105,107,109] It may be caused by a chromosome breakage or by a dysfunction in the mitotic system.[105,110] Since lagging chromosomes are detached from the mitotic spindle, they cannot travel into the direction of the poles in order to incorporate the new nucleus.[105,110] This lost genetic material is eventually enclosed by a nuclear membrane and is completely separated from the main nucleus, showing similar morphological features to the latter, except for the size that is quite smaller.[105] So being, the MN formation leads to the loss of genetic material by the micronucleated daughter cell.[110] Morphologically, MN show the following characteristics: the diameter of MN ranges $1/16^{th}$ to $1/3^{rd}$ of the mean diameter of the main nucleus or $1/256^{th}$ to $1/9^{th}$ of the area; MN are non-refractile, thus are not confoundable with artifacts resulting from the preparation; MN are not linked to the main nucleus, though they may be touching, but

not overlapping; the stain intensity of the MN is similar to the nucleus, though it may be slightly more intense.[106]

MN may also originate from fragments of broken anaphase bridges during chromosome rearrangements (e.g. dicentric chromatids, intermingled ring chromosomes or union of sister chromatids).[110] In these cases it may occur the formation of a nucleoplasmic bridge (NPB), surrounded by the nuclear membrane, which also represents a genetic defect and may lead to the occurrence of MN as well.[105,111] Another nuclear anomaly, the nuclear bud (NBUD), has a similar morphology to the MN, but it is still connected to the main nucleus. Under certain circumstances, too much DNA amplification occurs and the excess of genetic material concentrates at the periphery of the nucleus to be expelled during the S phase, thus forming the NBUD.[105,111] It may also be the product of the elimination of DNA repair complexes and excess of chromosomes in aneuploid cells.[105] In some situations the NBUD may be a precursor of MN.[105]

The causative agents of MN may be physical agents (ionizing radiation), oxidative stress, clastogen or aneugen agents, mutations in cell-cycle checkpoints or DNA repair mechanisms and nutritional deficiencies, such as folate and other co-factors, which are essential for DNA metabolism and mitosis.[106,112] These factors are capable of interacting with nuclear DNA and producing major genetic alterations in eukaryotic cells.[106] Therefore, MN (as well as NPB and NBUD) are not only important for biological risk assessment of genotoxic agents, but also as objective biomarkers of chromosomal instability, since it has been observed that malignant cells, cells with defects in the DNA repair system or impaired cell cycle checkpoints, present higher scores of MN.[105,113]

It took approximately 20 years for the *in vitro* mammalian cell micronucleus (MNvit) test to become accepted by the OECD as a reliable test method for the assessment of chromosomal damage, with the publishing of the OECD 487 guideline in July 2010.[114] This assay is not yet recommended by ISO 10993 Part 3, since this

standard was published some years earlier. The MNvit test allows the evaluation of the genotoxicity (objective observation of chromosomal aberrations) activity of chemicals and also assesses the cytotoxic effect of the tested material (as cell viability and proliferation).[107,109] The MNvit test is a robust effective and relatively accessible method, which can be applied to a great variety of cells,[107] though most studies have been performed in human peripheral blood lymphocytes [110] and rodent cell lines like CHO and V79, which are fully validated.[107] Moreover, when comparing with other methods such as chromosomal aberrations, it has shown to be simpler to perform, easier to learn how to count, time-effective, allowing the quantification of cells in the range of thousands, which increases its statistical sensitivity and has the possibility of becoming automated.[24,107,110,111] MNvit test has also evolved into a "cytome" method, since it is possible to use it as a biomarker to monitor chromosomal instability caused by genetic defects and/or external factors,[112] it is a multi-target genotoxic endpoint, predictive for *in vivo* genotoxicity and cancer in humans and allows extrapolation to potential limits of exposure or thresholds.[114] Moreover, it enables the assessment of other cellular events such as the mitotic rate, cell death (apoptosis and necrosis) in the same assay.[106,111]

The OECD 487 MNvit protocol requires that upon cells contact with the test substance, they should undergo a whole cell cycle, i.e. mitosis must occur so that damage is eventually induced on the chromosomes or mitotic spindle that leads to MN formation.[106,107] Since MN scoring is only valid for cells that completed mitosis during or after exposure to the test agent, one possibility is to employ a cytokinesis blocker (the most widely employed is the cytokinesis-block micronucleus (CBMN) assay, using an actin polymerisation inhibitor like cytochalasin B (CytoB) 3-6 µg/ml), leading to the formation of binucleated cells, clearly distinguishable from interphase cells.[106,107] In this case MN frequencies should be scored in at least 2000 binucleated cells per test concentration (if two replicas are used, then 1000 per culture).[106,107] Binucleated cells with irregular shapes, with more than two nuclei or with nuclei too divergent in size

should not be counted [106,107] However, it is possible as well to perform the MNvit test without employing a cytokinesis blocker, since that it has been assured that cells to be counted have undergone mitosis during or after the agent exposure, in order to avoid false positive results.[107] In this case, at least 2000 mononuclear cells per replica should be assessed.[107] Moreover, Parry *et al.* (2010) do not require the CBMN assay as a minimal performance criterion for MNvit, recommending it as a sensitive complement to detect some aneugens.[114]

Furthermore, the extent of cytotoxicity or cytostasis of the test agent should be assessed for that specific cell culture prior to the MNvit test, in order to determine the highest concentration of the test substance to be employed.[107] It is very important to avoid artifactual positive responses caused by excess of cytotoxicty, precipitation in the medium and changes in pH or osmolality.[107] Al least three test concentrations should be tested per chemical,[107] from little to no cytotoxic effect to the highest concentration tested that should not exceed 55 ± 5% cytotoxicity.[107] The OECD 487 protocol, also recommends that test procedures should be performed with and without an exogenous metabolic activation with post-mitochondrial S9 fraction from rat liver, since that some cell types have inadequate endogenous metabolic capacity.[107]

Untreated cultures should be employed as negative controls and give reproducible low and consistent MN counts. The cell types above mentioned typically show 5-25 cells with MN/1000 cells, though the counts for other cell types might be different.[107] Moreover, known inducers of small, but reproducible increases in MN formation (when compared with the negative control) should always be employed as positive controls in order to assess the efficiency and sensitivity of the test protocol and cell model used.[107]

In terms of results assessment, slides may be observed at the light microscope using the Romanowsky/Giemsa/Diff Quick stain [106,107,115-118] or at the fluorescence microscope, using fluorescent DNA specific stains such as acridine orange,[119] Hoechst

33258 plus pyronin-Y,[107] Schiff/Feulgen reagent [24,94,108] and propidium iodide (PI).[120] Some artifacts may be produced by not employing specific-DNA stains.[107,121]

One of the main advantages of the MNvit test is its ability to detect clastogenic (structural chromosome alterations) and aneugenic effects (numerical chromosome alterations).[110] However the MNvit test alone cannot distinguish between these two types of defects,[107] though this is an important information when screening chemicals and characterizing their genotoxic profile.[122] Moreover, the existence of a threshold has been suggested for aneugenic MN, since that spindle poisons do not interact directly with the DNA, but only with the mitotic apparatus.[122,123] Hence, aneugenic chemicals only show adverse effects upon a certain exposure level, representing a minimal biological risk after human exposure.[122,123] On the other hand, clastogens, including ionizing radiation, topoisomerase II inhibitors, inducers of reactive oxygen species (ROS) and alkylating agents, interfere directly with the DNA sequence and a threshold dose-effect relationship is not well established yet.[123] Therefore it is important to resort to immunofluorescence staining or fluorescence *in situ* hybridization (FISH), with DNA probes for human α-satellite DNA, to determine whether kinetochore proteins or centromeric DNA are present or absent inside the MN.[107,110,122] Since that acentric fragments do not contain centromeres nor kinetochores (clastogenic MN), if these are present then it confirms that it is an aneugenic MN.[107,110,122] However, this method is only well validated for human and V79 cell lines, since it is more difficult to get appropriate DNA probes for other cell types. Moreover, the FISH method implies the repetition of the slide preparation procedure, is time consuming and expensive.[122] Therefore, Hashimoto *et al*. (2010) proposed a new method to distinguish between aneugenic and clastogenic MN, by size-classifying them. MN showing less than ¼ the diameter of the main nuclei were considered clastogenic and those between ¼ to ½ the diameter of the main nuclei were aneugenic. Then they compared the sensitivity of this method with the traditional FISH procedure on chinese hamster lung fibroblasts to a

variety of chemicals and concluded that it was comparably reliable, quicker and simpler.[122]

B. JUSTIFICATION AND OBJECTIVES

A heat polymerizable denture base polymer (Type 1, Class 1) was selected as a test material since it is still widely employed mainly in the fabrication of dentures (either conventional of implant-supported removable of fixed, total or partial), which usually are used by the patients many hours per day for years or even decades. Moreover, these devices must be manufactured in such a way that there is as much base surface area as possible in close contact with the patient's oral mucosa (the palate and/or the gingiva). As stated previously, these materials have been constantly associated with allergic reactions and contact irritations. Therefore, it may be hypothesized that the chronic use of these devices, may lead to chronic damage to the oral tissues nearby.

Appropriate *in vitro* cytotoxicity and, eventually, genotoxicity testing of dental polymers is fundamental to establish the limits for clinical safety, requiring suitable cell models that closely simulate oral processes. Mammalian non-oral chinese hamster lung fibroblasts lines (V79) have been extensively used with this purpose, but to date, no *in vitro* studies were made concerning the objective quantification and relationship between acrylic resin residual monomer (MMA) leaching (in conditions comparable to routine clinical situations) and genotoxicity in an untransformed human gingival fibroblastic (HGF) cell line.

The main objectives of this research are to:

1. Quantify the MMA monomer concentration leached into artificial saliva from heat polymerizable denture base polymer pieces;
2. Characterize a human gingival fibroblast (HGF) line as an oral cell model for cyto and genotoxic testing;
3. Evaluate the cytotoxicity and genotoxicity effects in oral cells (gingival fibroblasts) leached residual monomer;
4. Optimize suitable techniques for the quantification of residual monomer.

C. MATERIALS AND METHODS

C.1. Acrylic Resin Sample Preparation: General Procedures

The characteristics of the acrylic resin used in this study are summarized in Table 6. The samples were produced by conventional prosthetic methods, respecting the manufacturer's instructions of use.

Table 6. Characterization of the denture acrylic resin tested (according to the manufacturer)

Products	Batch no.	Composition	Manufacturer	Powder/ Liquid Ratio	Standard polymerization cycle and cooling	Residual monomer
ProBase Hot Polymer (pink)	P49030	PMMA, plasticizer, pigments, BPO (< 1%)	Ivoclar Vivadent AG, Schaan, Liechtenstein	Powder: 22.5 g Liquid: 10 ml	Start with cold water. Heat up to 100ºC and let boil for 45 min. Cool at room temp. for 30 min and then with cold water.	< 2.2% (after standard polymeri-zation cycle)
ProBase Hot Monomer	P46582	MMA (50-100%), EGDMA (2.5-10%)				

Wax discs (1.5 mm height x 15 mm diameter) were cut manually from height calibrated 1.5 mm wax sheets (Anutex Toughened Wax, Kemdent, Purton, United Kingdom) using a metallic circular punch tool (Korff & Honsberg, Remscheid, Germany) with 15 mm of diameter. Each two discs were lightly heated and attached in order to produce single wax pieces measuring 3.0 ± 0.1 mm thick (using a manual thickness gauge) and 15.0 ± 0.1 mm of diameter (using a manual calliper). The best wax pieces were selected and mounted carefully to avoid air bubbles in type IV

gypsum (GC Fujirock EP, GC Ibérica, Madrid, Spain) inside one half of a conventional denture flask (Figure 2). The hardened gypsum was isolated with separating fluid (Ivoclar Vivadent AG, Schaan, Liechtenstein) and the other half of the flask was filled carefully with gypsum upon the wax discs. The flask was covered, put on a metallic press to remove the gypsum excesses and let harden. Afterwards, the ready closed flasks were immersed with the metallic press in boiling water for 5 minutes in order to eliminate the wax patterns. The flasks were opened and the wax rests were further washed with boiling water. According to the manufacturer instructions, both hot wet gypsum halves were isolated with 2 layers of separating fluid (Figure 3)

Fig. 2 – 3.0 x 15.0 mm wax patterns mounted in gypsum inside a conventional denture flask

Fig. 3 – Gypsum isolation with separating fluid

The polymer powder was weighted on a semi-analytic balance (Kern 440-33N, Kern&Sohn GmbH, Balingen, Germany) and the volume of monomer liquid was measured in a 10 ml glass graduated cylinder, according to the manufacturer 22.5 g : 10 ml mixing ratio. The two compounds were thoroughly mixed with a metallic spatula in a glass container and the resulting mass was left to mature in the closed container at room temperature for 8 to 10 minutes, until it did not stick to the fingers. The process of covering all patterns in the gypsum with the mass took no longer than 20 minutes

(Figure 4). Each flask was closed and loaded for approximately 5 minutes with 80 bar of pressure in a hydraulic press (Figure 5). Hereafter, the flasks were carried in pairs in the metallic press and completely immersed in cold water. The standard polymerization cycle recommended by the manufacturer was followed, as well as the gradual cooling procedure, as described in Table 6. In the end, each flask was opened and the gypsum was broke to liberate the resin discs.

Fig. 4 – Covering of patterns with the polymer mass

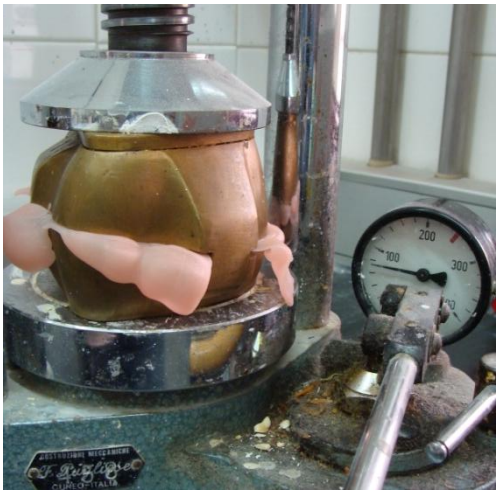


Fig. 5 – Flask loaded at 80 bar in hydraulic press

Since the manufacturer instructions of use did not specify the polishing procedure, the recommendations in the standard ISO 20795-1:2008 (section 8.8 – Test methods: Residual methyl methacrylate monomer) polishing method were followed. After the polymerization cycle was concluded, the discs were kept in a dark and dry place for 24 ± 5 hours at room temperature. The acrylic excesses and surface major irregularities were wet trimmed with a carbide bur at low speed. Afterwards, each disc was polished equally in both sides with a wet P 100 metallographic grinding paper (Klingspor AG, Haiger, Germany). Besides, both sides were wet polished with P 600 and P 1200 grinding impermeable papers (Klingspor AG) until the surface was smooth

and had minimal porosity at visual inspection. The periphery was also slightly wet abraded with the P 1200 grain paper.

In the end, an average of 3.0 mm height should be achieved in 3 different points of the discs measured with a manual thickness gauge. The 15 mm diameter was also verified using a manual caliper, each piece was weighted 3 times with an analytical balance (Kern 770-13, Kern&Sohn GmbH, Balingen, Germany) and all of these values were recorded for specimens' quality control. All specimens were kept in the dark at room temperature for approximately 24 hours before testing.

C.2. Extraction of the Leachable Residual Monomer

The extraction conditions and methods were based in ISO 10993-12:2007 recommendations.[80] Therefore, the standard surface area, which includes both sides of the sample and excludes minor irregularities, was used to determine the volume of extraction vehicle needed. ISO 10993-12:2007 rules that for larger moulded items with a thickness > 1.0 mm, the extraction ratio ± 10% (surface area/volume) is 3 cm^2/ml. Thus, the surface area of the discs was approximately 4.95 cm^2, corresponding to a vehicle volume of 1.65 ml, which was added with a micropipette to separate sterilized and light proof glass containers (Figure 6).

In order to simulate the oral conditions the polar extraction vehicle chosen was artificial saliva. Since in the published literature there are no consensual formulas for artificial saliva, this study followed the pre-standard DIN (Deutsches Institut für Normung) 53160-1:2002 (Table 7).[124] The solution was prepared in advance with sterilized distilled water and the pH was stabilized in the range of 6.8 ± 0.1. The solution was filtered with a 0.2 μm pore syringe filter (Puradisc 30, Whatman, GE Healthcare, Buckinghamshire, United Kingdom) and subsequently distributed in 10 ml aliquots, which were fronzen at -20ºC until use.

Fig. 6 – Acrylic resin sample submersed in artificial saliva inside a glass container (not opacified yet)

ISO 10993-12:2007 establishes that an appropriate exaggeration of the product use shall be conducted. Therefore, at 37ºC, discs (n = 6) (polished according to the standard ISO 20795-1:2008) were incubated in artificial saliva for 72 ± 2 hours. Negative controls of artificial saliva were also incubated for comparison. The containers were closed with the respective cover and stored in an incubator (IKA KS 4000 ic control, IKA® Werke GmbH & Co. KG, Staufen, Germany) with continuous agitation at 40 rpm.

In the end of each test period, the discs were collected with a sterile tweezers and the extraction samples inside the containers were frozen until the leached monomer analysis was performed.

Table 7. Artificial saliva formula (DIN 53160-1:2002) [124]	
Reagents	Mass concentration (g/L)
Magnesium chloride – $MgCl_2.6H_2O$	0.17
Calcium chloride – $CaCl_2.2H_2O$	0.15
Dipotassium hydrogen phosphate – $K_2HPO_4.2H_2O$	0.76
Potassium carbonate – K_2CO_3	0.53
Sodium chloride – NaCl	0.33
Potassium chloride – KCl	0.75
1% (m/m) Hydrochloric acid – added until the pH value 6.8 ± 0.1 is achieved. All reagents are from Sigma-Aldrich (St. Louis, MO, USA)	

C.3. HPLC Quantification of Leachable Residual Monomer

The analytical procedures described in this section were optimized and performed by the Laboratory of Applied Chemistry, Department of Chemistry-Physics, Faculty of Pharmacy of the University of Porto, Portugal. It was based on HPLC determination according to ISO 20795-1:2008 Annex A, but improved relatively to the method detection limit (MDL) and method quantification limit (MQL) by resorting to microextraction techniques.

Calibration solutions with known MMA concentrations ranging from 70 ppb to 14 ppm were prepared from pure MMA (99,4%, Sigma-Aldrich) in artificial saliva (composition in Table 7).

For the analysis, 1200 µl of each standard sample solution were transferred to 1500 µl microcentrifuge tubes and kept in ice during 10 minutes. Afterwards, 60 µl of 1-octanol were added and the mixtures were vortexed at 15 Hz for 20 seconds. Then, centrifugation was performed at 4000 rpm during 5 minutes, in refrigerated atmosphere (4ºC). The aqueous phase present in the bottom of the tube was removed and rejected with the help of a chromatographic syringe. The organic phase was injected directly in the chromatographic loop.

