CONTRIBUTIONS TO THE STUDY OF DENTAL DYSTROPHIES IN THE MIXED DENTITION

PhD THESIS ABSTRACT

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Scientific results obtained during doctoral studies are also due to my fellowship in the project “Program of Excellence in multidisciplinary doctoral and postdoctoral research in chronic diseases,” contract no. POSDRU/159/1.5/S/133377, project cofinanced by the European Social Fund Operational Programme “Human Resources Development” for 2007–2013, status that I had during July 2014 - November 2015.

The doctoral thesis includes: 156 pages, 34 tables, 86 figures, and 344 references.

This summary shows selective references and iconography, respecting numbering and content of the thesis.

Key words: dental dystrophies, fluorosis, molar incisor hypomineralization, scanning electron microscopy, amoxicillin, quality of life.
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CHAPTER 3
MOTIVATION AND GOALS OF THE DOCTORAL STUDY

Based on the existence of a considerable volume of scientific literature reserved to the dental dystrophies (more than 20,000 scientific articles on PubMed platform), we can say that they occupy an important place in a child's oral pathology.

This thesis, presented in a personal manner, is meant to bring a little light in such an ample field of dental dystrophies in the mixed dentition.

Thus, from the multitude of clinical forms of dental dystrophies that can affect mixed dentition we decided to confine us only to structural anomalies, namely to molar-incisor hypomineralization and dental fluorosis.

In the described context, the specific goals throughout the doctoral study were:

- Using small rodents detrimental to the retrospective studies in terms of assessment of the involvement of some substances/drugs in the etiology of dental dystrophies.
- Using current and accessible techniques, such as SEM and EDAX, for the quantification of possible micro-structural and compositional changes in dental hard tissues.
- *In vivo* evaluation of the involvement of chronic administration of different doses of amoxicillin/clavulanic acid in the etiology of molar incisor hypomineralization, by means of an evaluation of phenotypic, micro-structural, and mineral content
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changes of enamel induced by these drug formulation and by assessing histological changes occurred in the ameloblastic layer of the lower incisors of laboratory mice.

- Evaluation of the hypothesis that treatment with amoxicillin may cause transitory increase in blood glucose.
- In vivo chemical content variations and morphological changes evaluation of mice incisors’ enamel induced by chronic increased systemic intake of fluoride from drinking water.
- Assessment of the involvement of simultaneous administration of sodium fluoride and amoxicillin/clavulanic acid in the etiology of dental enamel dystrophies; through assessing the variations in enamel mineral content, evaluation of changes in outer enamel morphology, and evaluation of histological changes in the ameloblastic layer of mice lower incisors’ enamel.
- Optimization of the assessment of the child’s orodental health by taking into consideration its impact on the quality of life through cross-cultural adaptation and psychometric proprieties’ evaluation of the CPQ8-10 questionnaire.
CHAPTER 4
EVALUATION OF THE INVOLVEMENT OF CHRONIC ADMINISTRATION OF AMOXICILLIN/CLAVULANIC ACID IN THE ETIOLOGY OF MOLAR INCISOR HYPOMINERALIZATION

4.1 INTRODUCTION

One of the clinical forms of enamel hypomineralization is the so-called molar incisor hypomineralization (MIH), which affects most severely the first permanent molars and it is usually accompanied by less severe defects in the incisors.

Several retrospective and experimental studies suggested that amoxicillin can be involved in the etiology of MIH, but results were inconclusive, and the need for further controlled studies on test animals was highlighted (Hong et al., 2011, Ciarrocchi et al., 2012, Kumazawa et al., 2012).

The aim of our study was to assess variations in the enamel mineral content and to evaluate changes in outer enamel morphology and histological changes in the ameloblastic layer of mice lower incisors’ enamel induced by chronic administration of AMC in different doses.

4.2 MATERIAL AND METHODS

Twenty-eight C57BL/6 adult male mice of similar age, randomly divided into a control group (treated only with solvent – 0.1 mL distilled sterile water, once per day) and 3 treatment groups (n=7) which received subcutaneous injection once per day, for 60 days of 50 mg/kg BW (group II), 100 mg/kg BW (group III), and
150 mg/kg BW (group IV) of amoxicillin as amoxicillin/clavulanic acid (AMC, Amoxiplus®, Antibiotice SA, România). After 60 days of AMC treatment, the mice were deeply anesthetized with Isoflurane and sacrificed by decapitation (using sharp scissors). In order to assess the gross appearance of the incisors, photographs were taken with a D-SLR camera. The dental crowns were harvested and analyzed by SEM with attached EDAX module. The remaining mandibular bone (including the roots of the incisors) was used in order to perform histological analysis.

4.3 RESULTS

The incisors’ enamel of mice in the control group was dark yellow or orange-yellow, smooth, and translucent. In contrast, the lower incisors’ enamel of the AMC-treated mice appeared significantly different, with partial loss of translucency and yellow pigmentation, with chalky/whitish enamel in some areas (fig. 4.9).

![Fig. 4.9 Gross appearance and morphology of the incisors. (A) macroscopic aspect of lower incisors in control group (B-D) lower incisors of amoxicillin/clavulanic acid treated mice (group II, III, and IV respectively).](image)

Even though EDX analysis on the enamel in AMC treatment groups showed an increase in mean values of C, N, F, Na, and F/Fe ratio and a decrease in mean values of P, Cl, Ca, Fe, and Ca/P ratio as compared
to the control group, Mann–Whitney U test revealed significant differences only for F, P, and Ca (table 4.1). Since elements such as Mg, Se, Zn, K, and Al were present under 0.2%, and inconstant, we decide to exclude them from further EDX analysis.

