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Research Article

Accumulation of N-epsilon carboxymethyl lysine in various tissues and organs related to diet-induced aging process. State of the art and experimental animal study

[1]Anida-Maria Băbțan, [2]Bianca A. Boșca, [1]Nausica B. Petrescu, [3]Alina E. Pârvu, [3]Ana Uifălean, [4]Marian Taulescu, [4]Mihai Negru, [2]Maria Crișan, [1]Radu S. Câmpian, [1]Aranka Ilea

[1]Department of Oral Rehabilitation, Oral Health and Dental Office Management, Faculty of Dentistry, "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania; [2]Department of Histology, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, Cluj-Napoca, Romania; [3]Department of Physiopathology, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, Cluj-Napoca, Romania;[4]Department of Pathology, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania.

Abstract. Aging is a complex process that involves multiple pathogenic mechanisms leading to progressive morphological and functional alterations and increased risk of developing general pathology. Accumulation of deleterious substances, such as Advanced Glycation End Products (AGEs), in cells and tissues accelerates the multisystem functional decline that occurs with aging. Taking into consideration that Nε-carboxymethyl lysine (CML), one of the major AGEs, induces irreversible protein modifications and is implicated in various pathologies related to aging, our purpose was to assess the expression of CML in rat oral cavity and various organs, associated with the process of aging. The study was conducted on 8-month old and 2-year old rats, both females and males, that were fed standard pellet diet. The tissues harvested from the oral mucosa, dental and periodontal tissues, skin, salivary glands, and kidneys were processed using the standard histological technique and immunohistochemistry for CML. Our results indicated that CML expression was higher in the 2-year old rats and there was an association between CML accumulation in the oral cavity and other organs. These findings suggest that CML is implicated in the pathophysiology of aging, process which could be detected in oral cavity tissue samples harvested during various therapeutic procedures.

Key Words: N-epsilon carboxymethyl lysine (CML), Advanced Glycation End Products (AGEs), aging, diet, immunohistochemistry.

Corresponding Author: B. A. Boșca, email: biancabosca@yahoo.com

Introduction

Advanced Glycation End Products (AGEs) are glycated proteins, the result of the Maillard reaction between reducing sugars with an available amino (-NH2) or ketone [RC(=O)R'] group and proteins or lipids (Van Nguyen 2006). AGEs are classified according to their incoming basic structure, their ability to be identified using fluorescent techniques and to produce crosslink such as follows: GOLDIC, (2-ammonio-6-([2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4,5-dihydro1H-imidazol-5-ylidene] amino)hexanoate); MOLD, methyglyoxal-lysine dimmer; GOLD, glyoxal-lysine dimmer; MODIC, (2ammonio-6-([2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1Himidazol-5ylidene]amino)hexanoate); MRX, 8-hydroxy-5-methyldihydrothiazolo(3,2-alpha)pyridinium-3carboxylate, pentosidine, crossline and non-fluorescent non-crosslinked AGEs such as Nε-carboxymethyl lysine (CML), carboxyethyllysine (CEL), pyrraline, and imidazole (Aragno & Mastrocola 2017). From this glycated protein list, pentosidine, CML and pyrraline were the most investigated.

CML sources can be both exogenous and endogenous. The endogenous production is related to high levels of intracellular glucose and hyperglycemia conditions (Zhang et al 2017), while the exogenous ones are secondary to sun exposure, microwave ultrasounds, cigarette intake/tobacco smoke (Sanders et al 2017; Van Waateringe et al 2017) and food intake (Singh et al 2001). High-temperature and short-time food preparation results in a pleasant taste and an appealing presentation, as well a high CML formation. Due to the stable chemical bonds, their tissue accumulation is time and dose dependent, leading to multiple negative effects. Dietary CML have been intensely investigated

and the food content of CML is presented in the available databases (Hull al 2012; Scheijen et al 2016). Circulating CML is the result of the exogenous intake versus endogenous production, renal and feces elimination, and the presence of associated diseases represents an aggravating factor.

The effects of CML are receptor mediated (AGE-R1/OST-48, AGE-R2/80K-H, AGE-R3/galectin-3), scavenger receptors (SR-A, SR-B: CD36, SR-BI, SR-E: LOX-1; FEEL-1; FEEL-2) (Ott et al 2014), according to the targeted tissue. AGE-RAGE interaction activates NADPH-oxidase, which promotes NF-κB proinflammatory pathway, followed by the expression of Nitric oxide synthase (iNOS) and high levels of peroxynitrite ($ONOO^-$) (San Martin et al 2007). CML formation was proven to be stimulated in vitro by peroxynitrite and glucose enriched culture medium (Hofmann et al 2002). CML glycation products increase cyclooxygenase-2 (COX-2), prostaglandin-E2 (PGE2), IL-6, NF-κB-p65 and MMP-13 in human synoviocytes, maintaining a proinflammatory status (Su et al 2015). Oxidative stress impairs proteasomal activity, which promotes protein aggregate and covalent cross-links- lipofuscin, which accumulates in post-mitotic cells during aging, such as muscle fibers and cerebral tissue (Korovila et al 2017). During aging, human T-cells alter their chemotactic activity, while collagen fibers reduce T-cell migration, both mechanisms being mediated by CML accumulation (Sadowska-Bartosz & Bartosz, 2016). Continuous pro-inflammatory activity and the production of oxidant molecules induce protein damage and accelerate the aging process. Upon binding to the specific receptors, dietary CML increase TNF-α secretion from macrophage cells (van der Lugt et al 2018). Besides the RAGE group, there are two other components in CML modulating activity, the soluble RAGE (sRAGE) which can be provided either as an endogenous secretion (esRAGE) or by RAGE cleavage by metalloproteinases (Haddad et al 2016). These two forms of sRAGE have a competitive inhibitory effect on RAGE in cell signaling and bounding.

