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Effects of aging on DNA hydroxymethylation and methylation in human dental follicles

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ABSTRACT

Objective: Despite the high frequency of impacted teeth and increased frequency of lesions in dental follicles (DF) with aging, DF age-changes remain unclear. We compared the global methylation and hydroxymethylation profiles in DF in relation to age.

Design: DF associated with impacted lower third molars were obtained from 59 individuals. Global DNA methylation (5mC content) and hydroxymethylation (5hmC) were evaluated by ELISA. We tested the correlation between 5mC and 5hmC content, and the correlation of each with patients' age. The differences in age, 5mC, and 5hmC in DF from men/women, and location (left/right mandible) was tested.

Results: The mean age of the 59 individuals was 19.56 ± 3.92, ranging from 13 to 31 years, and most were women (n = 39). 5hmC content and age up to 19 years were inversely correlated (Spearman's correlation coefficient = -0.552, p = 0.0003, n = 38). There was no relationship between 5hmC and 5mC content. There was no difference in the medians of age (p = 0.25), 5hmC (p = 0.33) and 5mC (p = 0.86) between men/women, nor in the medians of age (p = 0.39), 5hmC (p = 0.99) and 5mC (p = 0.22) between the left/right side of the tooth extraction.

Conclusion: An inverse correlation between 5hmC and age was established, with no correlation between 5mC and 5hmC content in DF. The biological meaning of such a decrease of global DNA hydroxymethylation with age in DF remains to be clarified.

1. Introduction

Cases of impacted teeth are highly prevalent worldwide, showing an approximate prevalence of 16% in the adult population, with the third molars being the most frequently impacted teeth (Al-Zoubi, Alharbi, Ferguson, & Zafar, 2017; Dachi & Howell, 1961). Dental follicles (DF) or pericoronal follicular tissue are composed of epithelial (reduced enamel organ) and ectomesenchymal structures that surround the crown of the tooth after enamel formation. DFs are retained in cases of impacted teeth and can be studied when extracting third molars or other impacted teeth. Despite the high frequency of impacted teeth, there exists a paucity of research regarding DF biological changes.

After enamel formation, DF reduced epithelium suffers squamous metaplasia, while the remnants of the dental lamina tend to disappear with age (de Mello Palma et al., 2018; Stanley, Krogh, & Pannkuk, 1965). In retrospective studies of DF submitted for histopathological

Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; DF, dental follicles; ELISA, enzyme-linked immunosorbent assay; gDNA, genomic DNA; LOESS, locally estimated scatterplot smoothing; UFMG, Universidade Federal de Minas Gerais

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evaluation, odontogenic cysts and tumors are eventually diagnosed in addition to carcinomas. This indicates the significant role of these structures in the development of significant and even life-threatening diseases (Curran, Damm, & Drummond, 2002; Eversole, 1999; Slater, 2009; Yildirim, Ataoğlu, Mihmanli, Kiziloğlu, & Avunduk, 2008). A recent systematic review and meta-analysis concluded that the prevalence of odontogenic cysts and tumors associated with impacted third molars is 5.3%, and this prevalence is higher among individuals older than 40 years (Mello, Melo, Kammer, Speight, & Rivero, 2019).

Some advocate the elective removal of impacted teeth based on the significant risk of destructive cysts and tumor development from DF tissues (Assael, 2002). Additionally, the incidence of both locally destructive and life-threatening diseases originating from DF tissues increases with age. On this basis, the early removal of impacted teeth is desirable, and histopathological evaluation of the associated DF is highly recommended (Assael, 2002; Curran et al., 2002; Mello et al., 2019; Slater, 2009; Yildirim et al., 2008). However, managing impacted teeth is a clinical dilemma, and oral surgeons are frequently discouraged from sending DF for pathologic examination by insurance companies that may refuse to pay the pathology bill (Assael, 2002; Carl, Goldfarb, & Finley, 1995; Curran et al., 2002). Although it is known that the frequency of pathologic lesions in DF increases with aging, published information on the genetic and epigenetic background of DF is unavailable, and it remains unknown if there are considerable changes in this profile with aging. The occurrence of genetic and epigenetic alterations in DF with subjects' aging has never been explored, thereby remaining completely obscure.

We have previously assessed the presence of three hotspot mutations, *BRAF* p.V600E, *KRAS* p.G12 V, and p.G12R in DF associated with asymptomatic impacted teeth. These mutations are highly frequent in two epithelial odontogenic tumors—ameloblastoma and adenomatoid odontogenic tumor—and we hypothesized that they could be detected in some DF. However, our hypothesis was not confirmed since we did not identify these mutations (Coura et al., 2019).

Besides somatic mutations, epigenetic events, including DNA methylation, lead to inappropriate expression or silencing of genes without a change in their coding sequence (Egger, Liang, Aparicio, & Jones, 2004; Holliday & Pugh, 1975). Epigenetic patterns change during the aging process (Christensen et al., 2009) and, notably, comparing newborn CpG methylation and centenarian methylomes, a trend towards demethylation over a lifetime has been reported (Heyn et al., 2012). DNA global hypomethylation is usually accompanied by local hypermethylation during aging (Jung & Pfeifer, 2015). Some studies have investigated the methylation pattern of targeted genes in DF and observed the presence of methylation in the *P16, P27,* and *RB1* genes (Moreira et al., 2009). However, the pattern of global DNA methylation in DF has not been previously investigated. Methylation of the C^5 position of cytosine residues (5-methylcytosine, 5mC) in DNA has been recognized as an epigenetic silencing mechanism of fundamental importance (Holliday & Pugh, 1975; Valinluck & Sowers, 2007). 5-hydroxymethylcytosine (5hmC) results from the oxidation of 5mC, and its levels are approximately 0.1% in mammalian tissues, with great variation (Globisch et al., 2015). The functional significance of 5hmC as an epigenetic mark is poorly understood, but it seems to be associated with activated transcription (Breiling & Lyko, 2015).

Since alterations occur in the global methylation profile of different tissues with advancing age, we compared the global methylation and hydroxymethylation profiles in DF samples from young and older individuals. We hypothesized that the global DNA methylation and hydroxymethylation in DF can be changed throughout the aging process.