Table 4.1 Comparison between elemental content of control group incisors’ enamel versus AMC treatment groups incisors’ enamel.

<table>
<thead>
<tr>
<th>Element/ ratio</th>
<th>Control 0.1 mL/day solv. (I)</th>
<th>50 mg/kg BW/day AMC (II)</th>
<th>100 mg/kg BW/day AMC (III)</th>
<th>150 mg/kg BW/day AMC (IV)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>20.96 ± 3.91 (+) 21.05 ± 2.06 (+) 21.14 ± 3.21 (+) 23.96 ± 5.26</td>
<td>0.654</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1.35 ± 0.59 (+) 1.55 ± 0.72 (+) 1.59 ± 0.90 (+) 1.86 ± 0.74</td>
<td>0.645</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>30.96 ± 5.26 (+) 34.79 ± 4.45 (+) 31.32 ± 4.82 (-) 30.65 ± 6.10</td>
<td>0.383</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.59 ± 0.34 (+) 1.00 ± 0.24 (+) 0.73 ± 0.36 (+) 0.74 ± 0.27</td>
<td>0.105</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>0.48 ± 0.06 (+) 0.59 ± 0.17 (+) 0.55 ± 0.16 (+) 0.68 ± 0.27</td>
<td>0.619</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>14.32 ± 1.18 (-) 13.49 ± 0.61 (+) 14.33 ± 0.35 (-) 13.55 ± 1.24</td>
<td>0.189</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl</td>
<td>0.41 ± 0.08 (-) 0.37 ± 0.05 (-) 0.40 ± 0.06 (-) 0.37 ± 0.04</td>
<td>0.512</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>29.79 ± 4.73 (-) 25.88 ± 3.32 (-) 28.93 ± 2.86 (-) 27.33 ± 2.80</td>
<td>0.236</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>1.23 ± 0.41 (-) 1.10 ± 0.41 (-) 0.97 ± 0.22 (-) 0.83 ± 0.39</td>
<td>0.278</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/O</td>
<td>0.69 ± 0.18 (-) 0.61 ± 0.13 (-) 0.69 ± 0.19 (+) 0.84 ± 0.38</td>
<td>0.619</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca/P</td>
<td>2.07 ± 0.23 (-) 1.91 ± 0.19 (-) 2.01 ± 0.16 (-) 2.03 ± 0.30</td>
<td>0.430</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F/Fe</td>
<td>0.56 ± 0.38 (+) 0.91 ± 0.40 (+) 0.80 ± 0.48 (+) 1.17 ± 0.86</td>
<td>0.391</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Data represent mean±standard deviation values of percentage by mass (wt %) of 7 mice per group; (+) or (-) indicates an increase or decrease compared to the control group; p*- p value from Kruskal-Wallis test (p<0.05 statistically significant); solv. = solvent (sterile water for injections).

SEM (FEI Quanta 200 ESEM) observations of the incisors’ enamel in the control group showed homogeneous, regular, and smooth surfaces with several scarcely visible point-like pits. Morphology changes in the experimental groups ranged from scratched patterns, and small isolated pits-like enamel loss, to generalized demineralized enamel surface, giving a rough, foamy, scaly, or even cracked eggshell appearance to the affected areas (fig. 4.13, 4.17).

Histological analysis showed disturbances of maturation ameloblasts, which were less organized, with
increased amounts of clear vacuoles in the cytoplasm and slightly more elongated and less condensed nucleus, especially in groups III and IV. Additionally, they were often detached from the enamel matrix. Transitional ameloblasts formed underlying the cysts like lesions of varied sizes.

Fig. 4.13 Ultrastructure of lower incisors’ outer enamel by SEM in the treatment group no. II (50 mg/kg BW of AMC): We observed irregular scratched pattern in all specimens; isolated enamel loss with perfectly regular margins, smooth base, which extends from outer to inner enamel (A, D); conglomerate of superficial pits in outer enamel with regular margins with variable size and depth, giving a moth-eaten appearance to the affected areas (B); and grouped fissures in the outer enamel with variable size, depth, and irregular margins (C). Magnification: A, B =10000x, C, D =5000x.

4.4 DISCUSSION

The bioavailability of amoxicillin was reported to be more than 80% in humans, but only 44% in rats when administered orally (JECFA, 2012). Low bioavailability of the drug in small rodents (mice and rats) was related to presystemic degradation of amoxicillin in the intestine.
To avoid these inconveniences, we decided to use subcutaneous injections as route of administration.

In groups II and III, most of the defects were pits or fissures with variable incidence, size, depth, and usually with regular, smooth margins. These quantitative defects reveal enamel hypoplasia (dos Santos, Maia, 2012), which is inconsistent with characteristics of teeth affected by MIH (specific qualitative defects).