CML systemic effects

In human skin fibroblasts, phage display technology to identify which protein modification lead to malfunction of proteasome-one of the most effective defense against Nε-carboxymethyl-lysine (CML) accumulation (Gonzalez-Dosal et al 2006). Bacteriophage coat protein gene covers an interest protein gene, and the phage is stimulated to expose the analyzed protein, technique used in the identification of active ligands, enzymes, peptide, DNA interaction (Hoogenboom et al 1998).

CML aging-related process lead to an increase in damaged α-7 subunit of the proteasome along with the accumulation of damaged proteins in old cells. In fibroblast cell line, glyoxal (GO) increased CML expression in a time-dependent manner, with the consequent decrease in cell viability (Lee et al 2017). In the arteries, lungs, cardiac muscle, kidney, bone, cartilage, gastric mucosa and placenta, CML was negative, while in elders and in vascular pathology (atheromatosis and thickened vessels) CML was intensely positive (Kume et al 1995; Schleicher et al 1997), which proves that CML accumulates in a time and dose dependent manner.

Skin autofluorescence (SAF) was used to compare CML and pentosidine as a metabolic memory marker, in diabetic and chronical kidney disease elder patients (mean age- 82,4 years old), at baseline and after 10 years (Rajaobelina et al 2014). The association between SAF and renal and glycemic status was performed using multilinear regression adjustment for age, diet, and body mass index. Higher SAF was found in patients with chronical kidney disease and long-term diabetes. SAF was associated with the glomerular filtration rate, but not with glycemia during the investigated period, although SAF was correlated with both parameters at 10 years baseline. The authors hypothesized that the reason was the faster accumulation of AGEs in renal disorders compared with diabetes. CML was associated with the dysfunction of fasting glucose, which could increase diabetes incidence risk (Luft et al 2016).

In renal pathology, CML decreases glomerular filtration rate and impairs the renal function by altering the synthesis of extracellular matrix components and by promoting glomerular fibrosis (Semba et al 2015). Pilleron et al. in conducted a study on elderly healthy subjects and type I or II diabetes patients and compared the inner forearm SAF and skin biopsies in which glycated collagen, pentosidine and CML levels in were assessed. Their results showed a strong association between increased SAF, tissue frailty and polymedicated patients, diabetic patients and patients who did not practice physical activity (Pilleron et al 2017). Arterial stiffness increases with age and is associated with high cardiovascular risk. The stiffness of the arterial wall is the result of the collagen fibers alterations caused by decreased turnover of elastin and collagen, and CML accumulations. CML enhances lipid peroxidation and transports redox metal molecules, leads to the disfunction of the extracellular matrix by crosslinking type IV collagen fibers and laminin (Semba et al 2015). Since arterial normal function prevents mortality due to cardiac and endothelial disfunction and AGEs reflect the vascular modifications related to age, glycation showed that in elderly long-endurance training men, SAF was strongly associated with age and arterial stiffness but not with endothelial function; this suggests that the practice of endurance sport is not enough for reducing glycation, and the intensity of training plays an important role in reducing glycation (Couppé et al 2017). In mice, aging leads to an increased CML deposition in the cerebral cortex, midbrain, striatum and hippocampus which is directly correlated with the level of oxidative stress (Thangthaeng et al 2008).

In cardiac tissues collected from patients undergoing surgery, CML was highly expressed, and multiple linear regression showed that age, cardiac heart disease and diabetes were independent factors in CML accumulation; moreover, CML concentration was positively correlated with these factors (Hu et al 2013). Regarding red blood cell line, erythrocytes treated with CML-modified serum albumin showed a higher hemoglobin glycation compared with erythrocytes treated with glucose, showing that albumin glycation status could have a protective role in reducing hemoglobin glycation (Jagadeeshaprasad et al 2018). Considering that serum CML and AGEs receptors (RAGE) are associated with anemia and deformed erythrocytes, Roy et al. investigated the role of CML and selenium as risk factors for anemia in 1043 elderly volunteers (Roy et al 2012). Their findings showed that high CML, low circulating selenium and aging were independent factors leading to anemia in elderly adults. The possible mechanism involved CML accumulation on erythrocytes surface, CML-RAGE interaction

on the endothelial surface, and the additional oxidative stress induced by low selenium serum levels.

In a rodent experimental study, Kim et al. evaluated the expression of CML, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nitrotyrosine in retinal cells in old mice compared with young mice trained to treadmill, considering that retinal cells are highly active and indivisible cells exposed to oxidative stress (Kim et al 2015). The aging process was strongly correlated with the presence of CML in the retinal vessels and inner retina, where CML were three times higher in elderly mice compared with young mice, in which the treadmill exercise maintained oxidative stress markers at normal values. CML was also increased in retinal pericytes and vitreous body of diabetic patients, aggravating the microvascular retinopathy. Retinal pigment epithelium is altered in diabetic retinopathy, whereas CML is an independent factor in the epithelium degradation and visual acuity impairment (Mishra et al 2016), due to thickening and dysfunction of the extracellular matrix (Choudhuri et al 2013; Okubo et al 1999). The interaction between CML and the receptors induces oxidative stress, which triggers the proinflammatory pathways involving NF-kB; this mechanism could affect the endothelial retinal proliferation and could also stimulate platelet binding and fibrin formation, which are responsible for diabetic retinopathy (Bierhaus et al 1997; Yamagishi et al 1996). Thomas et al. used protein extraction techniques for CLM quantitative analysis in both non-demineralized and demineralized bone samples harvested from the distal diaphysal tibial bone. They reported that CML was more consistently found in insoluble collagen fibers, correlated with aging and bone frailty (Thomas et al 2018). The CML accumulation in collagen was due to the exposure of collagen to sugar and the formation of crosslinked fibers which could not be resorbed during bone metabolism. In samples of idiopathic pulmonary fibrosis human, CML and pentosidine were strongly expressed in the extracellular matrix of alveolar epithelial cells and bronchial epithelium; RAGE was concentrated in the membrane of alveolar epithelial cells, but was absent in fibroblasts foci and hyperplastic alveolar epithelial cells (Machahua et al 2016). CML is involved in carcinogenesis, by the interaction between the markers of oxidative stress and carbonyl stress (Vlková et al 2012). CML impairs the activity of growth factors and thus, cells proliferation and migration in the injured tissues is diminished (Quan et al 2014). RAGEs are highly expressed in premalignant oral lesions and squamous carcinoma cells (Chapman et al 2018; Sanders et al 2017). RAGE mRNA was reported in primary and metastatic melanoma; in oral squamous carcinoma cells, a higher expression was found in metastatic cells, with a direct correlation with the tumor depth and the low differentiation degree (Landesberg et al 2008). In pancreatic cancer, CML concentration was decreased in patients with Single nucleotide polymorphism (SNP) with the minor allele of rs640742 of *DDOST*; sRAGE concentrations were independently inversely correlated with the 82Ser allele of rs2070600 of *RAGE* and positively correlated with serum CML. The minor allele of rs1035786 of *RAGE* was associated with reduced risk of pancreatic cancer and *RAGE* rs1035798 SNP was associated with the risk of pancreatic cancer (Duan et al 2014). CC genotype rs2070600 (Gly82Ser) *RAGE* SNP was associated with higher sRAGE in human patients, where this SNP might make RAGE more sensitive to degradation proteins (Gaens et al 2009). In oral cavity, higher levels of CML were associated with periodontitis and periimplantitis, and morphophysiological changes in dental pulp and dentine collagen fibers, with an increase in dentine hardness and risk for fracture, alongside with the yellow-brown discoloration of dentine (Greis, Reckert, Fischer, & Ritz-Timme, 2017; Shinno et al 2016).