2. Materials and Methods

2.1. Sample selection and clinical data

The study was approved by the Ethics Committee of the Federal University of Minas Gerais (UFMG) (protocol 2.144.768). Written informed consent was obtained from all patients, and the study was performed in accordance with the Declaration of Helsinki. A convenience sample of DF associated with impacted lower third molars was obtained from 59 healthy individuals, irrespective of sex or age, or inclusion position at the Oral Surgery Clinics of UFMG. Individuals who did not want to participate in the study, reported antibiotics or anti-inflammatory use in the past six months, were smokers, showed systemic alterations or were immunocompromised, and cases of

Table 1
Clinical data of the 59 samples of dental follicles associated with impacted lower third molars.

Sample #	Clinical Information		
	Age	Sex	Side
1	21	Female	Right
2	16	Female	Right
3	25	Female	Left
4	20	Female	Left
5	23	Female	Left
6	17	Female	Right
7	17	Male	Left
8	17	Female	Right
9	18	Male	Right
10	17	Female	Right
11	19	Female	Left
12	16	Male	Left
13	20	Female	Right
14	30	Female	Right
15	31	Male	Right
16	27	Male	Left
17	17	Female	Left
18	17	Male	Left
19	20	Male	Right
20	17	Male	Right
21	17	Male	Right
22	17	Female	Right
23	17	Female	Right
24	20	Female	Left
25	19	Female	Right
26	16	Male	Left
27	16	Male	Right
28	15	Male	Left
29	17	Female	Right
30	19	Female	Left
31	23	Male	Right
32	17	Female	Right
33	17	Female	Left
34	23	Female	Left
35	25	Female	Left
36	19	Female	Left
37	15	Female	Left
38	17	Male	Right
39	22	Female	Right
40	13	Female	Right
41	19	Male	Right
42	18	Female	Right
43	18	Female	Left
44	24	Male	Left
45	17	Male	Left
46	22	Female	Right
47	18	Female	Left
48	27	Female	Left
49	18	Female	Left
50	17	Male	Left
51	22	Female	Left
52	24	Female	Left
53	19	Female	Right
54	22	Female	Right
55	18	Female	Right
56	17	Male	Left
57	18	Female	Left
58	30	Male	Left
59	17	Female	Right

Table 2
Percentage of DNA methylation and hydroxymethylation of the dental follicles, according to sex, age and side of extraction.

Variable	Group		n	Mean	SD	Median	Min	Max	Range	Obtained power
Hydroxymethylation %	Total		59	0.04	0.01	0	0	0.32	0-0.32	-
	Sex	Men	20	0.04	0.06	0	0	0.20	0-0.20	0.13[1]
		Women	39	0.03	0.07	0	0	0.32	0-0.32	
	Age (y.o.)	≤20	42	0.04	0.05	0	0	0.20	0-0.20	-[2]
		21-25	12	0.03	0.09	0	0	0.32	0-0.32	
		≥26	5	0.05	0.09	0.01	0	0.20	0-0.20	
	Side	Right	29	0.03	0.06	0	0	0.32	0-0.32	0.14[3]
		Left	30	0.04	0.07	0	0	0.20	0-0.20	
Methylation %	Total		59	65.82	4.31	66.89	0	100	0-100	-
	Sex	Men	20	65.86	34.76	67.44	0	100	0-100	0.05[4]
		Women	39	65.80	32.65	62.44	0	100	0-100	
	Age (y.o.)	≤20	42	70.58	30.94	74.11	0	100	0-100	-[2]
		21-25	12	56.37	31.42	31.42	21.33	100	21.33-100	
		≥26	5	48.53	49.61	49.61	0	100	0-100	
	Side	Right	29	71.32	30.31	68	0	100	0-100	0.34[5]
		Left	30	60.50	35.24	56.89	0	100	0-100	

[1] Sample size required to reach 80% power would be 813 women and 417 men (using 1.95 women: men observed ratio).
[2] Categorized age is presented for descriptive purposes only. Data analysis was performed using age as continuous variable. (Cohen, 1983).
[3] Sample size required to reach 80% power would be 560 right and 544 left teeth (using 1.03 right left observed ratio).
[4] Sample size required to reach 80% power would be 6,032,823 women and 3,093,755 men (using 1.95 women: men observed ratio).
[5] Sample size required to reach 80% power would be 123 right and 119 left teeth (using 1.03 right left observed ratio).

mandibular 3rd molars erupted or with semi-inclusion were excluded from the study.

Each DF sample was cut into two parts. One fragment was formalin-fixed paraffin-embedded for diagnosis confirmation. The other piece was stored in a tube containing 1 mL of nucleic acid preservation solution (RNAholder, BioAgency, Brazil) and stored at -80°C until the time of sample processing. Clinical data including age, sex, and location of the teeth were obtained from all patients.

2.2. DNA isolation

Genomic DNA (gDNA) was isolated from DF using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. gDNA concentration (ng/μL), as well as its purity considering the absorbance ratios A_{260nm}/A_{280nm} of ~1.8 and A_{260nm}/A_{230nm} of ~2.0 (Wilfinger, Mackey, & Chomczynski, 1997), were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The DNA was stored at -20 °C for subsequent analysis.

2.3. Global DNA methylation

The levels of 5mC and 5hmc were evaluated by submitting 100 ng of gDNA of each sample to an enzyme-linked immunosorbent assay (ELISA). The ELISA assay was carried out using the Quest 5mC™ DNA ELISA Kit (catalog number D5325) and Quest 5hmC™ DNA ELISA Kit (catalog number D5425), both from Zymo Research Corp. (Irvine, CA, USA), following the manufacturer's protocol. For each ELISA assay, 5mC and 5hmC, all samples were evaluated at the same time and in the same experiment. Colorimetric analysis was performed to identify the percentage of DNA methylation and hydroxymethylation of each sample. These percentual values were obtained based on the standard curve provided by the kits and the individual absorbance. To determine the absorbance of each sample, an Epoch™Microplate Spectrophotometer (BioTek, Winooski, USA) was used with absorbance read at 405 nm. Global DNA methylation and hydroxymethylation were expressed as the percentage of 5mc and 5hmc, respectively.