![Ultrastructure of lower incisors’ outer enamel by SEM in the treatment group no. IV (150 mg/kg BW of AMC). All examined surfaces were demineralized resulting in a rough, foamy (D), scaly (F), or even cracked eggshell (E) appearance in the affected areas. Magnification: A, C, D =10000x, B =1000x.](image)

Cracked eggshell appearance (fig. 4.17) indicates hypomineralization within inner enamel associated with breakdowns in outer enamel. The presence of hypoplastic lesions relates with results from several clinical studies, which suggest that amoxicillin may also cause fluorosis-like lesions (Laisi et al., 2009, Gottberg et al., 2014).
Through EDX analysis (table 4.I), we found that all AMC treatment groups tended to show the lower content of Ca and P in enamel, as a sign of hypomineralization. Also, all groups had almost similar Ca/P ratio, which indicates that calcium phosphate was a greater part of enamel inorganic content (Jalevik et al., 2001, Mahoney et al., 2004). AMC administration dose-dependently decreased the iron levels in the incisors’ outer enamel (table 4.I), which has led to partial loss of yellowish pigmentation (fig. 4.9). Moreover, increased levels of fluoride in AMC treatment groups could be involved in the translucency loss, as was previously reported by Everett (2011). Formation of the cyst-like lesions under the TA in the 100 and 150 mg/kg BW of AMC groups can be associated with local enamel hypoplasia seen as pits at the enamel surface of erupted teeth (Lyaruu et al., 2006). On the other hand, as our EDX analysis results suggest an increase in both carbonate and protein content in the outer enamel, we can assume that AMC has interfered with MA functions (like enamel matrix proteins removing and mineralization inducing) causing occurrence of the qualitative defects in enamel (hypomineralization).

4.5 CONCLUSIONS

Our results denote that chronic administration of AMC trough subcutaneous injection in C57BL/6 mice rise n-FBG levels, leads to disturbances in formation of lower incisors’ enamel, mainly as a dysfunction in the maturation and transitional ameloblasts, resulting in hypomineralized enamel, with quantitative and/or qualitative dose-dependent defects.
5.1 INTRODUCTION

Fluoride plays an important role in human health. It is known that fluoride ions participate actively in the enamel remineralization process and inhibit the cariogenic bacteria activity from the oral biofilm (Bălan et al., 2015).

Increased systemic intake of fluoride during critical periods of amelogenesis leads to dental fluorosis. Fluoride affects the ameloblasts function during the secretory and the maturation phase, causing poor mineralization and porous enamel formation, which are characterized by enlargement of the intercrystalline spaces that will be filled with proteins and water (Lyaruu et al., 2006; Warren et al., 2009).

The aim of this study was (1) to assess the morphology, clinical and compositional changes induced by chronic intoxication with low doses of NaF in mice enamel, (2) evaluation of systemic uptake of fluoride through EDX compositional analysis of the femur, and (3) evaluation of the histological changes induced by fluoride administration in ameloblastic layer.

5.2 MATERIAL AND METHODS

In this study we used 21 C57BL/6 inbred strain weanling male mice (8 to 10 weeks old), which were randomly divided into a control group and 2 experimental groups, consisting of 7 mice each. The
control group received only distilled water, group II received distilled water supplemented with 25 ppm fluoride, and group III received distilled water supplemented with 50 ppm fluoride. Fluoride was supplied as NaF through drinking water, for 60 days, and the mice had *ad libitum* access to standardized laboratory rodent diet and distilled water. After harvesting, fixation in glutaraldehyde, and dehydration in ethanol, lower incisors’ enamel was subjected to SEM and EDX analysis; the remaining mandibles were used in order to perform histological analysis.

5.3 RESULTS

Comparative examination of lower incisors’ photographs between the control group and groups treated with NaF, revealed changes in the translucency and color of the enamel (fig. 5.6).

![Fig. 5.6](image)

*Fig. 5.6* Clinical aspects and morphology of the incisors. (A) The incisors of mice in the control group were of a dark yellow or orange-yellow color, smooth and transparent. (B) Group II (25 ppm NaF): less yellow pigment, semi-opaque in some of the areas and exhibited slight roughness with regular, fine (scarcely visible) horizontal stripes of interleaved yellow and white colors. (C) Group III (50 ppm NaF): the incisors’ surface were notably rough, exhibiting changes to a chalk color, and the horizontal stripes of interleaved brown and white colors were more striking.
EDX elemental analysis in both experimental groups showed higher weight content of C, O, N, Na, and lower weight content of Ca, P, F, Fe, and Cl (table 5.I). SEM analyses showed morphological changes which ranged from small, isolated enamel pits with regular margins, to extensive and deep loss of dental hard tissues with irregular margins and dentine exposed areas, as well as multiple fissures in the enamel surface (fig. 5.9).

**Table 5.I Comparison of elemental content of control group incisors’ enamel versus experimental groups incisors’ enamel**