The chromatographic separation was performed using a Merck Hitachi LC system (Ltd. Tokyo, Japan), equipped with a LC pump L-7100, an interface D-7000 and a Diode Array Detector. A C18 column (Waters, Spherisorb ODS2, pore size 5 µm, 4.6 x 250 mm, Dublin, Ireland) with a security guard cartridge (4.0 x 3.0 mm, Phenomenex, USA) was used. An isocratic elution at 0.8 ml min^{-1} was performed using as MP methanol (MeOH) and water in a mixture 70:30 (v/v). The injection volume into the loop was 20 µl and the analysis was carried at room temperature (20 ± 1ºC). Chromatography Data Station Software was used for control and data processing. Spectrophotometric detection was carried out with a wavelength of 205 nm. Using the described experimental conditions, MMA monomers retention time was 5.20 minutes. MMA concentration in the artificial saliva solution was determined by interpolation using the calibration curve ($y = 4.03x10^{-8}\ x + 150000$).

C.4. Cell Culture Preparation and Maintenance

An untransformed human gingival fibroblast (HGF) commercial cell line (AG09429, Coriell Cell Repository, Camden, NJ, USA) was chosen as the main study model. Additionally, two other cell types were included in this study as control cell types: a chinese hamster lung fibroblast cell line (V79-4) (603371, CLS - Cell Lines

Service, Eppelheim, Germany) and an untransformed human fetal lung fibroblast cell line (WI-38) (90020107, Sigma).

Every procedure with cell cultures were performed at the Laboratory of Pharmacology and Biocompatibility of the Faculty of Dental Medicine of the University of Porto, Portugal. All cell cultures were acquired in cryovials and were unfroze and seeded in a sterile environment (inside a laminar flow cabinet) in 9 cm of diameter tissue culture plates (Orange Scientifique, Braine-l'Alleud, Belgium) with 10 ml of complete α-MEM (Table 8) at a concentration of $6x10^4$ cells/plate and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC. The culture medium was replaced 2 to 3 times a week.

The cells were removed from the culture plates by enzymatic digestion with 0.05% trypsin (Sigma) in 0.25% EDTA (Sigma) (10 minutes in humidified atmosphere of 95% air and 5% CO_2 at 37ºC) to detach adherent cells, when the monolayer cultures were 70-80% confluent, and were subsequently seeded at 6×10^4 cell/ml in 9 cm of diameter Petri plates.[125] HGF and WI-38 complete their cell cycles in about 24 hours, while V79-4 cells take only 12 hours. HGF and WI-38 cells took approximately 6-7 days to grow, while V79-4 cells were trypsinized in 3-4 days. The cultures were amplified and a few aliquots of each cell types were stored at -80ºC (if for only a few months) or -196ºC (if for several months) at a concentration of $2x10^6$ cell/ml in an appropriate medium composition with a cryoprotectant (45% α-minimal essential medium (α-MEM); 45% fetal bovine serum (FBS); 10% dimethyl sulfoxide (DMSO, Panreac Quimica, Barcelona, Spain))

Table 8. Complete cell culture medium (complete α-MEM) formula* [125]	
Reagents	Concentration
α-minimal essential medium (α-MEM, Gibco)	86% v/v
Fetal bovine serum (FBS; Gibco/BRL)	10% v/v
Ascorbic acid (Sigma)	50 μg/mL
Penicillin/Streptomycin (Gibco)	100 IU/mL/10 μg/mL
Amphotericin B (Fungizone, Gibco)	2.5 μg/mL
Glutamate (Sigma)	1% v/v
* Prepared fresh before use. Stored at 4ºC for no longer than 3 days.	

C.5. Cytotoxicity Test

MTT was the cytotoxicity test chosen and it was performed having in mind the general guidelines given in the Annex C of ISO 10993-5:2009.[83] Each experiment was performed 3 times. At least two passages after the cultures were unfroze and during the exponential growth phase, the 3 cell types were seeded in 96-well plates (Orange Scientifique) in 100 μl of complete α-MEM with a cell suspension of $1x10^5$ cells/ml ($1x10^4$ cells/well). According to the ISO recommendations, the cells were incubated for 24 hours in standard conditions before testing, in order to ensure that cells had time to adhere, return to their exponential growth phase and form a semi-confluent monolayer. Besides, the wells were observed at the inverted microscope (40x magnification) (Nikon TMS-F, Nikon, Japan) to confirm if the cultures growth was similar in all plates.

Just before the cytotoxicity testing started, the toxic agents were diluted in fresh complete cell medium, according to the conditions listed in Table 9. The initial culture medium was aspirated from the wells and replaced by 100 μl of treatment medium. The cells were incubated for another 24 hours in standard conditions.

Table 9. Experimental conditions of the cytotoxicity tests (MTT)

Experiment	Cells	Toxic agent	Manufacturer	Concentration (n = 6)		
1	HGF	Control (-)	_	0		
2	V79-4	EMS (μg/ml)	Sigma-Aldrich	600	1200	2400
3	WI-38	MMA (mM)		40	80	160
		Formaldehyde (μM)		400	800	1600

EMS – Ethyl methanesulfonate; MMA – Methyl methacrylate

Later on, the cultures were observed in the microscope in order to register the morphological alterations that occurred due to the toxic agents and to confirm the cell density in each well. Subsequently, 10 μl of the MTT solution (0.5 mg/mL; Sigma) was added to each well and the plates were incubated for 3-4 hours in standard conditions. Then, the culture medium was removed and the toxic waste was collected to separate recipients. 100 μl of DMSO were added to each well, even as two blanks of DMSO in each plate, and the plates were agitated for 5 minutes before being introduced in a microplate reader (Synergy HT, BioTek Instruments, Winoosky, VT, USA).

C.6. Genotoxicity Test

Based on the HPLC monomer quantification and cytotoxicity tests results, the *in vitro* mammalian cell micronucleus test was performed only for HGF and V79-4 cell lines, following the OECD 487 guideline for the testing of chemicals.[107] 2 separate experiments (A and B) were performed for each test group. No cytokinesis blocker was employed.

HGF and V79-4 cell types were seeded in 3.5 cm of diameter tissue culture plates (Orange Scientifique) at least two passages after the cultures were unfroze and during the exponential growth phase. HGF cells were cultured in 2 ml of complete α-MEM with a cell suspension of $1x10^5$ cells/ml (experiment A) and $5x10^4$ cells/ml (experiment B), while V79-4 were cultured at a concentration of $5x10^4$ cells/ml (experiment A) and $2.5x10^4$ cells/ml (experiment B). For experiment A, HGF were incubated for approximately 24 hours and V79-4 for about 12 hours, while for experiment B cells were incubated for an extra 24 hours.

Just before genotoxicity testing started, the toxic agents were diluted in fresh complete cell medium, according to the conditions listed in Table 10. The initial culture medium was aspirated from the wells and replaced by 1 ml of treatment medium. HGF and V79-4 cells were incubated for 1.5-2 normal cell cycles with the toxic agents.

After the toxicity testing, the culture medium was removed and the toxic waste was stored separately. Each culture plate was washed twice carefully with 1 ml of phosphate buffered saline (PBS, Sigma) in order not to remove the cells, followed by fixation with 1 ml of formaldehyde 3.7% for 30 minutes. Then, the cell membranes were permeabilized for 10 minutes with 1 ml of 0,5% tritonX100 (Sigma), to allow the action of 0.7 ml of RNase 100 μg/ml (Sigma) in PBS for 30 minutes. The nucleic material was stained with 0.7 ml of PI 10 μg/ml (Sigma) in PBS for 20 minutes. The excesses of

staining were washed 3 times with 1 ml of PBS and the plates were left to dry in the dark at room temperature.

The plastic walls of the dried plates were cut and a cover slip was mounted with a small drop of a mounting medium (Eukitt® O. Kindler GmbH, Sigma-Aldrich, St. Louis, EE. UU.). After drying, the margins of the cover slip were isolated using a transparent varnish, left to dry in the dark at room temperature and stored at 4ºC.

Table 10. Experimental conditions of the genotoxicity tests (MNvit)

Experiment	Cells	Toxic agent	Manufacturer	Concentration
		Control (-)	_	0
A	HGF	EMS (μg/ml)		600
B	V79-4	MMA (mM)	Sigma-Aldrich	0.0037* 40
		Formaldehyde (μM)		100

EMS – Ethyl methanesulfonate; MMA – Methyl methacrylate ; * Approximate value of the measured MMA at 72 hours (section D.1.) – $3.10x10^{-4}$ mg/ml ≈ 0.0037 mM

The plates were observed with a confocal multichannel microscope (Leica TCS SP2 AOBS, Leica Microsystems GmbH, Wetzlar, Germany) (Institute for Molecular and Cell Biology of the University of Porto) with fluorescence at 561 nm and a magnification of 400x. The number of micronuclei was counted in a minimum of 2000 cells per plate and recorded.[107] Simultaneously, clastogenic and aneugenic micronuclei were distinguished and counted, according to the size-classified micronucleus method proposed by Hashimoto *et al*. (2010).[122]

C.7. Statistical Analysis

The results from the HPLC quantification of residual monomer were analyzed using Excel®. Whenever necessary, the Dixon test was applied for the analysis of outliers. MDL was calculated as 3•(standard error of the calibration) = 3•(sy/x) and MQL was determined as 10•(standard error of the calibration) = 10•(sy/x).

The cytotoxicity and genotoxicity data were analyzed using Excel® and SPSS® V.18. The percent of cell viability was calculated for each well (n = 18) in relation to the mean absorbance of control wells. A descriptive analysis of the sample was performed and boxplots were analyzed for the existence of outliers. Additionally, agreement in controls' viabilities between different experiments was calculated with the Cronbach's Alpha. The normal distribution of the sample was confirmed by the Kolmogorov-Smirnov test (p>0,05).

Two-way ANOVA test was performed to assess if there were differences between the % of viabilities of the three cell types with increasing doses of each separate chemical substance. Dunnett post-hoc tests were performed considering as control groups 0 for the chemicals concentrations and the V79-4 for cells. Though the general significance level was p<0.01, a Bonferroni correction was applied, resulting in a p<0.05 significance level, since 5 comparisons were performed.

In relation to the genotoxicity tests, the percent of mononuclear cells with MN was calculated for the two experiments, as well as the proportion of clastogenic and aneugenic MN in 2000 cells each. The means of results from the two experiments performed were determined for the % of total cells with MN, cells with clastogenic MN and cells with aneugenic MN in 2000 cells. The cases (each experiment) were weighted for a frequency of 2000 cells (n = 2000), therefore the sample was assumed to follow a normal distribution.

Independent-samples t-test was performed to assess if there were differences between the 2 cell types in terms of their total scores of cells with MN (p<0.05).

Moreover, one-way ANOVA test was applied to assess if there were differences in the total number of micronuclei for the 2 cell types and different chemicals. Each chemical was considered with the respective concentration as independent groups and no comparisons between concentrations were performed. The Dunnett post-hoc test was executed to compare each test chemical group with the negative control group in terms of % of cells with MN with the negative control group. Though the general significance level was $p<0.01$, a Bonferroni correction was applied, resulting in a $p<0.05$ significance level, since 4 comparisons were performed.

D. RESULTS

D.1. Quantification of the Leachable Residual Monomer

A typical calibration curve obtained with the described analytical procedure is plotted in Figure 7. MQL was set at $1.17x10^{-4}$ mg/ml. Since that the calculated MDL presented a statistical error <10%, it was considered to be equal to the MQL. In the MMA concentration range between $9.30x10^{-4}$ and $1.86\ x10^{-2}$ mg/ml, good linear relationships were obtained. Figure 8 shows a typical MMA chromatogram, with a clean separated MMA peak detected around 5.20 minutes.

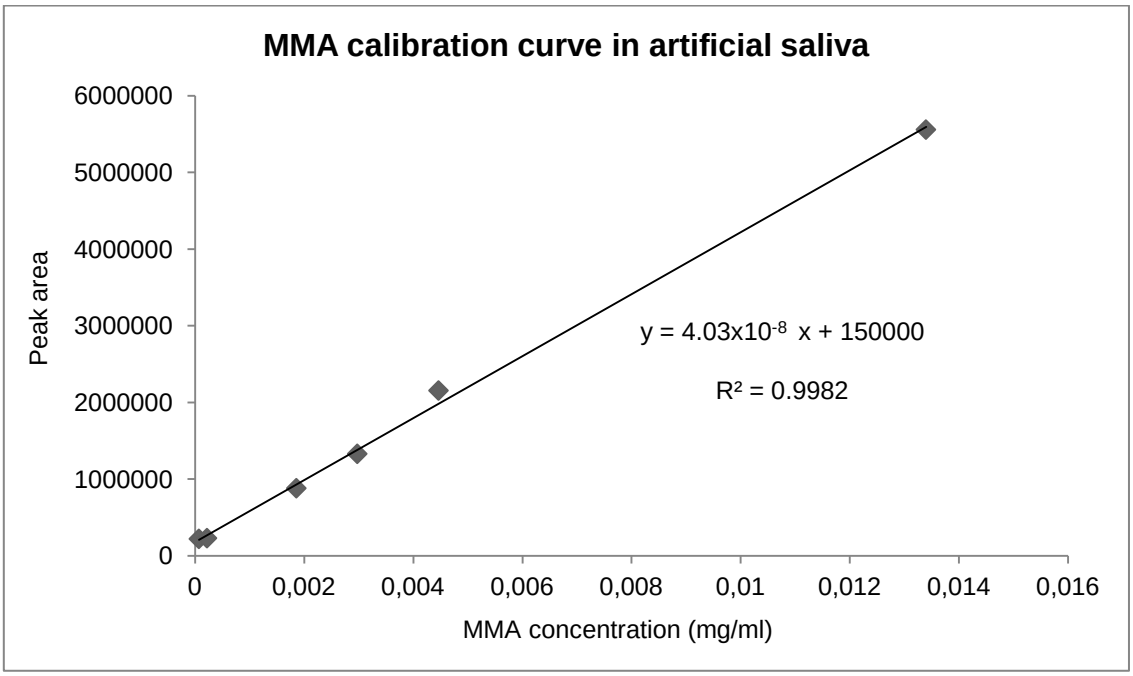


Fig. 7 – MMA calibration curve in artificial saliva for HPLC with 95% of confidence levels. The slope of the line has a SD value of ±0.07. The y-axis intercept has a SD of ±16000

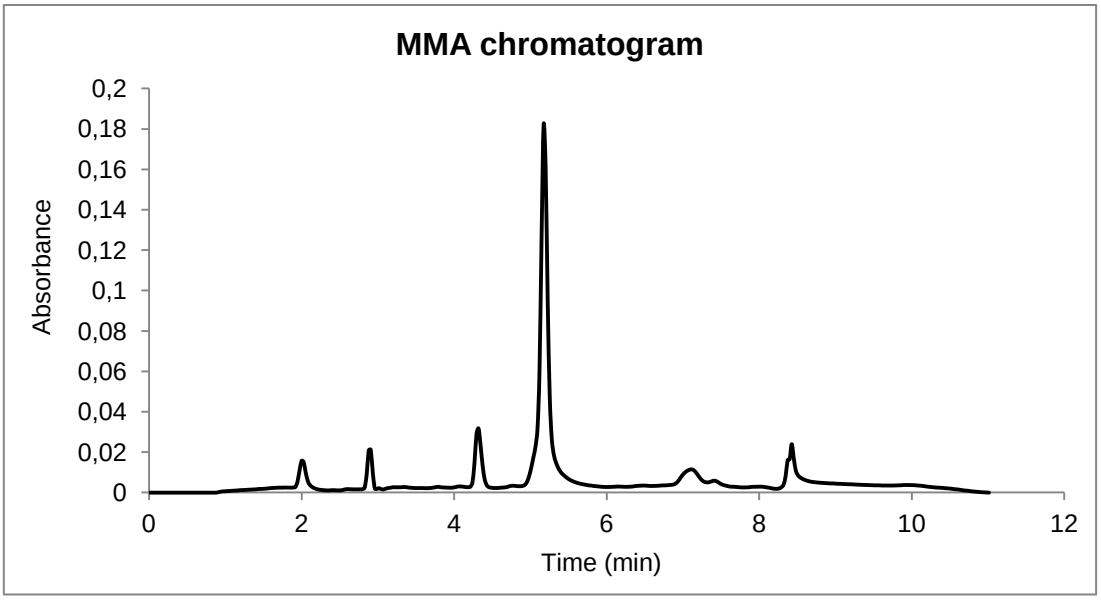


Fig. 8 – Typical MMA chromatogram (MMA concentration = 7.44×10^{-4} mg/ml)

Despite the monomer pre-concentration prior to the HPLC quantification, the leached residual monomer into artificial saliva is very low (Table 11). After 24 hours of sample submersion, the MMA concentration is below the MQL/MDL, thus it is not possible to determine it with precision. However, the 72 hour test group obtained a low, but quantifiable concentration of leached residual monomer. According to the Dixon test, no outliers were found, so all results from the 6 samples of each group were considered.

Table 11. Mean values of leached MMA into artificial saliva at 24 and 72 hours (n = 6, independent samples)

Time (hours)	24	72
Concentration (mg/ml)	< 1.17×10^{-4} (MQL)	3.10×10^{-4} (Standard relative error:10%)

D.2. Cytotoxicity Testing

Figure 9 illustrates the typical appearance of human fibroblasts at different steps of the cytotoxicity assay. Metabolically active fibroblast mitochondrias are capable of reducing the MTT into an insoluble formazan precipitate. Figure 10 shows a test 96-well plate prepared to be introduced in the microplate reader. It is observable that the fibroblasts viability decreases as the chemical concentration increases, since the color intensity of the solutions declines.

