COMPLEX MORPHOLOGIC INVESTIGATION ON
THE DEVELOPMENT OF THE STOMATOGNAT SYSTEM

SUMMARY OF THE PhD THESIS

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# COMPLEX MORPHOLOGIC INVESTIGATION ON THE DEVELOPMENT OF THE STOMATOGNAT SYSTEM

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This thesis is illustrated by 97 figures and 12 tables. The abstract includes a limited number from this total, while keeping the numbering in the thesis.
INTRODUCTION

A complex process that takes place over many successive stages, tooth development implies an epithelio-mesenchymal cellular dialogue between the oral epithelium and the subjacent mesenchyme. These functional interrelations have repercussions during the process of tooth development so the specific structural alterations, regardless of the type of dentition, decidual or permanent, are identical.

The regional characteristics of the teeth, which result from the development in vivo of the structural elements of the tooth, include changes in the configuration that correspond to the concepts (models) of induction, competence and differentiation (Ten Cate, 1998, Nanci, 2012).

Although the stages and the phases of the tooth development follow one another in dynamics, the strict delineation from a stage to another is impossible; moreover, in certain cases, there is a partial overlap between them. The names of stages are based on the morphology of the epithelial structure in the tooth germ. Consequently, the tooth development process has the following sequences: crown formation, root formation and, concomitantly, of the supporting structures.

In the tissues and structures characteristic for the developing and the mature tooth, the extracellular matrix has a series of structural and distribution particularities that ensure the specific functionality of the tooth.

Essential for the development of the tooth germ are: (i) the basal membrane, (ii) the enamel matrix and (iii) the dentin matrix – all these three structures having an important contents of matrix proteins. The matrix proteins are multifunctional, their role being, in correspondence with their location, extremely different: from promotion of the cell proliferation, migration and adhesion to the mineralization of the hard tissues of the tooth (Hu, Simmer, 2007, von Marschall, Fischer, 2008).

The bioethical principles that govern the research on the tooth development fall within the general framework of the research on human embryonic material. In this context, we perform a synopsis of the fundamental bioethical guidelines – which operate implicitly in the research focused on the development of the stomatognat system in general and of the tooth in particular. Starting from the necessity of embryologic studies, our presentation is oriented on (i) the conceptual framework of the definition of the embryo, (ii) the possibilities of procurement for the embryonic material, (iii) the translation of the scientific interest from embryo to embryonic stem cells, and (iv) international legislation implemented for the regulation of the research on embryos and embryonic stem cells. The experience of the research groups involved in the investigation of the complex mechanism of the tooth development must be valued also from the perspective of the bioethical aspects which control this field of biomedical research.

Within this vast context of the human embryology, the tooth development is, at its turn, well-known and thoroughly described in correlation with the chronological timeline, the eloquent proof being the comprehensive morphological characterization of bud, cap and bell stages.

In the field of dental medicine, the research focused on the complex mechanism of the tooth development involves both studies on embryos and on embryonic stem cells. Consequently, the bioethical principles that regulate these researches follow the general framework of the research on embryonic human material (Post, 2004).

Without a doubt, the research on the tooth development must respect the bioethical principles that operate in all medical fields where human embryonic material is used. Consequently, in the implementation of any research project, regardless of the potential benefits for the current state of knowledge, the moral value of the embryo must always come before everything.

CHAPTER 4. MOTIVATION AND OBJECTIVES

In the literature that covers the state of the art on tooth development two types of information can be distinguished:
- the information on the development and structure of stomatognat system, in general, and of the tooth, in particular, confirmed by numerous studies and integrated in the human biology, unanimously accepted by scientists;
- the information yielded by the recent studies of a small number of specialists that investigate tooth formation mechanisms from the perspective of the genetic and molecular events which determine the intiation, evolution and regulation of the histological and morphological tooth development.
Within this framework, the choice of the subject for the PhD research was motivated by the opportunities offered by the current international research directions on tooth development.

From the background of the investigations that start with the microscopical morphologic structures and go up to the molecular network which supports intercellular and intertissular communication, we started from the premise that the current PhD research will play a part in the deeper understanding of the morphological and functional relationships inside and outside the cells, the action mechanisms of the factors and mediators that intervene in the development and maturation of the definitive structures, the existent correlations between various tissue types and the temporal programation of the events in tooth genesis.

Thus, in this PhD research we focused on three main directions:

1. the investigation of the qualitative cell and tissue characteristics of the tooth germ through a classic analysis by light microscopy, centered on the appraisal of the dynamics of the phases and stages in tooth development, in correlation with the biologic age;
2. the investigation of the quantitative characteristics of the dental fibroblasts throughout tooth development, by using the facilities of computer-assisted image processing and computerized morphometry, concentrated on the establishment of a morphometric profile for the fibroblasts, in order to enrich the classic qualitative data on the stages of tooth development with quantitative information that has the ability to refine the characterization of the evolution in dental papilla and dental pulp;
3. the investigation of the molecular characteristics of tooth germ, through an immunocytochemical evaluation of certain markers whose expression reflect clearly defined processes or interactions: proliferation, apoptosis and matrix renewal and support, identified in the complex mechanism of the tooth germ development.

CHAPTER 5. CORELATIONS BETWEEN MORPHOLOGIC FEATURES AND EVOLUTION STAGES IN THE STRUCTURE OF THE TOOTH GERM

5.1. ARGUMENT

Tooth development represents a modulation process restricted to a certain point in space and time, characterized by induction, competence and differentiation and reflected in phases – stages which partially overlap, sometimes without the existence of a clear borderline (Ten Cate 1998, Nanci, 2003, Nanci, 2012).

Within this framework, the general objective was the investigation of the morphologic and histologic tooth genesis through a classic analysis by light microscopy. Namely, we defined as specific objectives the evaluation of the phases and stages in tooth development, centering on the appraisal of the dynamics in the evolution of the tooth germs correlated with the biological age. We must stress the fact that the value of the gathered data resides also in the fact that the study was performed on human material, the stomatognat territory being confronted, because of the bioethical implications, with major difficulties in the study of the normal status in humans.

5.2. MATERIAL AND METHOD

The study material, originating from the County Service of Forensic Medicine Vaslui and from the Pathology Laboratory of the Municipal Hospital Barlad, respectively, included human tissues corresponding to 20 cases (embryos and fetuses with a gestational age of at least 2 months, collected from medical or spontaneous abortions, and newborns deceased at birth). The gestational age of the biological material was established in accordance with maternal records (2 cases – 8 weeks i.u., 3 cases – 10 weeks i.u., 3 cases – 14 weeks i.u., 1 case – 16 weeks i.u., 2 cases – 18 weeks i.u., 3 cases – 22 weeks i.u., 4 cases – 24 weeks i.u., 2 cases – 40 weeks i.u.). The biological material was harvested, conserved and processed in accordance with the ethical principles that regulate these procedures, the collection of the specimens being performed only with the written consent of the family, certified by an informed consent protocol. The study was approved by the Ethical Committee of the “Grigore T. Popa” University of Medicine and Pharmacy, on the basis of the informed consent and of the PhD study protocol, defined at the completion of the first year of PhD studies.

The embryos were fixed in 10% neutral buffered formalin, fully embedded in paraffin, and sectioned under the microtome into 4 μm-thick serial sections. The cephalic extremities (fetuses aged from 10-16 weeks) and the maxillas and mandibles fragments (fetuses aged from 18-40 weeks) were carefully removed and dissected, fixed in 10% neutral buffered formalin and decalcified in 5% formic acid and 5 g sodium citrate solution. The decalcification time was variable (minimum 3 days, maximum 3 weeks), depending on the gestational age and, implicitly, on the mineralization degree. After demineralization, the fragments were routinely processed for the embedding in paraffin. The paraffin blocks were sectioned by microtome – serial...
cross sections of 4 μm thickness. All sections were stained with the standard haematoxylin and eosin (HE) staining and with special stains (trichrome Masson, trichrome Azan-Heidenhain, trichrome Szekely and PAS). The special stains were used in order to allow a precise identification and differentiation of the epithelial and mesenchymal or connective tissue structures in the tooth germs.

5.3. RESULTS

Identification of tooth germs

Our study facilitated a global assessment for each case. Excluding the artifacts due to the difficulties of harvesting and processing human embryonic and fetal material (including the orientation of the specimens, and the particularities of the decalcification time), we deemed available for the morphological analysis only the tooth germs that were completely sectioned and had preserved their structural integrity. For these tooth germs, we defined the histological stages of development.

Qualitative analysis of tooth germs: characteristic histologic elements

Cap stage

The tooth germs in cap stage had various shapes and sizes, without a direct correlation with the gestational age (as a uniformly defined interval) (Figures 5.10). The morphological structure included the three main components: the enamel organ, the dental papilla and the dental sac. The microscopic morphological evaluation of these components revealed details of the cell structure that contribute to the establishment of the typical architecture.

Bell stage

The tooth germs in the bell stage had various shapes and sizes. While their diversity could not be directly correlated with the gestational age (as a uniformly defined interval), it allowed, however, a dynamic evaluation for the three main components: enamel organ, dental papilla and dental sac (fig. 5.21, fig. 5.27, fig. 5.29). Obviously, the analysis of the enamel organ enabled the observation of the successive changes in shape and size which determine the passage from cap to bell stage.

Fig. 5.10. Primitive oral cavity: tooth germs in different phases of the cap and bell stage (PAS, x 2)

Fig. 5.21. Tooth germ in late bell stage – tip of the cusp (PAS, x 4)

Fig. 5.27. Tooth germ in late bell stage – horizontal section through the tip of the cusp (Szekely, x 10)
The dental papilla showed transformations directly correlated with the ones identified in the enamel organ. The dental sac was readily identifiable around the developing tooth germs, its structure being compact and denser.

5.4. DISCUSSION

The morphogenesis of the tooth and its supporting tissues is a component of the embryologic development of the individual and is in direct correlation with the development of the head.

Within this framework, the PhD study focused on the complex aspects in the development of the stomatognat system included, as initial approach, an investigation specific for the microscopic morphology that enabled the analysis of the phases and stages of the tooth development in correlation with the biologic age.

The value of microscopic examination in the characterization of the tooth development stages

The entire deciduous dentition is initiated between week 6 and 8 of embryonic development, the permanent successional dentition – between week 20 in utero and the 10th month after birth and the permanent molars – between week 20 in utero (for first molars) and the 5th year of life (for third molars). From the point of view of the age, the investigated cases covered the time span of the decidual dentition development, our interest being concentrated on the identification of the tooth germs in the preeruptive stage. Moreover, it was possible to reveal successional tooth germs corresponding to the permanent teeth. In the current study the ages of the embryos and foetuses enabled an investigation across many stages in the development of the deciduous dentition. However, the study focused on the identification of tooth germs in pre-eruptive stages. Depending on the biologic age it was possible to observe differences in the position of the developing tooth germs inside the maxillary bone in the areas equivalent to the dental alveoli. The tooth germs identified in the first trimester of the pregnancy were very small and were located at considerable distance from one another. The tooth germs identified in the second and third trimester of the pregnancy were larger, as a result of fast growth and, consequently, the space between them was considerably reduced, bringing them closer together.

The comparative microscopic examination revealed the asymmetrical growth of the components of the tooth germs, representative for the proliferation stage which leads to the cap stage. Transformations of the epithelial bud were noted, with a change in its shape from oval to round and later the central curving was observed, leading to the appearance of a “cap” or “bonnet” structure placed over a “ball” of condensed mesenchymal tissue.