<table>
<thead>
<tr>
<th>Element</th>
<th>Group I (distilled water)</th>
<th>Group II (25 ppm NaF)</th>
<th>Group III (50 ppm NaF)</th>
<th>$p^*$ Group I/III</th>
<th>$p^{**}$ Group I/II</th>
<th>$p^{**}$ Group I/III</th>
<th>$p^{**}$ Group II/III</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>20.96 ± 3.91</td>
<td>(-)19.42 ± 2.45</td>
<td>(-)18.66 ± 1.78</td>
<td>0.420</td>
<td>0.749</td>
<td>0.199</td>
<td>0.391</td>
</tr>
<tr>
<td>N</td>
<td>1.35 ± 0.59</td>
<td>(+)1.49 ± 0.42</td>
<td>(+)1.46 ± 0.52</td>
<td>0.916</td>
<td>0.688</td>
<td>0.775</td>
<td>0.886</td>
</tr>
<tr>
<td>O</td>
<td>30.96 ± 5.26</td>
<td>(+)33.16 ± 3.20</td>
<td>(+)35.07± 4.58</td>
<td>0.358</td>
<td>0.433</td>
<td>0.199</td>
<td>0.391</td>
</tr>
<tr>
<td>F</td>
<td>0.59 ± 0.34</td>
<td>(+)0.63 ± 0.17</td>
<td>(+)0.99 ± 0.38</td>
<td>0.088</td>
<td>0.936</td>
<td>0.086</td>
<td>0.046†</td>
</tr>
<tr>
<td>Na</td>
<td>0.48 ± 0.06</td>
<td>(+)0.56 ± 0.11</td>
<td>(+)0.60 ± 0.08</td>
<td>0.079</td>
<td>0.173</td>
<td>0.022†</td>
<td>0.568</td>
</tr>
<tr>
<td>P</td>
<td>14.32 ± 1.18</td>
<td>(+)14.85 ± 1.26</td>
<td>(+)14.58± 1.36</td>
<td>0.827</td>
<td>0.522</td>
<td>0.775</td>
<td>0.775</td>
</tr>
<tr>
<td>Cl</td>
<td>0.41 ± 0.08</td>
<td>(-)0.32 ± 0.06</td>
<td>(-)0.37 ± 0.10</td>
<td>0.259</td>
<td>0.078</td>
<td>0.474</td>
<td>0.473</td>
</tr>
<tr>
<td>Ca</td>
<td>29.79 ± 4.73</td>
<td>(-)28.83 ± 2.18</td>
<td>(-)27.77 ± 4.29</td>
<td>0.812</td>
<td>0.873</td>
<td>0.475</td>
<td>0.775</td>
</tr>
<tr>
<td>Fe</td>
<td>1.10 ± 0.41</td>
<td>(-)0.70 ± 0.36</td>
<td>(-)0.46 ± 0.29</td>
<td>0.029†</td>
<td>0.092</td>
<td>0.015†</td>
<td>0.198</td>
</tr>
<tr>
<td>C/O</td>
<td>0.69 ± 0.18</td>
<td>(-)0.59 ± 0.12</td>
<td>(-)0.53 ± 0.07</td>
<td>0.154</td>
<td>0.262</td>
<td>0.063</td>
<td>0.391</td>
</tr>
<tr>
<td>Ca/P</td>
<td>2.07 ± 0.23</td>
<td>(-)1.95 ± 0.23</td>
<td>(-)1.89 ± 0.11</td>
<td>0.262</td>
<td>0.337</td>
<td>0.116</td>
<td>0.475</td>
</tr>
<tr>
<td>F/Fe</td>
<td>0.56 ± 0.38</td>
<td>(+)1.09 ± 0.53</td>
<td>(+)2.69 ± 1.61</td>
<td>0.003†</td>
<td>0.109</td>
<td>0.003†</td>
<td>0.015†</td>
</tr>
</tbody>
</table>

*Note: Data represent mean ± standard deviation values of percentage by mass (wt %) of 7 mice per group. (+) or (-) indicates an increase or decrease compared to the control group; $p^*$ - $p$ value from Kruskal-Wallis test; $p^{**}$ - $p$ value from Mann-Whitney test; † statistically significant differences ($p<0.05$).*  

Histological analysis showed disturbances of SA, which had increased amounts of clear vacuoles in the cytoplasm. Occasionally SA formed underlying the cysts like lesions of varied sizes. MA were less organized, with increased amounts of clear vacuoles in the cytoplasm and slightly more elongated and inhomogeneous condensed...
nucleus, and often detached from the enamel matrix, especially in group III (50 ppm NaF) (fig. 5.16).

5.4 DISCUSSION
For our study we used mice because rodent incisor teeth grow continuously and exhibit all stages of tooth formation. Amelogenesis of mice incisors has been well studied (Smith, Nanci, 1996; Smith et al., 2005).

![Fig. 5.9 Ultrastructural appearances of mice lower incisors’ outer enamel in experimental group III (50 ppm NaF). It can be observed (A) a demineralized outer enamel with splotchy appearance; deep, narrow and elongated pits in outer enamel; (B) large outer and inner enamel loses with irregular margins, base and stair-stepped appearance; (C) conglomerate of irregular outer enamel loses with variable size, shape and depth giving a rough appearance to the affected areas; (D) isolated or multiple outer enamel cracks with irregular shape and antiparallel orientation. Analyzing the images obtained by SEM, we noticed that compared to control mice, the enamel surface in group II (25 ppm NaF) was much rougher and...](image-url)
covered by irregular "scratches" identified in all examined areas. The above described appearance is consistent with data reported by Chen et al., (2006).

Fig. 5.16 Photomicrographs of Masson’s trichrome-stained sections of mouse incisors. (A-B) Control group; secretory (A) and maturation (B) ameloblasts appearance. Maturation ameloblasts appearance in the group treated with 50 ppm NaF (C) and 100 ppm NaF (D). Original objective is 40x.