2.4. Statistical analyses

Descriptive and inferential statistics were carried out on R (version 3.6.1). Locally estimated scatterplot smoothing (LOESS) were created to evaluate the relationship between numerical variables. Spearman correlation coefficient was estimated where a linear association was observed. Normality was evaluated using Shapiro-Wilk test. Median differences of 5mC and 5hmC between sex and side of extraction of DF tissue were analyzed using Mann-Whitney test for independent samples. The level of statistical significance was set at 0.05. *A priori* and *post hoc* power analysis was conducted on G-power version 3.1.9.4 using observed ratios and effect-sizes to calculate sample size to reach 80% power and obtained power, respectively.

3. Results

The clinical data of the individuals from whom DF were collected are shown in Table 1. The mean age of the 59 individuals was 19.56 ± 3.92 (range 13-31) years, and most were women (n = 39). A similar proportion of cases occurred in each mandibular side (left n = 30; right n = 29). The median value of global DNA hydroxymethylation (5hmC) of all DF samples was zero, while the median value of global methylation (5mC) in DNA was 66.89% (Table 2).

Fig. 1 shows the relationship between 5hmC, 5mC, and the individuals' ages. The relationship between 5hmC *vs* age and 5mC *vs* age was not typically linear over time (Fig. 1A and B). An inversely proportional relationship was observed between 5hmC content and age up to 19 years (Spearman's correlation coefficient = -0.552, p = 0.0003, n = 38) (Fig. 1A). There was no relationship between 5hmC and 5mC content (Fig. 1C). Additionally, there was no difference in the medians of age (p = 0.25), 5hmC (p = 0.33) and 5mC (p = 0.86) between men and women (Fig. 2A and C). Similarly, there was no difference in the medians of age (p = 0.39), 5hmC (p = 0.99) and 5mC (p = 0.22) between the left and right sides of the tooth extraction (Fig. 2B and D). Obtained power ranged from 0.05 to 0.34 since reflecting low nominal differences between evaluated groups.

4. Discussion

Occasionally, DF cells for yet-unknown reasons lead to the formation of cysts and tumors, which include but are not restricted to dentigerous cysts, odontogenic keratocysts, and central epithelial odontogenic tumors, such as ameloblastomas and intraosseous squamous cells carcinomas (Araújo et al., 2014; Bodner, Manor, Shear, & van der Waal, 2011; Chretien, Carpenter, White, Harrah, & Lightbody, 1970; Colbert, Brennan, Theaker, & Evans., 2012; Gardner, 1975; Gay-Escoda, Camps-

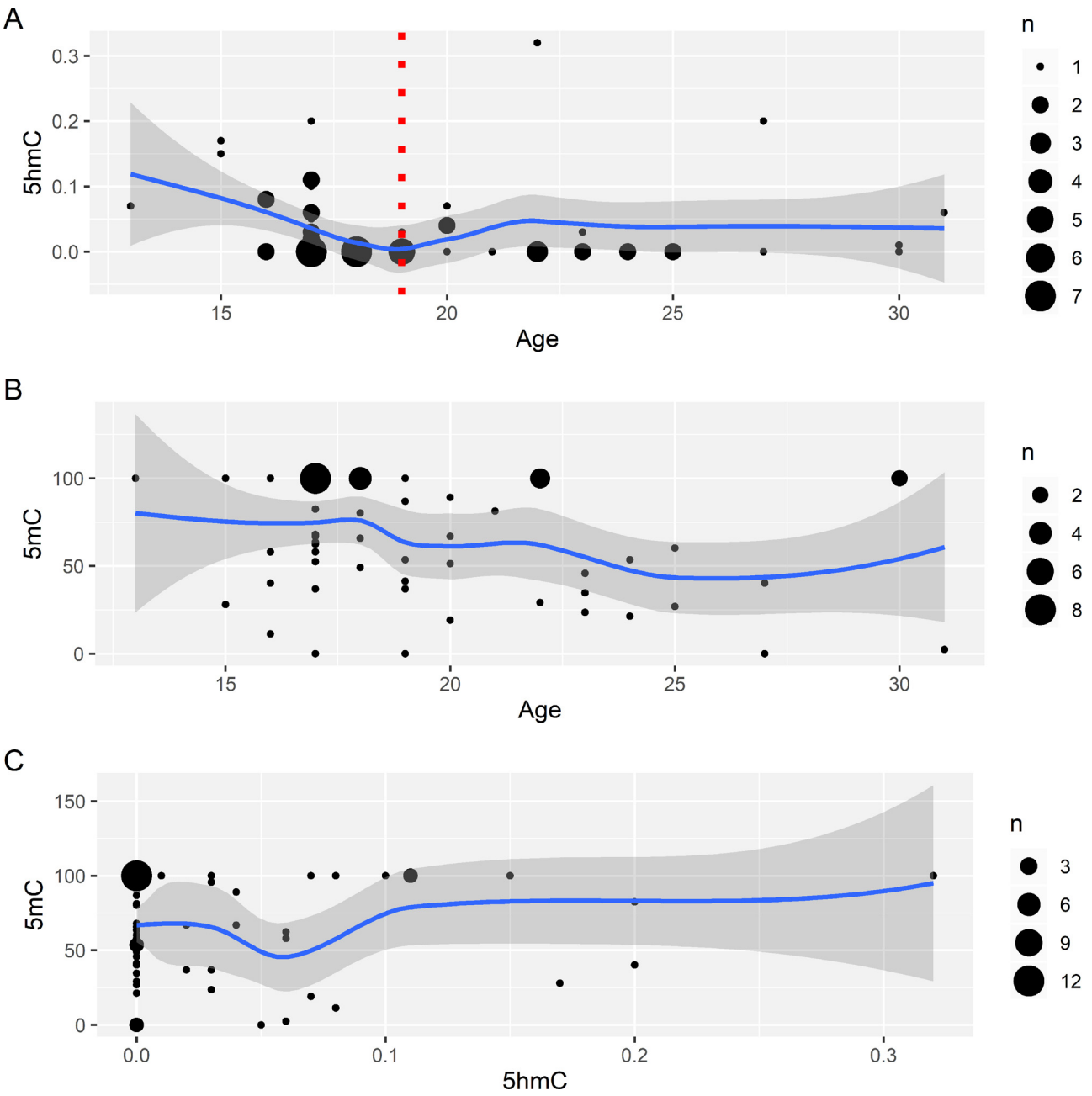

Fig. 1. Relationship between global DNA methylation, global DNA hydroxymethylation and the individuals' ages estimated using locally estimated scatterplot smoothing (blue line) and 95% confidence interval around smooth (grey area around the blue line). (A) The relationship between 5hmC and age was not linear over time (presence of sparse data points and the shape of blue curve). An inverse correlation was observed between 5hmC content and age up to 19 years (red dotted line) (Spearman's correlation coefficient = -0.552, p = 0.0003, n = 38). (B) The relationship between 5mC and age was not linear over time (presence of sparse data points and the shape of blue curve) and no correlation could be tested. (C) There was no relationship between 5hmC and 5mC content. The 5hmC and 5mC contents are expressed as percentages.