The analysis of the structural details of the investigated tooth germs led to an evaluation of the changes in the ameloblasts, as an essential morphological event in the tooth development process. Concurrently with the appraisal of the modifications in the ameloblasts it was also possible to observe the transformation of the odontoblasts placed at the periphery of the dental papilla turning into dental pulp. During the examination of the tooth germs the existing blood supply was also assessed in order to evaluate the differences between the cap and bell stages. These descriptive elements concentrated on the microscopic morphology of the tooth germ development, represent a distinct chapter of this PhD research. A solid documentation in the area of oral/dental histology reveals that the information is supported by a relatively small number of images – as opposed to the images for tissue or system histology – and frequently, the above-mentioned authors allow – through scientific courtesy – the joint usage of images picturing events,
processes or structures difficult to identify. Within this context we believe that the details observed in the investigated study group represent a consistent contribution to the current knowledge. We must underline the fact that the strictly descriptive approach will be deepened in the PhD research through the correlation between the identified morphologic changes with the biologic age of the cases in the study group, with the premise that their number will enable the realization of a detailed evolution profile.

**Difficulties in hard tissues processing: form issues – technical implications**

In our research, we used for the decalcification a solution with 5% formic acid and 5 g sodium citrate, because it preserves extremely well the cellular structures. The embedding of the harvested fragments was performed classically, in paraffin. On the basis of our results, from the point of view of the quality of the microscopic specimens, we believe that the decalcification technique we used offers optimal results and offers an adequate support for multiple special colorations. However, we must mention that at the international level, the techniques for the processing of the tooth and of the supporting structures profited recently from considerable updates through the implementation of performant technologies. Nevertheless, the costs for this technical equipment are extremely high and the staff involved is highly specialized. Consequently, there are very few laboratories with this type of equipment; they are oriented on the research activity in the field and provide access for the interested teams for the processing and the specific investigations. We deemed adequate the brief presentation of the modern techniques for hard tissue processing in order to be able to underline that in Romania there does not exist currently a laboratory with a specialized microtome for the sectioning of the highly mineralized tissues and able to use a processing technique without previous demineralization. Within this context, we must emphasize that although the investigations in the PhD study are supported by a classic examination modality, they are concentrated on morphologic elements that have alimited presence in the literature.

**CHAPTER 6. MORPHOMETRIC EVALUATION OF THE DEVELOPMENT OF THE PULP FIBROBLASTS IN THE TOOTH GERM**

6.1. ARGUMENT

In the research on the stomatognat system, the papers issued before the early eighties rely on the classical principles of morphometry, which imply manual identification of the interest regions and grid-based measurements. The more recent works benefit from the modern morphometry facilities, assisted by the computer and based on the development of softwares dedicated to identification and measurement that offer different automation degrees in identification of the interest regions and pixel-level accuracy in measurements.

The first papers, using classic morphometric methods, offer data on the differences between deciduous and, respectively, permanent dentition (Lichnovská et al., 1983, Lichnovská, 1984, Lichnovská, 1987), the superficial periodontal tissue (Schroeder, Munzel-Pedrazzoli, 1973) as well as the quantitative changes of the dentin (Vasilidiadis et al., 1983, Nitzan et al., 1986, Solheim, 1992) and cementum (Nitzan et al., 1986), in correlation with the aging process. The human dental pulp is a major subject, in terms of the quantitative information related to the odontoblasts (Benjamin et al., 1985, Warfvinge, 1987), the immune cells present in deciduous (Rodd,Boissonadé, 2006) and permanent teeth (Warfvinge, 1987, Pertot et al., 1997, Rodd,Boissonadé, 2006), in normal status and disease (Warfvinge, 1987, Pertot et al., 1997, Rodd,Boissonadé, 2006), and, finally, to the nervous elements (Matysiak, 1986, Matysiak et al., 1988).

Within this context, the general objective focused on the investigation of the quantitative features of the fibroblasts during tooth development. In accordance with the literature overview briefly presented above, investigations on this topic are completely absent – a fact which motivates our research and concomitantly ensures the novelty of the results. A key issue addressed here is the role of morphometry in detecting the differences between the development stages, strictly referring to dental papilla or pulp, respectively, which is a rather difficult task for the visual capacity of human observers. Namely, we defined as specific objective the design of a morphometric profile for the fibroblasts, in order to complement the classic qualitative knowledge about the development stages, by quantitative information able to refine the characterization of the dental papilla and dental pulp.

6.2. MATERIAL AND METHOD

6.2.1. CASE SELECTION

The cases were selected from the study group presented in detail in Chapter 5, section 5.2. Material and method.

Our study focused on the evaluation of the fibroblastic cell population present in mandibular central incisors. From the total number of 29 tooth germs (6 in cap stage, 6 in early bell stage and 14 in late bell stage)
identified as mandibular central incisors through microscopical examination, we selected 15 (5 cases for each stage) which were used for the development of our research. The selection relied on the quality of the histological images, in order to ensure the relevance of both biomedical information (necessary for the qualitative analysis – also including stage assessment) and numerical information (necessary for the quantitative analysis).

Consequently, the material was organized in 3 groups of study, as follows:
- group I included 5 tooth germs in cap stage (gestational age 10\textsuperscript{th} week i.u.)
- group II included 5 tooth germs in early bell stage (gestational age 14\textsuperscript{th} - 18\textsuperscript{th} week i.u.)
- group III included 5 tooth germs in late bell stage (gestational age 22\textsuperscript{nd} - 24\textsuperscript{th} week i.u.).

Each tooth germ defined a case; the germs (equivalently cases) belonging to a group were labeled from 1 to 5.

6.2. QUANTITATIVE COMPUTERIZED ANALYSIS

The computerized quantitative analysis was performed in the Zeiss KS400 software (Kontron Electronik GmbH, 1996).

The material for the computerized quantitative analysis consisted of digital images of the most meaningful zones of dental papilla / dental pulp, captured with the video camera of a Zeiss microscope. For each case belonging to a given group, 5 images (at x 200 magnification) were acquired in RGB format, from the serial sections. In the remainder of our paper, these images are referred to as “original images”. By using the automated image-processing techniques (detailed below), all pulp fibroblasts were identified on each image, and the following morphometric features / parameters were measured: area (A), perimeter (P) and form factor \((FF = 4\pi A/P^2)\) (Kontron Electronik GmbH, 1996). The results of the measurements were separately stored for each case, meaning about 500-700 records per case. Then, for each case, exactly 100 records were randomly selected, in order to define the sample vectors for A, P and FF (each of size 100).

We have designed and implemented three KS400 macros (IMAGMEAS – image measurement, CASESTAT – case statistics, GROUSTAT – group statistics) that enabled the automation of cell measurements, as well as the computation and graphical plotting of the statistical information derived from the measurement results.

All original images were preprocessed in order to increase the contrast of pulp fibroblasts relative to the extracellular matrix. Then a threshold segmentation technique was applied with appropriate values for the three RGB channels, which yielded the separation of regions occupied by the fibroblasts. The result is a binary image from which the regions with very small areas are removed – considered as scrap, such that the remaining white zones correspond to the fibroblastic cells in the original image.

6.2.3. DATA ANALYSIS

a) Statistical tests

For the intergroup statistical analysis of the morphometric features (i.e. compared analysis of the three groups), the ANOVA one-way test was applied by the help of the Matlab function \texttt{anova1} (The MathWorks Inc., 2010). A supplementary confirmation was proposed for these tests because, rigorously speaking, the ANOVA assumption on equal variance is slightly violated. For this purpose, we have used the Matlab function \texttt{ttest2} (The MathWorks Inc., 2010), which performs the Student’s test under the assumption of equal or unequal population variances. The \(p\) value was considered significant for \(p \leq 0.05\), highly significant for \(p \leq 0.01\) and very highly significant for \(p \leq 0.001\). The statistical analysis was also applied for intragroup investigations (i.e. between the cases belonging to the same group), but the intergroup and intragroup objectives were different (as commented in the Discussion section).

b) Mean-value-scaled relative distances (abbreviated as RDs)

The characterization of cases and, respectively, groups by the morphometric parameter mean-values allows the quantification of the similarity of the cases within a group and, respectively, of the differences between the three groups within the study. Two quantification procedures can be defined by relying on a single mathematical support explained below:

Let \(E = \{e_1, \ldots, e_n\}\) be a set of \(n\) elements and denote by \(M(E)\) their mean value, i.e. \(M(E) = \frac{e_1 + \cdots + e_n}{n}\). Introduce the transformation \(\tilde{e}_i = \frac{e_i - M(E)}{M(E)} = e_i - 1, \quad i = 1, \ldots, n\), and define the functions:
\[- RD_k(e_i) = |\tilde{e}_i| = \frac{|e_i - M(E)|}{M(E)}, \] called relative distance between \(e_i \in E\) and \(M(E)\).
- \( RD(e_i, e_j) = \left| \frac{e_i - e_j}{M(E)} \right| \), called relative distance between \( e_i, e_j \in E \).

In these forms, the numerator \( |e_i - M(E)| \) or \( |e_i - e_j| \) preserves the information about the distances between the initial values. At the same time, the denominator \( M(E) \) means a scaling operation that ensures the independence with respect to (w.r.t.) the concrete range of the initial values \( e_i, i = 1, \ldots, n \).

In our research we used the function \( RD_k(e_i) \) for the intragroup analysis and the function \( RD(e_i, e_j) \) for the intergroup analysis.

The function \( RD_k(e_i) \) measures \( / \) expresses the proximity of any element in the set \( E \) to \( M(E) \). Reasonably small values for any \( RD_k(e_i), e_i \in E, i = 1, \ldots, n, \) reflect a similarity between all the elements composing the set \( E \).

The function \( RD(e_i, e_j) \) measures \( / \) expresses the proximity of any two elements in the set \( E \). Let \( e_i, e_j, e_k \in E \) be three arbitrary elements of \( E \). If \( RD(e_i, e_j) \) is much larger than \( RD(e_i, e_k) \), we conclude that \( e_j \) is much closer to \( e_k \) than to \( e_i \); if \( RD(e_i, e_j) \) and \( RD(e_j, e_k) \) are rather equal, we conclude that \( e_j \) is as close to \( e_k \) than to \( e_i \).

At the intragroup level, a set of type \( E \) was defined for each group (I, II, III) and for each morphometric parameter (\( A, P, \) or \( FF \)). Such a set comprises five elements corresponding to the mean values of the five cases belonging to the considered group (and corresponding to the parameters \( A, P, \) or \( FF \)). Thus, we can calculate the relative distances:

- for intragroup I analysis: \( RD_{gI,A}(e_i), RD_{gI,P}(e_i), RD_{gI,FF}(e_i) \), \( i = 1, \ldots, 5 \) cases in group I;
- for intragroup II analysis: \( RD_{gII,A}(e_i), RD_{gII,P}(e_i), RD_{gII,FF}(e_i) \), \( i = 1, \ldots, 5 \) cases in group II;
- for intragroup III analysis: \( RD_{gIII,A}(e_i), RD_{gIII,P}(e_i), RD_{gIII,FF}(e_i) \), \( i = 1, \ldots, 5 \) cases in group III.

In the intergroup analysis, a set of type \( E \) was defined for each morphometric parameter (\( A, P, \) or \( FF \)) and comprises three elements corresponding to the mean values of the three groups investigated in our study. Thus, we can calculate the relative distances:

- between groups I and II: \( RD_A(gI,gII), RD_P(gI,gII), RD_{FF}(gI,gII) \);
- between groups II and III: \( RD_A(gII,gIII), RD_P(gII,gIII), RD_{FF}(gII,gIII) \).

