



UNIVERSITÀ POLITECNICA DELLE MARCHE
Repository ISTITUZIONALE

Differential expression of nicotinamide N-methyltransferase in primary and recurrent ameloblastomas and odontogenic keratocysts

This is the peer reviewed version of the following article:

Original

Differential expression of nicotinamide N-methyltransferase in primary and recurrent ameloblastomas and odontogenic keratocysts / Mascitti, M; Sartini, D; Togni, L; Pozzi, V; Rubini, C; Santarelli, A; Emanuelli, M. - In: EUROPEAN JOURNAL OF CLINICAL INVESTIGATION. - ISSN 0014-2972. - STAMPA. - 50:4(2020). [10.1111/eci.13220]

Availability:

This version is available at: 11566/278206 since: 2024-04-12T15:02:31Z

Publisher:

Published

DOI:10.1111/eci.13220

Terms of use:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. The use of copyrighted works requires the consent of the rights' holder (author or publisher). Works made available under a Creative Commons license or a Publisher's custom-made license can be used according to the terms and conditions contained therein. See editor's website for further information and terms and conditions.

This item was downloaded from IRIS Università Politecnica delle Marche (<https://iris.univpm.it>). When citing, please refer to the published version.

(Article begins on next page)

This is the peer reviewed version of the following article: Differential expression of nicotinamide N-methyltransferase in primary and recurrent ameloblastomas and odontogenic keratocysts / Mascitti, M; Sartini, D; Togni, L; Pozzi, V; Rubini, C; Santarelli, A; Emanuelli, M. - In: EUROPEAN JOURNAL OF CLINICAL INVESTIGATION. - ISSN 0014-2972. - STAMPA. - 50:4(2020), p. 13220. © 2020 Stichting European Society for Clinical Investigation Journal Foundation. Published by John Wiley & Sons Ltd, which has been published in final form at <https://dx.doi.org/10.1111/eci.13220>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions <https://authorservices.wiley.com/author-resources/Journal-Authors/licensing/self-archiving.html>. This article may not be enhanced, enriched or otherwise transformed into a derivative work, without express permission from Wiley or by statutory rights under applicable legislation. Copyright notices must not be removed, obscured or modified. The article must be linked to Wiley's version of record on Wiley Online Library and any embedding, framing or otherwise making available the article or pages thereof by third parties from platforms, services and websites other than Wiley Online Library must be prohibited.

Differential expression of **nicotinamide N-methyltransferase** in primary and recurrent
ameloblastomas and odontogenic keratocysts

Marco Mascitti ^{1#}; Davide Sartini ^{1#}; Lucrezia Togni ¹; Valentina Pozzi ²; Corrado Rubini ³;
Andrea Santarelli ^{1,4*}; Monica Emanuelli ^{1,2}

Affiliations.

¹ Department of Clinical Specialistic and Dental Sciences, Marche Polytechnic University,
Via Tronto 10, 60126, Ancona, Italy.

² New York-Marche Structural Biology Center (NY-MaSBiC), Marche Polytechnic
University, Via Tronto 10, 60126, Ancona, Italy.

³ Department of Biomedical Sciences and Public Health, Marche Polytechnic University, Via
Tronto 10, 60126, Ancona, Italy.

⁴ Dentistry Clinic, National Institute of Health and Science of Aging, INRCA, Via Tronto 10,
60126, Ancona, Italy.

These Authors contributed equally to this paper.

Corresponding author.

Andrea Santarelli

Via Tronto 10, 60126 Ancona - Italy

Phone +39-071-2206226 Fax +39-071-2206221

e-mail: andrea.santarelli@staff.univpm.it

Word count (main text): 2247

Abstract

Background. Odontogenic tumors are a group of rare heterogeneous diseases that range from hamartomatous tissue proliferations to benign and malignant neoplasms. Recurrences can occur after 10 years, so long-term clinical and radiological follow-up is required. The study of the molecular mechanisms involved in the development of these lesions is necessary to identify new prognostic markers. In this study we evaluate the possible role of nicotinamide N-methyltransferase (NNMT) in ameloblastomas (AM) and odontogenic keratocysts (OKC).

Materials and methods. 105 surgical specimens of primary and recurrent lesions were obtained from 55 patients (25 AM, 30 OKC). In particular, 50 AMs (25 primary, 25 recurrences) and 55 OKCs (30 primary, 25 recurrences) were retrieved. We carried out immunohistochemical analyses to evaluate the cytoplasmic expression of NNMT, measuring the percentage of positive cells and the value of NNMT expression intensity.

Results. NNMT expression was significantly higher in recurrent than primary AMs ($P = 0.0430$). This result was confirmed by staining intensity, showing more cases with moderate/intense staining in recurrent AMs ($P = 0.0470$). NNMT expression was significantly lower in recurrent than primary OKC ($P = 0.0014$). Staining intensity showed more cases with moderate/intense staining in primary OKCs ($P = 0.0276$).

Conclusions. This report is the first to evaluate NNMT expression in odontogenic lesions and to demonstrate a differential expression in recurrent AMs and OKCs, suggesting that there is potential for use of NNMT as prognostic marker.

Keywords.

Odontogenic tumors; ameloblastoma; odontogenic keratocyst; NNMT; immunohistochemistry.

Introduction.