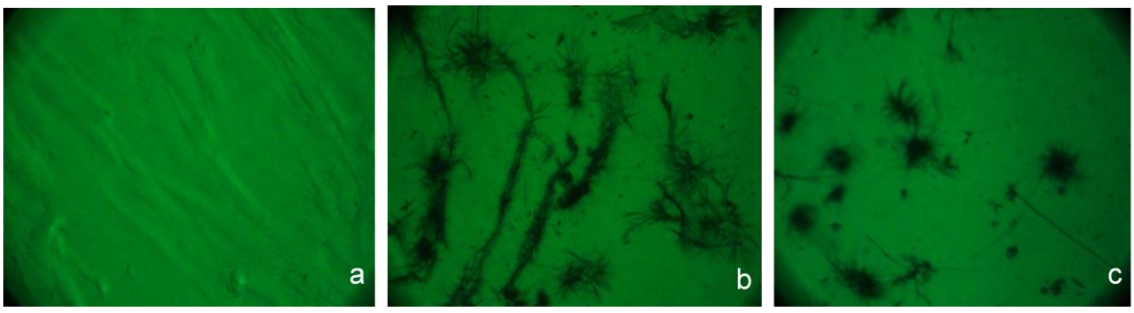


Fig. 9 – Human fibroblasts' morphology at different steps of the cytotoxicity testing (merely illustrative): a) cells during their exponential growth phase in normal culture conditions; b) negative control group of cells, showing extensive formazan precipitates upon MTT testing; c) test group of fibroblasts treated with cytotoxic concentrations of a chemical, presenting only a few small formazan precipitates upon incubation with MTT (a), b), c) 330x magnification, inverted microscope, no staining)

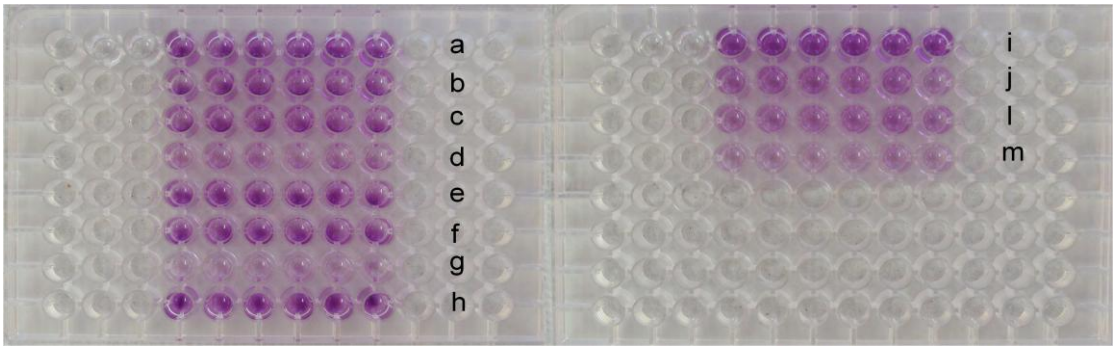


Fig. 10 – Illustrative 96-well plates MTT test for HGF cells (n = 6 for each test group in each microplate). The experiences were triplicated (final n = 18). Rows: a) negative control; b) EMS 600 µg/ml; c) EMS 1200 µg/ml; d) EMS 2400 µg/ml; e) MMA 40 mM; f) MMA 80 mM; g) MMA 160 mM; h) negative control; i) negative control; j) formaldehyde 400 µM; l) formaldehyde 800 µM; m) formaldehyde 1600 µM

The means and SDs for each test group are presented in Table 12.

Table 12. % of cells viability with the MTT cytotoxicity test for EMS, MMA and Formaldehyde

Toxic Agent	EMS			MMA			Formaldehyde		
Concentration	0			0			0		
Cell Type	HGF	WI-38	V79-4	HGF	WI-38	V79-4	HGF	WI-38	V79-4
% Viability (Mean (SD))	100.5	106.2	100.8	99.6	95.3m	99.2	100.0	101.8	100.0
	(8.3)	(8.0)	(6.3)	(7.8)	(9.6)	(5.6)	(8.5)	(10.9)	(11.9)
Concentration	600 µg/ml			40 mM			400 µM		
Cell Type	HGF	WI-38	V79-4	HGF	WI-38	V79-4	HGF	WI-38	V79-4
% Viability (Mean (SD))	95.6	95.0	86.1	84.5	88.4	98.0	57.7	67.4	38.6
	(13.3)	(15.0)	(8.7)	(11.2)	(11.5)	(13.6)	(9.3)	(10.0)	(12.8)

Table 12. Continuation									
Toxic Agent	EMS			MMA			Formaldehyde		
Concentration	1200 μg/ml			80 mM			800 μM		
Cell Type	HGF	WI-38	V79-4	HGF	WI-38	V79-4	HGF	WI-38	V79-4
% Viability (Mean (SD))	81.2 (7.7)	84.4 (11.4)	66.9 (8.4)	81.9 (7.6)	85.3 (13.2)	82.0 (12.3)	52.6 (9.2)	64.9 (10.9)	33.7 (5.2)
Concentration	2400 μg/ml			160 mM			1600 μM		
Cell Type	HGF	WI-38	V79-4	HGF	WI-38	V79-4	HGF	WI-38	V79-4
% Viability (Mean (SD))	42.4 (7.2)	67.7 (16.2)	38.4 (14.8)	39.6 (20.5)	47.8 (15.0)	34.9 (16.3)	42.2 (16.1)	48.5 (11.4)	28.9 (6.8)

The complete statistical analysis for this section is presented in the Annex section (H 1. Cytotoxicity Test Statistical Analysis Output). Firstly, the descriptive statistic analysis was made for each chemical. It showed that in the case of EMS (ethyl methanesulfonate) there was an outlier group of 6 wells (HGF at 600 µg/ml), which was considered missing, though it does not seem to affect much the outcome. Boxplot graphics (Figure 11) are presented to evaluate the outlier cases behavior. However, since there are n = 18 cases per toxic agent/concentration/cell type, the outliers highlighted in the boxplots were not excluded, since they do not affect the outcomes.

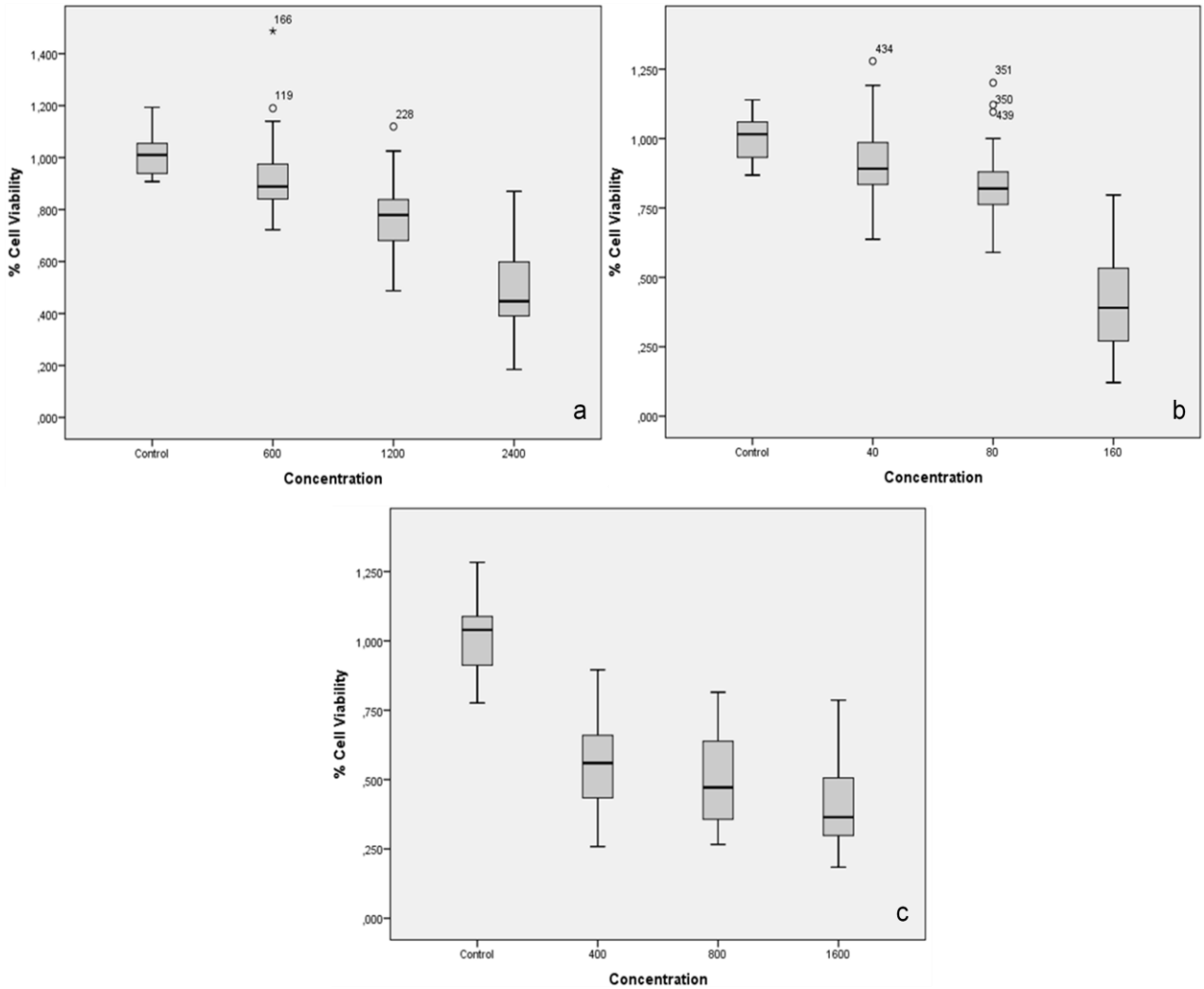


Fig. 11 – Boxplots per toxic agents with outliers: a) EMS; b) MMA; c) Formaldehyde.

The Cronbach's Alpha test showed that there was concordance between the three independent experiments (84.4%).

The two-way ANOVA test presented a high observed power (approximately 1.000). The cells' viability curves with the doubling toxic concentrations are plotted separately for each chemical agent (Figure 12 to 14), with 99% confidence intervals.

It was verified that for every chemical agents, there was a significant decrease (p<0.01) between the viabilities of the control groups and the other concentrations for all cell lines. On the other hand, despite an overall tendency of HGF and WI-38 to show higher viability values than V79-4, the effects on the three cell types are not statistically significant (p>0.01).

In general, the cells' viability seems to decrease almost linearly with doubling doses of EMS (Figure 12). MMA causes an initial progressive decrease in the cells' viability, but somewhere between 80 and 160 mM, the cells survival falls remarkably (Figure 13). Conversely, formaldehyde causes a marked decrease on viability right at the lowest concentration tested and then the toxic effect seems to be progress slower (Figure 14).

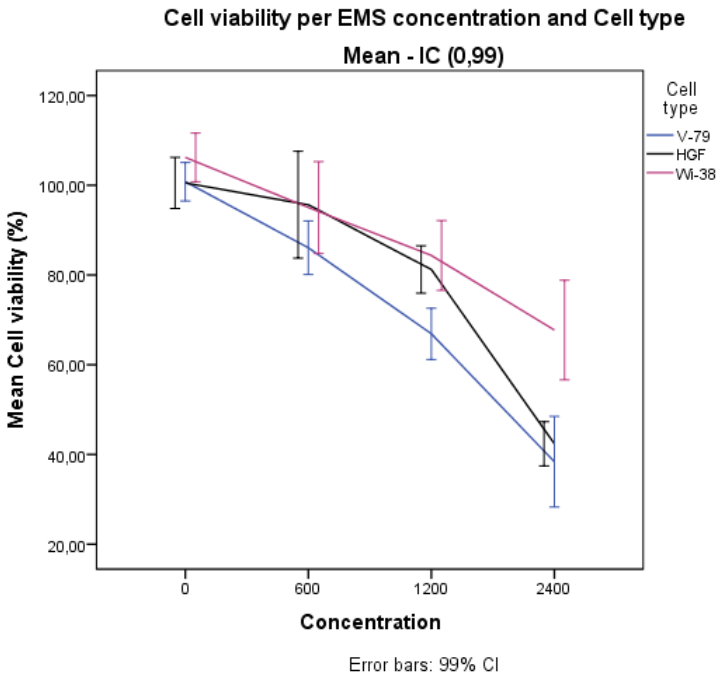


Fig. 12 – Separate cells viability curve for EMS with 99% of confidence levels

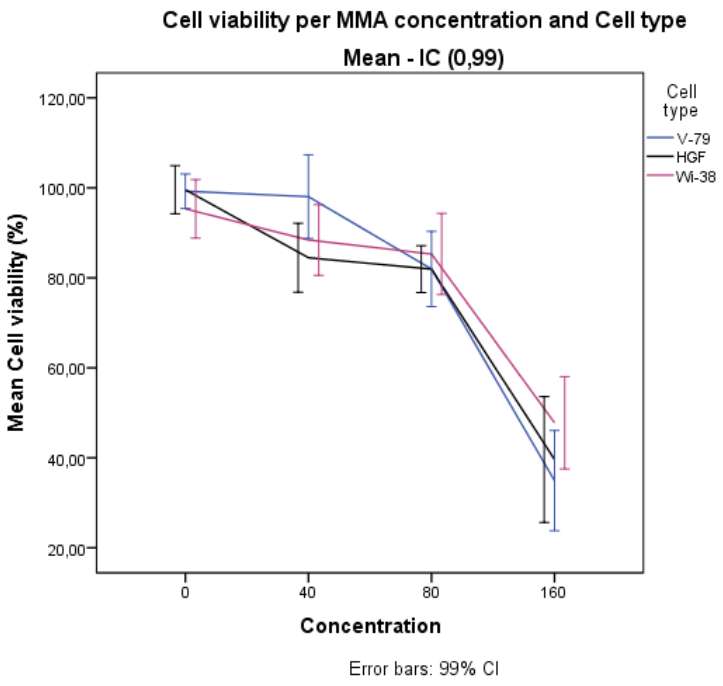


Fig. 13 – Separate cells viability curve for MMA with 99% of confidence levels

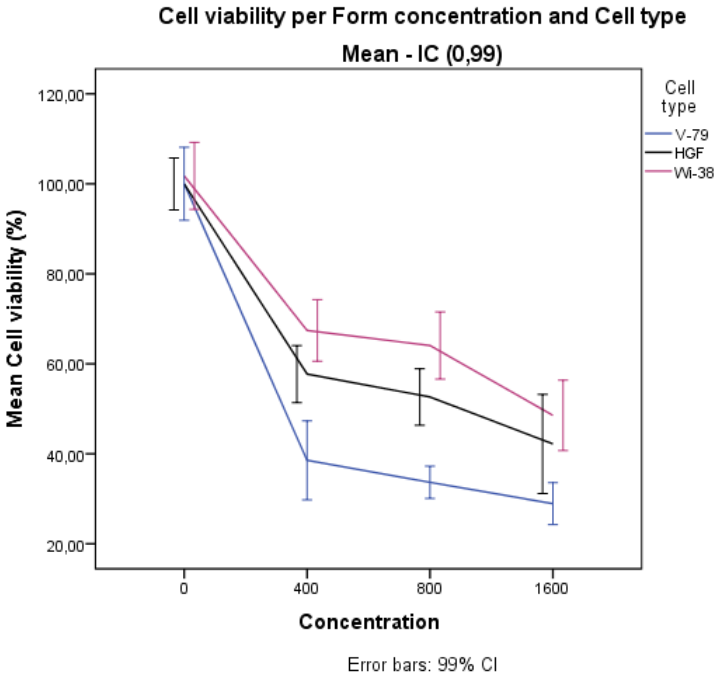


Fig. 14 – Separate cells viability curve for formaldehyde with 99% of confidence levels

D.3. Genotoxicity Testing

Figures 15 to 34 show examples of cell nuclei observed during the MN scoring at the confocal fluorescent microscope (PI staining).

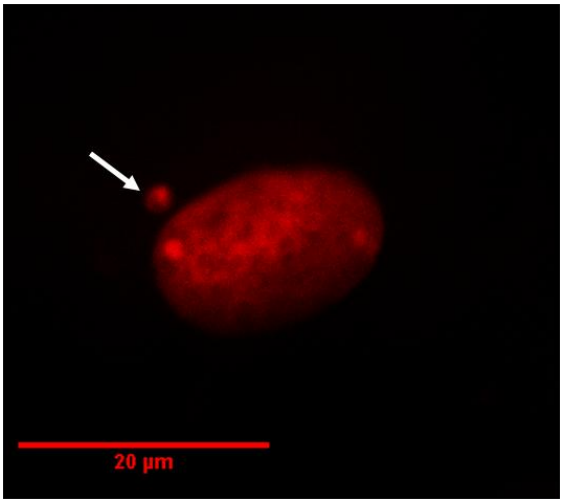


Fig. 15 – HGF cell with a clastogenic MN (arrow)

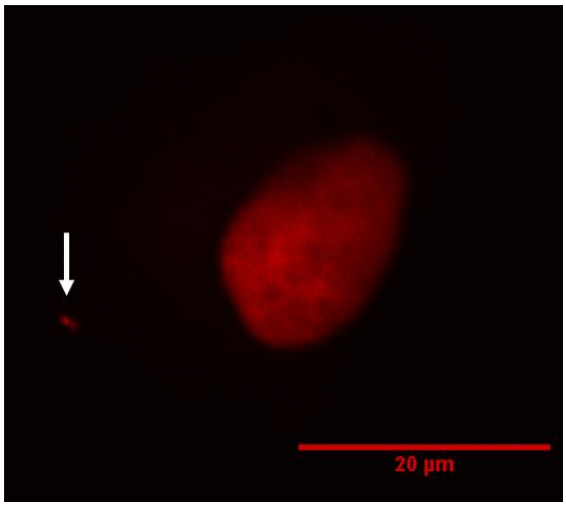


Fig. 16 – HGF cell with a borderline calstogenic MN (arrow)

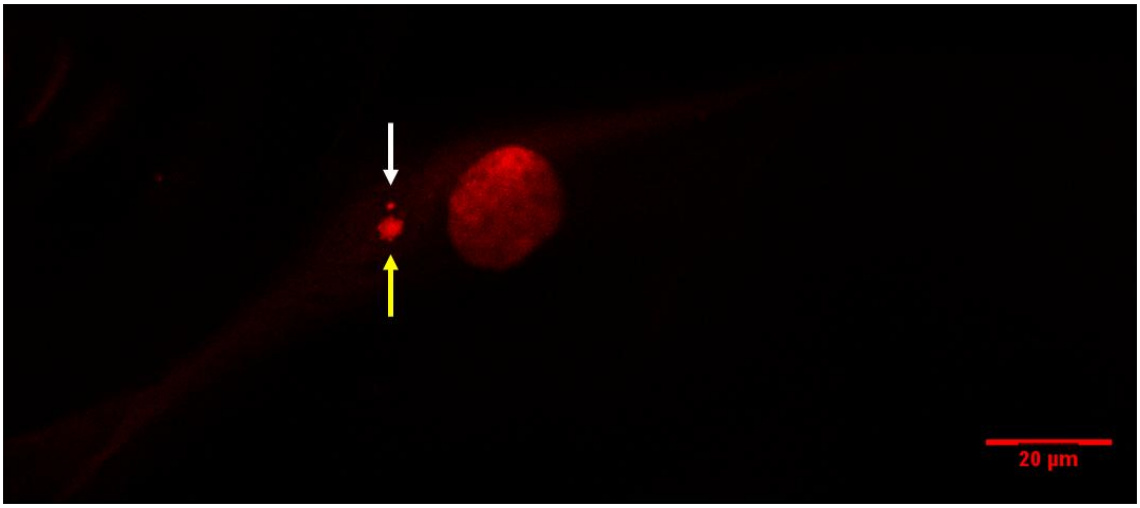


Fig. 17 – HGF cell with two clastogenic MN (arrows) inside the cytoplasm

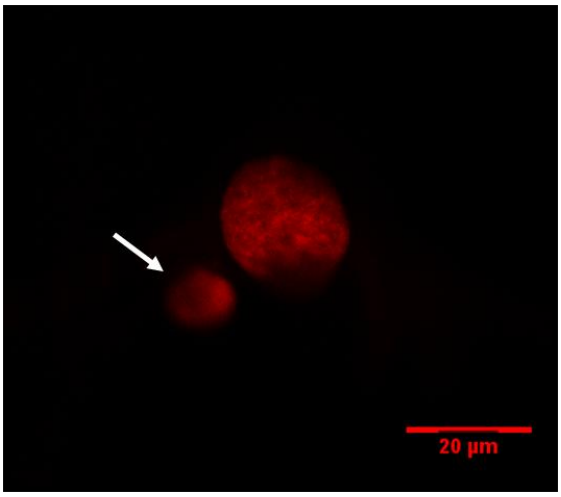


Fig. 18 – HGF cell with an aneugenic MN (arrow)