Font, López-Ramírez, & Vidal-Bel, 2015; Glosser & Campbell, 1999; Hankey & Pedler, 1957; Ide, Shimoyama, Horie, & Kaneko, 1999, Ide et al., 2008; Kramer & Scribner, 1965; Lee & Loke, 1967; Manganaro, Cross, & Startzell, 1997; Meadow, 1966; Panneerselvam, Parameswaran, Kavitha, & Panneerselvam, 2017; Reichart & Philipsen, 2004; Sciubba, Fantasia, & Kahn, 2001; Stephens, Kogon, & Reid, 1989; Waldron & Mustoe, 1989; Williams & Newman, 1963). Evidence of pathologic transformation of the DF tissues is derived either from case reports of cysts and tumors originating in these tissues or from observational clinicopathological investigations (Satheesan, Tamgadge, Tamgadge, Bhalerao., & Periera, 2016). The incidence of pathological alterations is higher among older individuals compared to younger ones (Baykul, Saglam, Aydin, & Başak, 2005), with a strong correlation between the incidence of cystic alterations in follicular tissues and age (Adelsperger, Campbell, Coates, Summerlin, & Tomich, 2000).

Aging is one of the main known risk factors for diseases since epigenetic patterns change over time (Lopez, Fernández, & Fraga, 2017). Age plays an important role in the changes that occur in DNA methylation patterns (Romanov & Vanyushin, 1981; Knapowski, Wieczorowska-tobis, & Witowski, 2002), which are related to the aberrant expression or silencing of genes (Sharma, Kelly, & Jones, 2010). DNA methylation is often dysregulated in tumor cells (Yoo & Jones, 2006), including odontogenic tumors (Moreira et al., 2009). Furthermore, dynamic changes in 5mC and 5hmC may be critical for

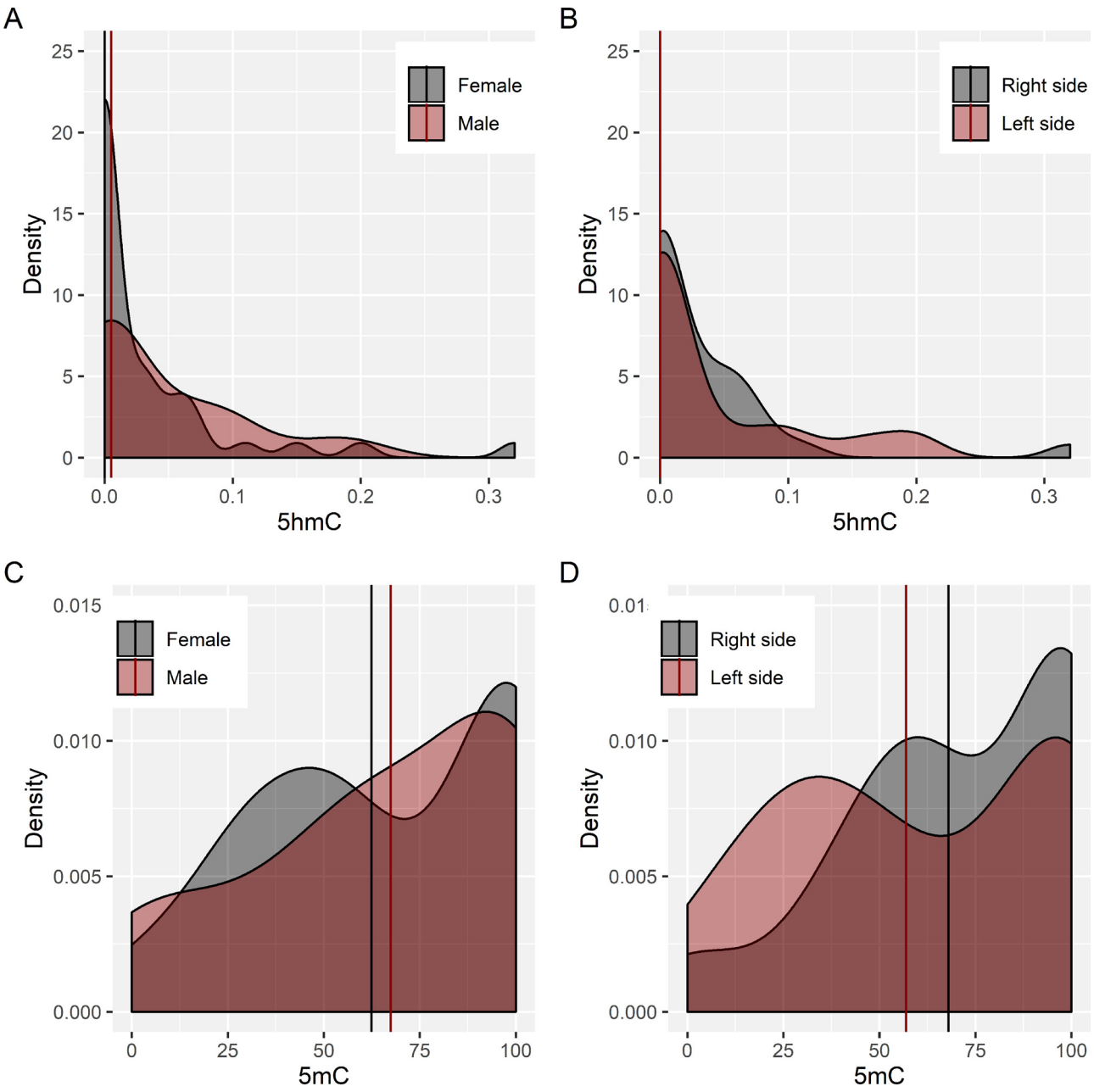

Fig. 2. Distribution of DNA methylation and global DNA hydroxymethylation across sex and side of extraction of impacted teeth/dental follicles. Vertical lines represent median values of 5hmC or 5mC percentage based on sex or side of extraction. (A) 5hmC median does not appear to be different according to sex (Mann-Whitney test, p = 0.33). (B) 5hmC median does not appear to be different according to the side of tooth extraction (Mann-Whitney test, p = 0.99). The median value lines are overlapped at 0. (C) 5mC median does not appear to be different according to sex (Mann-Whitney test, p = 0.86). (D) 5mC median does not appear to be different according to the side of tooth extraction (Mann-Whitney test, p = 0.22).

the regulation of amelogenesis (Yoshioka, Minamizaki, & Yoshiko, 2015).