### 6.3. RESULTS

The morphometric profiles of the cases belonging to groups I, II and III are summarized in Tables 6.1, 6.2 and 6.3, respectively. All these tables have the same organization, namely: three similar column blocks are allocated to the three morphometric parameters \( A, P, \) and \( FF \); the first five entries correspond to the five component cases; each case entry allocates two rows (the first row displays statistical information and the second one provides the relative distance from the group mean-value – expressed as percentage); the last entry contains a single row that displays statistical information referring to the whole group.

#### Table 6.1: Morphometric characterization of group I

<table>
<thead>
<tr>
<th>Case</th>
<th>Area ( A (\mu^2) )</th>
<th>Perimeter ( P (\mu) )</th>
<th>Form factor ( FF )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>min</td>
<td>max</td>
</tr>
<tr>
<td>1</td>
<td>18.755 ± 7.549</td>
<td>5.7%</td>
<td>7.129</td>
</tr>
<tr>
<td>2</td>
<td>14.635 ±5.903</td>
<td>17%</td>
<td>5.078</td>
</tr>
<tr>
<td>3</td>
<td>16.356 ±7.202</td>
<td>7.7%</td>
<td>5.317</td>
</tr>
<tr>
<td>4</td>
<td>20.335 ±7.730</td>
<td>14%</td>
<td>8.556</td>
</tr>
<tr>
<td>5</td>
<td>18.558 ±6.855</td>
<td>4.8%</td>
<td>7.530</td>
</tr>
<tr>
<td>Group I</td>
<td>17.733 ±7.328</td>
<td>5.078</td>
<td>43.034</td>
</tr>
</tbody>
</table>
Table 6.2. Morphometric characterization of group II

<table>
<thead>
<tr>
<th>Case</th>
<th>Area A (µm²)</th>
<th>Perimeter P (µm)</th>
<th>Form factor FF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
</tr>
<tr>
<td></td>
<td>RD_{A1} A(%)</td>
<td>RD_{P1} (%)</td>
<td>RD_{FF1} (%)</td>
</tr>
<tr>
<td>1</td>
<td>26.058 ±10.620</td>
<td>51.903 ±20.556</td>
<td>0.143 ±0.064</td>
</tr>
<tr>
<td></td>
<td>3.4%</td>
<td>0.2%</td>
<td>6.8%</td>
</tr>
<tr>
<td>2</td>
<td>24.212 ±10.647</td>
<td>51.065 ±18.990</td>
<td>0.129 ±0.049</td>
</tr>
<tr>
<td></td>
<td>3.8%</td>
<td>1.4%</td>
<td>3.2%</td>
</tr>
<tr>
<td>3</td>
<td>25.629 ±10.629</td>
<td>52.892 ±19.011</td>
<td>0.129 ±0.053</td>
</tr>
<tr>
<td></td>
<td>1.7%</td>
<td>2.1%</td>
<td>3.5%</td>
</tr>
<tr>
<td>4</td>
<td>24.499 ±10.562</td>
<td>53.228 ±19.847</td>
<td>0.120 ±0.042</td>
</tr>
<tr>
<td></td>
<td>2.7%</td>
<td>2.7%</td>
<td>10.4%</td>
</tr>
<tr>
<td>5</td>
<td>25.538 ±9.320</td>
<td>49.854 ±16.851</td>
<td>0.147 ±0.063</td>
</tr>
<tr>
<td></td>
<td>1.3%</td>
<td>3.7%</td>
<td>10.2%</td>
</tr>
</tbody>
</table>

Group II: 25.187 ±10.351 [0.07] 7.351 [0.02] 58.368 [0.04] 18.370 [0.01] 117.308 [0.03] 0.134 ±0.056 0.032 0.371

Table 6.3. Morphometric characterization of group III

<table>
<thead>
<tr>
<th>Case</th>
<th>Area A (µm²)</th>
<th>Perimeter P (µm)</th>
<th>Form factor FF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
</tr>
<tr>
<td></td>
<td>RD_{A1} A(%)</td>
<td>RD_{P1} (%)</td>
<td>RD_{FF1} (%)</td>
</tr>
<tr>
<td>1</td>
<td>32.847 ±11.928</td>
<td>67.560 ±23.797</td>
<td>0.105 ±0.046</td>
</tr>
<tr>
<td></td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>2</td>
<td>31.520 ±10.901</td>
<td>69.061 ±23.641</td>
<td>0.095 ±0.039</td>
</tr>
<tr>
<td></td>
<td>3.8%</td>
<td>3.0%</td>
<td>9.0%</td>
</tr>
<tr>
<td>3</td>
<td>30.783 ±11.880</td>
<td>64.058 ±22.609</td>
<td>0.106 ±0.041</td>
</tr>
<tr>
<td></td>
<td>6.1%</td>
<td>4.4%</td>
<td>1.6%</td>
</tr>
<tr>
<td>4</td>
<td>34.730 ±11.006</td>
<td>69.336 ±21.368</td>
<td>0.103 ±0.045</td>
</tr>
<tr>
<td></td>
<td>5.9%</td>
<td>3.4%</td>
<td>1.6%</td>
</tr>
<tr>
<td>5</td>
<td>34.060 ±9.390</td>
<td>65.089 ±19.183</td>
<td>0.114 ±0.044</td>
</tr>
<tr>
<td></td>
<td>3.8%</td>
<td>2.8%</td>
<td>8.7%</td>
</tr>
</tbody>
</table>

Group III: 32.788 ±11.115 [0.07] 10.719 [0.02] 68.401 [0.04] 26.432 [0.01] 125.166 [0.03] 0.104 ±0.043 0.038 0.289

6.3.1. STATISTICAL TESTS
Between any two groups, the statistical differences were very highly significant (p<0.001), for A, P and FF vectors.

At the intragroup level the statistical analysis revealed significant differences (p<0.05), for some pairs of cases, as follows: group I (A vectors – 1vs2, 1vs3, 2vs4, 2vs5, 3vs4, 3vs5; P vectors – 1vs2, 2vs4; FF vectors – 1vs4, 2vs4, 3vs4); group II (FF vectors – 1vs4, 4vs5, 2vs5, 3vs5); group III (A vectors – 2vs4, 3vs4, 5vs5; FF vectors – 2vs3, 2vs5). The abbreviation vs is used for versus.

6.3.2. MEAN-VALUE-SCALED RELATIVE DISTANCES
The RDs of any case correlated with the mean-value of the group to which the case belongs were provided by Tables 1, 2 and 3 (as explained above, in the presentation of those tables).

The RDs between the groups were calculated for each descriptor A, P, FF, by using the information in the last entry of Tables 1-3 (i.e. the mean values of the groups I, II, III) and yielded the following results – expressed as percentages from the global mean of each descriptor:
- $RD_A(gI, gII) = 29.45\%$, $RD_A(gII, gIII) = 30.12\%$, from the A global mean (25.24 µm²);
- $RD_P(gI, gII) = 43.97\%$, $RD_P(gII, gIII) = 30.71\%$, from the P global mean (49.59 µm);
- $RD_{FF}(gI, gII) = 88.29\%$, $RD_{FF}(gII, gIII) = 16.57\%$, from the FF global mean (0.176).

6.3.3. REGRESSION ANALYSIS
For each group we analyzed the linear regression $P$ vs A and we obtained the regression equations and the correlation factors reproduced below:
- group I: $P = 1.4263 A + 4.6868; A-P$ correlation factor = 0.9023;
- group II: $P = 1.6758 A+ 9.5794; A-P$ correlation factor = 0.9014;
- group III: $P = 1.7626 A + 9.2303; A-P$ correlation factor = 0.8825.
6.3.4. GRAPHICAL PLOTS

The construction of $A$ histograms (Fig. 6.3(a),(b),(c)), $P$ histograms (Fig. 6.4(a),(b),(c)) and $FF$ histograms (Fig. 6.5(a),(b),(c)) allowed a facile analysis of the differences between the fibroblastic populations in the three groups. Each histogram was complemented by a graphical plot of the Gaussian distribution with the same mean value and standard deviation of the empirical variable.

The scattergrams $P$ vs $A$ corresponding to groups I, II, III were plotted in Figure 6.6(a),(b),(c). We considered the same ranges for the scattergram axes in order to simplify the visual interpretation of the similarities and differences between the populations of the three groups from the point of view of the cell dimensions. Figure 6.6(a),(b),(c) also depicts the regression lines corresponding to the three equations given above. For a complete understanding of the differences between the regression equations associated with the three groups, Figure 6.7 presents a comparative plot of the three regression lines.

![Fig. 6.3. Histograms of $A$ (area) variable for the three groups: fig. 6.3.a – group I, fig. 6.3.b – group II, fig. 6.3.c – group III.](image)
COMPLEX MORPHOLOGIC INVESTIGATION ON THE DEVELOPMENT OF THE STOMATOGNAT SYSTEM

Fig. 6.4. Histograms of $P$ (perimeter) variable for the three groups: fig. 6.4.a – group I, fig. 6.4.b – group II, fig. 6.4.c – group III.

Fig. 6.5. Histograms of $FF$ (form factor) variable for the three groups: fig. 6.5.a – group I, fig. 6.5.b – group II, fig. 6.5.c – group III.
6.4. DISCUSSION

6.4.1. NOTICEABLE TRENDS IN FIBROBLAST RESEARCH – SCARCE MORPHOMETRIC SUPPORT

The pulp fibroblasts represent a class of the fibroblast population characterized by a high degree of heterogeneity. Their functional potential is extremely large, from the inductive role in tooth development to the repair function typical to mature teeth. In the successive stages of tooth development, the young fibroblasts organized as dental papilla and, later on, as dental pulp contribute to the differentiation of oral epithelium, and induce the formation and evolution of the enamel organ (Nanci, 2008). For the mature tooth, the fibroblast involvement in the pulp and dentin repair is a fact unanimously accepted (Palosaari et al., 2000, Smith, Lesot, 2001, Goldberg, Smith, 2004, Gruber et al., 2004, Goldberg et al., 2008).

The morphometric approach of the fibroblastic population is limited to two studies that refer to the repair function of dental pulp (Murray et al., 2002a, 2002b) – the first performed on human material, while the second was performed on animals. Both studies analyzed the mature dental pulp with the purpose to assess the changes in the cellular density (odontoblasts, subodontoblasts, fibroblasts), in the pulp total area and in the width of dentin, in correlation with the biological age. The results show that the patient aging implies the increase of dentinal thickness and the decrease of the density of odontoblasts, subodontoblasts, and pulp fibroblasts - both in the crown and root regions.

6.4.2. PROGRESS IN FIBROBLAST RESEARCH VIA MORPHOMETRY

Our investigation is founded on a quantitative point of view carefully built on the basis of relevant measurement results, which permits a deeper understanding of the fibroblast evolution in connection with tooth development stages. Our mathematical-type findings offer new and irrefutable support for an approach complementary to the traditional analysis based on the qualitative observations of cell transformation (i.e. size and shape modifications).

Key points in intergroup analysis

- The very highly significant differences between the A vectors (respectively P vectors) in any two groups are in full accordance with the class organization (cap, early bell, late bell stages) used in the tooth development. It is important to notice that the statistical difference (exclusively referring to the morphometric features of the fibroblasts) between the groups is in full accordance with the histological
differences (referring to the tooth germs, as complete entities) between those groups. This accor-
dance is explained by the inherent involvement of the morphometric features in the growing process.