Odontogenic tumors (OT) constitute a group of heterogeneous diseases that range from hamartomatous tissue proliferations to benign and to malignant **neoplasms** with metastatic potential. They derive from epithelial, ectomesenchymal and/or mesenchymal elements that are or have been part of the tooth-forming apparatus.¹ OTs are rare tumors with unknown etiology: most of them arise ex novo, while some OTs may originate from pre-existing odontogenic cysts.^{2,3}

Recently, the research is focusing on ameloblastoma (AM), due to the high of frequency oncogenic alterations reported in literature, and on odontogenic keratocyst (OKC), since its controversial nature. AM is a benign intraosseous epithelial odontogenic neoplasm characterized by expansion and a tendency for a local recurrence if not adequately removed. AM can occur at all ages, with a peak incidence of diagnosis in the IV-V decades of life.⁴ About 80% of lesions arise in mandible, especially in posterior region. The risk of recurrence varies from 15% to 80%, based on the meticulousness of the surgical treatment.⁵ More than 50% of relapses occur within 5 years, but can also occur beyond 20-25 years.^{6,7} Odontogenic keratocyst (OKC) is an odontogenic cyst characterized by a thin, regular lining of parakeratinized stratified squamous epithelium with palisading hyperchromatic basal cells. OKC was renamed as “keratocystic odontogenic tumor” in 2005 since then it had been reclassified as benign OT. This emphasized the intrinsic growth potential of the tumoral epithelium, the higher recurrence rate and the tendency to multiplicity, to distinguish it from other odontogenic cysts.⁸ However, the 2017 WHO guideline reclassified this entity as OKC, including in odontogenic cysts group, due to the lack of enough evidence to justify its classification as a neoplasm, stressing the issue is still highly debated.²

About 5% of all OKCs occur as part of naevoid basal cell carcinoma syndrome (NBCCS). These cases tend to be multiple and to arise in younger patients. The mandibula is the most affected site, especially its posterior region.

Because of the peak incidence in young adults, the high recurrence rate, local aggressiveness and lack of standardized management for OTs, it would be desirable to investigate the molecular mechanisms involved in their development. New sensitive molecular biomarkers and targets for molecular-based treatments are necessary to improve the diagnosis and prognosis of OTs. The present study focused on the enzyme nicotinamide N-methyltransferase (NNMT, EC 2.1.1.1), since its biological significance in odontogenic lesions has not examined yet.

NNMT is an S-adenosyl-L-methionine (SAM)-dependent cytoplasmatic enzyme, belonging to Phase II Metabolism.⁹ It is involved in the hepatic biotransformation and detoxification of many drugs and xenobiotics. In particular, NNMT catalyzes the N-methylation of nicotinamide and other structural related compounds as pyridines.^{10,11}

Although NNMT is mainly expressed in the liver, a low expression has been also detected in the kidney, lung, skeletal muscle, placenta, heart, and brain.⁹ Overexpression of NNMT has been reported in many cancers, such as glioblastoma, renal, lung and oral malignancies, suggesting that the enzyme may play an important role in malignant transformation.¹²⁻¹⁵ NNMT overexpression affects cell resistance to radiation-induced damage¹⁶ and is positively correlated with enhanced cancer cell migration and poor prognosis.^{17,18} These data seem to suggest the potential prognostic role of NNMT in several neoplasms. However, the function of NNMT in cancer cell metabolism is yet unclear.

The aims of the present retrospective study were to evaluate for the first time the NNMT expression in two odontogenic lesions, namely ameloblastoma (AM) and OKC, and to assess its role as prognostic marker of recurrence.

Materials and Methods.

Case selection

This retrospective study considered a total of 105 surgical samples obtained from a cohort of 55 randomly selected patients with AM (n. = 25) or OKC (n. = 30), who were treated at the Department of Maxillofacial Surgery, “Ospedali Riuniti” General Hospital, Ancona, Italy, between 1994 and 2017. In particular, 50 AMs (25 primary and 25 recurrent lesions) and 55 OKCs (30 primary and 25 recurrent lesions) were retrieved from the archives of the Institute of Pathology, Marche Polytechnic University, Ancona, Italy, by a single operator (L.T.), to ensure uniformity of the collected data. All patients received surgical treatment with curative intention. The clinical-pathological data of each patients had been collected and cataloged from clinical records of Department of Surgical and Special Odontostomatology and by the Department of Maxillofacial Surgery, Ospedali Riuniti of Ancona. Each sample was histologically re-evaluated to confirm the original diagnosis and reclassified according to the 4th Edition of the World Health Organization Classification of Head and Neck Tumors.² The study was conducted in accordance with the “Ethical Principles for Medical Research Involving Human Subjects” statement of the Helsinki Declaration. This study received exemption from the institutional review board because of its retrospective nature.

Immunohistochemical Analyses

For each case, 4- μ m serial sections from formalin-fixed, paraffin-embedded blocks of representative lesions areas were cut. Only sections containing sufficient odontogenic epithelium to assess the antibody reactivity with 1000 cells were considered for the investigation. Immunohistochemistry was performed on histological sections mounted on poly-L-lysine coated glass slides.

The sections were incubated with PT-Link, Low pH, (Dako, Agilent Technologies) to deparaffinization, rehydration and epitope retrieval, for 30 minutes at 97°C. After rinsing, the slides were transferred to the Autostainer Link 48 instrument (Dako, Agilent), for the immunohistochemical staining. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide for 7 minutes. Sections were rinsed with Tris-Buffered Saline (TBS) and incubated with rabbit polyclonal anti-human NNMT antibody, (Sigma-Aldrich, St. Louis, Missouri, USA), dilution 1:1500, or mouse monoclonal anti-human Ki-67 antibody (clone MIB-1; Dako, Glostrup, Denmark) dilution 1:50, for one hour in a humidified chamber, at room temperature.

