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## Impact of Claudin 10 deficiency on amelogenesis: lesson from a HELIX tooth

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## Impact of Claudin-10 deficiency on amelogenesis: lesson from a HELIX tooth

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**Running Head:** Claudin-10 deficiency and amelogenesis

**Disclosures:** none

**Key words:** Tight junctions; claudins; enamel; apatite; xerostomia; renal dysfunction

**ABSTRACT**

In epithelia, claudin proteins are important components of the tight junctions as they determine the permeability and specificity to ions of the paracellular pathway. Mutations in *CLDN10* cause the rare autosomal recessive HELIX syndrome (for: Hypohidrosis, Electrolyte imbalance, Lacrimal gland dysfunction, Ichthyosis, and Xerostomia), in which patients display severe enamel wear. Here, we assess whether this enamel wear is caused by an innate fragility directly related to claudin-10 deficiency in addition to xerostomia. A third molar collected from a female HELIX patient was analyzed by a combination of microanatomical and physico-chemical approaches (i.e., electron microscopy, elemental mapping, Raman micro-spectroscopy, synchrotron-based X-Ray fluorescence). The enamel morphology, formation time, organization, and microstructure appeared to be within the natural variability. However, we identified accentuated strontium variations within the HELIX enamel, with alternating enrichments and depletions following the direction of the periodical striae of Retzius. These markings were also present in dentin. These data suggest that the enamel wear associated with HELIX may not be related to a local disturbance of amelogenesis but rather to xerostomia. However, the occurrence of events of strontium variations within dental tissues might indicate repeated episodes of worsening of the renal dysfunction that may require further investigations.

## INTRODUCTION

Tooth enamel is the most mineralized structure of the organism and forms the outer layer of the dental crown. Amelogenesis is a complex process that occurs before tooth eruption and results from a complex epithelial mesenchymal cross talk between the enamel organ, which derives from the ectoderm, and the dental mesenchyme, which derives from the neural crest<sup>1-4</sup>. Enamel synthesis encompasses two major steps, namely the secretory stage during which the ameloblasts, the enamel-secreting cells of the enamel organ, secrete a template of enamel-specific extracellular matrix proteins, and the maturation stage during which most of this scaffold is replaced by hydroxyapatite<sup>5-9</sup>. Ameloblasts display a double set of tight junctions (TJs), both at their apical and basal ends; the apical TJs are subject to remodeling at the maturation stage depending on the cell phenotype<sup>9-11</sup>. Claudin proteins are the main components of the TJs that are either sealing the paracellular space or forming a channel, thus determining their permeability and ion specificity<sup>12</sup>. The expression of claudins has been reported in the ameloblast TJs<sup>13</sup>, including claudin-1, -3, -16, and -19<sup>10, 14-16</sup>. Along this line, claudin-10 was shown to be expressed in the enamel organ and more precisely in the stratum intermedium, a layer of epithelial cells located immediately adjacent to the ameloblast layer<sup>17</sup>. Two isoforms of claudin-10 are expressed in the kidney<sup>18</sup>, claudin-10a and claudin-10b. The expression of claudin-10a, which is anion-selective, is restricted to the proximal tubule<sup>19</sup>. Claudin-10b, which is cation-selective and may determine paracellular sodium permeability<sup>20, 21</sup>, is expressed not only in the thick ascending limb (TAL) of Henle's loop in the kidney<sup>19</sup> but also in many other epithelia<sup>22, 23</sup>.

Several genetic disorders affect the enamel structure of all the teeth from both dentitions, resulting in *Amelogenesis imperfecta* manifested by severe dental defects, which require complex restorations and significantly alter a patient's quality of life<sup>4, 5, 24-27</sup>. Among them, non-syndromic *Amelogenesis imperfecta* are due to pathogenic variants of genes that encode enamel-specific extracellular matrix proteins (*AMELX*, *ENAM* and *AMBN*), or proteins involved in enamel maturation (*MMP-20*, *KLK-4* and *SLC24A4*), or cell-cell and cell-matrix attachments (*ITGB6*, *COL17A1*, *LAMA3* and *LAMB3*)<sup>24, 28</sup>.