- For each group, the A histogram (respectively P histogram) shows that the above A interval
(respectively P interval) includes about 350 cases (i.e. approximately 70% from the population, which is
rather close to the percentage guaranteed by the ideal Gaussian distribution for the same type of intervals).

- Besides the dimension features (A and P), the FF feature is also involved in the analysis of the
fibroblast evolution. Tables 1-3 show that the mean value of FF decreases from 0.28 (group I) to 0.13 (group
II) and to 0.10 (group III). These values quantify important changes in the cell shape in the sense that the
ratio (perimeter/area-unit) increases from group I to group III. Subsequently, the FF values determined for
the three groups can be regarded as measures of the modifications in the cell shape, from star-like (in cap
stage) to almost fusiform (in late bell stage). It is worth noticing the refined action of FF as a shape-change
measure which is able to reveal that the modifications from group I to II (0.28 to 0.13 mean values) are more
significant than the modifications from group II to III (0.13 to 0.10 mean values). The mean values give a
significant, but punctual description of the discussed modifications between groups. A global picture is
offered by the three histograms in Figure 6.5, where the plots 5b and 5c have similar silhouettes, while plot
6.5a is drastically different. This remark has a solid motivation in descriptive tooth biology, in the sense that
cap and bell are stages completely separated (involving major differences), whereas early and late bell are
phases of the same stage (involving smaller differences).

- Another morphometric proof that the early bell stage is much closer to the late bell stage than to the
cap stage results from the comparison of the values calculated for RDs between the groups, w.r.t. FF
(16.57% between groups II, III unlike 88.29% between groups I, II). We must observe that the ratio of the
two RDs is 0.8829/0.1657 = 5.3283. At the same time, the RDs w.r.t. A (30.12% between groups II, III and
29.45% between groups I, II) or P (30.71% between groups II, III and 43.97% between groups I, II) are
unable to give such information. The mathematical merit of these relative distances consists in their
robustness w.r.t. the concrete size of the examined fibroblasts (due to the M(E) scaling used in the
definition of the RD(ei, ej) function – see “Material and method” section). In other words, for experiments
on new groups we expect intergroup relative distances with values similar to this study, regardless of the
inherent biological variation of the dimensions (possibly reflected by significantly different mean values for
A, P and FF).

**Key points in intragroup analysis**

- The existence of the statistical differences between some cases within the same group seems
surprising, if the understanding of case similarity is confined to the similarity of the morphometric
parameters. Essentially, the fundamental reason for the association of several cases within a group is the
histological profile of cells and structures, which does not necessarily involve the same numerical ranges for
all classes w.r.t. the morphometric features. Therefore, we consider the use of statistical tests at the
intragroup level is not relevant. Our research had a precise motivation for using these tests in order to prove
that human-coordinated selection of cases belonging to a group may ignore such differences (if they exist).

### 6.4.3. BRIEF COMMENTS ON THE MORPHOMETRIC TOOLS

The approach proposed in this work for the analysis of evolving fibroblasts is founded on our
experience in computerized morphometry (Cărintu, 2002; Cărintu, 2003). The technique used in (Cărintu,
2003) was considerably improved in order to process large sets of digital images in relatively short time
intervals. The segmentation strategy in (Cărintu, 2003) was replaced by a fully automated procedure, based
on color segmentation, which is able to operate successfully on all considered specimens, once their staining
difers within reasonable limits, predefined in RGB terms. Thus, during several work sessions, we could
extract the morphometric features for more than 8,000 cells (from which 1,500 cells were randomly selected
for the development of the study).

The principles at the basis of the design and implementation of our morphometric tools can be
transferred mutatis mutandis to investigations focused on other types of cells or structures belonging to the
tooth, provided that the staining allows a robust identification based on colour properties. It is worth also
mentioning that the Zeiss KS400 technology is not compulsory; we preferred it because of our extensive
experience with the system, but other software environments can be equally exploited for such applications.
All the above remarks show that our morphometric construction can serve as a basic and flexible guide for
researchers interested in addressing morphometry problems in the complex domain of oral biology.

### 6.4.4. ORIGINALITY

Our key objective was to provide meaningful quantitative criteria for testing the differences between
the evolutive stages (intergroup analysis) and the similarities of the cases belonging to the same stage
CHAPTER 7. MOLECULAR MARKERS IN TOOTH DEVELOPMENT

7.1. ARGUMENT

The modern concept of the cellular and molecular network that take action in predefined instances and locations in order to guide the pluripotent cells in their evolution towards the transformation in dental cells, with a rather limited functional potential, is already implemented (Thesleff, 2003, Bei, 2009). This network ensures cellular communication that plays an unanimously recognized role in the embryo development and in tooth development subsequently, through the intervention of dozens of small signal-molecules, receptors and genes transmitted from cell to cell or across the matrix – the most studied being the BMPs, FGFs, Wnt and Hedgehog (Hh) families (Thesleff, 2003, Zhang et al., 2006; D’Antò et al., 2010).

7.2. MATERIAL AND METHOD

The study group resulted after the selection of 5 cases out of the total of 20 cases presented in detail in Chapter 5, section 5.2. Material and method, from which we used the sections corresponding to the mandibular tooth germs. The selection relied on the gestational age and the quality of the existent biologic product, as follows:

- case 1 – 10th week i.u. with three tooth germs in cap stage, cooresponding to the medial incisor (I₁), lateral incisor (I₂) and molar 1 (M₁);
- case 2 – 14th week i.u., with five tooth germs in cap stage, corresponding to the medial incisor (I₁), lateral incisor (I₂), canine (C), molar 1 (M₁) and molar 2 (M₂);
- case 3 – 18th week i.u., with 2 tooth germs in early bell stage, corresponding to the medial (I₁) and lateral (I₂) incisors and three tooth germs in cap stage, corresponding to the canine (C), molar 1 (M₁) and molar 2 (M₂);
- case 4 – 22nd week i.u. with three tooth germs in late bell stage, corresponding to medioal and lateral incisors and canine and two tooth germs in the early bell stage, corresponding to molar 1 (M₁) and molar 2 (M₂);
- case 5 – 24th week i.u., with 5 tooth germs in late bell stage, corresponding to the medial (I₁) and lateral (I₂) incisors, canine (C), molar 1 (M₁) and molar 2 (M₂);

7.3. PROFILE OF PROLIFERATIVE ACTIVITY: CYCLIN D1

7.3.1. LANDMARKS

Cell cycle represents a sequential functional process, under a continous rigurous control by various molecules – such as tumor suppressor genes and/or oncogenes, members of the numerous complex signaling pathways, with a positive or negative role in the cellular events (Guertin et al., 2002). Cell growth and division is controlled by three main groups of regulator markers pro/anti-proliferative: (i) cyclin family, (ii) cyclin-dependent kinases (CDK) and (iii) cyclin-dependent kinase inhibitors (CDKI) (Pines, 1999, Ogasawara, 2013).

In correlation with the phase of the cell cycle cyclins are classified as (i) G1 cyclins (cyclin D and cyclin E, respectively) and (ii) S (DNA synthesis phase), G2 and M (mitosis) cyclins (cyclin A and cyclin B, respectively). Although expressed in a cell-specific manner and with characteristic distraction pattern, they are identical in structure and function (Cao et al., 2012). Cyclin D1 (CCND1) is a nuclear protein, a valuable marker for the control of the cell cycle, consisting of 295 aminoacids (Sherr, 1995).

From the point of view of the relationship between cyclin and the stomatognat system, the references in the literature are almost completely absent. There is, as far as we know, one single in vivo study,
performed on an animal model, which proved that cyclin D1 deficiency has repercussions on the development of the mandible and determines implicitly an uncontrolled growth of the incisors (Ogasawara, 2013). These results demonstrate the positive role of cyclins, mainly of cyclin D1 in the regulation of the development process of the mandible and maxilla, respectively (Ogasawara, 2013).

In this context, our study focused on the appraisal of cyclin D1 expression, as a reflection of the proliferative activity in the developing tooth germs.

7.3.2. CHARACTERISTICS OF THE QUANTIFICATION METHOD

The IHC reaction was interpreted on the basis of an evaluation and quantification system proposed in the literature (Choschzick et al., 2012), relying on two criteria: percentage of positive cells (P) and intensity of IHC reaction (I).

Thus, in correlation with the intensity of the reaction, the cases were divided into 4 categories: score 0 – lack of IHC reaction, score 1 – weak intensity (+), score 2 – moderate intensity (++), score 3 – strong intensity (+++). With respect to the estimated cell percentage, the cases were divided into 4 categories: score 0 – lack of IHC reaction, score 1 - ≤25% positive cells, score 2 – ≤ 50% positive cells, score 3 – ≤ 75% positive cells and score 4 – over 75% positive cells. The final results of this scoring system were the following: the reaction was considered negative for the nuclear aggregation of cyclin D1 when the final score was 0; weak, intensity 1 or 2 in ≤50% of the cells or intensity of 3 in ≤25% of the cells; moderate, 2 intensity in 50%-75% of the cells or 3 intensity in 25%-50% of the cells; strong : 2 intensity in >75% of the cells or 3 intensity in >50% of the cells.

7.3.3. RESULTS

The microscopic evaluation of the profile for cyclin D1, reflected in the nuclear positive reaction revealed in the entire study group a positive expression in the investigated tooth germs as follows. The characteristic nuclear pattern was apparent in tooth germs in week 10, 14 and 18 i.u. for the 22nd and 24th week, the expression of cyclin was identified in the nuclei from all the tooth germs in early bell stage and in the cytoplasm of the components in the enamel organ in late bell stage (ameloblasts, intermediate epithelium and only focal in the stellate reticum) – case where the reaction was interpreted as negative.

In correlation with the final score of the immunoexpression, cyclin D1 presented the following characteristics:

**Strong reaction:**
- 10th week i.u.:
  - cap stage (I,, M,,), in the internal epithelium, stellate reticulum and cervical loop;
  - cap stage (I,,), in the internal epithelium and stellate reticulum;
- 14th week i.u.:
  - cap stage (I,, C, M,), in the internal epithelium, stellate reticulum and cervical loop;
- 18th week i.u.:
  - early bell stage (I,, I,,), in the ameloblasts, intermediate epithelium, stellate reticulum and odontoblasts;
  - cap stage (C, M,), in the internal epithelium, stellate reticulum and cervical loop;
- 22nd week i.u.:
  - late bell stage (I,,), in the dental pulp – odontoblasts;
  - early bell (M,) in the ameloblasts, intermediate epithelium, stellate reticulum, dental pulp – odontoblasts;
  - early bell (M,), in the ameloblasts, intermediate epithelium and stellate reticulum;
- 24th week i.u.:
  - late bell stage (I,,), stellate reticulum, dental pulp – odontoblasts;
  - late bell stage (C), stellate reticulum;
  - late bell stage (M,), stellate reticulum, dental pulp – odontoblasts;
  - late bell stage (M,), stellate reticulum.