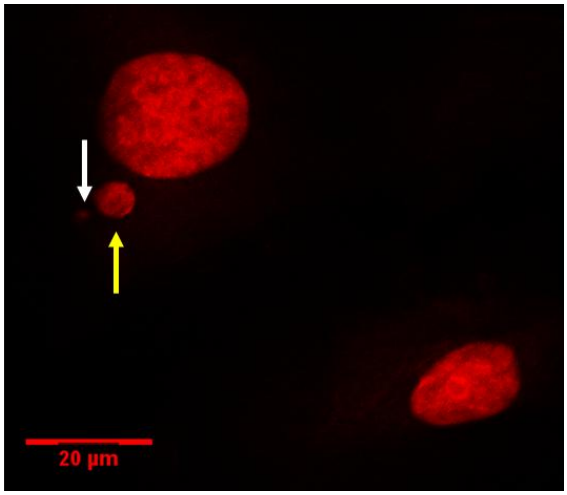


Fig. 19 – HGF cell with a very small borderline MN (white arrow) and a large clastogenic MN (yellow arrow), which was not considered aneugenic

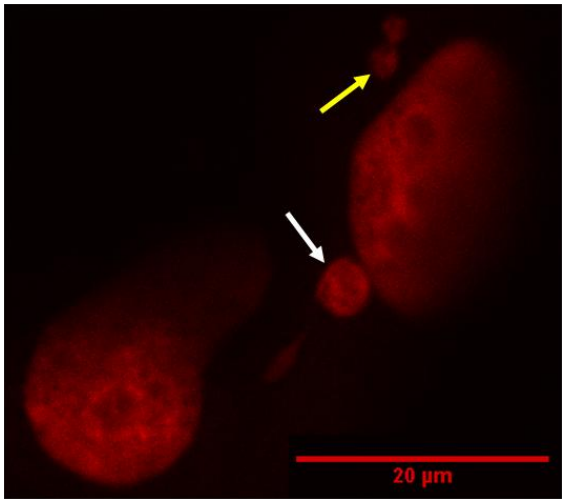


Fig. 20 – HGF cell with three clastogenic MN (arrows)

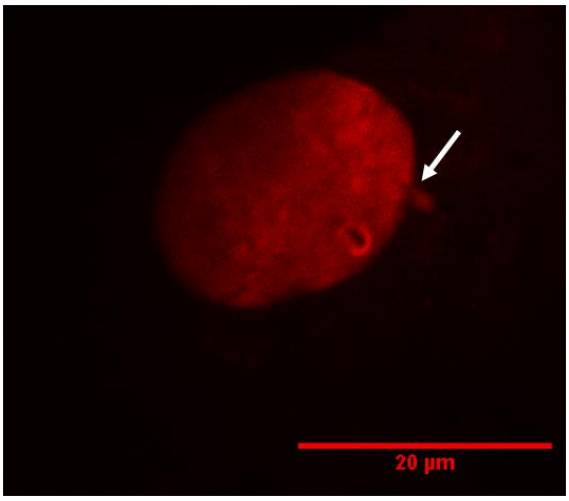


Fig. 21 – HGF cell with a NBUD (arrow) that could be easily confounded with a clastogenic MN

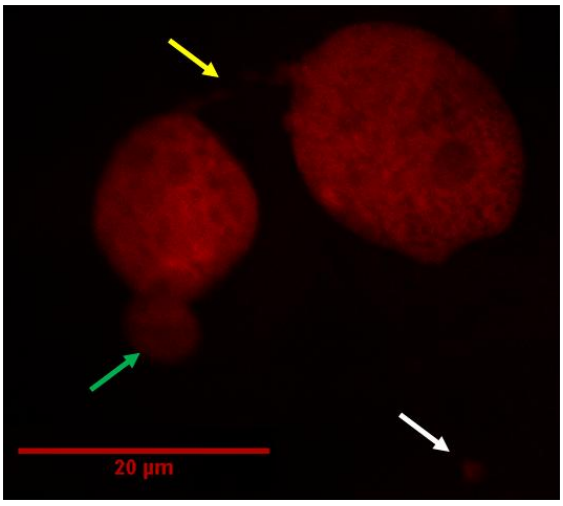


Fig. 22 – Two recently formed HGF cells, united by a NPB (yellow arrow), one of them has a large NBUD (green arrow) and the other has a clastogenic MN (white arrow)

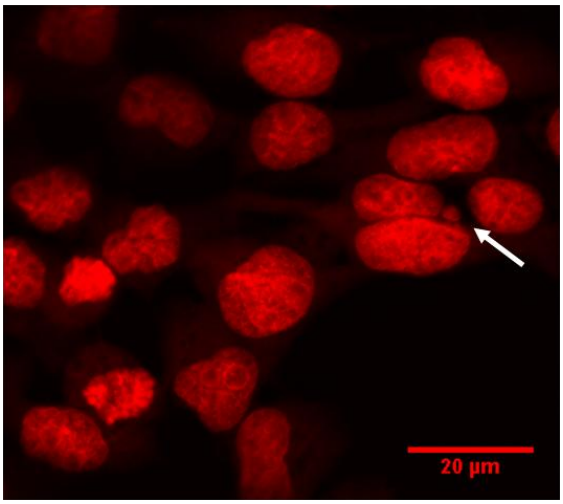


Fig. 23 – V79-4 cell with a clastogenic MN (arrow)

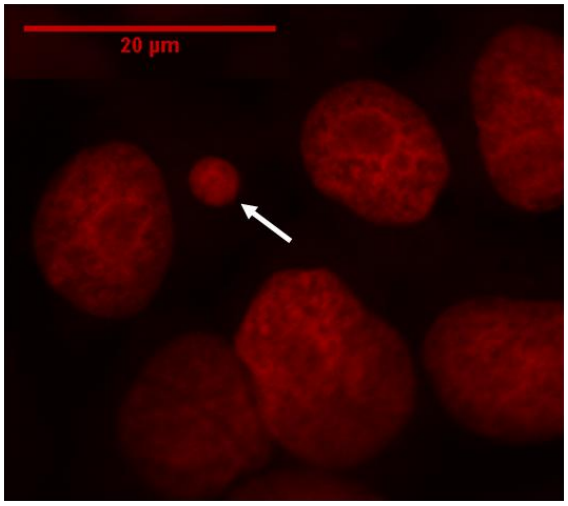


Fig. 24 – V79-4 cell with an aneugenic MN (arrow)

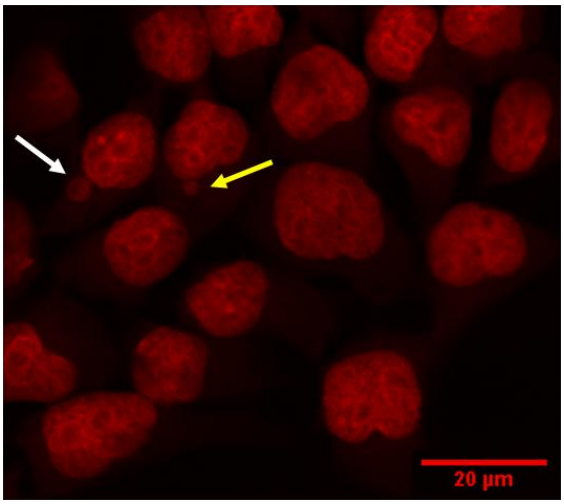


Fig. 25 – Two V79-4 cells each with its own MN (arrows)

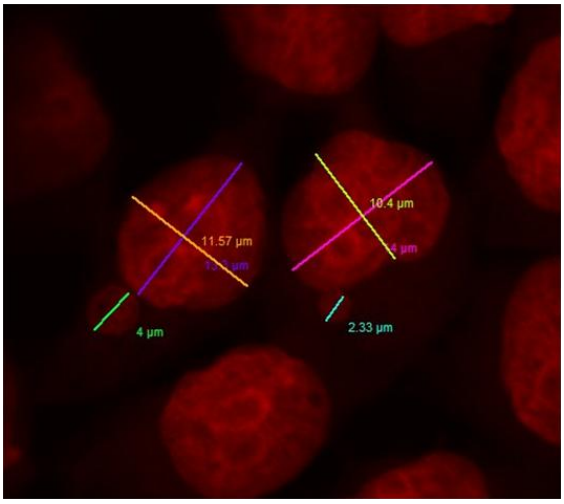


Fig. 26 – A magnification of the previous figure shows that when the MN are measured one of them is aneugenic (white arrow in Figure 25) and the other is clastogenic (yellow arrow in Figure 25)

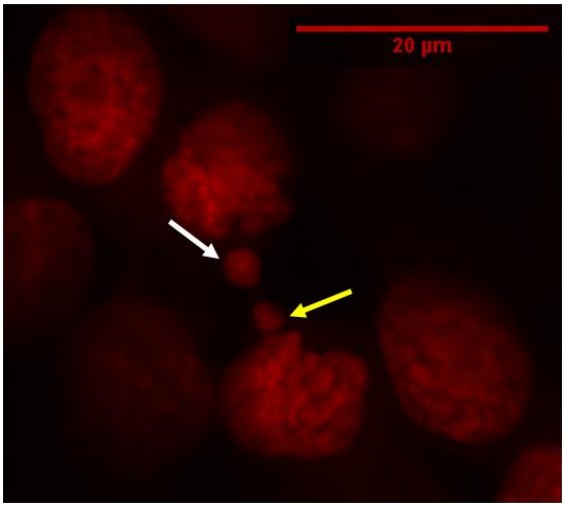


Fig. 27 – A defective mitosis (cytokinesis) resulted in the formation of two MN (arrow), one for each V79-4 daughter cell

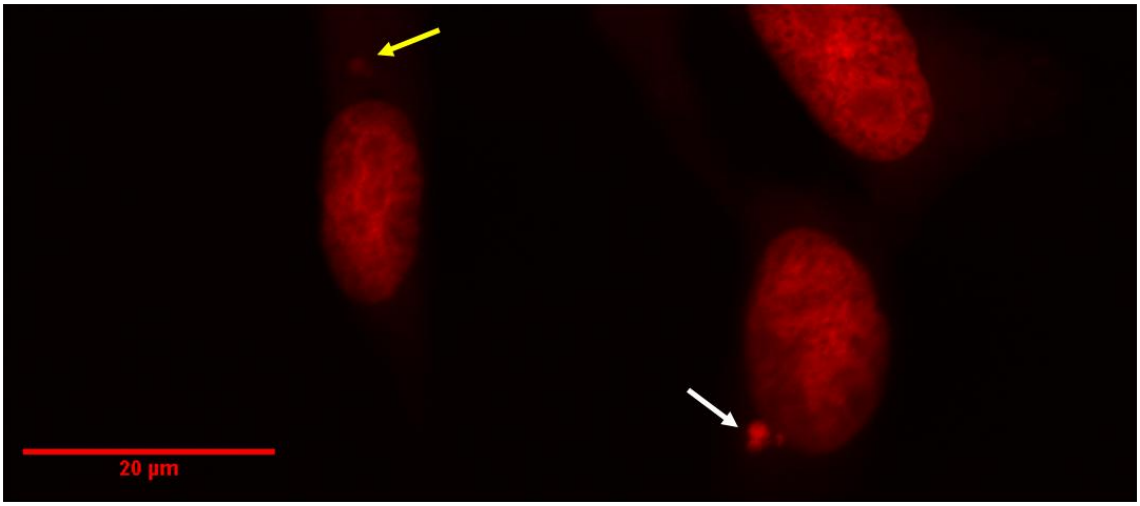


Fig. 28 – One of the V79-4 cells has a clastogenic MN (yellow arrow), while another has a small NBUD (white arrow)

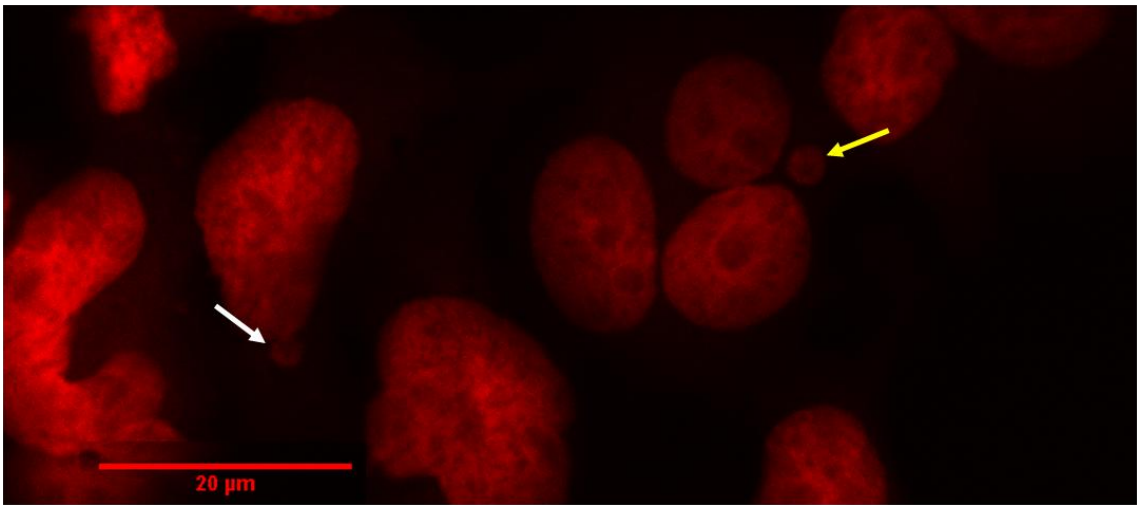


Fig. 29 – One of the V79-4 cells has an aneugenic MN (yellow arrow), while another has a small NBUD (white arrow)

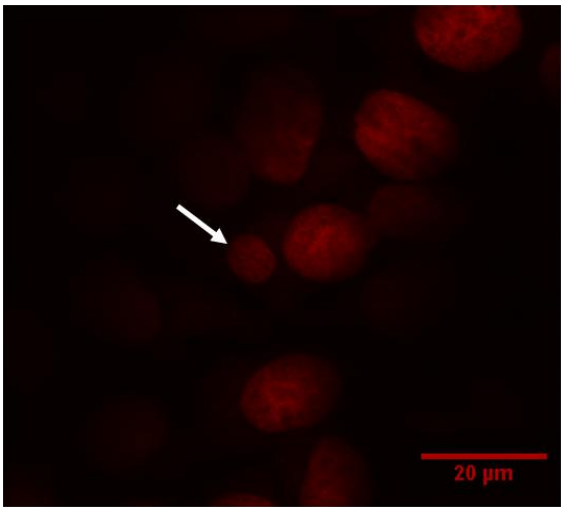


Fig. 30 – V79-4 cell with a large borderline aneugenic MN (arrow)

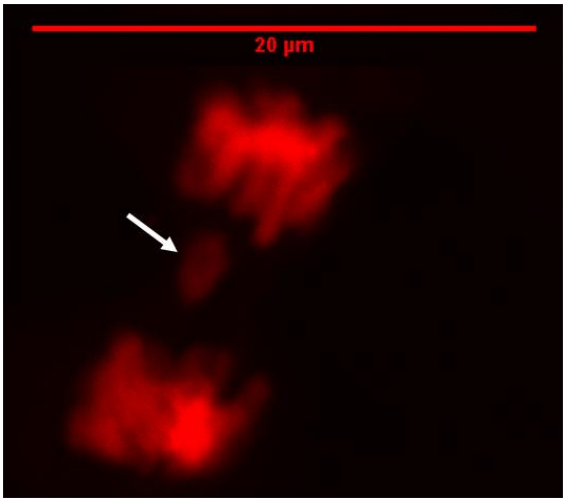


Fig. 31 – A defective mitosis (anaphase) of a V79-4 cell is resulting in the formation of an aneugenic MN (arrow), possibly a lagging chromosome

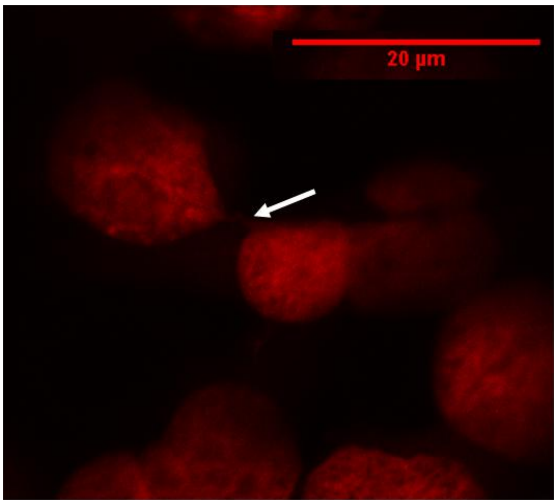


Fig. 32 – V79-4 cells united by a NPB (arrow)

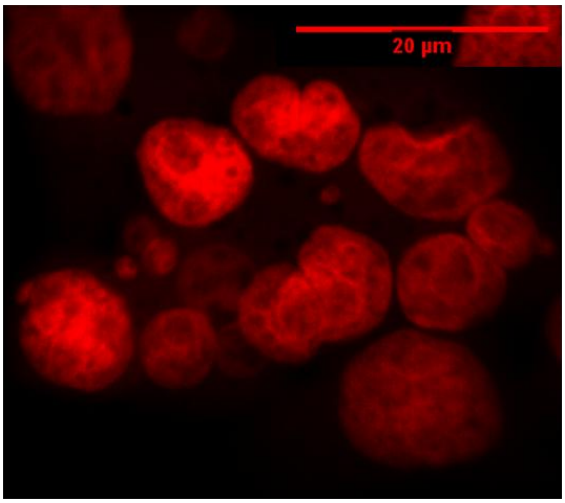


Fig. 33 – A group of V79-4 cells (possibly descendent from the same mother cell) where most of the cells have their own MN

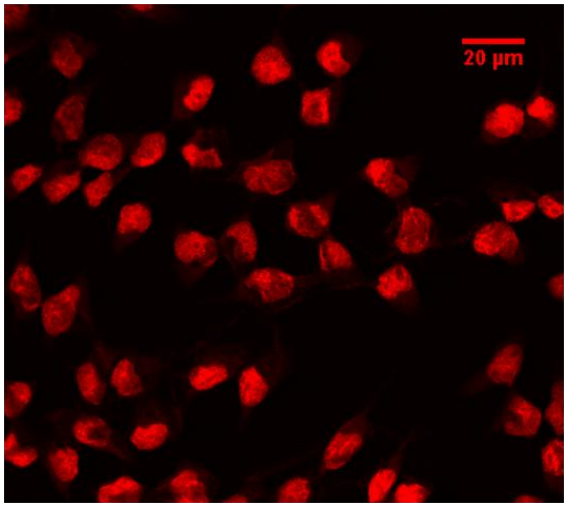


Fig. 34 – Overview of the great density of V79-4 cells

The detailed statistical analysis in presented in the Annex section (H 2. Genotoxicity Test Statistical Analysis Output). Table 13 presents the % of total cells with one or more MN in 2000 mononuclear cells.