The sections were washed three times with TBS and were incubated with Dako EnVision™ FLEX Horseradish Peroxidase (HRP), for 20 minutes. After rinsing, the sections were incubated with Dako EnVision™ FLEX 3.3'-Diaminobenzidine (DAB), for 10 minutes. Slides were rinsed with distilled water, manually counterstained with Mayer's hematoxylin (Bio-Optica, Milano, Italy) for 3-5 minutes, dehydrated, mounted with permanent mounting medium and examined by light microscopy. Positive controls consisted of tissue specimen sections of clear cell renal cell carcinoma, with known antigenic reactivity. A negative control was performed in all cases by substituting the primary antibody with normal mouse serum. Negative controls in all instances resulted in a negative immunoreactivity for NNMT. NNMT cytoplasmic staining of epithelial cells was evaluated, as previously reported.¹⁵ To evaluate NNMT expression, the mean percentage of positive cells was determined from the analysis of 100 cells in 10 random areas at x40 magnification. The percentage of positive cells was estimated per tenfold, from 0% to 100%, per image-field. For each patient, the mean value of scored percentages was considered. The staining intensity of NNMT was scored and reported into a dichotomous scale: “-” (no staining observed or incomplete, weak or barely perceptible cellular staining); and “+” (complete, moderate to intense,

circumferential cellular staining). The expression of Ki-67 was evaluated by assessing the percentage of cells displaying a positive-stained nucleus (estimated per tenfold, from 0% to 100%) and mean value of scored percentages was considered.

Two expert pathologists (C.R. and M.M.) independently assessed the positivity for NNMT, blinded to the clinical and pathological data. Each specimen was analyzed three times.

Statistical Analysis

Data were analyzed using GraphPad Prism software version 7.00 for Windows (GraphPad Software, San Diego, CA, USA). Data were presented as mean \pm SD.

Differences were determined using Mann-Whitney U test when 2 groups were compared, whereas Kruskal-Wallis test with Dunn post hoc test was used when 3 groups were compared. The χ^2 test and the Fisher exact test were used for grouped variables (staining intensity). Correlations between NNMT expression levels and continuous variables (age, size) were analyzed using the Spearman correlation coefficients. Weighted Cohen's kappa was used to determine interobserver agreement. A P value < 0.05 was accepted as statistically significant.

Results.

In this study, 105 surgical samples of odontogenic lesions were analyzed. Demographic and clinicopathological data of AM and OKC groups are listed in Table 1. AM group included 25 patients: 17 males and 8 females, with a male to female ratio of 2.1:1. The mean age was 51.4 years old (range 16-82). A total of 50 surgical samples of AM were collected (25 primary and 25 recurrences).

The mandible was the most affected site (mandible to maxilla ratio of 2.1:1). The size range varied between 0.4 and 8.5 cm, with mean diameter of 3.0 cm (Table 1).

OKC group included 30 patients: 21 males and 9 females, with a male to female ratio of 2.3:1. The mean age was 46.8 years old (range 8-81). A total of 55 surgical samples of OKC were collected (30 primary and 25 recurrences). The mandible was the most affected site (mandible to maxilla ratio of 2.1:1). The size range varied between 0.4 and 6.0 cm, with mean diameter of 2.3 cm (Table 1).

Cytoplasmic expression of NNMT was evaluated in the epithelial component of AM and OKC samples. The relationship between clinicopathological data and NNMT levels in AM was then analyzed. No statistical differences between NNMT expression and sex, age, site, size, and clinical variants were found ($P > 0.05$). A minor expression of NNMT was found in epithelial cells of Acanthomatous variant compared to Follicular variant ($P = 0.0411$).

Notably, the percentage of epithelial cells that showed NNMT positivity was significantly higher in recurrent than primary neoplasms (48.0% vs 34.6%, $P = 0.0430$) (Figure 1A and 2A-B, Table 2). This result was confirmed by staining intensity, showing more cases classified as “+” in recurrent than primary AMs (17 vs 10 cases, $P = 0.0470$) (Figure 1C).

Immunohistochemical analysis of Ki-67 showed no significant difference between primary and recurrent AMs (10.6 ± 7.5 vs 10.2 ± 8.6 , $P > 0.05$) (Figure 3).

Regarding OKC, the relationship between clinicopathological data and NNMT expression was also analyzed. No statistical differences between NNMT expression and sex, age, site, and size were found ($P > 0.05$). The percentage of epithelial cells that showed NNMT positivity was significantly higher in primary OKC than in recurrences ($P = 0.0014$) (Figure 1B and 2C-D, Table 2). Staining intensity of NNMT confirmed these results, with epithelial staining being more intense in primary OKCs (24 vs 6 cases, $P = 0.0276$) (Figure 1D). The evaluation of Ki-67 expression showed no significant differences between primary and recurrent OKCs (9.9 ± 1.8 vs 8.4 ± 1.7 , $P > 0.05$) (Figure 3).

Lastly, the investigators had excellent interobserver agreement, with a weighted Cohen's kappa of 0.885.

Discussion.

In 2005, World Health Organization classified OKC as a benign odontogenic **neoplasm**, based on several biological features, such as *PTCH* alterations.⁸ However, in 2017 OKC has been re-classified as cyst, due to insufficient evidence to support its neoplastic origin.² Indeed, although *PTCH* alterations are seen in 80% of OKCs, they are not specific, since the mutation of this tumor suppressor gene have been found in other odontogenic cysts.¹⁹ This debate is still open, and the classification system of OTs is subject to continuous and critical revision. To address this issue, part of the research efforts is currently focused on identifying molecular markers differentially expressed within odontogenic lesions and whose levels are significantly correlated with different tumor behaviors.