The presence of quantitative defects such as pits with smooth or irregular margins on the enamel surface, may suggest the influence of fluorides on the ameloblasts in the secretory phase. Even if such defects are characteristic of acute exposure to high doses of F (Bronckers et al., 2009), there are studies that claim that ameloblasts in the early secretory phase are highly susceptible to the action of fluoride. It was suggested that pits are associated with fluoride action on ameloblasts during transition from pre-secretory to secretory phase (Kierdorf, 1997).
In our study, EDX analysis revealed a decreasing trend of wt% for Ca, Fe, and an increase of wt% for C, P and F in outer enamel (table 5.1), which suggest formation of hypomineralized enamel. This is opposed to Suga's study, which reported an increase of Ca and F content (Suga et. al., 1987). Most of the studies have reported the formation of hypermineralized outer enamel and porous, hypomineralized inner enamel as result of chronic exposure to fluoride in drinking water. However, we have identified several signs of hypomineralization in the outer enamel as splotchy, furrowed, and fissured appearance in the affected areas, especially in group III (fig. 5.9). Moreover, superficial pits with conglomerate of irregular outer enamel loses of variable size, shape and depth giving a rough appearance to the affected areas, and the presence of horizontal white lines identified by us in both experimental groups, may suggest damage of ameloblasts in the late secretory or transition phase.

5.5 CONCLUSIONS
Chronic administration of 25 and 50 ppm NaF lead to chromatic and translucency changes of the incisors’ enamel in C57BL/6 mice, seen as reducing the amount of specific orange pigment and accentuated visibility of perikymata.

The severity of the morphological changes in mouse enamel varied with the supplied dose of NaF. The time period chosen to supply NaF was enough to induce lesions with uniform pattern in each experimental group. The SEM and EDX analysis confirmed the compositional and morphological changes specific to dental fluorosis.
6.1 INTRODUCTION

Systemic intake of fluoride higher than the optimal dose ($\geq 0.07$ mg F/kg/day) during critical periods of amelogenesis may lead to dental fluorosis (DF) (Warren et al., 2009; Everett, 2011).

Amoxicillin/clavulanic acid (AMC) is widely prescribed as a first choice antibiotic agent in pediatrics and pedodontics (Kumazawa et al., 2012; Gottberg et al., 2014), it was suggested that its use in early childhood is associated with MIH and similar to dental fluorosis defects (Hong et al., 2011). It is speculated that amoxicillin would interfere with ameloblasts functions in the secretory phase (Kumazawa et al., 2012).

In terms of associated action of amoxicillin and fluorides on amelogenesis, there exists a limited number of studies with some methodological problems, inconclusive results, and the need for further controlled studies on test animals was highlighted (Hong et al., 2011; Ciarrocchi et al., 2012; Kumazawa et al., 2012).

The aim of our study was to assess variations in enamel mineral content and to evaluate changes in outer enamel morphology and histological changes in the ameloblastic layer of mice lower incisors’ enamel induced by chronic and simultaneous administration of AMC+NaF in different doses.
6.2 MATERIAL AND METHODS

For our study we used 35 C57BL/6 inbred strain adult male mice (8 and 10 weeks old), randomly divided into:

- group I: control, received only solvent (0.1 ml distilled sterile water for once per day) and \textit{ad libitum} distilled water for drinking;
- group II: \textit{ad libitum} 25 ppm NaF in distilled water and 50 mg/kg BW/once per day of AMC, SC (subcutaneously);
- group III: \textit{ad libitum} 25 ppm NaF in distilled water and 100 mg/kg BW/once per day of AMC, SC;
- group IV: \textit{ad libitum} 50 ppm NaF in distilled water and 50 mg/kg BW/once per day of AMC, SC;
- group V: \textit{ad libitum} 50 ppm NaF in distilled water and 100 mg/kg BW/once per day of AMC, SC.

NaF extra pure powder (Scharlau® Spain), and amoxicillin/ clavulanic acid as powder for injections (Amoxiplus®, Antibiotice SA), purchased from a reputable pharmaceutical company, were administered for 60 days.

After 60 days of AMC+NaF treatment, the mice were deeply anesthetized with Isoflurane and sacrificed by decapitation. Incisors were photographed, harvested and subjected to SEM+EDX, histology analysis and clinical evaluation.

6.3 RESULTS

The enamel color of lower incisors of the AMC+NaF treated mice appeared significantly different, with partial loss of translucency and yellow pigmentation, more visible perikymata (especially at
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groups III-V), with chalky/whitish enamel in some of the areas (identified in all experimental groups) (fig. 6.1).

**Fig. 6.1** Clinical aspects and morphology of the incisors of mice in the control group and experimental groups at the end of treatment period with AMC+NaF. A - group I (control); B - group II; C - group III; D - group IV; E - group V

**Fig. 6.5** Ultrastructural appearances of mice lower incisors’ outer enamel in experimental group V (treated with 50 ppm NaF and 100 mg/BW of AMC). (A) an overall appearance, (B) demineralized enamel with corroded appearance, (C) conglomerates of deeper pits and fissures, (D) large pits, with furrowed appearance at base. Magnification: A=1000x, B, C=10000x, D=10000x.

SEM revealed morphology changes in the experimental groups which ranged from small superficial pits, with regular margins and isolated areas with evident
advanced demineralization (group II), to extended demineralized areas in the outer enamel, with corroded appearance (group V) (fig. 6.5).

EDX elemental analysis in the experimental groups tended to show the higher wt% of C, N, O, F, Na, C/O, F/Fe ratio and lower wt% of P, Cl, Ca, Fe, Ca/P ratio. Statistically significant differences were identified only for Fe (between groups I- III, IV; II-IV, decreased); for F (between groups II-III, V; IV-V, has increased); for N (II-III, has increased), and ratio F/Fe (between groups I-III-V; II-III-V, has increased).

Histological analysis revealed the most severe changes for both SA and MA in group V (fig. 6.16).