The aim of this study was to investigate the CML distribution in the oral cavity and various rodent model tissue structures accumulation after controlled food-intake and its correlation with the process of aging and chronical inflammation.

Material and method

The study was performed on Wistar rats, subdivided according to the age into young rats (aged 8 months), both females (n=5) and males (n=5) and adult rats (aged 2 years) both females (n=5) and males (n=5). The animals were previously housed in plastic cages in normal laboratory conditions (at a constant temperature of 22^0C, with a 12-hour light – 12-hour dark cycle) and fed with standard pellet food (20% proteins, 70% carbohydrates and 10% lipids) and water ad libitum. The animals were euthanized by deep anesthesia with ether and full necropsy examination was performed. The study was approved by the Ethics Committee, no. 79/3.03.2017.

Light microscopy

Samples of skin, oral mucosa (including gingiva), incisive teeth and periodontium, kidney, parotid and submandibular salivary glands were collected and fixed in 10% phosphate buffered formalin (pH=7.0) for 24 hours. The samples were processed by standard histological technique, included in paraffin blocks, sectioned at 3-4 microns (μm) and stained with hematoxylin and eosin (HE). Images were captured using an Olympus SP 350 digital camera and Stream Basic imaging software (Olympus Corporation, Tokyo, Japan).

Immunohistochemical technique

Serial 3μm sections were prepared from the same paraffin blocks that were used for the histological H&E examination and dried in an oven at 60°C overnight. An anti-carboxymethyl lysine NF-1G (Abcam, ab30917) primary antibody (dilution - 1: 250) was used for immunohistochemical staining. Negative controls for each sample were prepared by replacing the primary antibody with mouse IgG1 (Code X0931, Dako, Denmark). Antibody binding was visualized using the auto-immunostaining apparatus Leica BOND MAX system (Leica Biosystems, Germany) according to the manufacturer's instructions.

Immunopositivity for AGE-CML was scored using a semiquantitative grading system. The intensity of the CML staining was evaluated using a four step-wise system (0, unreactive; 1 (+) weak positive; 2 (++) moderately positive; and 3 (+++) strongly positive) (Schalkwijk et al 2004). The IHC assessment of the specimens was performed by three individual evaluators (M.T, M.N, B.A.B). The discrepancies in the CML quantification in the tissue samples were solved by the reassessment of the histological sections and the scoring consensus between the evaluators was achieved. The data were analyzed with GraphPad Prism 5 software (La Jolla, CA, USA). The comparisons between IHC scores was performed using the two-way ANOVA with the Bonferroni post-test (p value < 0.05).