**Moderate reaction:**
- 14th week i.u.:
  - cap stage (I,, M,), internal epithelium, stellate reticulum and cervical loop;
  - cap stage (I,, I,, C, M,, M,), fibroblasts in the dental pulp;
- 18th week i.u.:
  - early bell stage (I,,), dental pulp – fibroblasts;
  - early bell ((I,,), dental pulp – odontoblasts, fibroblasts;
  - cap stage (C), dental papilla – fibroblasts;
  - cap stage (M,), dental papilla – fibroblasts;
- cap stage (M2), internal epithelium, stellate reticulum, cervical loop and dental papilla – fibroblasts.
  - 22nd week i.u.: late bell stage (I2), in the dental pulp – odontoblasts and fibroblasts; late bell stage (C), stellate reticulum, and dental pulp – odontoblasts and fibroblasts; late bell stage (M2), dental pulp – odontoblasts;
  - 24th week i.u.: late bell stage (I2), in the stellate reticulum and dental pulp – odontoblasts; late bell stage (C), in the dental pulp – odontoblasts; late bell stage (M2), in the dental pulp – odontoblasts.

**Weak reaction:**
  - 10th week i.u.: cap stage (I1, M), in the dental papilla – fibroblasts; cap stage (I2), in the cervical loop and dental papilla – fibroblasts;
  - 22nd week i.u.; early bell stage (M2), dental pulp – fibroblasts.
  - 24th week i.u.; săptămâna a 24-a de viaţă intrauterină; late bell stage (I1, I2, C, M1, M2), dental pulp – fibroblasts

**Negative reaction:**
  - 22nd week i.u.; late bell stage (I1), in the ameloblasts, intermediate epithelium, stellate reticulum and dental pulp – fibroblasts; late bell stage (I2) in the ameloblasts, intermediate epithelium and stellate reticulum; late bell stage (C), in the ameloblasts and intermediate epithelium; early bell stage (M1) in the dental pulp – fibroblasts;
  - 24th week i.u.; late bell stage (I1, I2, C, M1, M2) in the ameloblasts and intermediate epithelium.
  - stadiul de clopot avansat (I1, I2, C, M1, M2), in ameloblaste, epiteliul intermediar;

7.3.4. **DISCUSSION**

The expression of cyclins represents the cyclical feature during the various phases of cell cycle, hence the generic term of “cyclin”. The values of cyclin D1 are correlated with the phase of the cell cycle where it develops activity with the ability to coordinate in time the cell cycle (Takahashi-Yanaga, Sasaguri, 2008). In order to synthesize and replicate DNA the level of cyclin D1 must be maintained at the same level or lowered for any overexpression causes the blockage of DNA synthesis, with a consequent impact on the increased, out of control proliferative activity (Fukami-Kobayashi, Mitsui, 1999).

The increased expression of cyclin D1 determines phase G1 to become shorter, the accumulation of damaged DNA and, consequently, the possibility of genetic errors or excessive proliferation (Li et al., 2006). The overexpression of cyclin D1 arises, from the point of view of the mechanism, through a damaged regulation at the post-translational level and not as an effect of a gene amplification (Russell et al., 1999).

Its regulatory role in the cell cycle is CDK-dependent. The cyclins cooperate with cyclin-dependent kinases: CDK4, CDK6 or CDK2 and function as regulators for the division of the cell cycle, as well (Aubry et al., 2009).

In our study the distribution of cyclin D1 was present in all the stages of tooth germ development from cap to late bell, in all categories of teeth (central and lateral incisors, canines, molars (M1, M2)), regardless of the embryonic age (week 10, 14, 18, 22 or 24 i.u.). However, there were recorded differences related to the distribution of the marker at the level of the epithelial and mesenchymal/connective cellular elements as well as to the intensity of the IHC expression.

Cyclin D1 presented a constant expression in the components of the enamel organ with a nuclear location in the cells from the internal and intermediate epithelium and from the stellate reticulum in the cap stage of the tooth germ. The same results were recorded in the structural elements from the early bell stage: ameloblasts, cells in the intermediate layer and cells in the stellate reticulum. We must mention the fact that the fibroblasts in the pulp ectomesenchyme presented an expression of cyclin D1 regardless of the stage of the tooth germ with a moderate intensity.

On the other hand, in the late bell stage, the pattern of IHC distribution of cyclin D1 reversed in favor of the odontoblasts, characterized by a nuclear positive reaction while the ameloblasts had a negative...
nuclear reaction but, extremely interesting a marked cytoplasmic immunoexpression. In the odontoblasts, cyclin D1 presented a moderate towards strong intensity. For the cells of the stellate reticulum as opposed to the cap stage and early bell stage, respectively, cyclin D1 was negative or positive only in focal areas.

These results can be interpreted from the point of view of the functional status of cyclin D1, namely that the nuclear expression in the incipient stages of the tooth germ development reflect the progression through cell cycle of the cells in the enamel organ, as a result of the reception of proliferation signals. Later, in the evolution of the tooth germ, in the late bell stage, the occurrence of cyclin D1 in the cytoplasm translates the termination of the proliferative activity in the ameloblasts, which, once mature and after their role is ended by the achievement of enamel production, enter in a short transitional stage before their complete disappearance.

Nevertheless, the late bell stage is characterized by an increased rate in the proliferation of the odontoblasts, where cyclin D1 was expressed with a moderate towards increased intensity. The expression of cyclin D1 is correlated with the division and proliferation of the odontoblasts, responsible for the dentinogenesis.

7.4. PRO AND ANTI-APOPTOTIC PROFILE: Bax and Bcl-2

7.4.1. LANDMARKS

Apoptosis is a mechanism with multiple phases, consisting of a series of events initiated in the extracellular environment and finalized in the genome; the process starts with the reception of external signals, continues with their transmission and analysis in the nucleus, resulting in the activation of genes responsible for the programmation of cell self-destruction (Schwartz, Osborne, 1993).

The Bcl-2 family represents a large range of molecules that act either as promoters or as tumor suppressors. While Bcl-2 is viewed as key regulator of apoptosis, functioning as tumor suppressor, Bax has the reverse effect, acting pro-apoptosis.

During the development of the stomatognat system, the structural components of the tooth germ go through various morpheni stages of proliferation, differentiation and/or programmed self-death. Thus apoptosis intervenes selectively in time and space on certain areas of differentiation of the dental structural elements (Kalibovic Govorko et al., 2010).

The data in the literature certifies the expression of Bcl-2 and Bax, on the basis of the results obtained from tooth germ of mouse embryos, but only in the periods of initiation of tooth germ development, without associating their expression with the succession of stages in tooth development (Krajewski et al., 1998). Very few published data are concentrated on the expression of Bcl-2 and Bax in the human odontogenesis, this information being obtained through the analysis of the tooth germs in fetuses with gestational age higher than 13 weeks (Slootweg, de Weger, 1994, Hatakeyama et al., 2000).

In this context, our study focused on the evaluation of Bax and Bcl-2 expression as a reflection of the pro and anti-apoptotic potential present in the developing tooth germs with an impact on the odontogenesis.

7.4.2. CHARACTERISTICS OF THE QUANTIFICATION METHOD

In order to evaluate the expression of Bax we used a semi-quantitative score (Zlobec et al., 2007b) based exclusively on the intensity of the IHC staining, the cases being divided into 3 classes: weak intensity (+), moderate intensity (++) and strong intensity (+++). The cases where the IHC reaction was absent (with the existence of a positive control) were considered negative.

The Bcl-2 IHC reaction was semi-quantitatively evaluated through a scoring system (Suzuki et al., 2002) based exclusively on the percentage of positive cells, as follows: 0 – < 1%, 1 – 1-25%, 2 – 26-50%, 3 – 51-75%, 4 – > 75%, without a correlation with the intensity of the immunoreactions. Score 0 was regarded as negative and scores 1, 2, 3 and 4 were regarded as positive.

7.4.3. RESULTS

The evaluation of Bax profile, represented by a diffuse cytoplasmic positive reaction revealed in the entire study group an expression present in all the investigated tooth germs, regardless of their position and stage of development.

The evaluation of the Bcl-2 profile, characterized by a predominantly apical location demonstrated an inconsistent distribution that differs in relation with the stage of development and cell type – epithelial/ameloblastic, ectomesenchymal/odontoblastic, without any differences with respect to the type of tooth.

In correlation with the intensity of the reaction, Bax presented the following characteristics:

**Strong reaction:**
- 14th week i.u.:
  - cap stage (I₁), in the stellate reticulum;
  - cap stage (I₂, C, M₁, M₂) in the internal epithelium and stellate reticulum;
22nd week i.u.
- late bell stage (I₁, I₂, C) in the ameloblasts, intermediate epithelium and stellate reticulum;
- early bell stage (M₁, M₂) in the ameloblasts, intermediate epithelium and stellate reticulum;

24th week i.u.:
- late bell stage (I₂, C, M₁, M₂) in the ameloblasts;
- late bell stage (I₂) in the ameloblasts, intermediate epithelium and stellate reticulum;

**Moderate reaction:**

- 10th week i.u.
  - cap stage (I₁, I₂, M₁) in the internal epithelium, stellate reticulum and cervical loop;
- 14th week i.u.:
  - cap stage (I₁), in the internal epithelium, cervical loop and dental papilla – fibroblasts;
  - cap stage (I₂, C, M₁) in the cervical loop and dental papilla – fibroblasts;
- 18th week i.u.
  - early bell stage (I₁, I₂), in the ameloblasts, intermediate epithelium and dental pulp – odontoblasts and fibroblasts;
  - cap stage (C, M₁, M₂) in the internal epithelium, stellate reticulum and dental papilla – fibroblasts;
- 22nd week i.u.
  - late bell stage (I₁, C), in the dental pulp – odontoblasts;
  - late bell stage (I₂), in the dental pulp – odontoblasts and fibroblasts;
  - early bell stage (M₂), in the dental pulp – odontoblasts;
- 24th week i.u.
  - late bell stage (I₁, C, M₁, M₂), in the intermediate epithelium and stellate reticulum;
  - late bell stage (M₂), in the dental pulp – odontoblasts.

**Weak reaction:**

- 10th week i.u.
  - cap stage (I₁, I₂, M₁), in the dental papilla – fibroblasts;
- 18th week i.u.:
  - early bell stage (I₁, I₂), in the stellate reticulum;
  - cap stage (C, M₁, M₂), in the cervical loop;
- 22nd week i.u.
  - late bell stage (I₁, C), in the dental pulp – fibroblasts;
  - early bell stage (I₁, I₂), in the dental pulp – odontoblasts and fibroblasts;
  - early bell stage (M₁), in the dental pulp – fibroblasts;
- 24th week i.u.
  - late bell stage (I₁, I₂, C, M₁, M₂), in the dental pulp – odontoblasts and fibroblasts;

The percentage ratio of Bcl-2 expression for the structural elements of the tooth germ yields two types of reaction: positive and negative. In correlation with the interpretation of the reaction, negative and positive, respectively, Bcl-2 presented the following characteristics:

**Positive reaction:**

- 10th week i.u.:
  - cap stage (I₁, I₂, M₁), in the internal epithelium, stellate reticulum, cervical loop and fibroblasts;
- 14th week i.u.:
  - cap stage (I₁, I₂, M₁, M₂), in the internal epithelium, stellate reticulum and cervical loop;
- 18th week i.u.:
  - cap stage (C, M₁, M₂), in the cervical loop and dental papilla – fibroblasts;
- 22nd week i.u.:
  - late bell stage (I₁, I₂, C), in the ameloblasts, intermediate epithelium and stellate reticulum;
  - early bell stage (M₁, M₂), in the ameloblasts and intermediate epithelium;
- 24th week i.u.:
  - late bell stage (I₁, I₂, C, M₁, M₂), in ameloblasts, intermediate epithelium and stellate reticulum.