In the present study, the global DNA methylation and hydroxymethylation patterns were evaluated in DF associated with impacted third molars. The global DNA methylation (percentage of 5mC) and global DNA hydroxymethylation (percentage of 5hmC) and the correlation between the values of both with age were tested in order to search for changes in this pattern during aging. An inverse correlation was observed between 5hmC content and age up to 19 years. This decline in 5hmC with aging is consistent with the established phenomenon of age-associated global hypo-hydroxymethylation previously observed in humans (Buscarlet, Tessier, Provost, Mollica, & Busque, 2016; Toraño et al., 2016; Xiong et al., 2015) as well as in mice (Kochmanski, Marchlewicz, Cavalcante, Sartor, & Dolinoy, 2018). Others, however, observed a positive correlation between age and 5hmC levels in healthy patients' blood (Jiang et al., 2017). This discrepancy may be due to several factors that are known to influence methylation, but most probably reflects the highly variable global contents of 5hmC content in different normal human tissues and cell lines previously reported (Kriaucionis & Heintz, 2009; Nestor et al., 2012). Notably, high 5hmC levels are observed in the brain (Kriaucionis & Heintz, 2009). Of note, DNA hydroxymethylation represents a strong and reproducible mark of chronological age (Xiong et al., 2015). In DF, it seems that the decrease in global DNA hydroxymethylation with age is also a remarkable phenomenon, however, its biological implications remain to be clarified.

Regarding global DNA methylation, a reduction in global 5mC levels from birth to old age has been previously reported (Buscarlet et al.,

2016). This decline is a well-established phenomenon of age-associated global hypomethylation previously observed in humans (Gonzalo, 2010). In our study, the percentage of 5mC and age was not associated with time, precluding the correlation evaluation.

Global DNA methylation and hydroxymethylation were not correlated in our study. This result is in line with previous studies that also did not observe a correlation of 5mC and 5hmC in blood and normal tissues (Godderis et al., 2015; Nestor et al., 2012). When comparing sex (women *vs.* men) with the percentage of 5hmC or 5mC, no difference was observed. Similar to our findings, other investigators did not detect any differences in sex in human cells, blood or saliva from healthy volunteers (Godderis et al., 2015; Weber et al., 2005). Considering previous work showed differences in third molar agenesis frequency between right and left jaw sides (Sujon, Alam, & Rahman, 2016; Trakinienė et al., 2018), we compared the tooth extraction side (right *vs.* left) with the percentage of 5hmC or 5mC and did not observe any difference. We aimed to test the correlation of global methylation and hydroxymethylation with age in human DF. Although global DNA methylation changes with chronological aging, global DNA methylation and hydroxymethylation can also be influenced by environmental exposures, including chemical and nonchemical stressors, in addition to nutritional factors (Dao, Cheng, Revelo, Mitzner, & Tang, 2014; Martin & Fry, 2018), which could not be addressed in our study.

Global DNA hydroxymethylation and methylation in DF have not been previously investigated. Besides global DNA methylation/hydroxymethylation changes during aging, gene-specific hypermethylation can lead to the silencing of several genes, including those responsible for cell cycle control (Burzynski, 2005). There are studies that investigated DNA methylation in target genes in odontogenic tumors (Costa et al., 2017; Gomes, Brito, Andrade, & Gomez, 2010; Moreira et al., 2009), and few studies have evaluated specific DNA methylation patterns of genes in DF (Ai et al., 2018; Gopinathan, Kolokythas, Luan, & Diekwisch, 2013; Li et al., 2018; Moreira et al., 2009).

In the present DF cohort, the relationship between 5mC and 5hmC with age was not linear over time, and an inverse correlation between DNA hydroxymethylation and age (up to 19 years) was found. This age-dependent decrease in 5hmC levels observed was not accompanied by changes in the levels of 5mC, as there was no correlation between 5mC and 5hmC content in DF. In conclusion, we provide evidence of global DNA hydroxymethylation changes with age in human DF. Further studies are necessary to determine the biological significance of decreased global DNA hydroxymethylation with age in DF.

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CRediT authorship contribution statement

Vinícius César Barbosa de Menezes: Data curation, Investigation, Writing - original draft. **Elisa Carvalho de Siqueira:** Investigation, Writing - original draft. **Sara Ferreira dos Santos Costa:** Investigation, Writing - review & editing. **Fabrício Tinôco Alvim de Souza:** Investigation, Writing - review & editing. **Renan Pedra de Souza:** Formal analysis, Methodology, Writing - review & editing. **Ricardo Santiago Gomez:** Conceptualization, Formal analysis. **Carolina Cavaliéri Gomes:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Adelsperger, J., Campbell, J. H., Coates, D. B., Summerlin, D. J., & Tomich, C. E. (2000). Early soft tissue pathosis associated with impacted third molars without pericoronal radiolucency. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 89*(4), 402–406. https://doi.org/10.1016/s1079-2104(00)70119-3.

Ai, T., Zhang, J., Wang, X., Zheng, X., Qin, X., Zhang, Q., ... Chen, F. (2018). DNA methylation profile is associated with the osteogenic potential of three distinct human odontogenic stem cells. *Signal Transduct Target Ther, 12*, 1. https://doi.org/10.1038/s41392-017-0001-6.

Al-Zoubi, H., Alharbi, A. A., Ferguson, D. J., & Zafar, M. S. (2017). Frequency of impacted teeth and categorization of impacted canines: A retrospective radiographic study using orthopantomograms. *Eur J Dent, 11*(1), 117–121. https://doi.org/10.4103/ejd.ejd_308_16.

