The importance of BRAF-V600E mutation to ameloblastoma metabolism

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Background: Ameloblastoma is a locally infiltrative, aggressive epithelial odontogenic neoplasm. BRAF-V600E mutation is frequently found in this tumor and has a pivotal role in its pathogenesis, but the consequences of this alteration need to be addressed. An untargeted metabolomics approach was applied to verify whether metabolic disturbances are related to tumor biology and whether BRAF-V600E mutation contributes to these alterations.

Methods: Formalin-fixed and paraffin-embedded tissue specimens from thirteen ameloblastoma and six dental follicles were included in this study. BRAF mutational status was determined by competitive allele-specific real-time PCR. Metabolite extracts were analyzed using gas chromatography coupled to mass spectrometry. Univariate and multivariate statistical methods were employed to compare the metabolic profiles of the samples.

Results: The abundance of eleven metabolites was significantly higher in ameloblastoma in relation to dental follicles, including amino acids, fatty acids, carbohydrates, inorganic acids, and organoheterocyclic compounds. The presence of BRAF-V600E mutations in ameloblastoma was related to decreased levels of glycerol in comparison with tumors carrying only wild-type alleles of this gene. No metabolic differences were observed between recurrent and primary manifestations of ameloblastoma.

Conclusions: Ameloblastoma exhibits a distinct metabolic profile from normal odontogenic epithelium. BRAF-V600E may contribute to metabolic alterations in ameloblastoma. Collectively, our findings suggest that metabolic alterations might play a role in tumor pathogenesis.

KEYWORDS
Ameloblastoma, BRAF, mass spectrometry, metabolomics, odontogenic tumor

1 | INTRODUCTION

Ameloblastoma is a benign odontogenic neoplasm, most commonly arising in the posterior region of the mandible. The tumor has a slow-growing development, but presents a locally aggressive behavior. According to the latest World Health Organization (WHO) classification of odontogenic tumors published in 2017, ameloblastoma was subdivided into three clinicopathological variants, that is,
ameloblastoma, unicystic ameloblastoma, and extraosseous/peripheral type. Microscopic examination of ameloblastoma specimens reveals structures that resemble tooth development apparatus, composed of islands or follicles of epithelial cells presenting peripherally columnar ameloblasts-like cells surrounding loosely arranged stellate cells. Treatment options range from conservative approaches (enucleation/curettage) to radical excision, though the former demonstrates a high recurrence rate, and both cause significant morbidity and facial deformity. Although rare, malignant transformation of ameloblastomas into ameloblastic carcinoma is also well known.

Although the molecular pathogenesis of ameloblastoma is not well understood, recent reports have suggested a prominent role of mitogen-activated protein kinase pathway dysregulation by recurrent activating mutations in BRAF. As metabolic disturbances have been shown to play a role in tumorigenesis, metabolomics represents a valuable tool to identify novel alterations that can be used for diagnosis and therapeutic purposes. However, there seems to be no study reporting the metabolic changes in ameloblastoma. Therefore, in the present study, a comparative untargeted metabolomics analysis using gas chromatography coupled to mass spectrometry (GC-MS) was performed on formalin-fixed paraffin-embedded (FFPE) tissues of ameloblastoma and dental follicles to identify relevant metabolites and metabolic pathways potentially dysregulated in the pathogenesis of this aggressive tumor and to verify whether BRAF-V600E mutation contributes to these alterations.

2 MATERIALS AND METHODS

2.1 Samples and subjects

The study was conducted in accordance with ethical standards and was approved by the local institutional review board and the Ethics Committee (CAAE: 97428718.5.0000.5149). A total of 19 FFPE tissue samples were retrieved from the files of the Oral Pathology Service at Universidade Federal de Minas Gerais, comprising of 13 ameloblastomas and six dental follicles obtained from healthy patients submitted to extractions of impacted third molars. Microscopic aspects of all cases were reviewed by two oral pathologists to confirm their diagnoses following current WHO guidelines.