**Negative reaction:**

- 14th week i.u.:
  - cap stage (I₁, I₂, C, M₁, M₂), in the fibroblasts;
Krajewski et al., from the stages of the apoptosis (Fumara et al., 2005; Silvaa et al., 2005) involved in the molecular processes from the stages of the apoptosis (Krajewski et al., 1998, Schorr, 1999, Korsmeyer et al., 2000, Correia-da-Silva et al., 2005). Due to its role in the maintenance of cell survival, Bcl-2 is regarded as proto-oncogene (Fumara et al., 2004, Gavathiotis et al., 2012). Bax, because of its pro-apoptotic role is believed to be a possible tumor suppressor (Cartron et al., 2002). The Bcl-2 gene was identified in 1984 at patients with lymphoid tumor pathologies (Schorr, 1999).

The specific arrangement of the cells in the tissue from the structure of the tooth germs, the shape, size, ulceration position and the number of teeth depend on the preservation of the functional balance in the apoptotic process between cell division/cell death (Matalova et al., 2004). Programmed cell death holds an important role in the elimination of the cellular excedent produced during the development of the tooth germ. At the same time, however, the inhibition of apoptosis, especially in its initial stages may determine a change in tooth shape or size, respectively (Matalova et al., 2004).

Our study is defined by the characterization of Bax/Bcl-2 balance in dynamics, at tooth germs in different weeks of embryonic development for the human species.

The evaluation of Bax – pro-apoptotic marker and Bcl-2 – anti-apoptotic marker, which was first performed separately, was later analyzed by comparison for investigated tooth germ, in order to reveal similarities and differences of expression and to understand the action mechanism of the two molecules.

In the initial stages of tooth germ development, namely cap stage, our results indicated that the Bax and Bcl-2 expression, respectively, was positive mainly in the epithelial cells (internal and intermediate epithelium and stellate reticulum) form the enamel organ and less in the mesenchymal/ectomesenchymal cells in the dental papilla. Although the evaluation was based on semi-quantitative scores, we can, however, note the existence of a slight predominance of Bax immunoexpression as opposed with Bcl-2 expression. This statement, although subjective, indicates that in the entire development of the tooth germ, the cap stage is defined by a genetically determined pro-anti-apoptotic balance that leads to the establishment of the architecture specific for each tooth type.

In the early bell stage, our data revealed a Bax expression in the ameloblasts – in accordance with the literature (Kondo et al., 2001, Kalibovic Govorko et al., 2010) but also in the odontoblasts, while for Bcl-2, the expression in the ameloblasts was either absent (in some tooth germs) or very weak (only 5% of cells were positive), while in the odontoblasts it was negative. Our results thus indicate very clearly that the early bell stage is governed by the pro-apoptotic ability, which ensures the life span of the ameloblasts and odontoblasts as they fulfill their roles, namely synthesis of enamel and dentin. Hence, the accelerated pro-apoptotic mechanism explains the initiation of the natural physiologic death of the ameloblasts, that will take place after the achievement of enamel production and of the odontoblasts as well – which will be replaced through the differentiation potential of the existent ectomesenchymal cells.

In the late bell stage, the ameloblasts were positive for Bax and for Bcl-2 as well, while the odontoblasts presented no expression for Bcl-2, only Bax expression, results that certify the intervention of Bax in the differentiation of the odontogenic epithelium. Our data suggests, with respect to the ameloblasts that the late bell stage is additionally characterized by the anti-apoptotic intervention of Bcl-2, which ensures a prolongation of cell survival up until the moment when the enamel deposition is finalized, and only later, before eruption, the pro-apoptotic will become dominant, leading implicitly to their complete disappearance. This result deserves a separate comment, because the literature reports only a pro-apoptotic profile for the ameloblasts (He et al., 2010) before eruption and not a phase of pro-antiapoptotic stability.

In the odontoblasts, the complete absence of Bcl-2 and the consequent pro-apoptotic activity coordinated by Bax could be explained by a necessity for a gradual disappearance of the odontoblasts in order to organize the architectural space, essential on one hand for dentin deposition and, on the other hand, for the delineation of the pulp chamber. Thus, through the inhibition of the anti-apoptotic effect, the odontoblasts suffer a programmed differentiation under the rigorous control of Bax.
In synthesis, the balance of these markers was maintained in the epithelial and mesenchymal component in the cap stage, while in the late bell stage it was preserved in the ameloblasts. In contrast, the pro-apoptotic activity was rather important, as opposed to the anti-apoptotic one, in the early bell stage, both for ameloblasts and odontoblasts, and in the late bell stage for the odontoblasts.

Thus, we must underline the fact that the development of the tooth germ is defined by the pro-apoptotic potential reflected in the Bax immunoexpression in all the cell components of the tooth germ and in all stages of development to the detriment of the anti-apoptotic ability, translated by the Bcl-2 immunoexpression. The only exception is the case of the ameloblasts in the late bell stage that express concomitantly Bax and Bcl-2, which suggests a moment of stability in the cellular evolution, possibly necessary for the maturation of the definitive enamel.

7.5. PROFILE OF MATRIX RENEWAL: MMP-9, TIMP-1

7.5.1. ARGUMENT

“Matrixins” is the generic term used for the description of the family of metalloproteinases (MMP) (Visse et al., 2003, Corotti et al., 2009), collagenolytic enzymes with wide structural specificity and a essential role in the degradation of damaged and native collagen, of non-collagenic proteins and of non-structural components of the extracellular matrix as well (Hannas et al., 2007, Nagase et al., 2006, Nascimento et al., 2011). Although the presence of MMP in the dental structures is intensely debated, it is not entirely elucidated their action mechanism in various tooth formation stages and in the evolution of the mature tooth (Porto et al., 2009). The reports in the literature concentrate on the role of MMP in bone remodeling and tooth eruption (Basi et al., 2011), the specificity of the distribution in the tissues during odontogenesis (Bourd et al., 2004) and on their relationship with the inflammatory and tumoral pathology of the tooth (Bourd-Boittin et al., 2005).

Within this context, our study focused on the evaluation of MMP-9 and TIMP-1 expression as a reflection of matrix remodeling potential in the developing tooth germs.

7.5.2. CHARACTERISTICS OF THE QUANTIFICATION METHOD

The IHC reaction was interpreted on the basis of a semi-quantitative evaluation system proposed in the literature (Xia et al., 2011) and relying on two criteria: percentage of positive cells (P) and intensity of IHC staining (I) with a final result in the form of a P+I score, with a maximum of 6 points. Thus, in correlation with the estimated percentage of positive cells, the cases were divided into 4 categories: score 0 – absent IHC reaction, score 1 - ≤25% of positive cells, score 2 – between 25% and 50% of positive cells, score 3 - >50% of positive cells. With respect to the staining intensity, the cases were divided into 4 categories: score 0 – absent IHC reaction, score 1 – weak intensity (+), score 2 – moderate intensity (++) and score 3 – strong intensity (+++). According to this quantification system, the reaction was considered negative when the final score was 0, positive when the final score was 1-2 and strongly positive for a final score between 3 and 6.

7.5.3. RESULTS

Microscopically, the evaluation of MMP-9 and TIMP-1 immunoexpression revealed the presence of cytoplasmic labelling in the epithelial and ectomesenchymal cells. Moreover, the positivity of the reaction was mostly constant, the staining pattern being generally homogenous; heterogenous isolated areas were identified as well, irregular and located in territories close to one another but of different origins (epithelial and mesenchymal, respectively).

On the basis of the scoring system mentioned above, the IHC reaction was quantified as very strong for all the evaluated tissue structures and cells, respectively, for all the investigated tooth germs. This is the reason for which we separated the results exclusively through a correlation with the intensity of the IHC staining, in order to be able to appraise the differences in the evolution of the development stages of the tooth germs.

In correlation with the intensity of the immunoexpression, MMP-9 and TIMP-1 presented the following characteristics:

Strong intensity:
- 10th week i.u.:
  - for MMP-9
  - cap stage (I1, I2, M1), in the internal epithelium, stellate reticulum and cervical loop;
  - for TIMP-1
  - cap stage (I1), in the internal epithelium and stellate reticulum;
  - cap stage (I2, M1), in the internal epithelium, stellate reticulum and cervical loop; dental papilla – fibroblasts;
- 14th week i.u.:
  - for MMP-9
- cap stage \( (I_1, I_2, C, M_1, M_2) \), in the internal epithelium, stellate reticulum and cervical loop;
- for \textit{TIMP-1}

- cap stage \( (I_1, I_2, C, M_1) \), in the internal epithelium, stellate reticulum and cervical loop; dental papilla – fibroblasts;
- cap stage \( (M_2) \), in the internal epithelium and stellate reticulum;

- \textbullet\ 18\textsuperscript{th} week i.u.:
- for \textit{MMP-9}
- early bell stage \( (I_1, I_2) \), in the ameloblasts;
- cap stage \( (C, M_1) \), in the internal epithelium, stellate reticulum, cervical loop and fibroblasts;
- for \textit{TIMP-1}
- early bell stage \( (I_1) \), in the ameloblasts;
- early bell stage \( (I_2) \), in the ameloblasts and fibroblasts;
- cap stage \( (C, M_1, M_2) \), in the internal epithelium and stellate reticulum;

- \textbullet\ 22\textsuperscript{nd} week i.u.:
- for \textit{MMP-9}
- late bell stage \( (I_1) \), in the ameloblasts and odontoblasts;
- late bell stage \( (I_2) \), in the odontoblasts;
- late bell stage \( (C) \), in the stellate reticulum;
- early bell stage \( (M_1, M_2) \), in the ameloblasts and odontoblasts;
- for \textit{TIMP-1}
- late bell stage \( (I_1) \), in the ameloblasts;
- late bell stage \( (I_2) \), in the odontoblasts;

- \textbullet\ 24\textsuperscript{th} week i.u.:
- for \textit{MMP-9}
- late bell stage \( (I_1, I_2, C, M_1, M_2) \), in the ameloblasts and odontoblasts;

\textbf{Moderate intensity:}

- \textbullet\ 10\textsuperscript{th} week i.u.:
- for \textit{MMP-9}
- cap stage \( (I_2, M_1) \), in the dental papilla – fibroblasts;
- for \textit{TIMP-1}
- cap stage \( (I_1) \), in the cervical loop;

- \textbullet\ 14\textsuperscript{th} week i.u.:
- for \textit{MMP-9}
- cap stage \( (I_1, I_2, C, M_2) \), in the dental papilla – fibroblasts;
- for \textit{TIMP-1}
- cap stage \( (M_2) \), in the cervical loop and dental papilla – fibroblasts;

- \textbullet\ 18\textsuperscript{th} week i.u.:
- for \textit{MMP-9}
- early bell stage \( (I_1, I_2) \), in the intermediate epithelium, stellate reticulum and odontoblasts;
- for \textit{TIMP-1}
- early bell stage \( (I_1, I_2) \), in the intermediate epithelium and stellate reticulum;
- cap stage \( (C, M_1, M_2) \), in the fibroblasts;

- \textbullet\ 22\textsuperscript{nd} week i.u.:
- for \textit{MMP-9}
- late bell stage \( (I_1) \), in the intermediate epithelium, stellate reticulum, odontoblasts and fibroblasts;
- late bell stage \( (I_2) \), in the ameloblasts, stellate reticulum and fibroblasts;
- late bell stage \( (C) \), in the intermediate epithelium and stellate reticulum;
- early bell stage \( (M_1, M_2) \), in the intermediate epithelium and stellate reticulum;
- for \textit{TIMP-1}
- late bell stage \( (I_1) \), in the intermediate epithelium, stellate reticulum, odontoblasts and fibroblasts;
- late bell stage \( (I_2) \), in the ameloblasts and fibroblasts;
- late bell stage \( (C) \), in the ameloblasts, intermediate epithelium and stellate reticulum;
- early bell stage \( (M_1, M_2) \), in the ameloblasts, intermediate epithelium and stellate reticulum;