Araújo, J. P., Kowalski, L. P., Rodrigues, M. L., de Almeida, O. P., Lopes Pinto, C. A., & Alves, F. A. (2014). Malignant transformation of an odontogenic cyst in a period of 10 years. *Case Rep Dent, 2014*, Article 762969. https://doi.org/10.1155/2014/762969.

Assael, L. A. (2002). Impacted teeth: reflections on Curran, Kugelberg, and Rood. *J Oral Maxillofac Sur, 60*(6), 611–612. https://doi.org/10.1053/joms.2002.33624.

Baykul, T., Saglam, A. A., Aydin, U., & Başak, K. (2005). Incidence of cystic changes in radiographically normal impacted lower third molar follicles. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 99*(5), 542–545. https://doi.org/10.1016/j.tripleo.2004.08.010.

Bodner, L., Manor, E., Shear, M., & van der Waal, I. (2011). Primary intraosseous squamous cell carcinoma arising in an odontogenic cyst: a clinicopathologic analysis of 116 reported cases. *J Oral Pathol Med, 40*(10), 733–738. https://doi.org/10.1111/j.1600-0714.2011.01058.x.

Breiling, A., & Lyko, F. (2015). Epigenetic regulatory functions of DNA modifications: 5-methylcytosine and beyond. *Epigenetics Chromatin, 8*, 24. https://doi.org/10.1186/s13072-015-0016-6.

Burzynski, S. R. (2005). Aging: gene silencing or gene activation? *Med Hypothese, 64*(1), 201–208. https://doi.org/10.1016/j.mehy.2004.06.010.

Buscarlet, M., Tessier, A., Provost, S., Mollica, L., & Busque, L. (2016). Human blood cell levels of 5-hydroxymethylcytosine (5hmC) decline with age, partly related to acquired mutations in TET2. *Exp Hematol, 44*(11), 1072–1084. https://doi.org/10.1016/j.exphem.2016.07.009.

Carl, W., Goldfarb, G., & Finley, R. (1995). Impacted teeth: prophylactic extractions or not? *N Y State Dent J, 61*(1), 32–35.

Chretien, P. B., Carpenter, D. F., White, N. S., Harrah, J. D., & Lightbody, P. M. (1970). Squamous carcinoma arising in a dentigerous cyst. Presentation of a fatal case and review of four previously reported cases. *Oral Surg Oral Med Oral Pathol, 30*(6), 809–816. https://doi.org/10.1016/0030-4220(70)90345-2.

Christensen, B. C., Houseman, E. A., Marsit, C. J., Zheng, S., Wrensch, M. R., Wiemels, J. L., ... Kelsey, K. T. (2009). Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. *PLoS Genet, 5*(8), Article e1000602. https://doi.org/10.1371/journal.pgen.1000602.

Cohen, J. (1983). The Cost of Dichotomization. *Appl Psychol Meas, 7*(3), 249–253.

Colbert, S., Brennan, P. A., Theaker, J., & Evans, B. (2012). Squamous cell carcinoma arising in dentigerous cysts. *J Craniomaxillofac Surg, 40*(8), 355–357. https://doi.org/10.1016/j.jcms.2012.01.020.

Costa, S., Pereira, N. B., Pereira, K., Campos, K., de Castro, W. H., Diniz, M. G., ... Gomez, R. S. (2017). DNA methylation pattern of apoptosis-related genes in ameloblastoma. *Oral Dis, 23*(6), 779–783. https://doi.org/10.1111/odi.12661.

Coura, B. P., de Resende, T. A. C., de Menezes, V. C. B., Bernardes, V. F., de Sousa, S. F., Diniz, M. G., ... Gomes, C. C. (2019). Assessing pathogenic mutations in dental follicles as an attempt to identify early events in odontogenic tumours tumourigenesis. *Arch Oral Biol, 14*, Article 104523. https://doi.org/10.1016/j.archoralbio.2019.104523.

Curran, A. E., Damm, D. D., & Drummond, J. F. (2002). Pathologically significant pericoronal lesions in adults: histopathologic evaluation. *J Oral Maxillofac Surg, 60*(6), 613–617. https://doi.org/10.1053/joms.2002.33103.

Dachi, S. F., & Howell, F. V. (1961). A survey of 3, 874 routine full-month radiographs. II. A study of impacted teeth. *Oral Surg Oral Med Oral Pathol, 14*, 1165–1169. https://doi.org/10.1016/0030-4220(61)90204-3.

Dao, T., Cheng, R. Y., Revelo, M. P., Mitzner, W., & Tang, W. (2014). Hydroxymethylation as a Novel Environmental Biosensor. *Current Environ Health Rep, 1*(1), 1–10. https://doi.org/10.1007/s40572-013-0005-5.

de Mello Palma, V., Danesi, C. C., Arend, C. F., Venturini, A. B., Blaya, D. S., Neto, M. M., ... Ferrazzo, K. L. (2018). Study of Pathological Changes in the Dental Follicle of Disease-Free Impacted Third Molars. *J Maxillofac Oral Surg, 17*(4), 611–615. https://doi.org/10.1007/s12663-018-1131-2.

Egger, G., Liang, G., Aparicio, A., & Jones, P. A. (2004). Epigenetics in human disease and prospects for epigenetic therapy. *Nature, 27*, 457–463. https://doi.org/10.1038/nature02625.