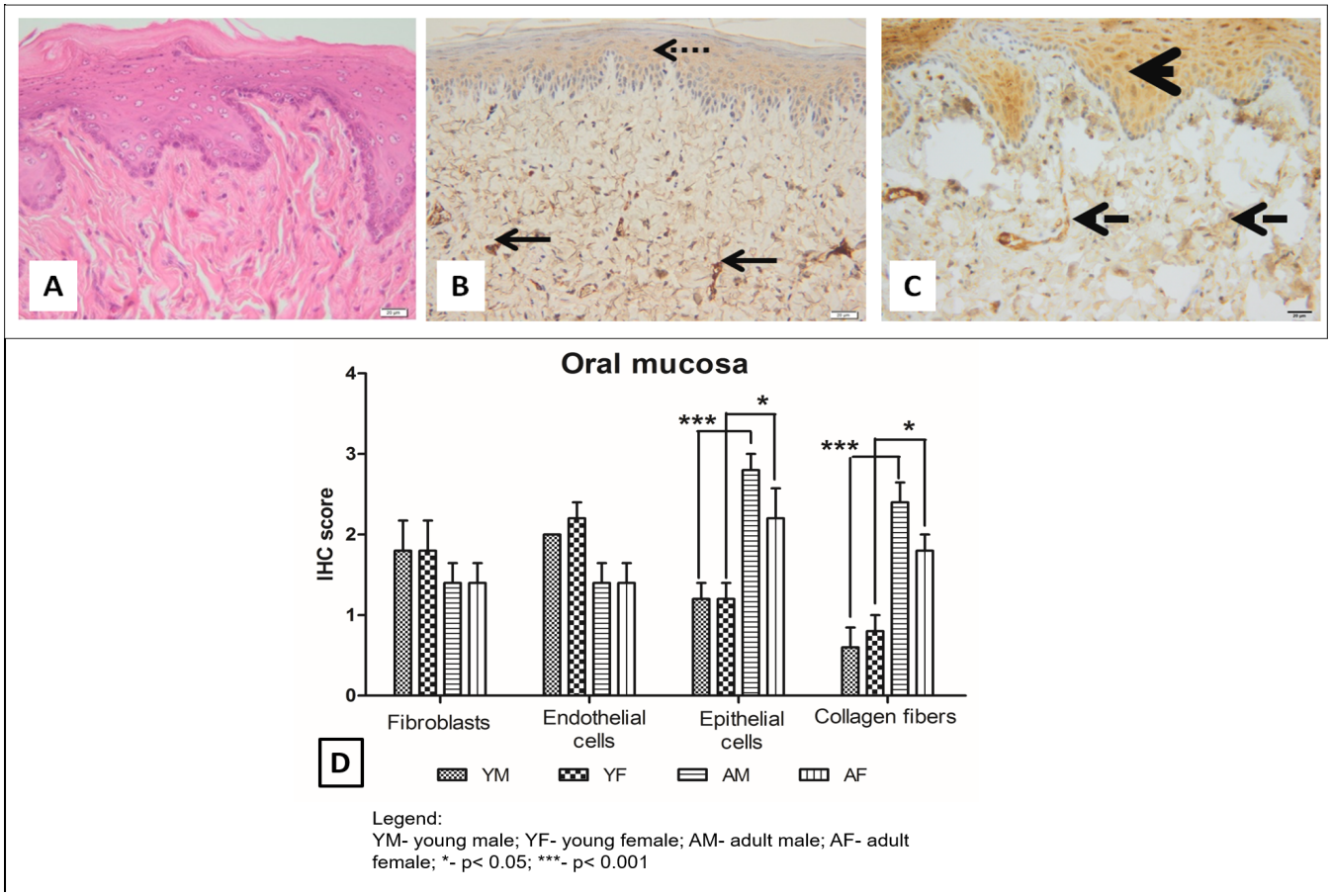

Fig. 1. Photomicrographs of the oral mucosa: A – stratified squamous epithelium and chorion, HE stain; B – young rat: CML expression in fibroblasts and capillaries in the chorion (arrows); negative CML reaction in the mucosal epithelium (dotted arrow), IHC for CML; C – adult rat: intense expression of CML in the epithelium (arrowhead) and in associated with the collagen fibers in the chorion (dashed arrows), IHC for CML; D – Comparison between the IHC expression for CML in the oral mucosa of young and adult, male and female rats.

Results

The immunohistochemical (IHC) technique revealed the variable distribution of CML in tissues and organs. IHC expression of CML was observed in the cytoplasm of epithelial cells, including the endothelial cells, connective tissue cells (fibroblasts and inflammatory cells) and associated with the collagen fibers. In the oral cavity, CML exhibited a diffuse expression in the oral mucosa and the dental and periodontal tissues (Fig. 1 and 2). In the young rats, the CML staining was more intense in the fibroblasts and endothelial cells lining the capillaries in the chorion (Fig. 1B). In the adult rats, CML expression was more intense in the epithelial cells in the stratified squamous epithelium and in the collagen fibers in the chorion, compared with the young rats (Fig. 1C). The CML expression in the epithelial cells and in the collagen fibers was significantly higher in the adult males compared with young males ($p < 0.001$) and in the adult females compared with the young females ($p < 0.05$) (Fig. 1D). There was no significant difference between males and females in the same age group.

There were no differences in the histological features of the dental tissues between the young and adult animals (Fig. 2A). In the young rats, CML expression was diffuse in the dental tissues, including the pulp and dentin, in the cementum, and the alveolar bone (Fig. 2B); the CML staining was more intense in the periodontal ligament (Fig. 2D). The CML accumulation in the periodontal ligament of adult males was significantly higher compared with young males ($p < 0.05$). There was no significant difference in CML expression in the dentine and the alveolar bone in adult and young animals. There was no significant difference between males and females in the two age groups (Fig. 2E).

In the skin, the epithelial cells in the epidermis and hair follicles contained a higher amount of CML than fibroblasts and collagen fibers in the dermis (Fig. 3). In young rats, IHC expression for CML was discrete, in the epidermal cels hair follicles and dermal fibroblasts (Fig. 3B). In adult individuals, CML expression was much more intense than in young rats with a diffuse distribution in the collagen fibers in the dermis and a deep staining in the epidermis and hair follicles (Fig. 3C). There were significant differences in the accumulation of CML in the dermal fibroblasts and collagen fibers and in the epithelial cells in the epidermis. In the adult males, the CML expression was significantly higher in the epithelial cells ($p < 0.01$) and in the collagen fibers ($p < 0.001$), compared with the young males. In the adult females, the CML expression was significantly higher in the fibroblasts ($p < 0.05$), in the epithelial cells ($p < 0.05$) and in the collagen fibers ($p < 0.001$), compared with the young females. There was no significant difference between males and females in the two age groups (Fig. 2E).