- \textbullet\ 24\textsuperscript{th} week i.u.:
- for \textit{MMP-9}
- late bell stage (I₁, I₂, C, M₁, M₂), in the intermediate epithelium and stellate reticulum;  
- for TIMP-1  
- late bell stage (I₁, I₂, C, M₁, M₂), in the ameloblasts, intermediate epithelium and stellate reticulum;

### Weak intensity:

- 10th week i.u.:  
  - for TIMP-1 and MMP-9  
  - cap stage (I₁), in the dental papilla – fibroblasts;  
- 18th week i.u.:  
  - for MMP-9  
  - early bell stage (I₁, I₂), in the dental pulp – fibroblasts;  
  - for TIMP-1  
  - early bell stage (I₁, I₂), in the odontoblasts;  
  - cap stage (C, M₁, M₂), in the cervical loop;  
- 22nd week i.u.:  
  - for MMP-9  
  - late bell stage (I₃), in the stellate reticulum;  
  - late bell stage (C), in the fibroblasts;  
  - early bell stage (M₂), in the fibroblasts;  
  - for TIMP-1  
  - late bell stage (I₃), in the stellate reticulum;  
  - late bell stage (C), in the odontoblasts and fibroblasts;  
  - early bell stage (M₁, M₂), in the odontoblasts and fibroblasts;  
- 24th week i.u.:  
  - for MMP-9  
  - late bell stage (I₁, I₂, C, M₁, M₂), in the dental pulp – fibroblasts;  
  - for TIMP-1  
  - late bell stage (I₁, I₂, C, M₁, M₂), in the odontoblasts and fibroblasts.

### 7.5.4. Discussion

Embryonic development, as morphogenesis and tissue remodeling requires not only the intervention of MMP but also the preservation of the balance between MMP versus TIMP quantity (Overall, Lopez-Otin, 2002). A review of the reports in the literature on the MMP-TIMP interactions in the stomatognat system reveals directions concentrated on the normal odontogenesis, but also on the deficiency in the MMP-TIMP equilibrium, which determine massive tissue destruction and compromises the formation of dental tissues and the mineralization process, eventually.

The role of MMP in the processes of epithelio-mesenchymal transition from the initial stages of odontogenesis is well documented (Sahlberg et al., 1999), the intervention of MMP being responsible for matrix degradation in the intial stages of the morphogenesis and cytodifferentiation of the tooth germ, respectively (Heikinheimo et al., 1995). During the development of the tooth germ, MMP are expressed in ameloblasts, odontoblasts and other cell components in the dental structure (Paiva et al., 2009, Gomes et al., 2011). Later, in the process of eruption, the MMP are involved in the resorption mechanism (Basi et al., 2011) – namely, MMP-9 was identified in the lamina propria from the areas adjacent to the eruptive teeth, which certifies the role in the degradation and remodeling of the connective tissue (Cerrì et al., 2010).

Our results have a high degree of originality for they reflect the simultaneous expression of a MMP (MMP-9) and of its specific tissue inhibitor (TIMP-1) in human tooth germs in the evolution of the odontogenesis – from 10th to 24th week i.u. our data indicate that MMP-9 and TIMP-1 are strongly expressed and balanced in cap stage, in the epithelial and mesenchymal component. The equilibrium between MMP-9 and TIMP-1 turns later, in the late bell stage, towards MMP-9 – especially in the odontoblasts. Specifically, our study indicated in all the investigated tooth germs, through the percentage of positive cells, a positivity for MMP-9 and TIMP-1 in the epithelial and mesenchymal cells in a ratio of 100%, with a few small exceptions: for MMP-9, in the pulp odontoblasts in early bell stage the positivity was 80% and for TIMP-1, in the pulp fibroblast in the late bell stage, the positivity was 30%.

We deem as relevant the intensity of the IHC reaction, evaluated as predominantly strong for all the cases investigated. However, small areas of moderate expression were noted for MMP-9 and TIMP-1 as well, restricted to the stellate reticulum of the enamel organ and to the fibroblasts in the dental pulp. Moreover, there were recorded in the late bell stage and especially for TIMP-1, areas with weak intensity in the odontoblasts and fibroblasts.
Our study can be commented in correlation with the data in literature that certifies: (i) at the animal experimental model, in the late stage of development – late bell stage, an increased expression for MMP-2 and MMP-9, concomitantly with a decreased expression for TIMP-1 and TIMP-2 at the dentino-enamel junction (Goldberg et al, 2003), and a decreased MMP-9 expression in the extomesenchyme of the dental sac (Sahlberg et al., 1999); (ii) at the animal experimental model, in the late stage of development – early bell and late bell stage, the presence of MMP-9 expression in the preameloblasts, preodontoblasts, mesenchymal cells in the pulp and in the apical areas of the ameloblasts and odontoblasts (Randall, Hall, 2002, Bourd-Boittin et al., 2005); (iii) in humans, on mature teeth, the immunoreactivity of MMP-9 in odontoblasts, predentin, medial coronal dentin and cells of the dental pulp: fibroblasts, mast cells and macrophages (Niu et al., 2011); (iv) the role of MMP in processing the organic matrix of enamel and dentin, resulting in the regulation of the mineralization process (Hannas et al., 2007, Brackett et al., 2011).

Very interestingly, in the early and late bell stage, the ameloblasts express strongly MMP-9 and much weaker TIMP-1 – a possible explanation for this immunoreactivity model being the possible ability of MMP produced by ameloblasts to (i) degrade the basal membrane at the interface with the dental pulp; (ii) degrade the matrix of the stellate reticulum in the enamel organ in order to facilitate their migration in a coronal direction and the enamel deposition and (iii) to regulate the turn-over of the macromolecules in the organic enamel matrix and the mineralization process.

The strong positivity of MMP-9 in the ameloblasts is considered as an important indicator for the differentiation process, in the context in which in the literature dedicated to this issue it is mentioned that the deficit or absence of MMP-9, demonstrated on an experimental model is associated with a leak of ameloblastic differentiation, absence of polarity and consequently, damage of the normal dental morphology (Yuan et al., 2009), possibly through the interference in the parallel synthesis of amelogenines (Feng et al., 2012).

Simultaneously, the odontoblasts present a similar model of MMP-9 – TIMP-1 expression to the ameloblasts – the excess of MMP-9 as compared with TIMP-1 suggesting the potential of erosion/degradation oriented towards the apical area, in order to (i) ensure the space for the retreat of the odontoblasts and the consequent dentin deposition and (ii) control the turnover of the proteoglycans in the organic matrix of dentin and its mineralization. The pulp fibroblasts present a weak intensity for MMP-9 and TIMP-1 which indicates that the dental pulp is in this stage a stable tissue, with a well-structured extracellular matrix, where there are no important reorganization processes.

A special mention must be made with respect to the negative aspect for MMP-9 and TIMP-1 in the developing dentin, while in the mature tooth MMP-2 and MMP-9 were identified in the deep and medial coronal dentin (Niu et al., 2011). In our opinion, the presence of the two MMP in mature dentin reflect the continuous dynamics of the dentinogenesis process (degradation, synthesis and deposition) specific for the mature tooth, whereas in the development stages of the tooth germ – early and late bell – the emphasis of the dentinogenesis process is on synthesis and deposition, the presence of MMP being thus justified.

**7.6. PROFILE OF MATRIX SUPPORT: FIBRONECTIN, COLLAGEN IV**

**7.6.1. ARGUMENT**

The extracellular matrix is organized as a result of certain events programmed in time and space, its component molecular categories being: (i) fibrillary proteins (collagen, laminin), (ii) glycosaminoglycans and proteoglycans and (iii) adhesion glycoproteins, where FN is an important member, because it promotes cell adhesion, migration, differentiation and cell signaling (González-García et al., 2013).

In the developing tooth germ, the extracellular matrix intervenes not only through its structural role of support but also through the differentiation potential of cell lineages (Tabata et al., 2003). Fibronectin (FN) is by far the most investigated multifunctional protein in the composition of the extracellular matrix and basal membranes. Alongside other macromolecules of mesenchymal origin, this one is in a permanent crossed dialogue with specific receptors such as integrins (Labat-Robert, 2012).

In correlation with the cell type that intervenes in the synthesis/production of FN, it can be of cellular (cFN) or plasmic type (pFN) – although only one gene is responsible for the coding (Zand, 2003). The differentiation with respect to its origin is reflected on the structural components of FN, so that some molecular regions (domains) will be specific for cFN while in the pFN form the same domains will be absent (Schwarzbauer, 1991).

The study of FN, as a relational molecule of cell adhesion and consequently tumor invasion is oriented most frequently towards the cFN form, the pFN form lacking cell specificity (Zand et al., 2003).

Type IV collagen, a triple helix molecular structure found in the components of the basal membrane consists of 6 protein chains numbered from α1(IV) to α6(IV) (Mariyama et al., 1994). The six types of proteins specific to type IV collagen are coded by six distinct genes (COL4A1 – COL4A6) (Zhou et al., 1994).
Type IV collagen ensures, due to its structure, the organization of a fibrillar network for the support of the cellular and extracellular elements in the extracellular matrix, with simultaneous roles of signal and adhesion mediator and of remodeling the extracellular matrix through the specific receptors.

In the literature, strictly with respect to the stomatognat system in general and to the tooth in particular there are few studies that analyze the FN profile in humans (Lukinmaa et al., 1991, Yoshiba et al., 1994), animal experimental models (Zhang et al., 2007) or cell cultures (Thesleff et al., 1987). Also, the reports on type IV collagen are limited.

In this context, our study focused on the evaluation of FN and type IV collagen expression, as molecules involved in the matrix structural support and intercellular communication in the developing tooth germs.

### 7.6.2. Characteristics of the Quantification Method

The IHC reaction for FN and type IV collagen was interpreted on the basis of an semi-quantitative evaluation and quantification system (Berkholtzet al., 2006, Kimet al., 2010). The grading was performed in correlation with the intensity of the immunostaining as follows: absent immunooexpression – 0, weak immunooexpression (+) – 1; moderate immunooexpression (++) – 2 and strong immunooexpression (+++) – 3.

### 7.6.3. Results

All the investigated tooth germs presented a positive immunocytochemical reaction for FN with the identification of differences in expression between epithelial and ectomesenchymal components, with respected to the evolution stage and gestational age, however without association with the type of tooth.