Table 13. Cells with MN in 2000 mononuclear cells (%) (mean of experiments A and B)

Cells	Toxic agent/Concentration	Total cells with MN	Cells with Clastogenic MN	Cells with Aneugenic MN
HGF	Control (-): 0	7,19%	6,89%	0,30%
	EMS: 600 µg/ml	9,72%*	9,34%	0,37%
	MMA: 0.0037mM	5,16%	4,64%	0,52%
	MMA: 40mM	6,16%	5,97%	0,20%
	Formaldehyde 100 µM	7,10%	6,97%	0,12%
V79-4	Control (-): 0	0,99%	0,82%	0,17%
	EMS: 600 µg/ml	4,57%*	4,02%	0,54%
	MMA: 0.0037mM	0,88%	0,73%	0,15%
	MMA: 40mM	1,00%	0,95%	0,05%
	Formaldehyde 100 µM	0,65%	0,62%	0,02%

***** statistically significant differences ($p<0.001$) in the % of total cells with MN between test groups and the respective negative control (One-way ANOVA analysis)

The sample was analyzed for outlier cases (Figure 35), though none were excluded, since the n was high (n = 2000) and the outcome was not affected.

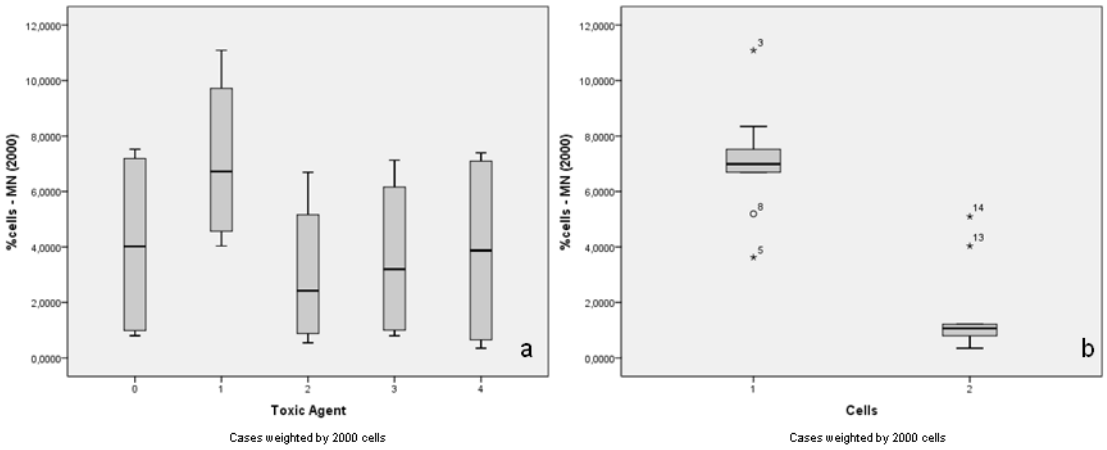


Fig. 35 – Boxplots with outliers: a) Toxic Agent (Labels: 0 – Control (-); 1 – EMS 600 μg/ml; 2 – MMA 0.0037 mM; 3 – MMA 40 mM; 4 – Formaldehyde 100 μM); b) Cells. (Labels: 1 – HGF; 2 – V79-4)

Though the equality of variances could not be assumed, independent-samples t-test showed that there is a statistically significant difference (p<0.05) in the % of total cells with MN between the two cell types: HGF presents constantly higher scores of MN than V79-4 (MN baseline 7.19% *vs.* 0.99%).

Although no homogeneity of variances was verified for this sample for one-way ANOVA analysis, the Brown-Forsythe robust test of equality of means was statistically significant (p<0.001). The Dunnett post-hoc test showed that for both cell types there are significantly higher scores of cells with MN in the EMS group (positive control) than the MN baseline (negative control) (p<0.001). This increasing is more notorious for the V79-4 cell type (approximately 4.6-fold) than for HGF (approximately 1.4-fold). However, no differences to the control group are verified for formaldehyde and MMA at the tested concentrations (p>0.01).

Figures 36 and 37 represent the ratio of cells with clastogenic and aneugenic MN for HGF and V79-4 cells, respectively, measured for the first time, with the

indicated toxic using the size method proposed by Hashimoto *et al*. (2010).[122] In both cases there is a clear predominance of clastogenic MN independently of test groups.

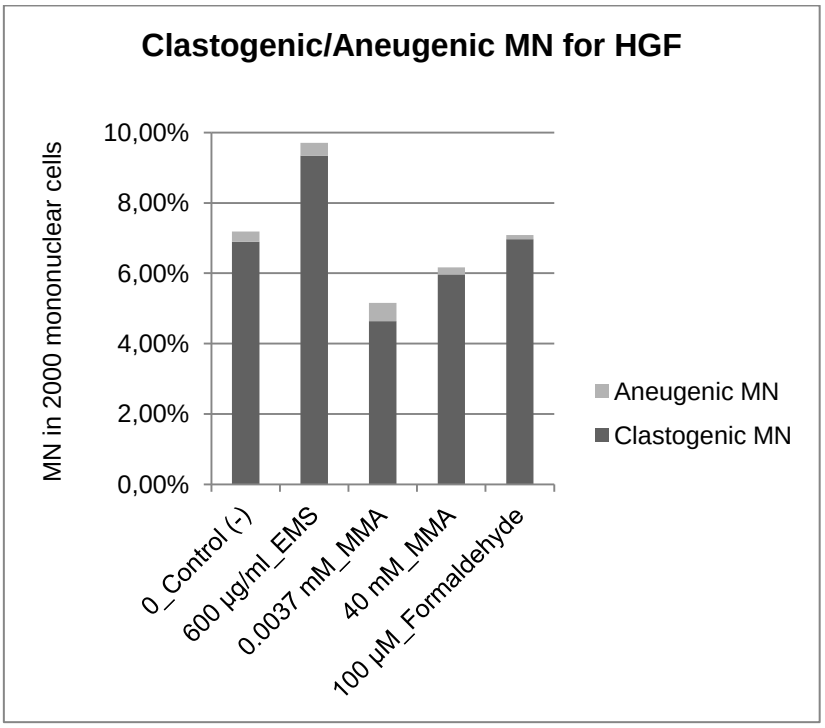


Fig. 36 – Ratio of clastogenic/aneugenic MN in 2000 cells scored for HGF cells (cell test group).

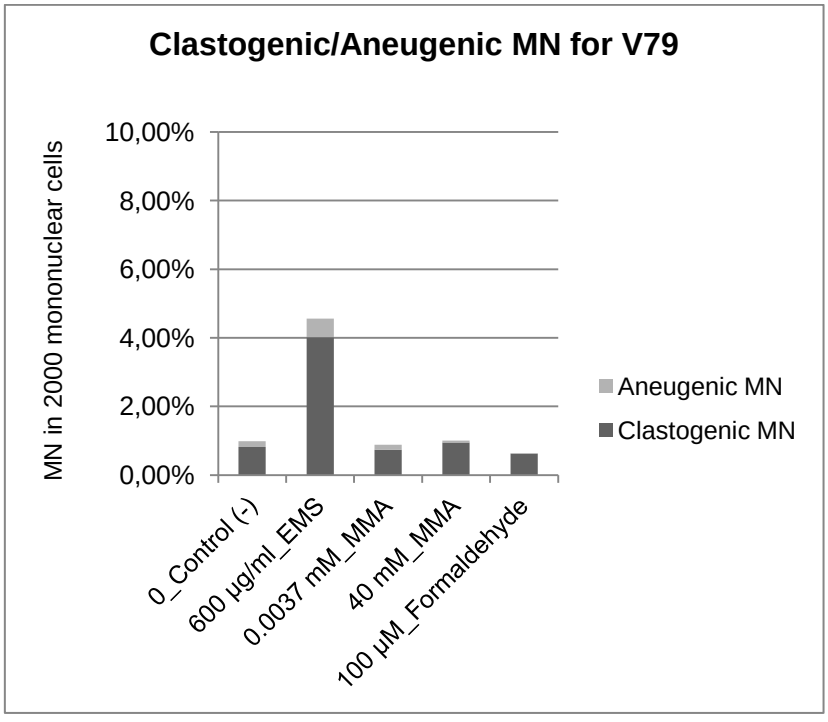


Fig. 37 – Ratio of clastogenic/aneugenic MN in 2000 cells scored for V79-4 cells (cell control group)

E. DISCUSSION

E.1. Leached Residual Monomer

As referred before, one of the main aims of the present research was to verify whether the residual monomer released in simulated oral conditions by a commercial heat polymerizable denture base polymer had genotoxic effects on oral fibroblasts. The first results obtained show that the resin studied leaches a very low quantity of MMA residual monomer (near the LOQ) into the artificial saliva formula used at 37ºC. As it will be further discussed bellow, the monomer extraction conditions followed the ISO standards and the quantification method has a very low MLD. It may be hypothesized that these results are due to the features of the material tested.

The acrylic resin chosen (ProBase Hot, Ivoclar) is one of the most widely used denture base polymers in the market. It is a denture base Type 1 powder-liquid (Class 1) material [5] composed essentially by the MMA monomer, though it also has a small proportion of EGDMA as a cross-linking agent (manufacturer instructions of use). The manufacturer of the acrylic resin chosen declares that the content of residual monomer of this material is lower than 2.2% (w/w) after following the standard polymerization protocol (manufacturer instructions of use), therefore complying with the ISO 20795-1:2008 recommendation. As stated previously, cross linked polymers have better physical and mechanical properties and are more difficult to expand and release molecules,[12,126] thus being the most plausible explanation for the low MMA concentration obtained in this study. Vallittu *et al.* (1998) determined the total content of residual monomer according to the ISO standard for precisely this brand of heat polymerizable material.[126] Interestingly, they found that when compared with other conventional brands of acrylic resins (Lucitone 199) cured in the same conditions, the total content of residual monomer was inferior for ProBase Hot.[126] Therefore, it would be expectable that the mount of leached monomer would also be low, which would be in agreement with our results in the leaching assay using this acrylic resin.

Urban *et al.* (2012, 2009) have been comparing the leaching properties of autopolymerizable hard relining denture polymers with and without cross linking features into artificial saliva. They came to the conclusion that materials with bifunctional monomers (such as 1,6-HDMA) promote the formation of a cross linked network and a higher degree of conversion, thus releasing less amounts of monomer [36] and byproducts.[127] Still, the concentration of leached monomer in artificial saliva from the cross linked materials is in the range of 1-6 μg/ml (ppm range).[36] These concentrations are higher than those obtained in the present research, probably due to the fact that autopolimerazable materials usually present a worst degree of conversion compared to heat polymerizable polymers. Moreover, Çelebi *et al.* (2008) refer to a study by Koda *et al.* (1990) in which it was not possible to determine the amount of leached monomer from heat and microwave cured resins into artificial saliva, since that the concentrations were below a reliable determination range.[128]

In terms of the test conditions, the protocol employed followed the recommendations in ISO 10993-12:2007, which provides a standardized approach that is an appropriate exaggeration of product use.[80] However, it was noticed that when assessing the state of the art in this field, there is no consensus between the different authors in terms of the followed methodologies. Hence, it becomes very difficult to compare the present results with theirs and to draw definite conclusions.

In the present research, an extraction ratio of 3 cm^2/ml was employed, respecting the guideline for larger moulded items (thickness > 1.0 mm), and the solvent volume used was a function of the surface area of the polymer discs.[80] Although suggested by the ISO 10993-12:2007 norm, the samples were not cut in pieces for this assay, because prosthetic devices are used by the patients as a single piece and there may be potential differences in extraction characteristics between intact and cut surfaces.[80] The extraction ratio is one of the most important variables in this type of study, since it influences directly the concentration of monomer quantified. Consequently, the effects of a given material may be under or over-estimated and, as a

result, misevaluated. Most published articles [19,27,32,36,41,43-45,48,52,102,127-132] present extraction ratios under 3 cm^2/ml, except for Danesh *et al.* (2011), whose extraction ratio is approximately 5 cm^2/ml.[30] As a consequence, in this particular case, there may be a higher concentration of leachable residual monomer in the solvent and, eventually, a worse toxic effect. Bural *et al.* (2011a,b) refers to a withdrawn ISO standard (ISO 10993-5:1999) [133] for cytotoxicity testing to justify the extraction ratio used (0,626 cm^2/mL).[32,102] However, this latter guideline refers to ISO 10993 Part 12 when it comes to prepare liquid extracts of a material.

Also, according to the ISO standard, the extraction period chosen for an assay is dependent on the temperature appropriated for the simulated test situation. In the case of the present work, 37ºC was the most appropriated temperature to simulate the oral conditions, thus an extraction period of 72 hours was recommended by the standard. Since it has been claimed by several authors that most of the leachable residual monomer is released in the first few hours of contact with the solvent,[27,30,36,41,128,129,132,134] samples were also evaluated at 24 hours.

Another source of variability comes from the fact that only a minority of authors performed the quantification of the extracts in independent samples [30] and most of the studies use the same sample for the subsequent measures. Moreover, in the latter cases, the solvent was sometimes maintained throughout the whole experience.[19,129,130,132] In other experiments the whole volume was substituted by fresh solvent after each test period,[27,32,41,43,102,128] or every 24 hours.[36,127,129,131]

On the other hand, the extraction conditions section in the ISO guideline recommended that an extraction period of 24 hours at 37ºC was only acceptable in case that the solvent chosen was tissue culture media for cytotoxicity testing. In the current investigation no extraction was performed with complete α-MEM. The quantification method was developed for inorganic artificial saliva, which composition is less complex than that of complete α-MEM. Further studies are needed to build a calibration curve for MMA in an organic matrix as complex as complete α-MEM, so as

to allow the quantification of leachable MMA. Culture medium with serum is appropriated for cell growth, as well as to extract both polar and non polar molecules.[83] However, this approach has not been explored very often. Bural *et al*. (2011a, 2011b) extracted the residual monomer for 24 hours at 37ºC using tissue culture medium but without serum, therefore not totally complying with the ISO 10993 Parts 5 and 12 recommendations.[32,80,83,102] According to the ISO recommendations, culture medium without serum is only specifically appropriated for polar substances such as ions.[83] They reported to add the serum just before cytotoxicity testing,[32,102] which probably caused a dilution of the leached residual monomer.

Though acceptable according to the ISO 10993-12:2007, another feature that contributes to the published methodologies' heterogeneity is the high variability of solvents used, particularly artificial saliva, whose composition diverged between studies from different authors [30,36,127,129] (see Annex H.3. Table1). Because of this notorious lack of agreement, a standardized formula was chosen for this research. On the contrary to Urban *et al.* (2012, 2009) [36,127] and in natural saliva, it must be noticed that this formula is totally inorganic and polar, which may have affected the capacity of this vehicle to penetrate the polymer matrix and dissolve the residual MMA.[132] Hence, it may be partially responsible for the low concentration of MMA in the samples, having in mind that in clinical conditions the amount of monomer leached may be higher.

Moreover, some studies use an ethanol solution (variable or unknown % v/v) as a solvent,[19,130,132] which used to be recommended by the U S Food and Drug Administration (FDA) as an oral environment simulator (at 75% v/v).[15,16,54,135] Yet it has been claimed by some authors that it promotes an excessive diffusion of monomers by expanding the polymer network.[17,30]

In order to quantify the released MMA monomer into artificial saliva, the chromatographic methods proposed by ISO 20795 were considered as the most indicated techniques to perform the exact quantification of the leachable residual monomer. In the published monomer leaching studies, though most authors agree in

using chromatography,[19,27,30,32,36,41,48,102,127-130,132] a minority preferred to quantify through UV spectrophotometry.[43-45,52,131] Despite this latter technique is valid for quantification in many situations, chromatography is more accurate and sensitive because there is a chemical separation and concentration of closely related but distinct monomer molecules, which may be present in the solution, before the quantification itself.[58] Furthermore, before MMA quantification, a microextraction pre-concentration technique was employed, which fairly improved the MDL and MQL. When compared to other studies,[30,128] the MDL and MQL of the present research is more favorable to detect minimal monomer concentrations.

E.2. MMA Cytotoxicity

Prosthodontic materials' clinical success is dependent not only on their physical/mechanical and chemical properties, but also on their biologic effects and safety. Since that one of the goals of this research was to evaluate the potential genotoxic effect of leachable MMA from a conventional prosthetic polymer, it was first necessary to assess the cytotoxic profile of MMA. As most *in vitro* models used to evaluate toxicity of dental base polymers rely on non-human immortalized cell lines from different (non-oral) tissues, in this study an untransformed HGF cell line was evaluated as a research oral cell model. For both purposes, control chemicals (EMS and formaldehyde) and control cell types (V79-4 and WI-38) were also tested.

EMS is an alkylatying agent (clastogen) [123] that has been very often used as a positive control in genotoxicity assays.[24,95-97,136,137] To our knowledge, this is the first report where EMS is tested in a commercial untransformed HGF cell line. In the present investigation there was a clear dose-dependent effect for all cell types tested. As the EMS doses doubled, the cells viability progressively diminished. On the other

hand, MMA and formaldehyde do not present such a predictable effect on cells. Although for lower MMA concentrations the cells viability decreased gradually, in the last doubling dose the viability sharply declined. Probably, when a certain degree of cell impairment is reached it causes irreversible and catastrophic damage leading to the death of most cells. In the case of formaldehyde, the results corroborate that it is a powerful cytotoxic agent at relatively low doses. It would have been interesting to evaluate the progression of cell viability for formaldehyde doses lower than 400 µM, though it is still predictable that the viability would diminish in an exponential fashion.

In terms of the influence of the cell types on the viability variation, no differences were found, i.e. for all chemicals the cells' viability response was the same. However, the data suggests that the human untransformed fibroblasts tend to be more resistant to MMA and formaldehyde than V79-4 cells, which may be nearer to a physiological response. It must be considered that V79-4 and WI-38 are widely used and recommended as *in vitro* test models for cytotoxicity [83] (and V79-4 for genotoxicity testing as well [107]). Therefore, if the HGF cell line presents a similar viability behavior, it may also be considered a reliable cell model. HGF has the advantages of being an untransformed human cell line, just like WI-38, and it came from an oral tissue, which, at least theoretically, should be closer to the oral physiological conditions than one of the most employed cell lines V79-4 (immortalized, non-human and non-oral).