![Fig. 6.16 Photomicrographs of Masson’s trichrome-stained sections of mouse incisors in the group IV (A, B) and V (C-E). Cystic defects and spotted staining of the enamel matrix (A, C); detaching of the SA layer and elongation of Tomes’ processes (D); Structural defects within the MA layer (E). Objectives: A: 10x; B, D, E: 40x; C: 20x.]

6.4 DISCUSSION

Even though most dental dystrophies have included in their etiology also a genetic component, some
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predisposing or etiological factors can be easily avoided by simply respecting some indications of preventing their occurrence.

In our study EDX analysis revealed Ca/P ratio of 2.07 in the control group, this varied insignificantly in the experimental groups (ranged between 1.97- 2.20). Thus, in terms of quality, the enamel’ structure was modified insignificantly, however there is the possibility of significant differences in terms of its quantity. Furthermore, C/O ratio of 0.69 for the control group and ranging between 0.73 and 0.94 in the experimental groups, along with increased values of wt% for C (20.96 in the control, and 22.11-25.21 in the experimental groups) and O (of 30.96 in the control and 28.30-33.00 in the experimental groups) suggest formation of excess carbonate hydroxyapatite Ca$_5$(PO$_4$,CO$_3$)$_3$(OH). Carbonate hydroxyapatite having reduced hardness may contribute to the occurrence of post eruptive defects in the enamel (as we noticed a "scratched" appearance of the enamel).

High wt% values for F in the groups III and V (0.59 control, and 0.70, 0.90 in the groups III and V) can suggest subsequent formation of carbonate fluorapatite Ca$_5$(PO$_4$,CO$_3$)$_3$F.

It is believed that the presence of fluoride in the enamel contributes to its strength of acid attack, but does not contribute to its mechanical strength, as it undermines it. This may explain the presence of enamel cracks observed by us during SEM images analysis.

Clinically observed roughness of the enamel in groups treated with AMC and NaF, may be associated with its inadequate maturation.
Great systemic intake of fluoride may increase blood glucose levels or may lead to impaired glucose tolerance and may aggravate the severity of some types of diabetes in both humans and tested animals (Trivedi et al., 1993; Xie et al., 2000; Chehoud et al., 2008; Vasant, Narasimhacharya, 2013).

Recently, the hypothesis stating that treatment with amoxicillin may increase blood glucose levels has already been raised in literature. However, there are no studies that confirm or disqualify this. In our study, blood glucose levels varied moderately intra- and inter-groups. We consider that further studies are necessary before enunciating some pertinent conclusions regarding this aspect.

6.5 CONCLUSIONS

Chronic administration of different doses of NaF (through drinking water) and AMC (through subcutaneous injections) in C57BL/6 mice lead to partial loss of specific orange pigmentation and changes in translucency of the incisors’ enamel, with occurrence of some areas of rough/whitish enamel, and more visible perikymata. These changes did not have a uniform distribution in the experimental groups.

Ultrastructural changes identified by SEM indicate outer enamel hypoplasia. EDX results indicate outer enamel hypomineralization.

We recommend avoiding prolonged or repeated treatments at short intervals of time with amoxicillin as AMC in the first 2 years of life of children, whether or not originating in areas with high levels of F in drinking water.
7.1 INTRODUCTION

The concept of oral health-related quality of life (OHRQoL) relates to the impact that oral conditions have on the individual’s daily functioning, well-being or quality of life. During the last ten years, researchers have developed different self-reported OHRQoL instruments for children and adolescents between 6 and 15 years old (Abanto et al., 2013).

The Child Perceptions Questionnaire (CPQ) represents one of these instruments, as a supplement to clinical indicators used to assess the child’s OHRQoL (Jokovic et al., 2002). Child Perceptions Questionnaires (CPQ) were developed for measurements, taking into account the children’s cognitive abilities and lifestyle for an age range from 6 to 7 years (CPQ6-7), 8 to 10 years (CPQ8-10) and from 11 to 14 years (CPQ11-14) (Jokovic et al., 2002, Jokovic et al., 2004).

The aim of the present study was to optimize the assessment of the child’s orodental health by taking into consideration its impact on the quality of life. The objectives of this study were (1) to translate the English version of CPQ8-10 into Romanian, (2) to adapt the questionnaire to the specifics of the Romanian culture, (3) to evaluate its comprehensibility through the means of a preliminary qualitative study, and (4) to evaluate its psychometric proprieties through the means of a validity and reliability evaluation.
7.2 MATERIAL AND METHODS

After translation and cross-cultural adaptation, performed in accordance with internationally accepted guidelines (fig. 7.1), CPQ$_{8-10}$ was applied to 120 children aged from 8 to 10 years in order to assess its validity and reliability.

![Diagram](image)

Fig. 7.1 Translation, cultural adaptation and psychometric proprieties evaluation chart of the questionnaire CPQ$_{8-10}$ for Romanian language.

The internal consistency of the instrument was assessed by Cronbach's Alpha Coefficient and the test-retest reliability by Intraclass Correlation Coefficient using one-way analysis of variance random effect. Spearman's correlation coefficients were calculated to
assess construct validity between the total and subscale scores and the respondents’ global ratings on oral health and well-being. Discriminant validity was analyzed using the Kruskal-Wallis or Mann–Whitney tests in groups defined by gender, family socioeconomic status, caries experience, place of residence (rural or urban) and presence of dental dystrophies.