With respect to the intensity of the cytoplasmic immunooexpression, FN showed the following characteristics:

**Strong intensity:**
- 10<sup>th</sup> week i.u.:  
  - cap stage (I<sub>1</sub>, I<sub>2</sub>, M<sub>1</sub>), in the dental papilla – fibroblasts;
- 14<sup>th</sup> week i.u.:  
  - cap stage (I<sub>1</sub>, I<sub>2</sub>, C, M<sub>1</sub>, M<sub>2</sub>), in the dental papilla – fibroblasts;
- 18<sup>th</sup> week i.u.:  
  - early bell stage (I<sub>2</sub>), in the stellate reticulum;
  - cap stage (C, M<sub>1</sub>, M<sub>2</sub>), in the dental pulp – fibroblasts;

**Moderate intensity:**
- 10<sup>th</sup> week i.u.:  
  - cap stage (I<sub>1</sub>, I<sub>2</sub>, M<sub>1</sub>), in the internal epithelium, stellate reticulum and cervical loop;
- 14<sup>th</sup> week:
  - cap stage (I<sub>1</sub>, I<sub>2</sub>, C, M<sub>1</sub>, M<sub>2</sub>), in the internal epithelium, stellate reticulum and cervical loop;
- 18<sup>th</sup> week i.u.:  
  - early bell stage (I<sub>1</sub>), in the ameloblasts, intermediate epithelium, stellate reticulum and dental pulp – fibroblasts;
  - early bell stage (I<sub>2</sub>), in the ameloblasts, intermediate epithelium and dental pulp – fibroblasts;
  - cap stage (C, M<sub>1</sub>, M<sub>2</sub>), in the internal epithelium, stellate reticulum and cervical loop;
- 22<sup>nd</sup> week i.u.:  
  - late bell stage (I<sub>1</sub>, I<sub>2</sub>, C, M<sub>1</sub>, M<sub>2</sub>), in the ameloblasts, intermediate epithelium, stellate reticulum and dental pulp – odontoblasts;
- 24<sup>th</sup> week i.u.:  
  - late bell stage (I<sub>1</sub>, I<sub>2</sub>, M<sub>1</sub>, M<sub>2</sub>), in the ameloblasts, intermediate epithelium and stellate reticulum;
  - late bell stage (C, M<sub>1</sub>, M<sub>2</sub>), in the ameloblasts, intermediate epithelium, stellate reticulum and dental pulp – odontoblasts;

**Weak intensity:**
- 18<sup>th</sup> week i.u.:  
  - early bell stage (I<sub>1</sub>, I<sub>2</sub>), in the dental pulp – odontoblasts;
- 22<sup>nd</sup> week i.u.:  
  - late bell stage (I<sub>1</sub>, I<sub>2</sub>, C, M<sub>1</sub>, M<sub>2</sub>), in the dental pulp – fibroblasts;
- 24<sup>th</sup> week:  
  - late bell stage (I<sub>1</sub>, I<sub>2</sub>), in the dental pulp – odontoblasts and fibroblasts;
  - late bell stage (C, M<sub>1</sub>, M<sub>2</sub>), in the dental pulp – fibroblasts.

Microscopically, the analysis of type IV collagen immunophenotype revealed the complete absence of the labelling in the structural components of the tooth germs, regardless of the evolution stage or tooth type, with the exception of the basal membrane of the capillaries in the dental pulp – early and late bell stage,
respectively. On the other hand, type IV collagen was identified in the extracellular compartment around the developing tooth germs as early as the 10th week i.u.

In the study group, the expression of type IV collagen was evaluated as moderate, as follows:

- **18th** week i.u.:
  - early bell stage (I₁, I₂), in the capillary endothelium;
- **22nd** week i.u.:
  - late bell stage (I₁, I₂), in the capillary endothelium;
  - early bell stage (M₁, M₂), in the capillary endothelium;
- **24th** week i.u.:
  - late bell stage (I₁, I₂, C, M₁, M₂), in the capillary endothelium.

### 7.6.4. DISCUSSION

Given that the data on the presence of FN in the developing tooth germ is limited, we deem appropriate a short review of the main structural and functional landmarks characteristic for this adhesion molecule. The FN molecule has approximately 5-10 nm in width (Singh et al., 2010) and consists of 2 structural subunits of approximately 250 kD, similar but not identical, joined together by two disulphidic bridges at the C-terminal end (Pankov, Yamada, 2002).

Through the contacts with the specific receptors on the surface of the fibroblasts, especially integrins, FN mediates cell motility and dissemination (Zand et al., 2003, Hynes, 2004), and thus intervenes in the embryogenesis in general through matrix modeling and especially in the angiogenesis (pFN has a role in the formation of the vascular system and cFN – in its maturation) (Astrof, Hynes, 2009).

In the case of the dental structures, FN was identified in human species both in the structural elements of the tooth germ and in the gingival and periodontal ligament (Lukinmaa et al., 1991). Its presence in excess in the periodontal tissues is associated with the pathogenesis of the periodontal disease and represents the substrate for the invasion of the bacterial proteinases (Feghali, Grenier, 2012).

The information in the literature certifies on the experimental model, the accumulation of FN from the bud stage in the epithelial and mesenchymal structures as well (Zhang et al., 2007). In the bell stage, the expression of FN is characteristic especially for the areas of ameloblast and odontoblast differentiation – namely, the apical pole of these cells (Zhang et al., 2007).

Our study revealed on human tooth germs in different developmental stages, in accordance with the gestational age results similar with the ones reported in the literature, on experimental animals (Zhang et al., 2007).

Our results revealed the cytoplasmic expression of FN especially in the enamel organ in cap stage, in all the epithelial components: internal, intermediate and outer epithelium. The IHC distribution of FN in the dental papilla in cap stage was less apparent by comparison with the one in the epithelium, where the immunolabelling presented a homogenous diffuse expression, moderate and in certain areas, strong.

The location of FN at the apical pole of the ameloblasts and odontoblasts suggests a possible role not only in their differentiation, but also in the establishment of the dentino-enamel junction, through functional connexions between the epithelial and mesenchymal elements that regulate the process of deposition of the organica matrix and its later mineralization. The positive expression of FN, apparent in the predentin adjacent to the internal epithelium of the enamel organ/ameloblasts, but absent in the dentin, indicate the stage where the mineralization process is not yet initiated. The maxim synthesis of FN is hence associated with the period when the progressive production and deposition of the dentin matrix occurs, before the triggering of the events specific for the mineralization. It is also possible for the expression of FN in the odontoblasts, in the early bell stage, to be a supplementary factor in the finalization of the differentiation of the preameloblasts in the ameloblasts.

Moreover, we believe that the intense immunoexpression which characterizes the periphery of the dental pulp reflects not only cell-cell interactions but also the cell-matrix ones, through which the remodulation of the connexive tissue is achieved in the dental pulp, in the evolution of the tooth germ.

The presence of the collagene component in the development of the tooth germ is undisputed, given the implication of the mesenchyme and ectomesenchyme in the odontogenesis. Type IV collagen however requires a separate discussion, because, as opposed to the other members of the collagene protein family which includes 28 types (Bächinger et al., 2010), it has an epithelial origin and is synthesized exclusively by the epithelial cells of the tooth germ and located predominantly in the basal membranes of the tooth germ (Thesleff et al., 1981, Tabata et al., 2003). In this context, the function of type IV collagen is still unclear in the tooth morphogenesis (Tabata et al., 2003).

Our study revealed the absence of type IV collagen in the investigated tooth germs, regardless of the developmental stage, both in the epithelial and mesenchymal stage. The positive reaction was identified
exclusively in the capillary basal membrane, with a moderate intensity. Extremely interesting, the reaction to type IV collagen was negative in the basal membranes of the internal and external epithelium of the enamel organ – area where its presence was expected. The comment on this negative result of definite originality focuses on a possible particularity of this membrane with transitional character in the evolution of the tooth germ. Thus, the absence of type IV collagen might represent an element that contributes to the deciphering of the fragmentation mechanism for the basal membrane between the epithelial and mesenchymal / ectomesenchymal component of the tooth germ.

The immunoreaction of type IV collagen detected only in the basal membrane of the vascular endothelium in the dental pulp may be explained as a result of the development of the vascular structures, as the tooth germ evolves from cap to bell stage and of the transformation of the dental papilla in dental pulp. A supplementary aspect that must be underlined resides in the presence of the capillaries with the basal membrane positive to type IV collagen mainly in the central area of the pulp and not at the periphery – a possible explanation is the gradual maturation of the capillary component, from the center to the periphery. Nevertheless, the IHC exam revealed a strong expression of type IV collagen in the mesenchymal structures adjacent to the tooth germ even from the cap stage – 10th week i.u. this particularity may be interpreted from the point of view of the major differences between the tooth formation mechanism and that of the adjacent structures – which leads to the same supposition, namely that the epithelial cells of the tooth germ have a different potential of type IV collagen production as compared with other types of epithelial cells.

Finally, we must underline the fact that the two investigated molecules revealed completely different aspects, despite our expectations founded on the theoretical data according to which FN and collagen are frequently associated, even through they have different origins (Hurmerinta et al., 1986 4, Thesleff et al., 1987). Definitely, their behaviour in the developing tooth germs is different as compared with the one in the common, ordinary epithelial and connective tissues.

CHAPTER 8. CONCLUSIONS

1. Tooth germs in cap and bell stage, respectively, presented various morphologic aspects without a precise overlap between morphology and a uniformly defined time lapse in correlation with the gestational age. The pronounced diversity in shape and size of the identified tooth germs proves the influence of the genetic factors and supports the existence of specific microenvironmental interactions in the tooth development.

2. The morphometric analysis of the fibroblasts at the intergroup level, performed with the concomitant usage of the geometric features area (A), perimeter (P) and form factor (FF) and the exploitation of the FF set of values through the relative distances scaled with respect to the average values proved that the early bell stage is 5.48 times (5.32, respectively) closer to late bell stage than to cap stage. At the same stage, we proved that the procedure, based on the relative distances, applied with respect either to A or P fails in the quantitative discrimination inter-stage.

3. The morphometric analysis of the pulp fibroblasts at the intragroup level, based on the relative distances instrument that allowed the evaluation of the similarities through the variations in the average values of each case around the average value of the group, applied with respect to all three parameters A, P and FF, proved the existence of a 10% threshold that separates the human border between the acceptance and rejection of region similarities, on the basis of the caprisons between areas, perimeters and contour types.

4. Cyclin D1 expression represents a significant indicator of proliferation in the cellular differentiation stages, the IHC profile being correlated with the evolution of the cells with epithelial and mesenchymal origin towards a morphologic specialisation that ceases either after the achievement of the functional mission (ameloblasts) or goes on through the permanent character of the new role (odontoblasts).

5. The presence of Bax in all the developmental stages of the tooth germs indicates that the pro-apoptotic process is essential for the development and morphogenesis of the stomatognat system. In the ameloblasts from the late bell stage the simultaneous expression of Bax and Bcl-2 suggests a moment of stability in the evolution of the cells, possibly necessary for the maturation of the definitive enamel. In the odontoblasts from the late bell stage, the decrease of Bcl-2 suggests an insufficient anti-apoptotic opposition against the pro-apoptotic factors, which reflects the renewal dynamics of the odontoblasts, necessary for the formation and deposition of dentin.

6. MMP-9 and TIMP-1 are expressed from the beginning of the odontogenesis, without differences between the decidual tooth germs. Cap stage is characterized by a balance between MMP-9 and TIMP-1, the expression of the molecules being strong in the enamel organ epithelia and weak in the cells of the dental papilla. For the bell stage, the balance between MMP-9 and TIMP-1 turns in favor of MMP-9. The
expression of MMP-9 in the ameloblasts and odontoblasts represents an important indicator for the cell differentiation process of these cells, with direct repercussion on the organic matrix synthesis and normal enamel and dentin mineralization.

7. All the investigated tooth germs presented a positive IHC reaction for the fibronectin, with the identification of differences in expression between the epithelial and eutomesenchymal component, in correlation with the evolution stage and gestational age, but without association with the correspondent tooth type. The presence of fibronectin reflects the matrix stability necessary for the organization of the tooth germs in shape and size throughout the developmental evolution stages.

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