The nuclear protein Ki-67 is a well-known cellular marker strictly associated with cell proliferation.²⁰ Previous studies reported a low Ki-67 expression in odontogenic cyst and neoplasms.²¹ Furthermore, data reported in literature seem to demonstrate that Ki-67 expression do not differ between OKC and AM, while other odontogenic cysts display low Ki-67 levels.^{21,22} Another study found no differences in Ki-67 expression between primary and recurrent OKCs, suggesting that incomplete removal, rather than intrinsic growth rate, was likely to be responsible for recurrences.²² In the present study, no differences were found in Ki-67 expression between primary and recurrent odontogenic lesions, confirming what is reported in literature about the limited usefulness of this marker in OKC and AM (Figure 3). Indeed, it is assumed that the low expression of Ki-67 may hinder the evaluation of the proliferation activity in these lesions.²³

Results obtained in the present study showed that there were no differences in NNMT expression between primary OKCs and AMs. On the contrary, enzyme levels within recurrent lesions were significantly lower in OKCs than in AMs, suggesting the presence of different features underlying metabolism as well as cell proliferation activity.

NNMT was found to be upregulated in a wide variety of neoplasms, including clear cell renal cell carcinoma (ccRCC),^{24,25} oral squamous cell carcinoma (OSCC),^{17,26,27} non-small cell lung cancer,²⁸ bladder urothelial carcinoma,^{13,29} and cutaneous malignant melanoma.³⁰ However, the biological effects related with enzyme overexpression in cancer have not been totally disclosed and the role played by NNMT in **cancer** cell metabolism remains partly unclear.

A great bulk of studies explored the involvement of NNMT in proliferative capacity of tumor cell. shRNA-mediated gene silencing of NNMT led to a significant inhibition of *in vitro* cell proliferation and anchorage-independent cell growth, both in HeLa-derived KB cancer cells³¹ and in human lung cancer cell line A549.¹¹ Moreover, NNMT knockdown efficiently reduced cell growth and tumor formation ability of 786O ccRCC cells³² and PE/CA PJ-15 OSCC cell line,⁹ upon subcutaneous injection into athymic mice, thus suggesting the potential role played by the enzyme in *in vivo* tumorigenicity.

A recent work showed that NNMT is selectively expressed in some breast cancer cell lines. Subsequent enzyme downregulation was significantly associated with inhibition of cell growth *in vitro* and tumor formation in mice in Bcap-37 and MDA-MB-231 cell lines, originally displaying high NNMT expression levels. On the contrary, the induction of NNMT upregulation in MCF-7 and SK-BR-3 cells, lacking endogenous enzyme expression, led to opposite effects with respect to those obtained upon NNMT silencing.³³

NNMT overexpression was also found to be associated with cancer stem cells (CSCs), acting as important determinants in cancer initiation, progression and recurrence. In particular,

enzyme upregulation was reported in CSC-enriched cell populations obtained from Hep-2 laryngeal carcinoma cell line. Moreover, CSC-enriched cells exhibited a strong ability to form tumor *in vivo*. Considering the fundamental role played by CSCs in carcinogenesis, these results candidate NNMT as a leading molecule affecting metabolism and proliferation capability of cancer cell.³⁴

In HSC-2 OSCC cell line, NNMT upregulation significantly increased cell growth *in vitro*. Subsequent molecular analyses were carried out to explore whether enzyme overexpression was associated with the involvement of specific cellular pathways. Concerning cell proliferation, Ki-67 expression was therefore investigated. Results obtained showed that Ki-67 levels did not undergo dysregulation upon induction of NNMT upregulation, thus suggesting that proliferative capacity of cancer cell is a complex phenotypical aspect regulated by the combined effects of different molecular determinants.³⁵

In conclusion, this report is the first to evaluate NNMT expression in odontogenic lesions and to demonstrate a differential expression between recurrent AMs and OKCs.

These preliminary results seem to indicate that there is potential for the use of NNMT as prognostic marker in odontogenic lesions. **However, statistical analyses performed using data obtained from a small cohort of samples display limited power, even when results are significant. For this reason,** larger studies are needed to confirm these findings and to explore the biological role of NNMT.

Compliance with ethical standards

The study was conducted in accordance with the “Ethical Principles for Medical Research Involving Human Subjects” statement of the Helsinki Declaration.

This study received exemption from the institutional review board because of its retrospective nature and because tissue samples were de-identified. The study did not include animals; therefore, issues relating to animal welfare do not apply.

Funding

None declared.

Conflict of interest

The authors declare that they have no conflicts of interest.

Author contribution.

AS and ME contributed to the design and concept of the study. MM and CR contributed to histological analysis and writing of the manuscript. DS, VP, and LT contributed to the collection of the data and statistical analysis. All authors revised the manuscript and contributed to the final approval. AS takes full responsibility for the work as a whole, including the study design, access to data and the decision to submit and publish the manuscript.

References.