As referred previously, most studies concerning the cytotoxicity of MMA employed either immortalized cell lines or primary cells and a wide variety of cytotoxicity tests have been applied. In the present study, the 50% cell viability was reached with a concentration between 80 and 160 mM. Lai *et al*. (2004) had similar results to those of the present study. For a 50% primary HGF and PDL viability (determined by the MTT assay), they had a MMA concentration of 1.2%,[14] corresponding approximately to 120 mM. The same authors also tested 1,6-HDMA and isobutyl methacrylate (IBMA) and stated that since most monomers are released in the first hour after polymerization, the direct application of uncured relining materials in the

oral cavity may seriously irritate the mucosa and cause cell death by either necrosis or apoptosis.[14]

Conversely, some authors found that lower concentrations of MMA were necessary to reach 50% cell viability.[24,35,68,79,101] It might be explained by possible differences in the type of cytotoxicity assays employed, different cell types and growth rates at the moment of the test that may influence the outcomes.

In a systematic review by Chaves *et al.* (2012), the cytotoxicty of denture base and hard chairside resins was evaluated. They found some evidence that heat-polymerizable resins (thermo and microwave) are less cytotoxic than autopolymerizable and light or dual-cured materials.[18] However, the conclusions were not definitive, since there are too many variables and heterogeneity in the studies analyzed, in terms of the cell types, cytotoxicity tests employed, brands and polymerization cycles of the resins evaluated.[18] Bural *et al.* (2011a, 2011b) studied the effects of different polymerization cycles and post polymerization treatments in the elution and cytotoxicity on L929 cells of autopolymerizable and heat-polymerizable resins, respectively.[32,102] In the autopolymerizable materials study, a higher degree of conversion and a lower leached MMA did not always mean that the cytotoxicity would be lower.[102] Conversely, a reduction in the leached MMA increased the L929 viability for heat-polymerizable resins.[32] In general it was considered that the materials were slightly cytotoxic.[32,102]

However, it seems that the cells viability is not only dependent on the quantity of residual monomer released. In a study by Rose *et al*. (2000) found that despite light-cured orthodontic resins released less UDMA than the autopolymerizable leached MMA, the MTT test on L929 cells showed a lower viability caused by UDMA.[27] Therefore, UDMA is more cytotoxic than MMA even at lower concentrations.[27] Atsumi *et al.* (2006) showed that when compared with other hydrophobic monomers like BA (*n*-butyl acrylate), BMA (*n*-butyl methacrylate), IBMA, HMA (*n*-hexyl methacrylate) and DMA (*n*-dodecyl methacrylate), MMA was the less cytotoxic.[35] These results were

comparable to those found for hydrophilic dental molecules in the same study, which are not as cytotoxic since that they have more difficulty to cross the lipid bilayer cell membrane to penetrate the cells.[35] Furthermore, Lai *et al.* (2004) explain that the monomers 1,6-HDMA are more lipophilic and more cytotoxic than IBMA, which in turn is more hydrophobic and toxic than MMA.[14] Schweikl *et al*. (2001) found that MMA and HEMA, which are monofunctional monomers, were less toxic than the composite resins' bifunctional monomers like TEGDMA, UDMA and bis-GMA.[24]

Similarly to Yang *et al*. (2003),[68] the results obtained by Jorge *et al*. (2004) show how different cytotoxity tests employed may have totally different outcomes.[84] They found that independent on the materials used and test groups, the resins had a cytotoxic effect when evaluated through the ^{3}H-thymidine assay, but were not cytotoxic when the MTT test was applied.[84]

To our knowledge, cytotoxic testing using untransformed HGF cell line is very limited and there are very few reports regarding dental polymers. Reichl *et al*. (2006) used a HGF untransformed cell line to evaluate the response to amalgam and to co-monomers from restorative dental composites.[138] A dose-dependent loss of cell viability was observed and the XTT assay also showed that HEMA was the less cytotoxic substance, followed by TEGDMA, UDMA, bis-GMA and the mercury molecules.[138] In another study, Reichl *et al.* (2010) found that the same HGF cell line expresses an enzyme from the cytochrome P450 superfamily and is capable of metabolizing methacrylic acid (MA), which is a toxic subproduct of the metabolization of methacrylic co-monomers.[139] It may be hypothesized that the HGF cell line tested in the present research also expresses an enzyme from the cytochrome P450 superfamily and may be capable of metabolizing methacrylic subproducts. This may explain why these cells seem to be more resistant to MMA aggressions than the immortalized cell lines previously referred.

Furthermore, the viability of the present HGF cell line had already been evaluated for nicotine [115] and for single-wall carbon nanotubes.[118] In both cases it was found that its response depended on the doses employed.

E.3. MMA Genotoxicity

The final purpose of this research was to evaluate whether MMA was a genotoxic molecule. This issue has been gaining relevance because viable cells with serious DNA lesions are likely to initiate a carcinogenesis process.[68] Besides, the HGF profile as an oral cell model for the MNvit test was evaluated because, according to Parry *et al.* (2010), it is important to use cells as similar as possible to the *in vivo* tissue that contacts more directly with the chemical that is being studied.[114] The cells should be as closer to the human *in vivo* cells as possible and should at least be capable of expressing the p53 gene or other genes essential for normal genotoxic responses.[114]

The MMA concentration (0.0037mM) close to the value determined by HPLC for the 72 hours group was chosen for the genotoxicity test. Moreover, based on the results obtained in the MTT assay, non-cytotoxic MMA, EMS and formaldehyde concentrations were compared in terms of the MN frequencies in 2000 cells. In the genotoxicity section, the MNvit assay was performed with the HGF cell line and with only one control, the immortalized V79-4 cell line, which was the most referenced and recommended by the international guidelines.[107]

Minimal criteria proposed by Parry *et al* (2010) were accomplished in this MNvit assay: duplicate cultures were performed; positive (EMS 600 μg/ml) and negative controls were used; good quality of the cells preparation, with visible cell membrane at the microscope; validated guidelines for MN scoring were followed [106] and an adequate cell number was counted.[114]

Interestingly, the amount of MN/2000 cells in the negative control of HGF was significantly higher than the baseline MN score in V79-4. In fact, OECD suggested that in negative control groups for the recommended cell types, such as V79-4, the count should be 5-25/1000 cells with MN,[107] which corresponds approximately to 0.5-2.5% MN score. The result of the V79-4 negative control group (0.99%) fits perfectly this interval, thus validating the procedures performed. In the HGF negative control group the MN score was quite higher (7.19%).

Since that at 600 µg/ml EMS did not register a significant difference in the cells' viability from the negative controls, it was selected as the positive control drug for the MNvit assay. The present results confirm that EMS works well as a positive control in both cell types, given that the MN count increased significantly when compared with the negative control. However, in relation to the HGF cells, the increasing in the total score of MN was not as marked as in V79-4 cells.

In the V79-4 group no differences were found for none of the MMA concentrations and formaldehyde in relation to the control group. Therefore it may be assumed that these chemicals at the tested concentrations are not genotoxic for these cells. Despite that in the lowest MMA concentration tested in the HGF group the MN scores are bellow the scores in the negative control, this difference is not meaningful.

EMS has been previously classified as a clastogen agent by the FISH method.[123] However, the present study employed the size-classification method of MN proposed by Hashimoto *et al*. (2010).[122] For the first time, EMS was confirmed to be a clastogen with the latter method. Thus, the present report corroborates the usefulness of the size-classification method.

To our knowledge, only a research group has been testing this particular HGF cell line for MNvit testing, though the substances tested are not related to dental resin acrylics.[115,116,118] In general, it was noticed that the percents of MN in the present study (5.16 to 9.72%) are slightly higher than in all nicotine groups (>1.5 to <6%) tested by Argentin *et al.* (2004, 2006).[115,116] In the case of the study with single-wall carbon

nanotubes all the MN scores (>0.05 to <3.5%) were equally lower than in the present study.[118] One of the factors that might explain these differences is that the materials tested are different; even between the referred studies, which followed the same MNvit protocol, the MN scores varied in slightly different ranges. Another relevant fact is that the protocol followed by Argentin and Chichetti *et al.* was different from the present procedures since they followed the CBMN protocol and counted only 1000 binucleated cells.[115,116,118] Though both procedures are considered valid by the OECD 487 guideline in terms of the relative MN scores (negative controls *vs*. test group), there may be differences in the absolute number of MN counted between assays with and without cytokinesis blocker. More importantly, it has been reported that the Giemsa stain employed by the former authors is not the most reliable, as it may not detect all MN present in the cells.[121] Conversely, in the present research the DNA was specifically stained with PI and was observed at the confocal fluorescence microscope. This observation technique is more specific and sensitive.[121] thus probably more MN were distinguished and scored. Finally, despite the MN morphologic features are well described and scoring guidelines are well established, there might be slight differences in the scoring criteria too.

Since the 90's that an increasing concern on the genotoxicity of dental medical products has lead to the publication of several studies. Root canal sealers, dentin bondings, composite resins and their components have been target materials. Denture and orthodontic base polymers have not been as studied. Yang *et al.* (2003) tested the genetoxic profile of MMA on CHO cells through the CA and SCE assays, which detected structural defects in chromosomes, just like MN.[68] On the contrary to the results of the present study, they registered a dose-dependent increase in the number of CAs (chromatid-type aberrations – gaps and breaks) and SCEs (intra and interstrand cross-links), suggesting MMA as a potential clastogen.[68] Schweikl *et al.* (2001) cited that an approximate 100% correlation had been established between the CA assay and the MNvit test.[24] However, the CA assay is not considered as predictive of *in vivo*

genotoxic or carcinogenic activity as the MNvit assay,[114] so the clinical relevance of these results might be minimal.

Composite resin monomers have also been studied for their genotoxicty. Schweikl *et al.* (2001) reported a dose-related increase in the numbers of MN for unpolymerized TEGDMA, HEMA and glycidyl methacrylate (GMA) under physiological conditions; also high concentrations of MMA and bisphenol A induced elevated numbers of MN associated with cytotoxicity, and a very low mutagenic activity for Bis-GMA and UDMA.[24]

Dorn *et al.* (2008) extended the investigations on the non-specific mechanisms that explain the dental monomers' genotoxic profile. The monomers' lipophilicity has a great impact on disturbing the hydrophilic processes that occur during the cell cycle. It interferes with karyokinesis by disturbing DNA synthesis, which may be sensitive for hydrophobic interactions; it may induce lysosomal breakdown and subsequent DNase release from lysosomes, thus causing DNA double strand breaks and chromosomal aberrations, leading to clastogenicity. Moreover it perturbs cytokinesis, leading to rearrangement processes of actin and astral and interzonal microtubules and consequent aneugenic defects.[140]

In addition, authors have been deeply studying the possibility that apoptosis and mutagenicity induced by resin monomers could be mediated by oxidative stress. A 2006 study on V79-4 fibroblasts and RPC-C2A pulp cells revealed that GMA, TEGDMA and HEMA induced cytotoxicity, apoptosis and genotoxicity was dose-dependent, but both were significantly decreased by co-treatment with *N*-acetylcystein (NAC), an antioxidant.[141] Further studies, where the MNvit test was also applied, found similar results for TEGDMA, HEMA [142] and bonding agents of dental adhesives in V79 cells,[137] as well as for camphorquinone in CHO cells in co-treatment with the reducing agent *N,N*-dimethyl-*p*-toluidine.[109] Recent investigations with resinous canal sealers concluded that the formation of MN was induced by the generation of ROS,[95] whereas pulp capping materials like castor oil bean cement and mineral trioxide aggregate

(MTA) were considered safe.[96] In another research in this field in 2010, TEGDMA was confirmed to arrest cell cycle by the production of ROS, by comparison with adriamycin and mitomycin C, two chemotherapeutic agents, which arrest the cell cycle through different mechanisms.[143] Therefore, when the production of ROS exceeds the cell redox balance capacity, strand DNA breaks may happen, activating the genetic repair mechanisms, programmed cell death or, at worst, the occurrence of mutagenesis.[95,137,142,144]

E.4. Perspectives For Future Research

Biocompatibility and biological risk assessment of medical devices is a never ending science issue, since that more than ever it is an absolute requirement when providing the best human health care. The same principle applies in prosthodontics.

In terms of the present study, it would be important to further develop and validate the analytical procedures of quantification of residual monomer with the microextraction technique and HPLC, namely in more complex solvents such as complete α-MEM or even natural saliva. Moreover, it would be interesting to investigate the amount of other potentially toxic leachable molecules. Once these procedures are validated and systematized, it will be easier to apply them to a wider variety of commercially available polymeric materials or before being launched into the market.

Moreover, the present study design included tests on extracts, but ISO 10993-5:2009 also recommends other types of methodological approaches such as direct and indirect contact between the test material and the cells.[83] These experiments could provide further information about the interactions between the cells, the polymer piece and its leachable molecules. Besides, the results already obtained should be consolidated and expanded, in order to obtain a thoroughly characterization of the suitability of HGF as an oral cell model for *in vitro* genotoxicity testing.

Furthermore, when compared to *in vitro* experiments, *in vivo* studies in these fields are virtually inexistent.[9] Therefore, the next step in terms of investigation should include clinical evaluations on the human exposure and the real biological risks of leachable molecules from dental devices. More specifically, studies are already being developed to evaluate the levels of MN in exfoliated cells caused by oral conditions such as periodontitis.[94] It could be very useful to objectively measure the levels of leached molecules from polymer based devices and the scores of MN.

More studies are also needed to keep on developing the promising MNvit method. When it is fully characterized and validated, automatic systems could be developed for a simpler, faster and even more reliable and reproducible scoring of MN. Moreover, further studies should explore and validate the size-classification method of MN, as well as other practical techniques for distinguishing clastogenic and aneugenic MN. Since it is considered a “cytome” assay of chromosomal instability, mitotic dysfunction and cell death,[145] in the near future MNvit will probably become very helpful in the assessment of cancer risk.

F. CONCLUSIONS

The main purpose of the present study was to evaluate *in vitro* the potential genotoxic effects of acrylic resins on human gingival fibroblasts. It may be stated that all objectives proposed for this research were accomplished. The main conclusions that can be drawn are:

1. The newly developed microextraction technique in association with an optimized HPLC methodology reduced the MQL and MDL of MMA in artificial saliva;
2. MMA residual monomer in artificial saliva is only detectable after 72 hours of extraction and the concentration is around the MQL;
3. MMA decreases the viability of the HGF , V79-4 and WI-38 cell types for concentrations between 40 and 160 mM;
4. MMA did not induce genotoxic damage on the HGF or V79-4 cell lines for the concentrations assessed;
5. A properly fabricated cross-linking heat-polymerized appliance does not represent a high toxic risk in terms of the leached MMA;
6. The HGF cell line proved to be a suitable and useful tool as a model for *in vitro* cytotoxicity testing;
7. Further investigations need to be planned using HGF cells for the MNvit assay.

G. REFERENCES

1. The Glossary of Prosthodontic Terms. 8th ed: The Academy of Prosthodontics; 2005.

2. Felton DA. Edentulism and comorbid factors. J Prosthodont. 2009;18(2):88-96.

3. Turker SB, Sener ID, Kocak A, Yilmaz S, Ozkan YK. Factors triggering the oral mucosal lesions by complete dentures. Arch Gerontol Geriatr. 2010 Jul-Aug;51(1):100-104.

4. Carr AB, McGivney GP, Brown DT. McCracken's removable partial prosthodontics. 11th ed. St. Louis: Mosby; 2004.

5. International Organization for Standardization. ISO 20795-1:2008(E). Dentistry — Base polymers — Part 1: Denture base polymers. Switzerland. 2008

6. International Organization for Standardization. ISO 10993-1:2009(E). Biological evaluation of medical devices — Part 1: Evaluation and testing within a risk management process. Switzerland. 2009

7. Darvell BW, Clark RK. The physical mechanisms of complete denture retention. Br Dent J. 2000 Sep 9;189(5):248-252.

8. Bettencourt AF, Neves CB, de Almeida MS, Pinheiro LM, Oliveira SAe, Lopes LP, et al. Biodegradation of acrylic based resins: a review. Dent Mater. 2010;26(5):e171-180.

9. Gautam R, Singh RD, Sharma VP, Siddhartha R, Chand P, Kumar R. Biocompatibility of polymethylmethacrylate resins used in dentistry. J Biomed Mater Res B Appl Biomater. 2012;100B(5):1444-1450.

10. Leggat PA, Smith DR, Kedjarune U. Surgical applications of methyl methacrylate: a review of toxicity. Arch Environ Occup Health. 2009;64(3):207-212.

11. International Organization for Standardization. ISO 10993-13:2010(E). Biological evaluation of medical devices — Part 13: Identification and quantification of degradation products from polymeric medical devices. Switzerland. 2010

12. Anusavice KJ, Phillips RW. Phillips' science of dental materials. 11th ed. St. Louis: W.B. Saunders; 2003.

13. Ferracane JL. Hygroscopic and hydrolytic effects in dental polymer networks. Dent Mater. 2006 Mar;22(3):211-222.

14. Lai YL, Chen YT, Lee SY, Shieh TM, Hung SL. Cytotoxic effects of dental resin liquids on primary gingival fibroblasts and periodontal ligament cells in vitro. J Oral Rehabil. 2004;31(12):1165-1172.

15. Lee SY, Huang HM, Lin CY, Shih YH. Leached components from dental composites in oral simulating fluids and the resultant composite strengths. J Oral Rehabil. 1998 Aug;25(8):575-588.

16. Polydorou O, Trittler R, Hellwig E, Kummerer K. Elution of monomers from two conventional dental composite materials. Dent Mater. 2007 Dec;23(12):1535-1541.

17. Tabatabaee MH, Mahdavi H, Zandi S, Kharrazi M. HPLC analysis of eluted monomers from two composite resins cured with LED and halogen curing lights. J Biomed Mater Res B Appl Biomater. 2009;88(1):191-196.

18. de Andrade Lima Chaves C, Machado AL, Vergani CE, de Souza RF, Giampaolo ET. Cytotoxicity of denture base and hard chairside reline materials: A systematic review. J Prosthet Dent. 2012;107(2):114-127.

19. Kawahara T, Nomura Y, Tanaka N, Teshima W, Okazaki M, Shintani H. Leachability of plasticizer and residual monomer from commercial temporary restorative resins. J Dent. 2004;32(4):277-283.

20. Urban VM, Machado AL, Oliveira RV, Vergani CE, Pavarina AC, Cass QB. Residual monomer of reline acrylic resins:: Effect of water-bath and microwave post-polymerization treatments. Dent Mater. 2007;23(3):363-368.