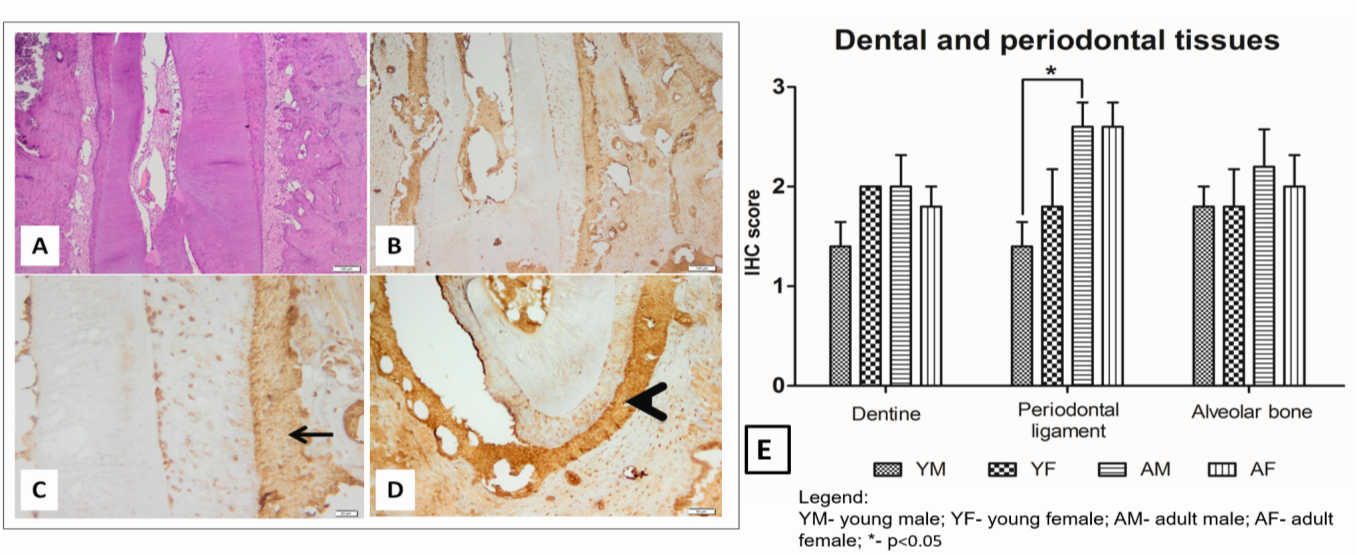

Fig. 2. Photomicrographs of the dental and periodontal tissues: A – longitudinal section through the tooth, periodontal ligament and alveolus, HE stain; B – Young rats: diffuse expression of CML IHC for CML: the arrow; C –Young rats: intense CML staining in the periodontal ligament (arrow), IHC for CML; D – Adult rat: increased expression of CML in the periodontal ligament (arrowhead), IHC for CML; E – Comparison between CML expression in dental and periodontal tissues between young and adult, male and female rats

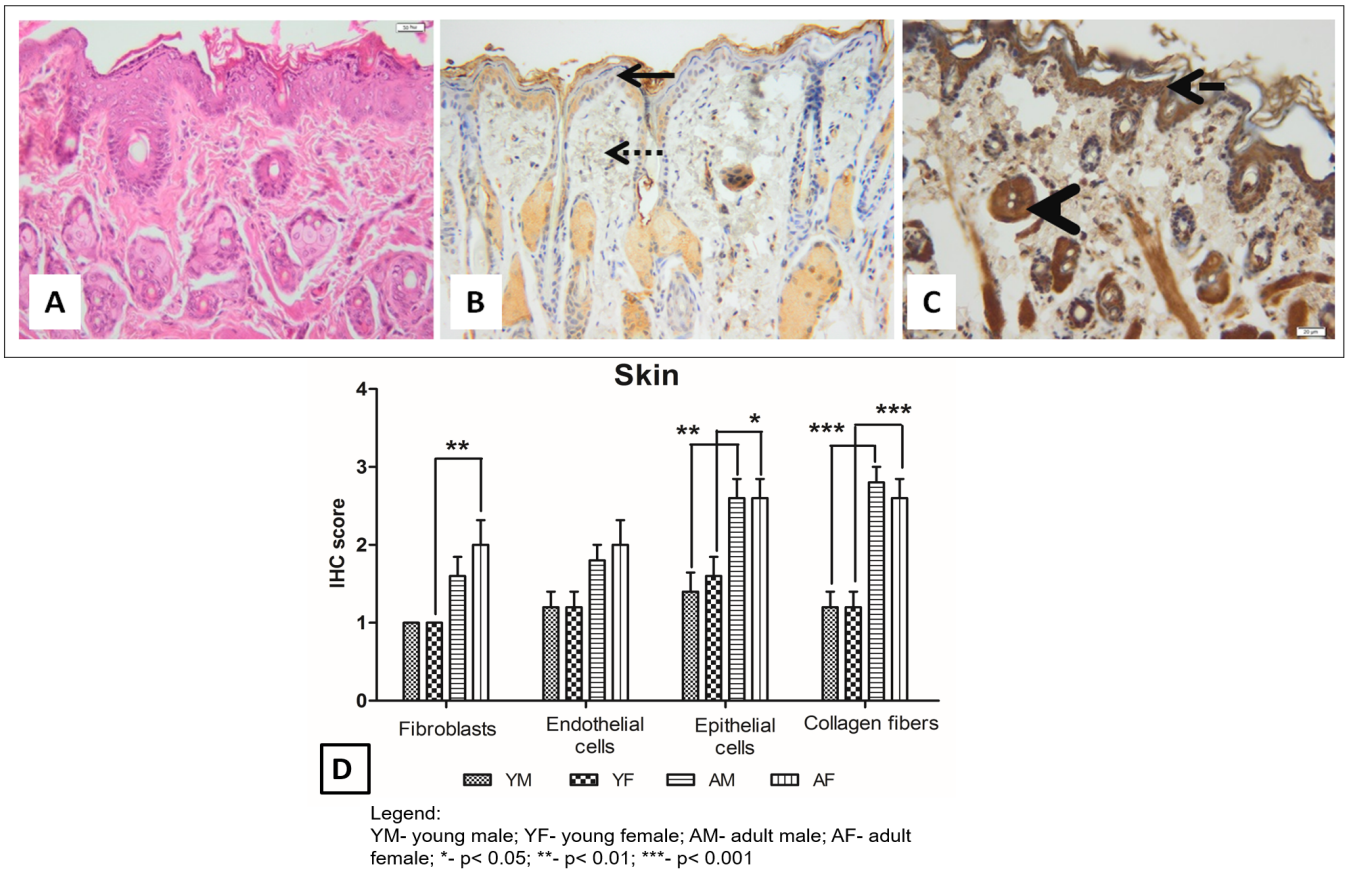

Fig. 3. Photomicrographs of the skin: A- Epidermis and dermis, HE stain; B – Young rats: low CML expression in the epidermis (arrow) and dermis (dotted arrow), IHC for CML; C – Adult rats: - intense CML expression in the epidermis (dashed arrow) and hair follicles (arrowhead), IHC for CML; D – Comparison between CML expression in epidermis and dermis of young and adult, male and female rats

In the parotid and in the submandibular salivary glands, a multifocal distribution of CML was observed in the serous acini compared with the mucous acini, the excretory ducts and the connective tissue in the stroma (Fig. 4). The IHC expression was more discrete in young individuals (Fig. 4B) compared with the adults (Fig. 4C). In the adult animals, CML was intensely expressed in the serous acini (IHC score = 3) compared with young animals which showed a moderate (IHC score = 2) or a low CML expression (IHC score = 1).