Eversole, L. R. (1999). Malignant epithelial odontogenic tumours. *Semin Diagn Pathol,*

16(4), 317–324.
Gardner, A. F. (1975). A survey of odontogenic cysts and their relationship to squamous cell carcinoma. *Dent J, 41*(3), 161–167.
Gay-Escoda, C., Camps-Font, O., López-Ramírez, M., & Vidal-Bel, A. (2015). Primary intraosseous squamous cell carcinoma arising in dentigerous cyst: Report of 2 cases and review of the literature. *J Clin Exp Dent, 7*(5), 665–670. https://doi.org/10.4317/jced.52689.
Globisch, D., Munzel, M., Muller, M., Michalakis, S., Wagner, M., Koch, S., ... Carell, T. (2015). Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. *PLoS One, 5*(12), Article e15367. https://doi.org/10.1371/journal.pone.0015367.
Glosser, J. W., & Campbell, J. H. (1999). Pathologic change in soft tissues associated with radiographically' normal' third molar impactions. *Br J Oral Maxillofac Surg, 37*(4), https://doi.org/10.1054/bjom.1999.0061 2592-60.
Godderis, L., Schouteden, C., Tabish, A., Poels, K., Hoet, P., Baccarelli, A. A., ... Van Landuyt, K. (2015). Global Methylation and Hydroxymethylation in DNA from Blood and Saliva in Healthy Volunteers. *Biomed Res Int, 2015*, Article 845041. https://doi.org/10.1155/2015/845041.
Gomes, C. C., Brito, J. A., Andrade, C. I., & Gomez, R. S. (2010). DNA methyltransferase expression in odontogenic cysts and tumours. *Oncol Lett, 1*(1), 143–146. https://doi.org/10.3892/ol_00000026.
Gonzalo, S. (2010). Epigenetic alterations in aging. *Journal of applied physiology (1985), 109*(2), 586–597. https://doi.org/10.1152/japplphysiol.00238.2010.
Gopinathan, G., Kolokythas, A., Luan, X., & Diekwisch, T. G. (2013). Epigenetic marks define the lineage and differentiation potential of two distinct neural crest-derived intermediate odontogenic progenitor populations. *Stem Cells Dev, 22*(2), 1763–1778. https://doi.org/10.1089/scd.2012.0711.
Hankey, G. T., & Pedler, J. A. (1957). Primary squamous-cell carcinoma of mandible arising from epithelial lining of dental cyst. *Proc R Soc Med, 50*(9), 680–681.
Heyn, H., Li, N., Ferreira, H. J., Moran, S., Pisano, D. G., Gomez, A., ... Esteller, M. (2012). Distinct DNA methylomes of newborns and centenarians. *Proc Natl Acad Sci U S A, 109*(26), 10522–10527. https://doi.org/10.1073/pnas.1120658109.
Holliday, R., & Pugh, J. E. (1975). DNA modification mechanisms and gene activity during development. *Science, 187*, 226–232.
Ide, F., Mishima, K., Yamada, H., Horie, N., Saito, I., Shimoyama, T., ... Kusama, K. (2008). Unsuspected small ameloblastoma in the alveolar bone: a collaborative study of 14 cases with discussion of their cellular sources. *J Oral Pathol Med, 37*(4), 221–227. https://doi.org/10.1111/j.1600-0714.2007.00628.x.
Ide, F., Shimoyama, T., Horie, N., & Kaneko, T. (1999). Primary intraosseous carcinoma of the mandible with probable origin from reduced enamel epithelium. *J Oral Pathol Med, 28*(9), 420–422. https://doi.org/10.1111/j.1600-0714.1999.tb02114.x.
Jiang, T., Zong, L., Zhou, L., Hou, Y., Zhang, L., Zheng, X., ... Zhao, C. (2017). Variation in global DNA hydroxymethylation with age associated with schizophrenia. *Psychiatry Res, 257*, 497–500. https://doi.org/10.1016/j.psychres.2017.08.022.
Jung, M., & Pfeifer, G. P. (2015). Aging and DNA methylation. *BMC Biol, 31*, 13–17. https://doi.org/10.1186/s12915-015-0118-4.
Knapowski, J., Wieczorowska-Tobis, K., & Witowski, J. (2002). Pathophysiology of ageing. *J Physiol Pharmacol, 53*(2), 135–146.
Kochmanski, J., Marchlewicz, E. H., Cavalcante, R. G., Sartor, M. A., & Dolinoy, D. C. (2018). Age-related epigenome-wide DNA methylation and hydroxymethylation in longitudinal mouse blood. *Epigenetics, 13*(7), 779–792. https://doi.org/10.1080/15592294.2018.1507198.
Kramer, H. S., & Scribner, J. H. (1965). Squamous-cell carcinoma arising in a dentigerous cyst. Report of a case. *Oral Surg Oral Med Oral Pathol, 19*, 555–561. https://doi.org/10.1016/0030-4220(65)90019-8.
Kriaucionis, S., & Heintz, N. (2009). The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science, 15*, 929–930. https://doi.org/10.1126/science.1169786.
Lee, K. W., & Loke, S. J. (1967). Squamous cell carcinoma arising in a dentigerous cyst. *Cancer, 20*(12), 2241–2244. https://doi.org/10.1002/1097-0142(196712)20:12<2241::aid-cncr2820201226>3.0.co;2-f.
Li, Q., Guo, Y., Yao, M., Li, J., Chen, Y., Liu, Q., ... Feng, Y. (2018). Methylation of Cdkn1c may be involved in the regulation of tooth development through cell cycle inhibition. *J Mol Histol, 49*(5), 459–469. https://doi.org/10.1007/s10735-018-9785-0.
Lopez, V., Fernández, A. F., & Fraga, M. F. (2017). The role of 5-hydroxymthylcytosine in development, aging and age-related diseases. *Ageing Res Rev, 37*, 28–38. https://doi.org/10.1016/j.arr.2017.05.002.
Manganaro, A. M., Cross, S. E., & Startzell, J. M. (1997). Carcinoma arising in a dentigerous cyst with neck metastasis. *Head Neck, 19*(5), 436–439. https://doi.org/10.1002/(sici)1097-0347(199708)19:5<436::aid-hed12>3.0.co;2-5.
Martin, E. M., & Fry, R. C. (2018). Environmental Influences on the Epigenome: Exposure-Associated DNA Methylation in Human Populations. *Annu Rev Pub Health, 39*, 309–333. https://doi.org/10.1146/annurev-publhealth-040617-014629.
Meadow, S. R. (1966). Malignant change in a dental cyst. *Oral Surg Oral Med Oral Pathol, 21*(3), 282–285. https://doi.org/10.1016/0030-4220(66)90058-2.