1. Rubini C, Mascitti M, Santarelli A, et al. Odontogenic tumors: a retrospective clinicopathological study from two Italian centers. *Pathologica*. 2017;109(1):35-46.
2. El-Naggar A, Chan J, Rubin Grandis J, Takata T, Slootweg P, eds. *WHO classification of head and neck tumours*. 4 ed. Lyon: International Agency for Research on Cancer; 2017. World Health Organization classification of tumours.
3. Lo Muzio L, Mascitti M, Santarelli A, et al. Cystic lesions of the jaws: a retrospective clinicopathologic study of 2030 cases. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2017;124(2):128-138.
4. Mascitti M, Togni L, Troiano G, et al. Odontogenic tumours: a 25-year epidemiological study in the Marche region of Italy. *Eur Arch Otorhinolaryngol*. 2019.
5. Buchner A, Merrell PW, Carpenter WM. Relative frequency of central odontogenic tumors: a study of 1,088 cases from Northern California and comparison to studies from other parts of the world. *J Oral Maxillofac Surg*. 2006;64(9):1343-1352.
6. Hayward JR. Recurrent ameloblastoma 30 years after surgical treatment. *J Oral Surg*. 1973;31(5):368-370.
7. Al-Moraissi EA, Dahan AA, Alwadeai MS, et al. What surgical treatment has the lowest recurrence rate following the management of keratocystic odontogenic tumor?: A large systematic review and meta-analysis. *J Craniomaxillofac Surg*. 2017;45(1):131-144.
8. Barnes L, Eveson JW, Reichart P, Sidransky D, eds. *Pathology and genetics of head and neck tumours*. Lyon: IARC Press; 2005. World Health Organization classification of tumours.

9. Aksoy S, Szumlanski CL, Weinshilboum RM. Human liver nicotinamide N-methyltransferase. cDNA cloning, expression, and biochemical characterization. *J Biol Chem*. 1994;269(20):14835-14840.
10. Pozzi V, Sartini D, Morganti S, et al. RNA-mediated gene silencing of nicotinamide N-methyltransferase is associated with decreased tumorigenicity in human oral carcinoma cells. *PLoS One*. 2013;8(8):e71272.
11. Peng Y, Sartini D, Pozzi V, Wilk D, Emanuelli M, Yee VC. Structural basis of substrate recognition in human nicotinamide N-methyltransferase. *Biochemistry*. 2011;50(36):7800-7808.
12. Sartini D, Seta R, Pozzi V, et al. Role of nicotinamide N-methyltransferase in non-small cell lung cancer: in vitro effect of shRNA-mediated gene silencing on tumourigenicity. *Biol Chem*. 2015;396(3):225-234.
13. Sartini D, Muzzonigro G, Milanese G, et al. Upregulation of tissue and urinary nicotinamide N-methyltransferase in bladder cancer: potential for the development of a urine-based diagnostic test. *Cell Biochem Biophys*. 2013;65(3):473-483.
14. Seta R, Mascitti M, Campagna R, et al. Overexpression of nicotinamide N-methyltransferase in HSC-2 OSCC cell line: effect on apoptosis and cell proliferation. *Clin Oral Investig*. 2018.
15. Mascitti M, Santarelli A, Sartini D, et al. Analysis of nicotinamide N-methyltransferase in oral malignant melanoma and potential prognostic significance. *Melanoma Res*. 2019;29(2):151-156.
16. D'Andrea FP, Safwat A, Kassem M, Gautier L, Overgaard J, Horsman MR. Cancer stem cell overexpression of nicotinamide N-methyltransferase enhances cellular radiation resistance. *Radiother Oncol*. 2011;99(3):373-378.

17. Emanuelli M, Santarelli A, Sartini D, et al. Nicotinamide N-Methyltransferase upregulation correlates with tumour differentiation in oral squamous cell carcinoma. *Histol Histopathol.* 2010;25(1):15-20.
18. Kim J, Hong SJ, Lim EK, et al. Expression of nicotinamide N-methyltransferase in hepatocellular carcinoma is associated with poor prognosis. *J Exp Clin Cancer Res.* 2009;28:20.
19. Speight PM, Takata T. New tumour entities in the 4th edition of the World Health Organization Classification of Head and Neck tumours: odontogenic and maxillofacial bone tumours. *Virchows Arch.* 2018;472(3):331-339.
20. Rahmanzadeh R, Huttmann G, Gerdes J, Scholzen T. Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis. *Cell Prolif.* 2007;40(3):422-430.
21. da Silva LP, Serpa MS, Santana T, do Nascimento GJ, de Souza Andrade ES, Sobral AP. Clinicopathological and cell proliferation evaluation of ameloblastomas and keratocystic odontogenic tumors: a 10 year retrospective study. *Eur Arch Otorhinolaryngol.* 2017;274(2):1089-1095.
22. Li TJ, Browne RM, Matthews JB. Epithelial cell proliferation in odontogenic keratocysts: a comparative immunocytochemical study of Ki67 in simple, recurrent and basal cell naevus syndrome (BCNS)-associated lesions. *J Oral Pathol Med.* 1995;24(5):221-226.
23. Jaafari-Ashkavandi Z, Mehranmehr F, Roosta E. MCM3 and Ki67 proliferation markers in odontogenic cysts and ameloblastoma. *J Oral Biol Craniofac Res.* 2019;9(1):47-50.