2.2 Metabolomics on FFPE tissue samples

2.2.1 Sample preparation

The extraction of metabolites was performed according to modifications on the protocol described by Wojakowska et al. Tissue regions of interest were pre-defined during microscopic examination of hematoxylin-eosin-stained sections. The first and second 10-μm-thick slices were discarded, and subsequently, three tissue sections with 10 μm thick and 0.3 cm² of area were collected into microtubes. Technical triplicates were produced for each sample. Tissues were deparaffinized by washing twice with 1 mL of xylene (Merck, Darmstadt, Germany) followed by centrifugation at 4°C, 10 minutes. The residual solvent was evaporated in a thermoblock. Polar metabolites were extracted with 250 μL of methanol (Sigma-Aldrich, St. Louis, MO, USA): water 1:1 (v/v), followed by 5 minutes of vortexing and sonication for 10 minutes in an ultrasonic bath. The resulting mixture was centrifuged for 15 minutes, 4°C at 13 250 g, and the supernatant was collected into another tube. To the pellet, 250 μL of dichloromethane: methanol 3:1 (v/v) (Sigma-Aldrich) were added in order to obtain apolar metabolites and were also subjected to 5 minutes of vortexing and 10 minutes of sonication in an ultrasonic bath. The supernatant was collected by centrifugation (13 250 g, 15 minutes, 4°C) and combined with the polar fraction. Subsequently, 250 μL of the resulting mixture was transferred to a glass insert and were completely evaporated in a speedvac concentrator at 30°C.

Methoximation was performed by the addition of 30 μL of methoxamine (Sigma-Aldrich) in pyridine (Sigma-Aldrich) [15 mg/mL]. The vials were vortexed for 5 minutes, and the reaction was carried out for 2 hour at 60°C. For silylation, 30 μL of MSTFA (Sigma-Aldrich) was added and the reaction was carried out for 1 hour at 60°C. Finally, 1 μL of C18:0 methyl stearate (Sigma-Aldrich) [1000 ppm] and 59 μL of heptane (Sigma-Aldrich) were added to each sample. Quality controls (QCs) were prepared by pooling the same amount from each sample and were analyzed throughout the run, after every five samples. A blank sample was included in the study and was obtained from sections of a paraffin block prepared without tissues, following the same extraction and derivatization procedures applied to the samples.

2.2.2 GC-MS system

Analyses were performed with Gas Chromatography system 7890 C coupled with a quadrupole Mass Detector 5975 A (Agilent Technologies®, Santa Clara, CA, USA) equipped with automatic injector combiPAL autosampler. The samples (2 μL each) were injected in split mode (ratio 1:10) in capillary column DB-5MS (30 m length, 0.25 mm internal diameter, and 0.25 μm thickness film) from Agilent Technologies®. Ultrapure helium (99.9999%) was used as carrier gas at a flow rate of 1.0 mL/min. The injector temperature was maintained at 250°C. The oven temperature was programmed at 60°C (1.0 minutes) with gradual increase at a rate of 10°C/min up to 325°C. The mass detector was operated in electron impact ionization at 70 eV and in full-scan mode at the range 50-600 m/z.

2.2.3 GC-MS data processing

Data processing was performed in XCMS software package (version 1.24.1)® running at R platform (version 3.2.2). Data extraction was performed by matched filter method. The parameters were as follows: (a) peak width (fwhm) = 4; (b) signal-to-noise ratio (snt-thresh) = 1.5; and (c) maximum number of peaks per extracted ion chromatogram (max) = 40. Peak grouping was based on bandwidth correction (bw) = 5 and two for the first and second grouping, respectively, the width of the overlapped bands of m/z (mzwid) = 0.25, and the minimum fraction of samples necessary in at least one group to be
valid (minfrac) was set at 0.1. The “fillPeaks” tool was used to remove missing values, and default “retcor” method was applied with non-linear alignment and degree of smoothing for local polynomial regression fitting (span) = 0.5 to correct the retention time. Annotation of metabolites using AMDIS (version 2.71) was searched against Fiehn GC/MS RLT Library for identification of co-eluted compounds based on the retention index.10 The data set was normalized by the C18:0 methyl stearate internal standard before statistical analyses.