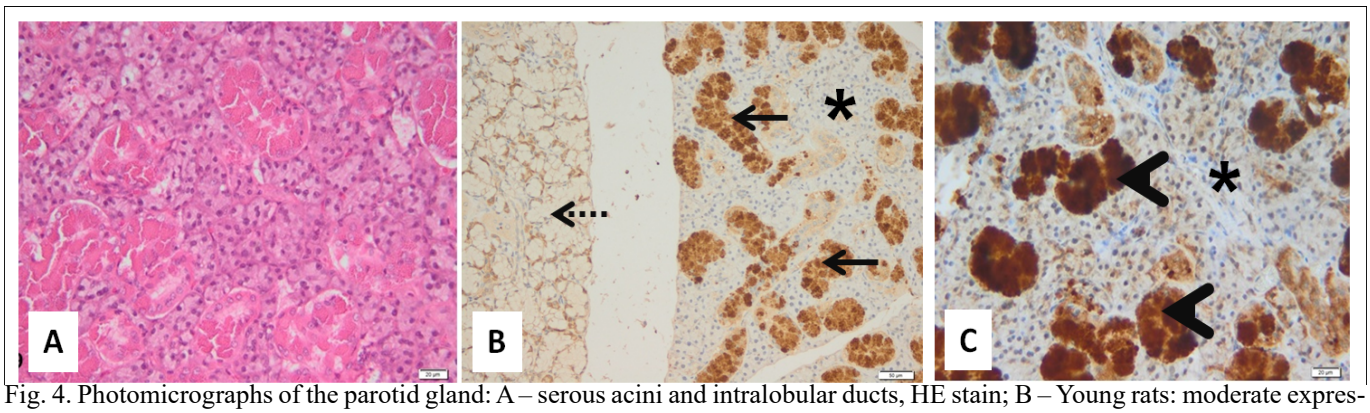

Fig. 4. Photomicrographs of the parotid gland: A – serous acini and intralobular ducts, HE stain; B – Young rats: moderate expression of CML in the serous acini (arrows) and low expression in the mucous acini (dotted arrow) and the stroma (asterisk), IHC for CML; C – Adult rats: intense IHC reaction for CML in the serous acini (arrowheads) and negative reaction in the intralobular excretory ducts and stroma (asterisk), IHC for CML