24. Sartini D, Muzzonigro G, Milanese G, Pierella F, Rossi V, Emanuelli M. Identification of nicotinamide N-methyltransferase as a novel tumor marker for renal clear cell carcinoma. *J Urol.* 2006;176(5):2248-2254.
25. Campagna R, Cecati M, Pozzi V, et al. Involvement of transforming growth factor beta 1 in the transcriptional regulation of nicotinamide N-methyltransferase in clear cell renal cell carcinoma. *Cell Mol Biol (Noisy-le-grand).* 2018;64(7):51-55.
26. Sartini D, Santarelli A, Rossi V, et al. Nicotinamide N-methyltransferase upregulation inversely correlates with lymph node metastasis in oral squamous cell carcinoma. *Mol Med.* 2007;13(7-8):415-421.
27. Sartini D, Pozzi V, Renzi E, et al. Analysis of tissue and salivary nicotinamide N-methyltransferase in oral squamous cell carcinoma: basis for the development of a noninvasive diagnostic test for early-stage disease. *Biol Chem.* 2012;393(6):505-511.
28. Sartini D, Morganti S, Guidi E, et al. Nicotinamide N-methyltransferase in non-small cell lung cancer: promising results for targeted anti-cancer therapy. *Cell Biochem Biophys.* 2013;67(3):865-873.
29. Pozzi V, Di Ruscio G, Sartini D, et al. Clinical performance and utility of a NNMT-based urine test for bladder cancer. *Int J Biol Markers.* 2018;33(1):94-101.
30. Ganzetti G, Sartini D, Campanati A, et al. Nicotinamide N-methyltransferase: potential involvement in cutaneous malignant melanoma. *Melanoma Res.* 2018;28(2):82-88.
31. Pozzi V, Mazzotta M, Lo Muzio L, et al. Inhibiting proliferation in KB cancer cells by RNA interference-mediated knockdown of nicotinamide N-methyltransferase expression. *Int J Immunopathol Pharmacol.* 2011;24(1):69-77.
32. Tang SW, Yang TC, Lin WC, et al. Nicotinamide N-methyltransferase induces cellular invasion through activating matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells. *Carcinogenesis.* 2011;32(2):138-145.

33. Zhang J, Wang Y, Li G, Yu H, Xie X. Down-regulation of nicotinamide N-methyltransferase induces apoptosis in human breast cancer cells via the mitochondria-mediated pathway. *PLoS One*. 2014;9(2):e89202.
34. Pozzi V, Sartini D, Rocchetti R, et al. Identification and characterization of cancer stem cells from head and neck squamous cell carcinoma cell lines. *Cell Physiol Biochem*. 2015;36(2):784-798.
35. Seta R, Mascitti M, Campagna R, et al. Overexpression of nicotinamide N-methyltransferase in HSC-2 OSCC cell line: effect on apoptosis and cell proliferation. *Clin Oral Investig*. 2019;23(2):829-838.

Figure legends.

Figure 1 Graphs showing NNMT levels expressed as percentage of positive cells (mean \pm SD) in AMs (A) and OKCs (B). Bar charts showing NNMT levels expressed as staining intensity (“-” negative/weak staining, “+” moderate/intense staining) in AMs (C) and OKCs (D) (* P < 0.05)

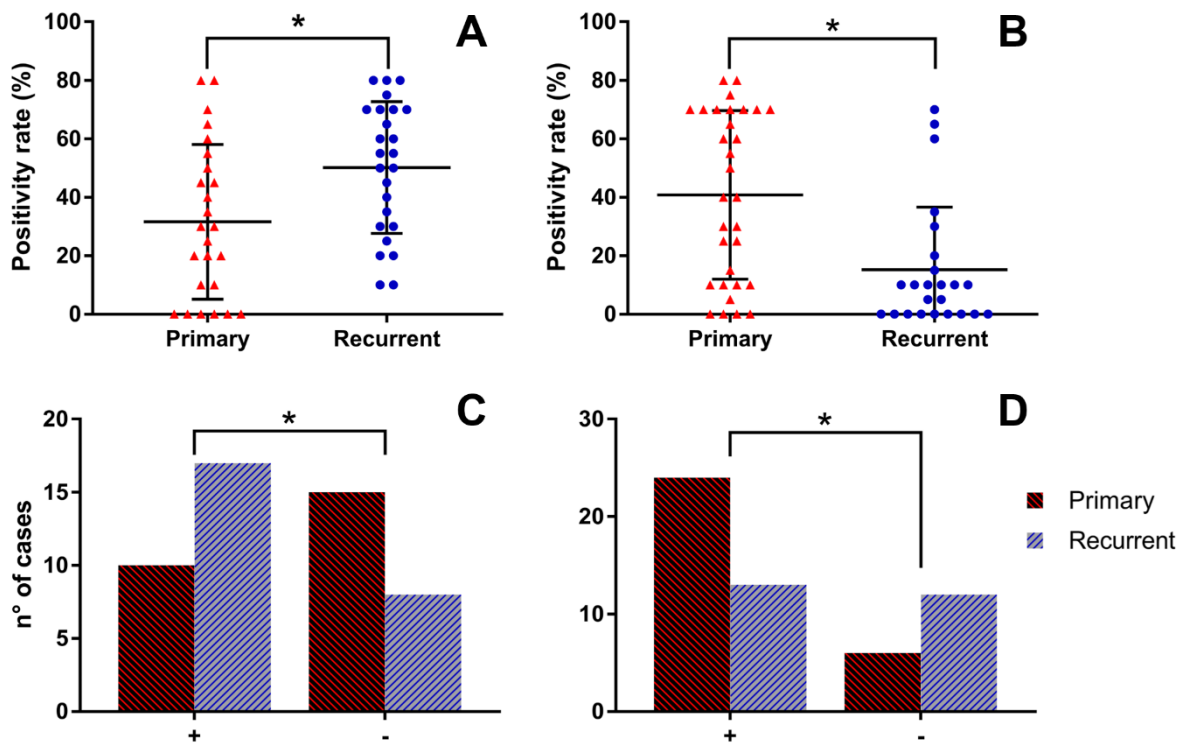


Figure 2 NNMT cytoplasmic expression in AMs and OKCs. **Primary (A) and recurrent (B)** plexiform AM consisting of strands and cords of odontogenic cells that form anastomoses, surrounded by loose connective tissue (x20 magnification). The inset areas of greater magnification showed that the cytoplasmic staining of NNMT is significantly lower in primary AMs than recurrences. **Primary (C) and recurrent (D)** OKC consisting of parakeratinized epithelium without rete ridges, with palisaded basal layer and focal areas showing reversed nuclear polarity (x20 magnification). The inset areas of greater magnification showed that the cytoplasmic staining of NNMT is significantly higher in primary OKCs than recurrences.