2.3 | DNA extraction and BRAF-V600E mutation assessment

Genomic DNA was extracted from the samples using QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The presence of BRAF-V600E mutation was evaluated by competitive allele-specific real-time PCR using TaqMan probe (BRAF_476_mu) (Life Technologies, CA, USA), as previously described.11

2.4 | Statistical analysis

Data were Pareto scaled and log2 transformed before multivariate statistical analyses. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were conducted in SIMCA 14.1. Pairwise comparisons using either Student’s t test or Mann-Whitney U test for independent data were performed on Statistica version 10, depending on the normality distribution assessment (Kolmogorov-Smirnov, Lilliefors and Shapiro-Wilk tests). A P-value <0.05 was used as the threshold for significance. Pathway analyses were performed on MBrole 2.0 and Metaboanalyst 4.0.12

3 | RESULTS

3.1 | Clinicopathological features

Ameloblastoma cases included in the study were comprised of eight men and five women, with age ranging from 13 to 81 years old, and the mean age was 35 years old. Eleven cases affected the mandible and two cases affected the maxilla. Nine cases were primary tumors, three were recurrences and one was unknown. Dental follicles were collected from four males and two female patients with a mean age of 16 years old (ranged from 9 to 21 years). Ameloblastoma samples were comprised of multiple histopathological types. In seven cases, plexiform type prevailed, with one of them showing expressive additional areas of acanthomatous pattern. In the other six cases, the most abundant type was follicular, with three cases demonstrating expressive additional areas of acanthomatous or granular pattern.

3.2 | BRAF mutational status of the samples

Real-time PCR was performed to evaluate the presence of BRAF-V600E mutation in twelve ameloblastoma cases with functional DNA available. Nine of 12 analyzed ameloblastomas (82%) showed the BRAF-V600E mutation.

3.3 | Analysis of the metabolic profile of FFPE specimens

PCA and total ion chromatogram (TIC) overlay were applied to overview the quality of the system. A tight clustering of QCs in a well-defined area of the plot (Figure S1a) and a proper overlay of the chromatograms (Figure S1b) was observed, confirming the robustness and stability of the procedures.

Mass spectrometry analysis of sample extracts allowed the identification of 26 metabolites. The list of the compounds along with their respective biochemical and physical properties are shown in Table 1. Most of the identified metabolites were classified as amino acids, peptides, and conjugates, accounting for 23.08% of the total, and all the classes of the identified compounds are represented in Figure S2.

3.4 | Detecting metabolic differences between ameloblastoma and dental follicles

In order to pinpoint the metabolic differences and similarities between ameloblastoma and dental follicles, comparisons were performed using a PLS-DA built with the identified metabolites. Tumor specimens were shown to cluster apart from normal tissue. The quality parameter, Q2, which was 0.708, indicated a good fit and prediction ability of the model (Figure 1A). A CV-ANOVA was done to evaluate the statistical significance of the analysis, and a P-value of 0.005 was obtained, indicating a significant separation of the groups (Figure 1B). To avoid model overfitting, a permutation test with 100 interactions was used. The intercepts of R2 and Q2 were, respectively, 0.448 and –0.295, demonstrating a valid PLS-DA model.

An unsupervised hierarchical clustering analysis was performed using the mean ionic abundance of the identified metabolites in each patient. A result similar to the observed in PLS-DA was obtained (Figure 1C). Dental follicles formed a separate cluster containing almost all normal samples, except for one case. In contrast, all tumor specimens were grouped together in the same cluster. To identify the metabolites that contributed most to the discrimination of groups within the PLS-DA model, metabolites with variable importance in projection (VIP) above 1.0 and/or a P-value below 0.05 calculated by univariate analysis, including Student’s t test or Mann-Whitney U test, were identified. In total, 11 metabolites showed significant differences in abundance between ameloblastoma and dental follicles (Table 2). All of these compounds were increased in tumor specimens, with fold changes ranging from 1.03 to 5.04.

3.5 | Alterations in metabolic pathways in ameloblastoma

To determine the biological pathways that are altered in ameloblastoma in comparison with follicles, a metabolic pathway enrichment
analysis was performed using the metabolites that differed significantly in abundance between these samples (Table 3). Only pathways enriched with at least three metabolites from the input and a P-value < 0.05 were selected. Metabolic pathways involved in aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, and ABC transporters were found to distinguish tumor and normal tissue.

### 3.6 | Comparative evaluation of the metabolic profile of primary and recurrent manifestations

As ameloblastoma is an odontogenic tumor with high recurrence risk, we compared the metabolic profile of primary and recurrent tumors to verify whether these manifestations could present different profiles in metabolism. However, no metabolic signature of recurrence could be defined in the present study, according to multivariate and univariate statistical analyses.

### 3.7 | Relationship between BRAF-V600E and metabolic profile

In order to verify whether the BRAF-V600E mutation contributes to the alterations detected in ameloblastoma, the metabolic profiles of ameloblastoma samples with and without this mutation were compared. In univariate analysis, glycerol (P = 0.02) levels were significantly higher in tumors with wild-type alleles. However, no significant difference was observed in PLS-DA and CV-ANOVA.