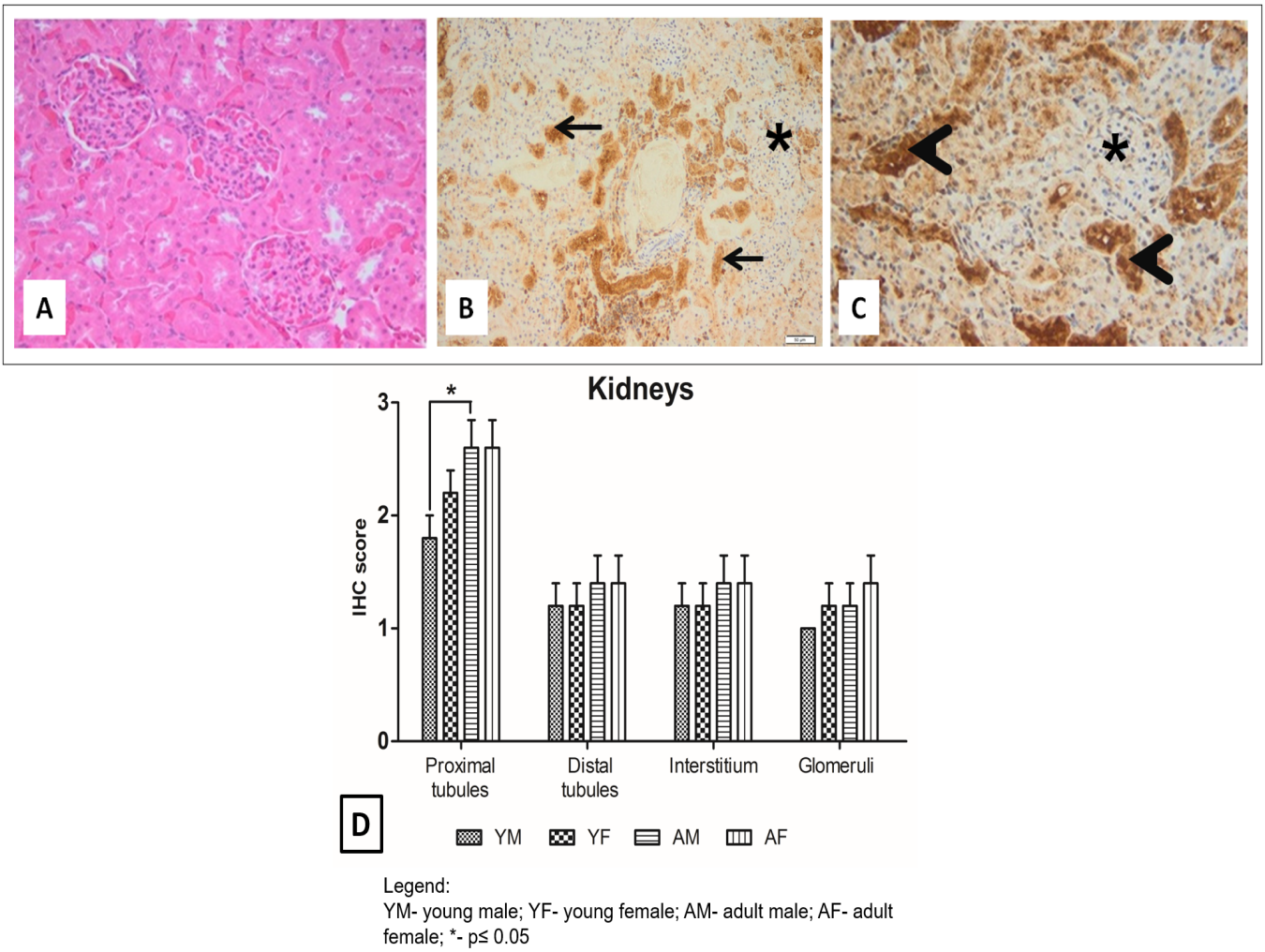

Fig. 5. Photomicrographs of the kidney: A – the renal cortex, HE stain; B – Young rats: moderate CML reaction in the proximal renal tubules (arrows) and negative reaction in the distal tubules and glomeruli (asterisk), IHC for CML; C – Adult rats: intense positive multifocal IHC reaction for CML in renal proximal tubules (arrowheads) and negative in the glomeruli (asterisk), IHC for CML; D – Comparison between CML expression in renal tubules, interstitium and glomeruli between young and adult, male and female rats.

No histological changes were identified within the renal tissues (Fig. 5A). In the renal cortex, the epithelial cells in the proximal convoluted renal tubules showed a higher intensity of CML expression than the distal tubules, glomeruli and interstitium (Fig. 5). The reaction for CML in the proximal renal tubules was moderate in young individuals (Fig. 5B), but more intense in adult rats (Fig. 5C). The CML reaction in the renal medulla and the renal pelvis was negative. The CML expression was significantly higher in the renal proximal tubules of adult males compared with young males ($p< 0.05$). There was no significant difference between males and females in the two age groups (Fig. 5D).

Discussions

According to the literature, a diffuse and intense expression of CML in various tissues is associated with chronic inflammation, diabetes and/or aging. In mice, aging leads to an increased CML deposition in the cerebral cortex, midbrain, striatum and hippocampus which is directly correlated with the level of oxidative stress (Thangthaeng et al 2008). In our study, most likely, the diffuse and intense distribution of CML in different organs was associated with the age of the rats. Moreover, our results revealed that CML had similar distribution in the oral cavity and in various organs. These findings suggest that CML is implicated in the pathophysiology of aging, and this process could be detected in oral cavity tissue samples. The clinical significance of our findings is that the tissues harvested during various therapeutic procedures in human patients (e.g. hard dental tissues harvested during tooth extraction or the gingiva harvested during gingivectomy) could be used in order to assess the aging process and the risk for developing associated general diseases. Recent clinical and in vitro studies reported that besides CML, other AGEs also exert systemic effects and are implicated in various pathologies. Satake et al. used high-performance liquid chromatography (HPLC) to assess the accumulation of hydroxyproline and pentosidine in tibia and femur and to demonstrate their implication in the development of osteoarthritis (Satake et al 2018). Aging was correlated with cartilage browning and degeneration, but there was no association between the accumulation of pentosidine and tissue degradation. Sarcopenia, as a mechanism related to aging and its correlation with AGEs accumulation, was investigated by skeletal muscle mass index (SMI), SAF on the upper arm and correlated with the biological samples (Isami et al 2018). SMI was higher in men, and significantly higher SAF was an independent factor in low SMI subjects. This association was explained by the fact that oxidative stress activates ubiquitin-proteasome system, which induces muscle protein disruption. In UVA photoaged human fibroblasts and fibroblasts lysosomes, AGEs had higher levels compared with non-photoaged fibroblasts; in UVA exposed fibroblasts deterioration was noticed due to the impairment of proteasome and protein degradation (Xu et al 2018). In patients undergoing hemodialysis diagnosed with chronic kidney disease (CKD), high levels of AGEs - SAF were age-related and negatively correlated with serum parathormone, which suggested the AGEs implication in chronic bone disease (França et al 2017). The effect of AGEs - age dependent accumulation on collagen mechanical properties was assessed in skin biopsies harvested from healthy young (less than 30 years old) and elder (more than 60 years old) volunteers (Ahmed et al 2017). In elders, the dermis was porous with gaps between the collagen layers, whereas in young individuals the dermis was dense, with discrete gaps. The quaternary structure showed an age-related collagen matrix density decrease. Moreover, the mechanical properties influenced by hydration showed that Young's modulus was increased in dehydrated fibrils, due to the loss of glycoaminoglycans (GAG) and HLA proteoglycan's components. Gautieri et al. found 14 pairs of arginine-lysine in the extracellular matrix and collagen fibrils that form AGE- glucosepane by their tridimensional structure (Gautieri et al 2014). They described approximately 10 intermolecular crosslinks for each collagen molecule, which is the main mechanism in loss of elasticity and collagen stiffening. Couppé et al. assessed the mechanical effect of physical exercise in AGE - pentosidine accumulation in skin, femoral muscle and patellar tendon, and the structural modifications induced by pentosidine in the tendons (Couppé et al 2014). In vivo MRI investigations showed a 30% more intense signal for elderly trained men compared with elderly untrained and young men. Tendon stress was associated with the physical performance, and the fibril density in tendons was more reduced in elderly subjects. The size of the patellar tendon was 30% larger and pentosidine cross-linking was lower in master athletes, showing a diminished mechanical stress.