7.3 RESULTS

Both initial translations were almost similar. After review, the Committee decides to make several changes by taking into consideration the cultural particularities of the studied population and the social variability of the possible target group. The results concerning cultural adaptation (pre-test) show that the Romanian version of the CPQ\textsubscript{8-10} questionnaire was well understood by the all 20 children who participated in this stage of the study. The purpose of this stage was to assess the questionnaire’s readability, comprehension and ease of application. No item exceeded the admitted limit of misunderstanding by the respondents (15%), so a further review of any of the questions from the questionnaire was not necessary.

CPQ\textsubscript{8-10} psychometric proprieties evaluation study was conducted on a convenience group, consisting of 123 children which was formed based on the variable "area of origin" (urban/rural). In order to assess the test-retest reliability, randomly selected subgroup (N = 41) of the children were invited again to answer the questionnaire after a 10-day period. CPQ\textsubscript{8-10} global score was calculated by summing in advance coded answers to all
25 questions of the questionnaire, also were calculated scores for each of the 4 evaluated domains.

The mean score on the CPQ8-10 was 14.91 (SD = 1.06), and the scores on all the domains were found to be highly skewed and platykurtic. Cronbach's alpha coefficient ranged from 0.67 to 0.88, showing acceptable to good internal consistency for the evaluated domains and excellent consistency for the global CPQ8-10 score (table 7.VIII).

**Table 7.VIII** Internal consistency of the CPQ8-10 scores

<table>
<thead>
<tr>
<th>Number of items</th>
<th>Cronbach’s alpha (n=123)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total scale</td>
<td>25</td>
</tr>
<tr>
<td><strong>Subscales</strong></td>
<td></td>
</tr>
<tr>
<td>Oral symptoms</td>
<td>5</td>
</tr>
<tr>
<td>Functional limitations</td>
<td>5</td>
</tr>
<tr>
<td>Emotional well-being</td>
<td>5</td>
</tr>
<tr>
<td>Social well-being</td>
<td>10</td>
</tr>
</tbody>
</table>

Test-retest reliability revealed almost perfect reproducibility (ICC = 0.98) (table 7. IX). The global ratings on oral health and well-being were correlated to the total score (r_s = 0.87 and 0.72 respectively) and to the sub-scores (varied between 0.56 and 0.82). The CPQ8-10 score was able to discriminate between all defined groups, except gender group that did not reach statistical significance.

**7.4 DISCUSSION**

In order to be used in a non-English speaking population and to ensure the equivalency with the original, any instrument must be translated, adapted and validated in accordance with internationally accepted procedures (Herdman et al., 1998; Widenfelt et al.,
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2005). We have followed the steps outlined in the guidance issued by Guillemin et al. (1993); this prevents reducing the potential of the original instrument or occurrence of biases in interpretation due to incorrect adaptation of tools at distinct cultural peculiarities of the target country. Cultural adaptation is a key step in developing a tool for measuring skills and attitudes. Often this process is long, but allows developing a tool equivalent to the original and accepted to be used in international comparative studies (Barbosa et al., 2011).

| Table 7.IX Test-retest reliability statistics of the CPQ8-10 |
|-----------------------------------------------|-------------------|-----------------|-----------------|-----------------|
| Mean-test (DS) | Mean-retest (DS) | ICC* (N=41) | inf. limit | sup. limit |
| Total scale      | 14.1(12.3)       | 14(11.3)         | 0.989         | 0.977         | 0.995         |
| Subscales        |                   |                  |               |               |               |
| Oral symptoms    | 6.1(3.9)         | 5.7(3.8)         | 0.973         | 0.944         | 0.987         |
| Functional limitations | 3.2(3.6) | 3.2(3.3)         | 0.982         | 0.962         | 0.991         |
| Emotional well-being | 2.6(3.1) | 2.6(2.7)         | 0.975         | 0.949         | 0.988         |
| Social well-being  | 2.2(3.5)         | 2.6(3.2)         | 0.974         | 0.946         | 0.988         |

*Intraclass correlation coefficient

Another issue that must be considered in the process of adaptation is the formation and implication of the so-called Committee of Review. In our study, this Committee was formed by a multidisciplinary team that compared the original instrument to the two initial translations, the preliminary version, and the version resulted after the backward translation. This Committee also verified the correctness of the instructions for completing the questionnaire and the exactitude of the response scales.

The qualities of a questionnaire are usually tested by a cross-sectional study comprising a group of 50 to
200 respondents, while evaluating the test-retest reliability requires a sub-group of about 10% (approx. 30 individuals) (Herdman et al., 1998; Widenfelt et al., 2005; Abanto et al., 2013).

The reliability evaluation through test-retest method revealed almost perfect concordance in our study (Landis, Koch, 1997), ICC=0.989 (0.973-0.982). Similar results were reported by other authors: 0.96 (0.89-0.98) (Wogelius et al., 2009), 0.96 (0.79-0.95) (Torres, 2008).

7.5 CONCLUSIONS

Testing the questionnaire in a group of children showed that the Romanian version of the CPQ8-10 seems to be a valid instrument for assessing quality of life in children associated with oral health.

CPQ8-10 may have important implications in both research and practice. First, it can provide insight into the impact of disturbances induced by oral diseases and its perception by children at everyday life. Secondly, it could be used by clinicians to assess success of orodental treatment. Also, the questionnaire can be used as an indicator for monitoring social change, for evaluating social intervention programs and policies and the efficiency of oral health programs, as well as dental treatment needs for this age group.

In children with dental dystrophies, the questionnaire proved to be a good instrument to assess the impact of dental dystrophies on their state of emotional and social well-being.