### 4 | DISCUSSION

Ameloblastoma is associated with high local morbidity, and a better understanding of its molecular alterations may provide useful information for the identification of biomarkers with prognostic potential and new drugs for the treatment of more aggressive tumors. In this
In our study, we investigated the metabolic profile of ameloblastoma in comparison with normal odontogenic tissue. Since metabolite levels reflect the interaction between environmental factor, genetic background, enzymatic function, and metabolic processes, metabolomics is a valuable tool to provide new insights into the molecular pathogenesis of diverse neoplasias. FFPE samples were used in this study, considering their wide availability and that they are frequently accompanied by corresponding patient clinical history.

Although formalin fixation with paraffin embedding causes a global depletion of metabolites in comparison with fresh frozen tissues, tissue-based metabolomics on FFPE samples is still feasible and useful for the identification of novel metabolic biomarkers, to predict...
and discriminate biological states and to study relevant metabolic pathways.\textsuperscript{14} In the present study, we were able to distinguish ameloblastoma specimens from dental follicles using FFPE samples, pointing out metabolic pathways that are potentially altered in this tumor.

One key finding of this study was the identification of 11 metabolites that were upregulated in ameloblastoma in comparison with normal odontogenic tissue. The use of these metabolites in pathway analysis enabled the identification of the metabolic pathways of aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, and ABC transporters as metabolic processes potentially upregulated in this odontogenic tumor.

Changes in the mechanisms involved in translation have been described in cancer, and the overexpression of tRNAs is one of the main processes associated with a high translational activity of tumor cells.\textsuperscript{15,16} In breast cancer, the higher expression of the tRNA\textsubscript{Asn}, tRNA\textsubscript{Thr}, tRNA\textsubscript{Ser}, and tRNA\textsubscript{Val} is required to support the protein production that is necessary for tumor growth.\textsuperscript{17} In ameloblastoma, the aminoacyl-tRNA biosynthesis was found to be upregulated, suggesting an increased translational activity needed for tumor development.

The reprogramming of cell metabolism is frequently observed in cancer, with an intense breakdown of glucose for lactate production, despite sufficient oxygen levels for tumor growth and survival, a phenomenon known as Warburg effect.\textsuperscript{18} The glycolysis-diverting pathways contribute to sustain the anabolic phenotype of cancer cells.\textsuperscript{19} The serine/glycine biosynthesis is a component of this pathway and has a pivotal role in tumor biology, contributing to the synthesis of biomolecules, that is, nucleic acids, proteins, and lipids.\textsuperscript{20} The metabolite 3-phosphoglycerate, arising from the glycolytic pathway, is oxidized to serine by phosphoglycerate dehydrogenase (PHGDH), and then, serine is converted to glycine. In previous studies, the overexpression of PHGDH was observed in melanoma,\textsuperscript{21} thyroid cancer types,\textsuperscript{22} and colorectal cancer.\textsuperscript{23} In the present study, serine and glycine were found to have a higher abundance in ameloblastoma in comparison with dental follicles. Consistently, the levels of PHGDH were found to be significantly increased in ameloblastoma in comparison with dental follicles by proteomic analysis (data not published). The increase of these compounds suggests a potential upregulation of serine/glycine biosynthesis in ameloblastoma, providing the building blocks necessary for tumor growth.

The BRAF-V600E mutation is present in high frequency in ameloblastoma, ranging from 46% to 82% of the cases.\textsuperscript{3} In agreement, this mutation was observed in 82% of the samples included in the present study. Since BRAF-V600E mutations have been implicated as important regulators of metabolic processes in other neoplasms,\textsuperscript{23} we hypothesized that this could also take place in ameloblastomas. Our results demonstrated significantly decreased levels of glycerol in tumor samples with this mutation in comparison with those carrying only wild-type allele of BRAF. The decreased levels of glycerol in ameloblastomas with BRAF-V600E might result from increased glycolysis rate. Glycerol can be incorporated into this glycolytic pathway after its conversion to dihydroxyacetone phosphate. Addiction to glycolysis was observed in melanoma,\textsuperscript{24} colorectal,\textsuperscript{25} and thyroid cancers cell models with mutated BRAF.\textsuperscript{26} Although our results suggest that BRAF-V600E may contribute to some of the metabolic alterations found in ameloblastoma and are in agreement with previous studies on malignant diseases harboring this mutation, it is also known that the effects of BRAF mutations are dependent on the genetic context;\textsuperscript{23} therefore, the metabolic disturbances associated with this mutation in the ameloblastoma context need to be further addressed using a larger set of patients and different analytical platforms.