21. Puska MA, Lassila LV, Aho AJ, Yli-Urpo A, Vallittu PK, Kangasniemi I. Exothermal characteristics and release of residual monomers from fiber-reinforced oligomer-modified acrylic bone cement. J Biomater Appl. 2005 Jul;20(1):51-64.

22. Keh ES, Hayakawa I, Takahashi H, Watanabe A, Iwasaki Y, Akiyoshi K, et al. Improving a self-curing dental resin by eliminating oxygen, hydroquinone and water from its curing process. Dent Mater J. 2002;21(4):373.

23. International Organization for Standardization. ISO 20795-2:2010(E). Dentistry — Base polymers — Part 2: Orthodontic base polymers. Switzerland. 2010

24. Schweikl H, Schmalz G, Spruss T. The induction of micronuclei in vitro by unpolymerized resin monomers. J Dent Res. 2001;80(7):1615-1620.

25. Jorge JH, Giampaolo ET, Machado AL, Vergani CE. Cytotoxicity of denture base acrylic resins: a literature review. J Prosthet Dent. 2003;90(2):190-193.

26. Bayraktar G, Duran O, Guvener B. Effect of glass fibre reinforcement on residual methyl methacrylate content of denture base polymers. J Dent. 2003;31(4):297-302.

27. Rose EC, Bumann J, Jonas IE, Kappert HF. Contribution to the Biological Assessment of Orthodontic Acrylic Materials. J Orofac Orthop. 2000;61(4):246-257.

28. Köroğlu A, Özdemir T, Duru Pamir A, Usanmaz A. Residual acrylic monomer content of denture base resins with different fiber systems. J Appl Polym Sci. 2012;125(1):471-476.

29. Spasojević P, Adnađević B, Veličković S, Jovanović J. Influence of microwave heating on the polymerization kinetics and application properties of the PMMA dental materials. J Appl Polym Sci. 2011;119(6):3598-3606.

30. Danesh G, Hellak T, Reinhardt KJ, Vegh A, Schafer E, Lippold C. Elution characteristics of residual monomers in different light- and auto-curing resins. Exp Toxicol Pathol. 2011 Apr 27.

31. Bayraktar G, Guvener B, Bural C, Uresin Y. Influence of polymerization method, curing process, and length of time of storage in water on the residual methyl methacrylate content in dental acrylic resins. J Biomed Mater Res B Appl Biomater. 2006 Feb;76(2):340-345.

32. Bural C, Akta, E, Deniz G, Ünlüçerçi Yi, Bayraktar G. Effect of leaching residual methyl methacrylate concentrations on in vitro cytotoxicity of heat polymerized denture base acrylic resin processed with different polymerization cycles. J Appl Oral Sci. 2011;19:306-312.

33. Hochman N, Zalkind M. Hypersensitivity to methyl methacrylate: mode of treatment. J Prosthet Dent. 1997;77(1):93-96.

34. International Organization for Standardization. ISO 10993-17:2002(E). Biological evaluation of medical devices — Part 17: Establishment of allowable limits for leachable substances. Switzerland. 2002

35. Atsumi T, Fujisawa S, Tonosaki K. (Meth) acrylate monomer-induced cytotoxicity and intracellular Ca2+ mobilization in human salivary gland carcinoma cells

and human gingival fibroblast cells related to monomer hydrophobicity. Biomaterials. 2006;27(34):5794-5800.

36. Urban VM, Machado AL, Vergani CE, Giampaolo ET, Pavarina AC, de Almeida FG, et al. Effect of water-bath post-polymerization on the mechanical properties, degree of conversion, and leaching of residual compounds of hard chairside reline resins. Dent Mater. 2009;25(5):662-671.

37. Boeckler AF, Morton D, Poser S, Dette KE. Release of dibenzoyl peroxide from polymethyl methacrylate denture base resins: an in vitro evaluation. Dent Mater. 2008 Dec;24(12):1602-1607.

38. Jorge JH, Giampaolo ET, Vergani CE, Machado AL, Pavarina AC, Carlos IZ. Effect of post-polymerization heat treatments on the cytotoxicity of two denture base acrylic resins. J Appl Oral Sci. 2006;14(3):203-207.

39. Hashimoto Y, Kawaguchi M, Miyazaki K, Nakamura M. Estrogenic activity of tissue conditioners in vitro. Dent Mater. 2003 Jun;19(4):341-346.

40. Shintani H. Pretreatment and chromatographic analysis of phthalate esters, and their biochemical behavior in blood products. Chromatographia. 2000;52(11):721-726.

41. Kopperud HM, Kleven IS, Wellendorf H. Identification and quantification of leachable substances from polymer-based orthodontic base-plate materials. Eur J Orthod. 2011;33(1):26-31.

42. Mikai M, Koike M, Fujii H. Quantitative analysis of allergenic ingredients in eluate extracted from used denture base resin. J Oral Rehabil. 2006;33(3):216-220.

43. Azzarri M, Cortizo M, Alessandrini J. Effect of the curing conditions on the properties of an acrylic denture base resin microwave-polymerised. J Dent. 2003;31(7):463-468.

44. Braun KO, N. Mello JA, N. Rached R, Del Bel Cury AA. Surface texture and some properties of acrylic resins submitted to chemical polishing. J Oral Rehabil. 2003;30(1):91-98.

45. Del Bel Cury AA, Rached RN, Ganzarolli SM. Microwave-cured acrylic resins and silicone–gypsum moulding technique. J Oral Rehabil. 2001;28(5):433-438.

46. Hiromori K, Fujii K, Inoue K. Viscoelastic properties of denture base resins obtained by underwater test. J Oral Rehabil. 2000;27(6):522-531.

47. Lassila LVJ, Vallittu PK. Denture base polymer Alldent Sinomer®: mechanical properties, water sorption and release of residual compounds. J Oral Rehabil. 2001;28(7):607-613.

48. Lee S-y, Lai Y-l, Hsu T-s. Influence of polymerization conditions on monomer elution and microhardness of autopolymerized polymethyl methacrylate resin. Eur J Oral Sci. 2002;110(2):179-183.

49. Pfeiffer P, Rosenbauer EU. Residual methyl methacrylate monomer, water sorption, and water solubility of hypoallergenic denture base materials. J Prosthet Dent. 2004;92(1):72-78.

50. Spasojevic P, Stamenkovic D, Pjanovic R, Boskovic-Vragolovic N, Dolic J, Grujic S, et al. Diffusion and solubility of commercial poly(methyl methacrylate) denture base material modified with dimethyl itaconate and di-n-butyl itaconate during water absorption/desorption cycles. Polym Int. 2012 Aug;61(8):1272-1278.

51. Usanmaz A, Ateş J, Doğan A. Thermal and mechanical properties of microwave- and heat-cured poly (methyl methacrylate) used as dental base material. J Appl Polym Sci. 2003;90(1):251-256.

52. Nunes de Mello JA, Olmedo Braun K, Nunes Rached R, Del Bel Cury AA. Reducing the negative effects of chemical polishing in acrylic resins by use of an additional cycle of polymerization. J Prosthet Dent. 2003;89(6):598-602.

53. Urban VM, Cass QB, Oliveira RV, Giampaolo ET, Machado AL. Development and application of methods for determination of residual monomer in dental acrylic resins using high performance liquid chromatography. Biomed Chromatogr. 2006 Apr;20(4):369-376.

54. Bakopoulou A, Papadopoulos T, Garefis P. Molecular Toxicology of Substances Released from Resin–Based Dental Restorative Materials. Int J Mol Sci. 2009;10(9):3861-3899.

55. Bartoloni J, Murchison D, Wofford D, Sarkar N. Degree of conversion in denture base materials for varied polymerization techniques. J Oral Rehabil. 2000;27(6):488-493.

56. Viljanen EK, Skrifvars M, Vallittu PK. Dendritic copolymers and particulate filler composites for dental applications: degree of conversion and thermal properties. Dent Mater. 2007;23(11):1420-1427.

57. Harris DC. Quantitative chemical analysis. 7th ed. New York: W.H. Freeman & Co.; 2007.

58. Skoog DA, Holler FJ, Crouch SR. Principles of instrumental analysis. 6th ed. Belmont: Cengage Learning; 2007.

59. Miller JN, Miller JC. Statistics and chemometrics for analytical chemistry. 5th ed. Essex: Pearson Education Limited; 2005.

60. Carlsson GE. Clinical morbidity and sequelae of treatment with complete dentures. J Prosthet Dent. 1998;79(1):17-23.

61. Emami E, de Grandmont P, Rompré PH, Barbeau J, Pan S, Feine JS. Favoring Trauma as an Etiological Factor in Denture Stomatitis. J Dent Res. 2008 May 1, 2008;87(5):440-444.

62. Gendreau L, Loewy ZG. Epidemiology and etiology of denture stomatitis. J Prosthodont. 2011 Jun;20(4):251-260.

63. Koutis D, Freeman S. Allergic contact stomatitis caused by acrylic monomer in a denture. Australas J Dermatol. 2001;42(3):203-206.

64. Durner J, Spahl W, Zaspel J, Schweikl H, Hickel R, Reichl FX. Eluted substances from unpolymerized and polymerized dental restorative materials and their Nernst partition coefficient. Dent Mater. 2010;26(1):91-99.

65. Freitas JB, Gomez RS, De Abreu MH, Ferreira EFE. Relationship between the use of full dentures and mucosal alterations among elderly Brazilians. J Oral Rehabil. 2008 May;35(5):370-374.

66. Lung C, Darvell B. Minimization of the inevitable residual monomer in denture base acrylic. Dent Mater. 2005;21(12):1119-1128.

67. Lung C, Darvell B. Methyl methacrylate monomer-polymer equilibrium in solid polymer. Dent Mater. 2007;23(1):88-94.

68. Yang HW, Chou LSS, Chou MY, Chang YC. Assessment of genetic damage by methyl methacrylate employing in vitro mammalian test system. Biomaterials. 2003;24(17):2909-2914.

69. Biocina-Lukenda D, Gregurek-Novak T, Cekic-Arambasin A. Denture stomatitis associated with allergic reaction to teeth prostheses. J Eur Acad Dermatol Venereol. 2004;18(2):227-229.

70. Gawkrodger DJ. Investigation of reactions to dental materials. Br J Dermatol. 2005;153(3):479-485.

71. Gonçalves TS, Morganti MA, Campos LC, Rizzatto SMD, Menezes LM. Allergy to auto-polymerized acrylic resin in an orthodontic patient. Am J Orthod Dentofacial Orthop. 2006;129(3):431-435.

72. Leber AP. Human exposures to monomers resulting from consumer contact with polymers. Chem Biol Interact. 2001 Jun 1;135-136:215-220.

73. Piirilä P, Hodgson U, Estlander T, Keskinen H, Saalo A, Voutilainen R, et al. Occupational respiratory hypersensitivity in dental personnel. Int Arch Occup Environ Health. 2002;75(4):209-216.

74. Piemonte ED, Lazos JP, Brunotto M. Relationship between chronic trauma of the oral mucosa, oral potentially malignant disorders and oral cancer. J Oral Pathol Med. 2010 Aug 1;39(7):513-517.

75. Rosenquist K, Wennerberg J, Schildt EB, Bladstrom A, Goran Hansson B, Andersson G. Oral status, oral infections and some lifestyle factors as risk factors for oral and oropharyngeal squamous cell carcinoma. A population-based case-control study in southern Sweden. Acta Otolaryngol. 2005 Dec;125(12):1327-1336.

76. Vaccarezza GF, Antunes JL, Michaluart-Junior P. Recurrent sores by ill-fitting dentures and intra-oral squamous cell carcinoma in smokers. J Public Health Dent. 2010 Winter;70(1):52-57.

77. Tomenson JA, Bonner SM, Edwards JC, Pemberton MA, Cummings TF, Paddle GM. Study of two cohorts of workers exposed to methyl methacrylate in acrylic sheet production. Occup Environ Med. 2000 Dec;57(12):810-817.

78. Tomenson JA, Carpenter AV, Pemberton MA. Critical review of the epidemiology literature on the potential cancer risks of methyl methacrylate. Int Arch Occup Environ Health. 2005 Sep;78(8):603-612.

79. Fujisawa S, Atsumi T, Kadoma Y. Cytotoxicity of methyl methacrylate (MMA) and related compounds and their interaction with dipalmitoylphosphatidylcholine (DPPC) liposomes as a model for biomembranes. Oral Dis. 2000;6(4):215-221.

80. International Organization for Standardization. ISO 10993-12:2007(E). Biological evaluation of medical devices — Part 12: Sample preparation and reference materials. Switzerland. 2007

81. Jorge JH, Giampaolo ET, Vergani CE, Machado AL, Pavarina AC, Carlos IZ. Biocompatibility of denture base acrylic resins evaluated in culture of L929 cells. Effect of polymerisation cycle and post polymerisation treatments. Gerodontology. 2007;24(1):52-57.

82. Karp G. Cell and molecular biology: concepts and experiments. 5th ed. Toronto: John Wiley & Sons, Inc.; 2007.

83. International Organization for Standardization. ISO 10993-5:2009(E). Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity. Switzerland. 2009

84. Jorge JH, Giampaolo ET, Vergani CE, Machado AL, Pavarina AC, Carlos IZ. Cytotoxicity of denture base resins: effect of water bath and microwave postpolymerization heat treatments. Int J Prosthodont. 2004 May-Jun;17(3):340-344.

85. International Organization for Standardization. ISO 10993-3:2003(E). Biological evaluation of medical devices — Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity. Switzerland. 2003

86. Schweikl H, Schmalz G, Bey B. Mutagenicity of dentin bonding agents. J Biomed Mater Res. 1994;28(9):1061-1067.

87. Schweikl H, Schmalz G, Federlin M. Mutagenicity of the root canal sealer AHPlus in the Ames test. Clin Oral Investig. 1998 Sep;2(3):125-129.

88. Schweikl H, Schmalz G, Gottke C. Mutagenic activity of various dentine bonding agents. Biomaterials. 1996;17(14):1451-1456.

89. Heil J, Reifferscheid G, Waldmann P, Leyhausen G, Geurtsen W. Genotoxicity of dental materials. Mutat Res. 1996;368(3-4):181-194.

90. Kleinsasser NH, Schmid K, Sassen AW, Harreus UA, Staudenmaier R, Folwaczny M, et al. Cytotoxic and genotoxic effects of resin monomers in human

salivary gland tissue and lymphocytes as assessed by the single cell microgel electrophoresis (Comet) assay. Biomaterials. 2006;27(9):1762-1770.

91. Kleinsasser NH, Wallner BC, Harréus UA, Kleinjung T, Folwaczny M, Hickel R, et al. Genotoxicity and cytotoxicity of dental materials in human lymphocytes as assessed by the single cell microgel electrophoresis (comet) assay. J Dent. 2004;32(3):229-234.

92. Schweikl H, Schmalz G. Glutaraldehyde containing dentin bonding agents are mutagens in mammalian cells in vitro. J Biomed Mater Res. 1997;36(3):284-288.

93. Schweikl H, Schmalz G, Rackebrandt K. The mutagenic activity of unpolymerized resin monomers in Salmonella typhimurium and V79 cells. Mutat Res. 1998;415(1-2):119-130.

94. Bastos-Aires D, Azevedo Á, Pereira ML, Perez-Mongiovi D, Teixeira A. Estudo preliminar dos tipos celulares da mucosa oral em pacientes com doença periodontal. Rev Port Estomatol Med Dent Cir Maxilofac. 2011;53(2):99-102.

95. Camargo CHR, Camargo SEA, Valera MC, Hiller KA, Schmalz G, Schweikl H. The induction of cytotoxicity, oxidative stress, and genotoxicity by root canal sealers in mammalian cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;108(6):952-960.

96. Camargo S, Camargo C, Hiller KA, Rode S, Schweikl H, Schmalz G. Cytotoxicity and genotoxicity of pulp capping materials in two cell lines. Int Endod J. 2009;42(3):227-237.

97. Schweikl H, Schmalz G. The induction of micronuclei in V79 cells by the root canal filling material AH Plus. Biomaterials. 2000;21(9):939-944.

98. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cell. 6th ed. New York: Garland Science, Taylor and Francis Group; 2007.

99. Cooper GM, Hausman RE. The cell: a molecular approach. 3rd ed. Boston: ASM Press; 2004.

100. Eagle H. Nutrition needs of mammalian cells in tissue culture. Science. 1955 Sep 16;122(3168):501-514.

101. Thonemann B, Schmalz G, Hiller KA, Schweikl H. Responses of L929 mouse fibroblasts, primary and immortalized bovine dental papilla-derived cell lines to dental resin components. Dent Mater. 2002;18(4):318-323.

102. Bural C, Aktas E, Deniz G, Unlucerci Y, Kizilcan N, Bayraktar G. Effect of post-polymerization heat-treatments on degree of conversion, leaching residual MMA and in vitro cytotoxicity of autopolymerizing acrylic repair resin. Dent Mater. 2011 Nov;27(11):1135-1143.

103. Azevedo C, Sunkel CE. Biologia celular e molecular. 5th ed. Porto: Lidel; 2012.

104. Balmain A. Cancer genetics: from Boveri and Mendel to microarrays. Nat Rev Cancer. 2001 Oct;1(1):77-82.

105. Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, et al. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis. 2011 Jan;26(1):125-132.

106. Fenech M. The in vitro micronucleus technique. Mutat Res. 2000 Nov 20;455(1-2):81-95.

107. Organisation for Economic Co-operation and Development. OECD 487. OECD guideline or the testing of chemicals — In vitro mammalian cell micronucleus test. 2010

108. Fenech M, Holland N, Zeiger E, Chang WP, Burgaz S, Thomas P, et al. The HUMN and HUMNxL international collaboration projects on human micronucleus assays in lymphocytes and buccal cells--past, present and future. Mutagenesis. 2011 Jan;26(1):239-245.

109. Li YC, Huang FM, Lee SS, Lin RH, Chang YC. Protective effects of antioxidants on micronuclei induced by camphorquinone/N, N dimethyl p toluidine employing in vitro mammalian test system. J Biomed Mater Res B Appl Biomater. 2007;82(1):23-28.

110. Norppa H, Falck GC. What do human micronuclei contain? Mutagenesis. 2003 May;18(3):221-233.

111. El-Zein R, Vral A, Etzel CJ. Cytokinesis-blocked micronucleus assay and cancer risk assessment. Mutagenesis. 2011 Jan;26(1):101-106.

112. Iarmarcovai G, Bonassi S, Botta A, Baan RA, Orsiere T. Genetic polymorphisms and micronucleus formation: a review of the literature. Mutat Res. 2008 Mar-Apr;658(3):215-233.

113. Terradas M, Martin M, Tusell L, Genesca A. Genetic activities in micronuclei: is the DNA entrapped in micronuclei lost for the cell? Mutat Res. 2010 Jul-Sep;705(1):60-67.