The main purpose of this study was to make the questionnaire CPQ8-10 ready for use in Romania, and it was successfully conducted.
1. The doctoral thesis represents an experimental study that analyzes the effects of some chemicals on the enamel development, and their involvement in the etiology of dental dystrophies. Also, the present work has pursued optimization of orodental health assessment of children with dental dystrophies in terms of quality of life.

2. The results of conducted studies attest that C57BL/6 inbred strain mice is an appropriate model for studying the involvement of several substances in the etiology of dental dystrophies generally, and dental fluorosis with molar-incisor hypomineralization particularly.

3. Chronic administration of AMC in concentrations of 50/100/150 mg/kg BW/day using subcutaneous injection induces phenotypic, morphologic, histologic, and compositional changes in lower incisors of laboratory mice.

4. Depending on the administered doses, AMC causes partial loss of translucency, yellow pigmentation and the emergence of some chalky/whitish areas in mice incisors’ enamel.

5. SEM and EDX analysis performed at the middle ⅓ of the lower incisors’ enamel of AMC treated mice,
revealed hypomineralized enamel formation with quantitative and/or qualitative morphological defects.

6. The histological analysis performed at the level of bone portions of the lower incisors in groups treated with AMC revealed disturbances predominantly at MAs. They were less organized and had increased amounts of clear vacuoles in the cytoplasm. Additionally, the nuclei were slightly elongated and less condensed. Also, MAs were often detached from enamel matrix and from neighboring cells. Moreover, TAs were disorganized and had formed underlying cyst-like lesions of varied sizes.

7. Supplementing drinking water with 25 and 50 ppm of NaF led to chromatic changes in mice lower incisors by reducing the amount of specific orange pigment, and accentuated perikyma visibility.

8. SEM and EDX analysis performed at the middle $\frac{1}{3}$ of the lower incisors’ enamel of NaF treated mice, revealed hypomineralized enamel formation with a high content of $\text{F}^-$, and presence of some morphological quantitative defects, which severity varied depending on administered doses of NaF.

9. MAs seem to be most sensitive to chronic exposure of $\text{F}^-$ in low doses. A characteristic defect of this stage is the development of very porous and hypomineralized inner enamel layer.

10. Simultaneous administration of AMC and NaF in mice revealed the existence of a linear relationship
between severity of enamel defects and administered doses of AMC+NaF.

11. In groups treated with the AMC + NaF in addition to the partial loss of translucency, yellow pigmentation, the emergence of some chalky/whitish areas, and more accentuated perikyma visibility in mice lower incisors; the SEM and EDX analyses revealed some changes specific to enamel hypoplasia (through presence of quantitative defects), and enamel hypomineralization (through downward trend for wt% of P, Cl, Ca, Fe, and Ca/P ratio).

12. We recommend avoiding prolonged or repeated treatments at short intervals of time with amoxicillin as AMC in the first 2 years of life of children, whether or not originating in areas with high levels of F in drinking water.

13. Cross-cultural adaptation and psychometric proprieties evaluation of the Romanian version of the CPQ8-10 questionnaire revealed good construct validity (convergent and discriminant), test-retest reliability, and internal consistency, recommending it as a suitable instrument for assessing OHRQoL in Romanian children aged from 8 to 10 years.

14. CPQ8-10 may have important implications in both research and practice. First, it can provide insight into the impact of disturbances induced by oral diseases and its perception by children at everyday life. Secondly, it could be used by clinicians to assess success of
orodental treatment. Also, the questionnaire can be used as an indicator for monitoring social change, for evaluating social intervention programs and policies and the efficiency of oral health programs, as well as dental treatment needs for this age group.

15. In children with dental dystrophies, the questionnaire proved to be a good instrument to assess the impact of dental dystrophies on their state of emotional and social well-being.
CHAPTER 9
FUTURE PERSPECTIVES OF THE DOCTORAL STUDY

The doctoral research may be further developed by following a series of goals:

1. Our study regarding the involvement of AMC administration in the etiology of molar-incisor hypomineralization having a unique design can be considered a pilot study and can be continued by:
   - immunohistochemical evaluation of qualitative and quantitative distribution of the proteins involved in amelogenesis (amelogenin, enameline, ameloblastine, tufteline, etc.);
   - accomplish SEM and EDX analysis at the level of inner enamel;
   - evaluation of AMC effects on dentine and dentinogenesis;
   - interdisciplinary study regarding the blood glucose level fluctuations caused by treatment with AMC and/or other antibiotics commonly prescribed in children;

2. Considering internationally highlighted decline in dental caries prevalence and an increasing of dental fluorosis prevalence, it is required rigorous monitoring of overall daily fluoride intake especially in children. The first steps in this direction can be represented by developing "fluoride maps" of Romania, by evaluation of F⁻ concentrations in the primary sources of drinking
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water: surface, underground, and bottled waters from commerce.

3. Collaboration with other country universities in order to perform a national epidemiological study regarding dental dystrophies in children and adolescents.

4. CPQ$_{8-10}$ questionnaire can be used as a standard scale for rating COHRQoL in national and/or international studies deployed in different cultural contexts. However, due to regional socio-cultural differences, to be used nationwide, and in order to confirm presented by us properties, additional transverse studies conducted in other regions of the country are needed.

5. Also, to form an overview of COHRQoL in Romania, it is necessary to adapt and evaluate CPQ questionnaires suitable for the other stages of age (CPQ$_{6-7}$ and CPQ$_{11-14}$).
REFERENCES


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