CML and lysine were quantified in chocolate-flavor mixed drinks (hot chocolate, hot malt drink) using liquid chromatography coupled to ion trap tandem mass spectrometry (Niquet-Léridon & Tessier, 2011). Quantifications showed high CML levels (CML mg/kg of protein) dependent of their nutritional input and thermic treatment to which they were exposed during processing: from 26 ± 1.7 and 130.5 ± 27.4 µg/g powder; researchers also found that the powder mixed with high temperature milk increases CML concentration in comparison with the theoretical mixing of hot liquid and protein powder, suggesting that during high temperature processing, new CML are formed. CML was quantified in minced-meat before and after 190°C frying, and they found that CML concentration increased in a time dependent manner, 0.28 mg/100 g of meat to 4.82 mg/100 g of minced-meat (Scheijen et al 2016); they found that peanuts, biscuits, multicereals and nutrients exposed to high temperature have a high CML concentration, from 2 to 5 mg/100 of product. Uribarri et al. (2010). extended a food database presenting dAGEs in 549 nutrients. CML was assessed as kU/100g of food. Regarding meat and its derivates, 450°F broiled beef, beef steak, roasted/deep fried chicken, fried bacon had the highest CML content, ranging from 7.487 kU/100 g to 91.577 kU/100 g, correlated to the time and cooking temperature. In fat nutrients, the highest CML contents were found in butter ~23.340 kU/100 g, oleaginous compounds, margarine, and oil ~ 21.680 kU/100g. In C57BL/6 mice, food intake of powder was compared with pelleted form to assess the differences in body fat increase (Yan et al 2011). Pellets were obtained by cooking red wheat at 98°C for 45 minutes, then mixed with water and air-dried to 29°C. Mice fed with pellets showed a lower body weight, body mass and body fat compared with powder fed mice, but an increased weight of the internal organs (liver, kidney, spleen). The high temperature pelleting process might have reduced protein digestion by AGEs formation alongside with the oxidation induced by the time storage. Ford et al. showed that mice eating pelleted nutrients had a higher water consume and ate in higher quantities pellets compared to meal diet (Ford & Ward, 1983). According to the literature, a diffuse and intense expression of CML at the levels of various tissue structures is associated with chronic inflammatory processes, diabetes and / or aging. In the case of individuals evaluated by us, most likely, the diffuse and intense distribution of CML in different organs is associated with the older age of the rats. Our study investigated CML distribution using IHC investigation in a determined time period. Our results indicated a significant age-dependent CML accumulation, especially in the epithelial cells ($p < 0.01$) and collagen fibers ($p < 0.001$), in both oral mucosa and skin. The periodontal ligament showed a moderate CML immunopositivity, significantly

higher in adult males ($p < 0.05$) compared with young males, maybe due to the collagen component. There was no significant age-related accumulation of CML in the hard-dental tissues and the alveolar bone. For reliable results and considering AGEs as accurate/proven biomarkers, future studies will investigate if CML expression and accumulation in the oral tissues influences or is influenced by the salivary levels of CML (Băbţan et al 2019; Ciui et al 2019; Ilea et al 2018).

Conclusions

CML had similar distribution in oral cavity and in various organs and was associated with the animals' age. These findings suggest that CML is implicated in the pathophysiology of aging, process which could be detected in oral cavity tissue samples. The clinical significance of our findings is that the tissues harvested during various therapeutic procedures (e.g. hard dental tissues harvested during tooth extraction or the gingiva harvested during gingivectomy) could be used in order to assess the aging process and the risk for developing associated general diseases.

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Authors

•Anida-Maria Băbțan, Department of Oral Rehabilitation, Oral Health and Dental Office Management, Faculty of Dentistry, "Iuliu Haţieganu" University of Medicine and Pharmacy, Victor Babeș Street, no 15, Postcode: 400012, Cluj-Napoca, Cluj, Romania, EU, e-mail; anida.baezamica@yahoo.ro

•Bianca Adina Boșca, Department of Histology, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Louis Pasteur Street, No 4, Cluj-Napoca, Postcode: 400349, Cluj, Romania, EU, e-mail: biancabosca@yahoo.com

•Nausica Bianca Petrescu, Department of Oral Rehabilitation, Oral Health and Dental Office Management, Faculty of Dentistry, "Iuliu Haţieganu" University of Medicine and Pharmacy, Victor Babeș Street, no 15, Postcode: 400012, Cluj-Napoca, Cluj, Romania, EU, e-mail: nausica_petrescu@yahoo.com

•Alina Elena Pârvu, Department of Physiopathology, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Victor Babeș Street, no 2-4, Postcode: 400012, Cluj-Napoca, Cluj-Napoca, Romania, EU, e-mail: parvualinaelena@umfcluj.ro

•Ana Uifălean, Department of Physiopathology, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Victor Babeș Street, no 2-4, Postcode: 400012, Cluj-Napoca, Cluj-Napoca, Romania, EU, e-mail: alina.uifalean@umfcluj.ro,

•Marian Tăulescu, Department of Pathology, University of Agricultural Sciences and Veterinary Medicine, Mănăștur Street, No 3-5, Postcode: 400372, Cluj-Napoca, Romania, EU, e-mail: taulescumarian@yahoo.com

•Mihai Negru, Department of Pathology, University of Agricultural Sciences and Veterinary Medicine, Mănăștur Street, No 3-5, Postcode: 400372, Cluj-Napoca, Romania, EU, e-mail: mihaingr@yahoo.com

•Maria Crișan, Department of Histology, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Louis Pasteur Street, No 4-6, Cluj-Napoca, Postcode: 400349, Cluj-Napoca, Cluj-Napoca, Romania, EU, e-mail: mcrisan@yahoo.com

•Radu Septimiu Câmpian, Department of Oral Rehabilitation, Oral Health and Dental Office Management, Faculty of Dentistry, "Iuliu Haţieganu" University of Medicine and Pharmacy, Victor Babeș Street, no 15, Postcode: 400012, Cluj-Napoca, Cluj, Romania, EU, e-mail: rcampian@email.com

•Aranka Ilea, Department of Oral Rehabilitation, Oral Health and Dental Office Management, Faculty of Dentistry, "Iuliu Haţieganu" University of Medicine and Pharmacy, Victor Babeș Street, no 15, Postcode: 400012, Cluj-Napoca, Cluj, Romania, EU, e-mail: arankailea@yahoo.com

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