Ameloblastoma exhibits a higher membrane and cytoplasmic GLUT-1 expression, the most important transmembrane glucose transporter protein associated with energy production through the glycolytic pathway, in relation to dental germ. However, the levels

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>FC</th>
<th>VIP score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-hydroxyypyridine</td>
<td>1.04</td>
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<td>β-cyano-L-alanine</td>
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<td>1.10</td>
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<td>1.7E-04</td>
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<td>Glycerol</td>
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<tr>
<td>Glycine</td>
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<td>1.42</td>
<td>2.0E-06</td>
</tr>
<tr>
<td>L-glutamic acid</td>
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<td>3.42</td>
<td>1.0E-07</td>
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<tr>
<td>L-serine</td>
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<td></td>
<td>0.0036</td>
</tr>
<tr>
<td>Phosphoric acid</td>
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<td>1.01</td>
<td>0.0142</td>
</tr>
<tr>
<td>D-(+)-trehalose</td>
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<td>1.02</td>
<td>0.0410</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>1.04</td>
<td></td>
<td>0.0405</td>
</tr>
</tbody>
</table>

FC, fold change; VIP, variable importance in projection. Fold changes were determined by dividing the mean ionic abundance of ameloblastoma samples to those of dental follicles.

<table>
<thead>
<tr>
<th>Metabolic pathways</th>
<th>P-value</th>
<th>FDR correction</th>
<th>Metabolites in pathway (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC transporters</td>
<td>1.04E-06</td>
<td>3.9E-05</td>
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<tr>
<td>Aminoacyl-tRNA biosynthesis</td>
<td>6.74E-04</td>
<td>1.4E-02</td>
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</tr>
<tr>
<td>Cyanoamino acid metabolism</td>
<td>3.85E-05</td>
<td>3.0E-03</td>
<td>3</td>
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</tbody>
</table>

P-value and FDR demonstrate the significance of pathway analysis (P < 0.05).

| Metabolic pathways enriched from metabolites significantly differing between ameloblastoma and dental follicle samples |
of hypoxia-inducible factor-1α (HIF-1α), which is a transcription factor involved in the adaptation of stressful condition such as hypoxia, were shown to be altered in 27.3% of 57 cases investigated by Sánchez-Romero et al., with only one case displaying nuclear positivity. These findings suggested that mechanisms other than hypoxia might be involved in the differential expression of GLUT-1 in ameloblastoma.\textsuperscript{23} Interestingly, colorectal cancer cells with BRAF-V600E mutation show increased levels of GLUT-1 irrespective of HIF-1α regulation.\textsuperscript{25} In addition, the analysis of glucose metabolism in ameloblastoma using \textsuperscript{18}F-fluorodeoxyglucose (FDG) and FDG positron emission tomography demonstrated active uptake of FDG in tumor tissues,\textsuperscript{26} further corroborating an important role of glucose metabolism in ameloblastoma pathophysiology. Therefore, the comprehensive evaluation of the glycolytic process in this disease, as well as its association with BRAF mutational status, is issues that still need to be resolved in ameloblastoma.

Despite the evidences obtained in this study demonstrating the metabolic disturbances in ameloblastomas, these metabolic alterations identified here lack functional validation, although many of the results are in agreement with the literature. Moreover, GC-MS is a robust platform with high accuracy and reliability in the identification of compounds; however, it presents the drawback that only a few compounds can be volatized and analyzed.\textsuperscript{29} Therefore, to get a wider view of the alterations in ameloblastoma metabolic profile and to identify a recurrence metabolic signature, other analytical platforms should be employed to complement our results.

In conclusion, ameloblastoma shows a different metabolic profile from dental follicles, presenting a higher abundance of most metabolites. There was no difference in the molecular signature between primary and recurrent tumors. The BRAF-V600E mutation is associated with metabolic changes in ameloblastoma. Although this study presents novel aspects related to ameloblastoma metabolism, further studies are necessary to clarify their role in tumor origin and progression.

**CONFLICT OF INTEREST**

None declared.

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**REFERENCES**


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