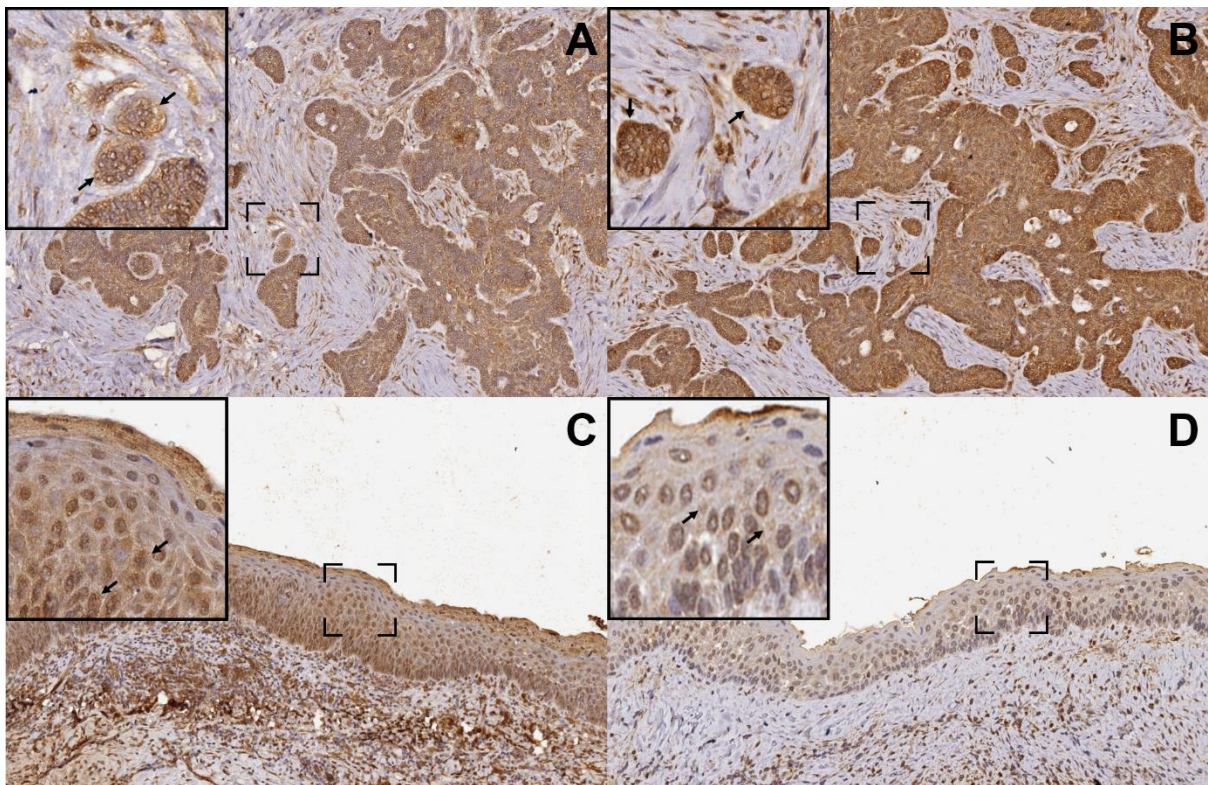


Figure 3 Ki-67 expression in AMs and OKCs. Primary (A) and recurrent (B) plexiform AM (x20 magnification). The inset areas of greater magnification showed no difference in nuclear staining of Ki-67 between primary and recurrent AMs. Primary (C) and recurrent (D) OKC (x20 magnification). The inset areas of greater magnification showed no difference in nuclear staining of Ki-67 between primary and recurrent OKCs.