114. Parry JM, Kirsch-Volders M. Special issue on in vitro MN trial. Mutat Res. 2010 Oct 29;702(2):132-134.

115. Argentin G, Cicchetti R. Genotoxic and antiapoptotic effect of nicotine on human gingival fibroblasts. Toxicol Sci. 2004;79(1):75-81.

116. Argentin G, Cicchetti R. Evidence for the role of nitric oxide in antiapoptotic and genotoxic effect of nicotine on human gingival fibroblasts. Apoptosis. 2006;11(11):1887-1897.

117. Bloching M, Reich W, Schubert J, Grummt T, Sandner A. Micronucleus rate of buccal mucosal epithelial cells in relation to oral hygiene and dental factors. Oral Oncol. 2008 Mar;44(3):220-226.

118. Cicchetti R, Divizia M, Valentini F, Argentin G. Effects of single-wall carbon nanotubes in human cells of the oral cavity: geno-cytotoxic risk. Toxicol In Vitro. 2011;25(8):1811-1819.

119. Sobol Z, Homiski ML, Dickinson DA, Spellman RA, Li D, Scott A, et al. Development and validation of an in vitro micronucleus assay platform in TK6 cells. Mutat Res. 2012 Jul 4;746(1):29-34.

120. Thomas P, Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, et al. Buccal micronucleus cytome assay. Nat Protoc. 2009;4(6):825-837.

121. Heddle JA, Fenech M, Hayashi M, MacGregor JT. Reflections on the development of micronucleus assays. Mutagenesis. 2011 Jan;26(1):3-10.

122. Hashimoto K, Nakajima Y, Matsumura S, Chatani F. An in vitro micronucleus assay with size-classified micronucleus counting to discriminate aneugens from clastogens. Toxicol In Vitro. 2010 Feb;24(1):208-216.

123. Elhajouji A, Lukamowicz M, Cammerer Z, Kirsch-Volders M. Potential thresholds for genotoxic effects by micronucleus scoring. Mutagenesis. 2011 Jan;26(1):199-204.

124. DIN Deutsches Institut für Normung e. V. DIN 53160-1 (2002-10-00). Determination of the colour fastness of articles for common use — Part 1: Resistance to artificial saliva. Germany. 2002

125. Costa-Rodrigues J, Fernandes MH. Paracrine-mediated differentiation and activation of human haematopoietic osteoclast precursor cells by skin and gingival fibroblasts. Cell Prolif. 2011 Jun;44(3):264-273.

126. Vallittu PK, Ruyter IE, Buykuilmaz S. Effect of polymerization temperature and time on the residual monomer content of denture base polymers. Eur J Oral Sci. 1998 Feb;106(1):588-593.

127. Urban VM, Machado AL, Vergani CE, Giampaolo ET, Pavarina AC, Cass QB. Leachability of degradation products from hard chairside reline resins in artificial saliva: Effect of water-bath post-polymerization treatment. J Appl Polym Sci. 2012;123(2):732-739.

128. Çelebi N, Yüzügüllü B, Canay Ş, Yücel Ü. Effect of polymerization methods on the residual monomer level of acrylic resin denture base polymers. Polym Adv Technol. 2008;19(3):201-206.

129. Alawi M, Amin W. Effect of ageing on monomer elution from poly(methyl methacrylate) resin under simulated intra-oral conditions. Fresenius Environ Bull. 2007;16(4):408-414.

130. Buyukerkmen B, Ozturk AN. Effect of polymerisation techniques on residual monomer amount released from different acrylic resins. Mater Res Innov. 2010;14(5):381-384.

131. Oliveira VM, Leon BL, Del Bel Cury AA, Consani S. Influence of number and position of flasks in the monomer release, Knoop hardness and porosity of a microwave-cured acrylic resin. J Oral Rehabil. 2003 Nov;30(11):1104-1108.

132. Sofou A, Tsoupi I, Emmanouil J, Karayannis M. HPLC determination of residual monomers released from heat-cured acrylic resins. Anal Bioanal Chem. 2005;381(7):1336-1346.

133. International Organization for Standardization. ISO 10993-5:1999(E). Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity. Switzerland. 1999

134. Huang FM, Tai KW, Hu CC, Chang YC. Cytotoxic effects of denture base materials on a permanent human oral epithelial cell line and on primary human oral fibroblasts in vitro. Int J Prosthodont. 2001 Sep-Oct;14(5):439-443.

135. Sideridou ID, Achilias DS. Elution study of unreacted Bis GMA, TEGDMA, UDMA, and Bis EMA from light cured dental resins and resin composites using HPLC. J Biomed Mater Res B Appl Biomater. 2005;74(1):617-626.

136. Bin CV, Valera MC, Camargo SE, Rabelo SB, Silva GO, Balducci I, et al. Cytotoxicity and genotoxicity of root canal sealers based on mineral trioxide aggregate. J Endod. 2012 Apr;38(4):495-500.

137. Demirci M, Hiller KA, Bosl C, Galler K, Schmalz G, Schweikl H. The induction of oxidative stress, cytotoxicity, and genotoxicity by dental adhesives. Dent Mater. 2008;24(3):362-371.

138. Reichl FX, Esters M, Simon S, Seiss M, Kehe K, Kleinsasser N, et al. Cell death effects of resin-based dental material compounds and mercurials in human gingival fibroblasts. Arch Toxicol. 2006;80(6):370-377.

139. Reichl FX, Seiss M, Buters J, Behrendt H, Hickel R, Durner J. Expression of CYP450-2E1 and formation of 2, 3-epoxymethacrylic acid (2, 3-EMA) in human oral cells exposed to dental materials. Dent Mater. 2010;26(12):1151-1156.

140. Dorn SB, Degen GH, Bolt HM, van der Louw J, van Acker FAA, den Dobbelsteen DJ, et al. Some molecular descriptors for non-specific chromosomal genotoxicity based on hydrophobic interactions. Arch Toxicol. 2008;82(5):333-338.

141. Lee DH, Lim BS, Lee YK, Ahn SJ, Yang HC. Involvement of oxidative stress in mutagenicity and apoptosis caused by dental resin monomers in cell cultures. Dent Mater. 2006;22(12):1086-1092.

142. Schweikl H, Hartmann A, Hiller KA, Spagnuolo G, Bolay C, Brockhoff G, et al. Inhibition of TEGDMA and HEMA-induced genotoxicity and cell cycle arrest by N-acetylcysteine. Dent Mater. 2007;23(6):688-695.

143. Eckhardt A, Müller P, Hiller KA, Krifka S, Bolay C, Spagnuolo G, et al. Influence of TEGDMA on the mammalian cell cycle in comparison with chemotherapeutic agents. Dent Mater. 2010;26(3):232-241.

144. Eckhardt A, Gerstmayr N, Hiller KA, Bolay C, Waha C, Spagnuolo G, et al. TEGDMA-induced oxidative DNA damage and activation of ATM and MAP kinases. Biomaterials. 2009;30(11):2006-2014.

145. Fenech M. Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. Mutat Res. 2006 Aug 30;600(1-2):58-66.

H. ANNEXES

H.1. Cytotoxicity Test Statistical Analysis Output

For further details on the statistical analysis, please consult the respective file on the annexed CD.

Univariate Analysis of Variance EMS

Tests of Between-Subjects Effects

Dependent Variable: Cell viability (%)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power[b]
Corrected Model	98206,812[a]	11	8927,892	75,300	,000	828,299	1,000
Intercept	1341622,857	1	1341622,857	11315,560	,000	11315,560	1,000
Concentration	84864,435	3	28288,145	238,589	,000	715,766	1,000
Celltype	8453,138	2	4226,569	35,648	,000	71,296	1,000
Concentration * Celltype	5088,777	6	848,129	7,153	,000	42,920	,997
Error	23475,755	198	118,564				
Total	1465747,319	210					
Corrected Total	121682,567	209					

a. R Squared = ,807 (Adjusted R Squared = ,796)

b. Computed using alpha = ,01

Post Hoc Tests

Concentration

Multiple Comparisons

Dependent Variable: Cell viability (%)

Dunnett t (<control)

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval
					Upper Bound
600	0	-10,6763*	2,16003	,000	-4,8161
1200	0	-25,0116*	2,09554	,000	-19,3263
2400	0	-53,0029*	2,09554	,000	-47,3176

Based on observed means.

The error term is Mean Square(Error) = 118,564.

*. The mean difference is significant at the ,01 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Homogeneous Subsets

Cell type

Multiple Comparisons

Dependent Variable: Cell viability (%)

Dunnett t (<control)

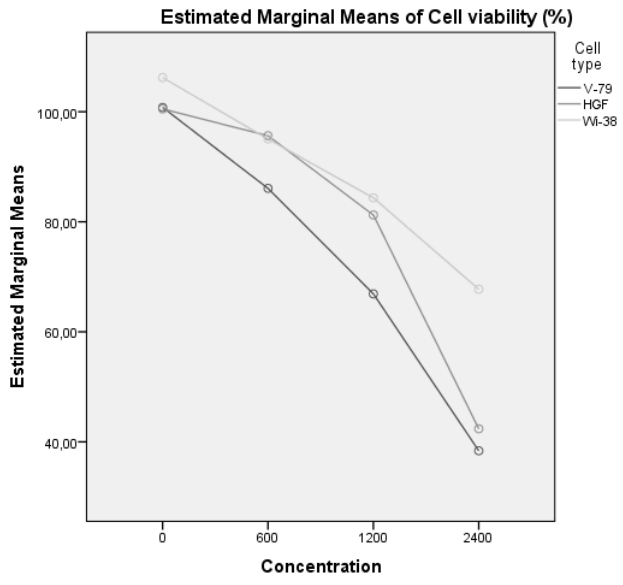
(I) Cell type	(J) Cell type	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval
					Upper Bound
HGF	V-79	5,4767	1,85558	1,000	10,2690
Wi-38	V-79	15,3011	1,81479	1,000	19,9880

Based on observed means.

The error term is Mean Square(Error) = 118,564.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Profile Plots



Univariate Analysis of Variance MMA

Tests of Between-Subjects Effects

Dependent Variable: Cell viability (%)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power[b]
Corrected Model	109693,709[a]	11	9972,155	62,317	,000	685,485	1,000
Intercept	1315632,271	1	1315632,271	8221,494	,000	8221,494	1,000
Concentration	106077,307	3	35359,102	220,962	,000	662,886	1,000
Celltype	309,591	2	154,796	,967	,382	1,935	,079
Concentration * Celltype	3306,811	6	551,135	3,444	,003	20,665	,827
Error	32644,795	204	160,024				
Total	1457970,775	216					
Corrected Total	142338,504	215					

a. R Squared = ,771 (Adjusted R Squared = ,758)

b. Computed using alpha = ,01

Post Hoc Tests

Concentration

Multiple Comparisons

Dependent Variable: Cell viability (%)

Dunnett t (<control)

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval
					Upper Bound
40	0	-7,7500*	2,43450	,002	-1,1503
80	0	-14,9831*	2,43450	,000	-8,3834
160	0	-57,2715*	2,43450	,000	-50,6718

Based on observed means.

The error term is Mean Square(Error) = 160,024.

*. The mean difference is significant at the ,01 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Homogeneous Subsets

Cell type

Multiple Comparisons

Dependent Variable: Cell viability (%)

Dunnett t (<control)

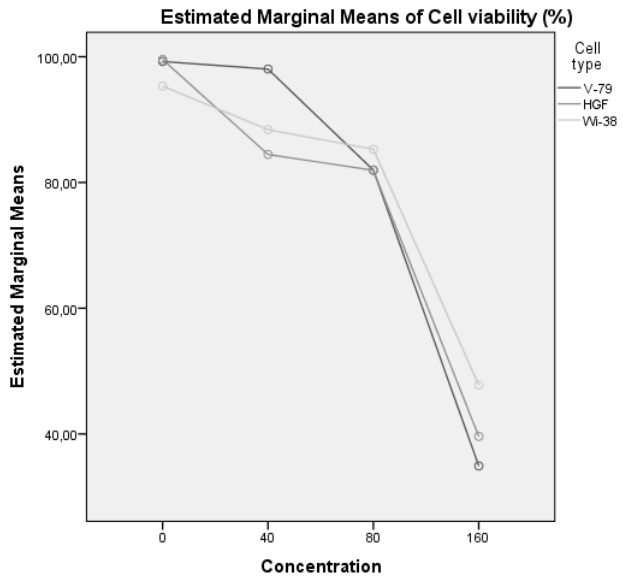
(I) Cell type	(J) Cell type	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval
					Upper Bound
HGF	V-79	-2,1470	2,10834	,249	3,2946
Wi-38	V-79	,6564	2,10834	,780	6,0980

Based on observed means.

The error term is Mean Square(Error) = 160,024.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Profile Plots



Univariate Analysis of Variance Form

Tests of Between-Subjects Effects

Dependent Variable: Cell viability (%)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power[b]
Corrected Model	137201,554[a]	11	12472,869	110,647	,000	1217,118	1,000
Intercept	811302,491	1	811302,491	7197,083	,000	7197,083	1,000
Concentration	117295,729	3	39098,576	346,844	,000	1040,533	1,000
Celltype	14997,079	2	7498,539	66,520	,000	133,039	1,000
Concentration * Celltype	4908,746	6	818,124	7,258	,000	43,546	,998
Error	22996,221	204	112,727				
Total	971500,266	216					
Corrected Total	160197,775	215					

a. R Squared = ,856 (Adjusted R Squared = ,849)

b. Computed using alpha = ,01

Post Hoc Tests

Concentration

Multiple Comparisons

Dependent Variable: Cell viability (%)

Dunnett t (<control)

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval
					Upper Bound
400	0	-46,0211*	2,04330	,000	-40,4819
800	0	-50,4621*	2,04330	,000	-44,9229
1600	0	-60,6983*	2,04330	,000	-55,1592

Based on observed means.

The error term is Mean Square(Error) = 112,727.

*. The mean difference is significant at the ,01 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Homogeneous Subsets

Cell type

Multiple Comparisons

Dependent Variable: Cell viability (%)

Dunnett t (<control)

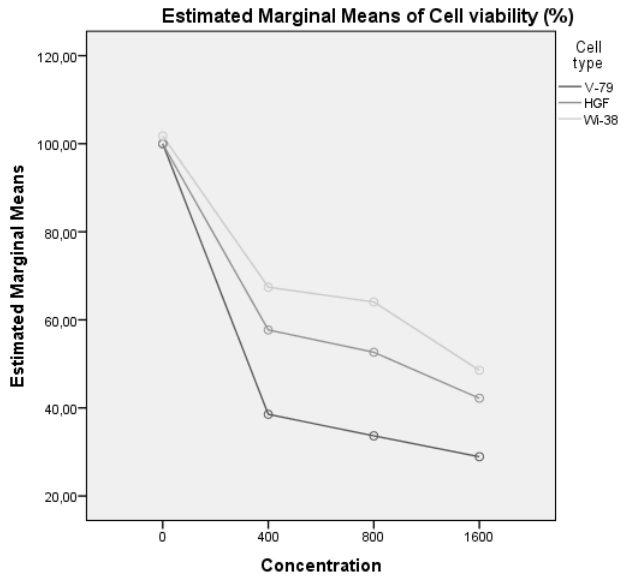
(I) Cell type	(J) Cell type	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval
					Upper Bound
HGF	V-79	12,8409	1,76955	1,000	17,4081
Wi-38	V-79	20,1599	1,76955	1,000	24,7271

Based on observed means.

The error term is Mean Square(Error) = 112,727.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Profile Plots



H.2. Genotoxicity Test Statistical Analysis Output

For further details on the statistical analysis, please consult the respective file on the annexed CD.

T-Test

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
%cells - MN (2000)	Equal variances assumed	19,306	,000	323,336	39998	,000	5,4478118	,0168488	5,4147878	5,4808357
	Equal variances not assumed			323,336	38623,580	,000	5,4478118	,0168488	5,4147878	5,4808357

ANOVA

%cells MN/2000 cells

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	83613,345	4	20903,336	2558,857	,000
Within Groups	326719,687	39995	8,169		
Total	410333,031	39999			

Robust Tests of Equality of Means

%cells MN/2000 cells

	Statistic[a]	df1	df2	Sig.
Welch	2845,078	4	19940,349	,000
Brown-Forsythe	2558,857	4	38335,124	,000

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

%cells MN/2000 cells

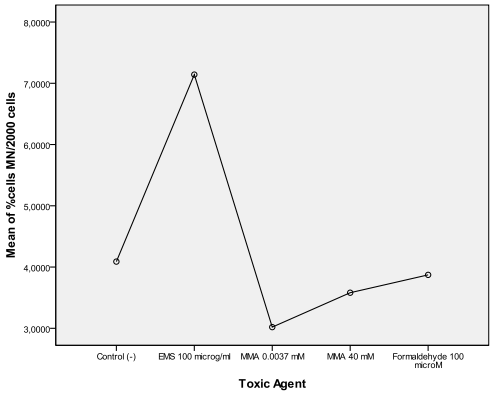
Dunnett t (>control)[a]

(I) Toxic Agent	(J) Toxic Agent	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval Lower Bound
EMS 100 microg/ml	Control (-)	3,0526508*	,0451913	,000	2,927394
MMA 0.0037 mM	Control (-)	-1,0697749	,0451913	1,000	-1,195032
MMA 40 mM	Control (-)	-,5079951	,0451913	1,000	-,633252
Formaldehyde 100 microM	Control (-)	-,2168691	,0451913	1,000	-,342126

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

*. The mean difference is significant at the 0.01 level.

Means Plots



H.3. Discussion Tables

Table 1. Artificial saliva formulas published in residual monomer leaching studies		
Reference	Composition	
Urban VM *et al.* Dent Mater. 2009;25(5):662-671. Urban VM *et al.* J Appl Polym Sci. 2012;123(2):732-739.	NaCl	0.4 g/L
	KCl	0.4 g/L
	$CaCl_2 \cdot 2H_2O$	0.795 g/L
	$NaH_2PO_4 \cdot 2H_2O$	0.78 g/L
	$Na_2S \cdot 9H_2O$	0.005 g/L
	Urea	1.0 g/L
Danesh G *et al*. Exp Toxicol Pathol. 2011 Apr 27.	$CaCl_2 \cdot 2H_2O$	0.7 mmol/L
	$MgCl_2 \cdot 6H_2O$	0.2 mmol/L
	KH_2PO_4	4 mmol/L
	HEPES buffer	20 mmol/L
	KCl	30 mmol/L
Alawi M *et al.* Fresenius Environ Bull. 2007;16(4):408-414.	NH_4Cl	233 mg/L
	$MgCl_2 \cdot 6H_2O$	43 mg/L
	KCl	1162 mg/L
	KSCN	222 mg/L
	$CaCl_2 \cdot 2H_2O$	210 mg/L
	KH_2PO_4	354 mg/L
	Sodium citrate	13 mg/L
	$NaHCO_3$	535 mg/L
	Na_2HPO_4	375 mg/L