Mello, F. W., Melo, G., Kammer, P. V., Speight, P. M., & Rivero, E. R. C. (2019). Prevalence of odontogenic cysts and tumors associated with impacted third molars: A systematic review and meta-analysis. *J Craniomaxillofac Surg, 47*(6), 996–1002. https://doi.org/10.1016/j.jcms.2019.03.026.
Moreira, P. R., Guimarães, M. M., Gomes, C. C., Diniz, M. G., Brito, J. A., de Castro, W. H., ... Gomez, R. S. (2009). Methylation frequencies of cell-cycle associated genes in epithelial odontogenic tumours. *Arch Oral Biol, 54*(10), 893–897. https://doi.org/10.1016/j.archoralbio.2009.07.006.
Nestor, C. E., Ottaviano, R., Reddington, J., Sproul, D., Reinhardt, D., Dunican, ... Meehan, R. R. (2012). Tissue type is a major modifier of the 5-hydroxymethylcytosine content of human genes. *Genome Res, 22*(3), 467–477. https://doi.org/10.1101/gr.126417.111.
Panneerselvam, K., Parameswaran, A., Kavitha, B., & Panneerselvam, E. (2017). Primary intraosseous squamous cell carcinoma in a dentigerous cyst. *South Asian J Cancer, 6*(3), 105. https://doi.org/10.4103/2278-330X.214579.
Reichart, P. A., & Philipsen, H. P. (2004). *Odontogenic tumors and allied lesions.* London: Quintessence Publishing Co25–270.
Romanov, G. A., & Vanyushin, B. F. (1981). Methylation of reiterated sequences in mammalian DNAs. Effects of the tissue type, age, malignancy and hormonalinduction. *Biochim Biophys Acta, 27*, 204–218. https://doi.org/10.1016/0005-2787(81)90156-8.
Satheesan, E., Tamgadge, S., Tamgadge, A., Bhalerao, S., & Periera, T. (2016). Histopathological and Radiographic Analysis of Dental Follicle of Impacted Teeth Using Modified Gallego's Stain. *J Clin Diagn Res, 10*(5), 106–111. https://doi.org/10.7860/JCDR/2016/16707.7838.
Sciubba, J. J., Fantasia, J. E., & Kahn, L. B. (2001). *Atlas of tumor pathology, 3rd series: tumors and cysts of the jaw.* Washington, DC: Armed Forces Institute of Pathology71–127.
Sharma, S., Kelly, T. K., & Jones, P. A. (2010). Epigenetics in cancer. *Carcinogenesis, 31*(1), 27–36. https://doi.org/10.1093/carcin/bgp220.
Slater, L. J. (2009). Comments on "Pathologic changes in the soft tissues associated with asymptomatic impacted third molars". *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 107*(1), 5. https://doi.org/10.1016/j.tripleo.2008.08.055.
Stanley, H. R., Krogh, H., & Pannkuk, E. (1965). Age changes in the epithelial components of follicles (dental sacs) associated with impacted third molars. *Oral Surg Oral Med Oral Pathol, 19*, 128–139. https://doi.org/10.1016/0030-4220(65)90226-4.
Stephens, R. G., Kogon, S. L., & Reid, J. A. (1989). The unerupted or impacted third molar–a critical appraisal of its pathologic potential. *J Can Dent Assoc, 55*(3), 201–207.
Sujon, M. K., Alam, M. K., & Rahman, S. A. (2016). Prevalence of Third Molar Agenesis: Associated Dental Anomalies in Non-Syndromic 5923 Patients. *PLoS One. 11*(8), Article e0162070. https://doi.org/10.1371/journal.pone.0162070.
Toraño, E. G., Bayón, G. F., Del Real, Á., Sierra, M. I., García, M. G., Carella, A., ... Fernández, A. F. (2016). Age-associated hydroxymethylation in human bone-marrow mesenchymal stem cells. *J Transl Med, 14*(1), 207. https://doi.org/10.1186/s12967-016-0966-x.
Trakinienė, G., Šidlauskas, A., Andriuškevičiūtė, I., Šalomskienė, L., Švalkauskienė, V., Smailienė, D., ... Trakinis, T. (2018). Impact of genetics on third molar agenesis. *Scientific reports, 8*(1), 8307. https://doi.org/10.1038/s41598-018-26740-7.
Valinluck, V., & Sowers, L. C. (2007). Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. *Cancer Res, 67*(3), 946–950. https://doi.org/10.1158/0008-5472.CAN-06-3123.
Xiong, J., Jiang, H. P., Peng, C. Y., Deng, Q. Y., Lan, M. D., Zeng, H., ... Yuan, B. F. (2015). DNA hydroxymethylation age of human blood determined by capillary hydrophilic-interaction liquid chromatography/mass spectrometry. *Clin Epigenetics, 23*, 72. https://doi.org/10.1186/s13148-015-0109-x.
Waldron, C. A., & Mustoe, T. A. (1989). Primary intraosseous carcinoma of the mandible with probable origin in an odontogenic cyst. *Oral Surg Oral Med Oral Pathol, 67*(6), 716–724. https://doi.org/10.1016/0030-4220(89)90014-5.
Weber, M., Davies, J. J., Wittig, D., Oakeley, E. J., Haas, E. M., Lam, W. L., ... Schübeler, D. (2005). Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet, 37*(8), 853–862. https://doi.org/10.1038/ng1598.
Wilfinger, W. W., Mackey, K., & Chomczynski, P. (1997). Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity. *Biotechniques, 22*(3), 474–481. https://doi.org/10.2144/97223st01.
Williams, I. E., & Newman, C. W. (1963). Squamous-cell carcinoma associated with a dentigerous cyst of the maxilla. Review and report of a case. *Oral Surg Oral Med Oral Pathol. 16*, 1012–1016. https://doi.org/10.1016/0030-4220(63)90206-8.
Yildirim, G., Ataoğlu, H., Mihmanli, A., Kiziloğlu, D., & Avunduk, M. C. (2008). Pathologic changes in soft tissues associated with asymptomatic impacted third molars. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 106*(1), 14–18. https://doi.org/10.1016/j.tripleo.2007.11.021.
Yoo, C. B., & Jones, P. A. (2006). Epigenetic therapy of cancer: past, present and future. *Nat Rev Drug Discov, 5*(1), 37–50. https://doi.org/10.1038/nrd1930.
Yoshioka, H., Minamizaki, T., & Yoshiko, Y. (2015). The dynamics of DNA methylation and hydroxymethylation during amelogenesis. *Histochem Cell Biol, 144*(5), 471–478. https://doi.org/10.1007/s00418-015-1353-z.