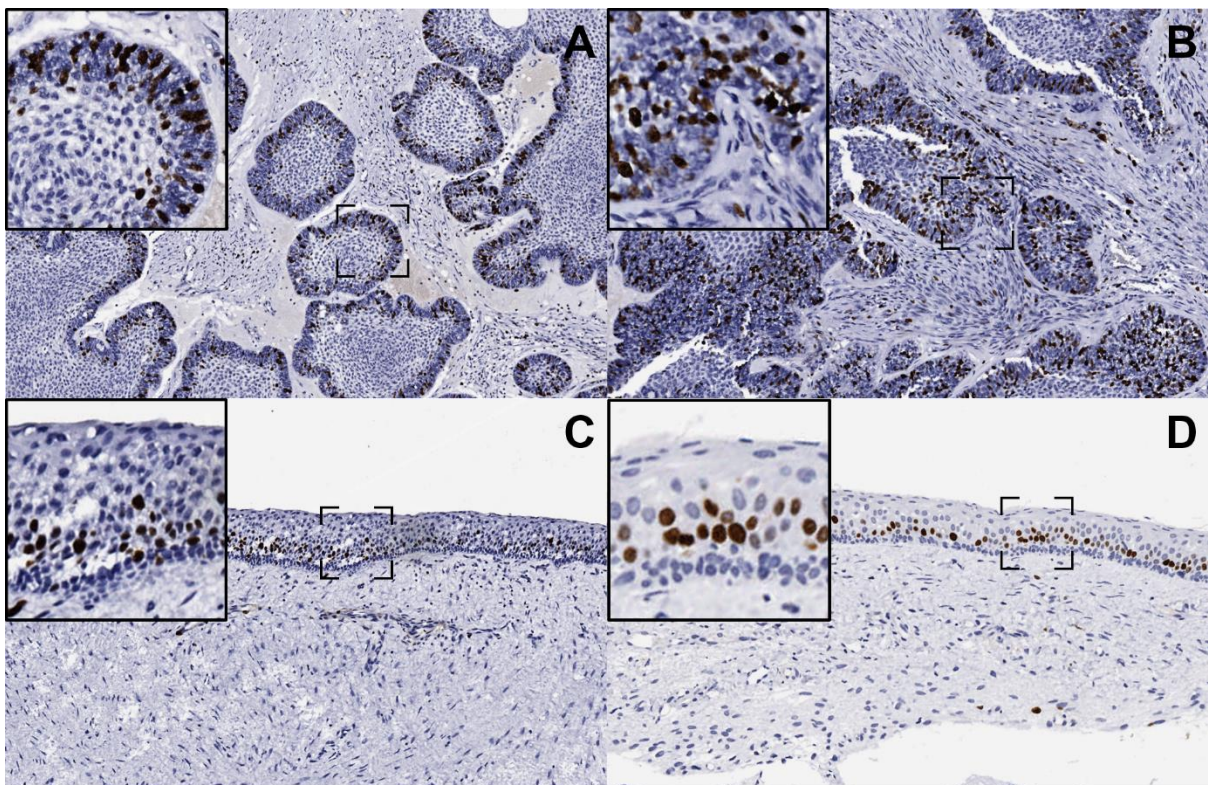


Table 1. Clinicopathological data of AM and OKC samples. “Sex” category reports the number of patients (n. = 25 for AM; n. = 30 for OKC).

Parameter	Ameloblastoma				OKC
	Classic	Unicystic	Peripheral	Total	
<i>Sex</i>					
- Male	8	8	1	17	21
- Female	3	3	2	8	9
<i>Age</i>	59.6 ± 15.8	40.5 ± 19.1	66.0 ± 5.7	51.4 ± 19.5	46.8 ± 17.6
<i>Site</i>					
- Maxilla	7	6	3	16	18
- Mandible	16	16	2	34	37
<i>Size</i>	3.3 ± 1.6	2.8 ± 2.3	2.1 ± 0.4	3.0 ± 1.9	2.3 ± 1.2
<i>Histological type</i>					
- Follicular	13	10	1	24	/
- Plexiform	7	8	1	16	/
- Acanthomatous	3	4	3	10	/
<i>Clinical form</i>					
- Primary	12	11	2	25	30
- Recurrence	11	11	3	25	25

Table 2. Differences in clinicopathological data and NNMT expression at epithelial level of AM and OKC. “n. cases” refers to the number of surgical samples (AM = 50; OKC = 55), except for “Sex” and “Age” categories, which report the number of patients (AM = 25; OKC = 30).

Parameter	AM			OKC		
	n. cases	mean \pm SD	P-value	n. cases	mean \pm SD	P-value
<i>Sex</i>						
- Male	17	40.6 \pm 25.5	> 0.05 ^a	21	27.4 \pm 30.6	> 0.05 ^a
- Female	8	43.1 \pm 29.8		9	30.6 \pm 31.5	
<i>Age</i>						
- < 65 years	16	43.3 \pm 26.4	> 0.05 ^{a,c}	24	26.7 \pm 30.7	> 0.05 ^{a,c}
- > 65 years	9	37.5 \pm 27.7		6	38.1 \pm 29.8	
<i>Site</i>						
- Maxilla	16	48.1 \pm 27.1	> 0.05 ^a	18	35.3 \pm 29.4	> 0.05 ^a
- Mandible	34	38.2 \pm 26.3		37	25.0 \pm 31.0	
<i>Variants</i>						
- Classic	23	37.0 \pm 29.5	> 0.05 ^b			
- Unicystic	22	40.0 \pm 21.9				
- Peripheral	5	68.0 \pm 19.2				
<i>Histological type</i>						
- Follicular	24	48.0 \pm 27.5	> 0.05 ^{b*}			
- Plexiform	16	41.0 \pm 29.6				
- Acanthomatous	10	23.1 \pm 25.6				
<i>Size</i>						
- \leq 2.5 cm	26	42.0 \pm 29.2	> 0.05 ^{a,c}	40	25.4 \pm 28.9	> 0.05 ^{a,c}
- > 2.5 cm	24	40.8 \pm 24.5		15	36.3 \pm 34.5	
<i>Clinical form</i>						
- Primary	25	34.6 \pm 29.5	0.0430^a	30	40.0 \pm 13.3	0.0014^a
- Recurrence	25	48.0 \pm 22.4		25	31.0 \pm 22.9	

^a Mann-Whitney U test.

^b Kruskal-Wallis test with Dunn’s post hoc test.

^c Lack of significance was confirmed by Spearman correlation test ($\rho < |0.18|$, $p > 0.05$).

* Significant differences in NNMT expression in epithelial cells (Follicular vs Acanthomatous; $P = 0.0411$).