*Amelogenesis imperfecta* is also frequently found in patients with genetic disorders related to kidney, skin and many other organs<sup>14, 24, 28, 29</sup>. A disorder of this sort was recently found to be

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3 associated with loss-of-function variants of *CLDN10*, resulting in HELIX syndrome characterized  
4 by Hypohidrosis, Electrolyte imbalance, Lacrimal gland dysfunction, Ichthyosis, and Xerostomia  
5 and the autosomal recessive inheritance (OMIM 617671; Prevalence: <1 /1,000,000)<sup>30-35</sup>. In  
6 addition, it has been reported that the patients with HELIX syndrome displayed a very early and  
7 severe enamel wear<sup>32</sup>. As claudin-10 is expressed in the forming tooth germ<sup>13,17</sup>, it is difficult to  
8 determine whether this severe enamel wear results from the impaired salivary secretion, one of the  
9 main manifestations of the HELIX syndrome<sup>30</sup>, or from an intrinsic enamel fragility directly  
10 related to claudin-10 deficiency. In the present study, the examination of the enamel of a retained  
11 third permanent molar, which was in a submucosal position and therefore partially exposed to the  
12 oral environment, was used to explore this question. The tooth was removed for orthodontic  
13 therapeutic reasons from a young female adult patient with HELIX syndrome. By combining  
14 microanatomical and physico-chemical approaches, we could evidence that neither the rate of  
15 enamel formation nor its morphology, organization and structure were significantly impacted by  
16 claudin-10 deficiency. However, we identified the occurrence of random events of strontium  
17 variations within both enamel and dentin that may reflect a disorder in strontium handling,  
18 potentially caused by the renal dysfunction.  
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## 34 MATERIALS AND METHODS

### 36 Samples

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38 An impacted right permanent third lower molar was collected from a 19-year old female patient  
39 with the HELIX syndrome. This patient was already reported as patient A-IV-2<sup>32</sup>. This family  
40 presented a missense variation c.386C >T (NM\_182848), p.S129L in claudin-10a (c.392C>T  
41 (NM\_006984), and p.S131L in claudin-10b. A 3D Cone Beam Computed Tomography (CBCT)  
42 exam of the lower jaw was performed to prepare the surgery. Since this impacted third molar was  
43 massive, the surgeon decided to section it to limit post-operative adverse events. Three impacted  
44 age-matched third molars were gathered and were randomly used as control for the various  
45 experiments. All teeth were extracted at the request of an orthodontist in the context of a treatment  
46 plan and were collected with the informed consent of the patients, in accordance with the ethical  
47 guidelines laid down by French law (agreement IRB 00006477 and n° DC-2009-927, Cellule  
48 Bioéthique DGRI/A5). All teeth were fixed in 70% ethanol for a week.  
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### Preparation of the tooth sections

Sections of the crown of the HELIX patient's molar and control third molars were carried out to study the microanatomy of dental tissues by optical and scanning electron microscopy (SEM) and to perform chemical analyses by Raman micro-spectroscopy, Energy-Dispersive-X-ray (EDX) microanalysis and Synchrotron X-Ray Fluorescence (SXRF) imaging. For the HELIX molar, since the distal half of the crown was fragile and easily broken, and preserved both enamel and dentin, the section was performed on that fragment, thus yielding a bucco-distal crown thin section. A bucco-lingual section through the mesial cusps was performed on the control lower third molars.

For preparation of the thin sections for micro-anatomy, Raman and SXRF analyses, the teeth were embedded in cyanoacrylate and fixed with wax on the glass slide. We used a saw equipped with a diamond disk (Struers, Champigny-sur-Marne, France) under a continuous water spray. After the first cut, the surface of the block was polished with carbide grinding paper (Grit 600/P1200) and Chemomet paper with 1  $\mu\text{m}$  aluminum powder (Bühler, Uzwil, Switzerland). This surface was glued onto the slide with Araldite 2020 (Huntsman corporation, The Woodlands, TX). The block was then sectioned into  $\sim 300$   $\mu\text{m}$  slices and polished (Grit 600/P1200) to reach an average thickness of  $\sim 160$   $\mu\text{m}$  for the HELIX molar and  $\sim 60$   $\mu\text{m}$  for the control third molar. The polishing process was kept minimal for the HELIX tooth because of its fragility. Finally, the sections were polished again with Chemomet paper with 1  $\mu\text{m}$  aluminum powder until a completely flat surface was obtained.

For preparation of the sections for SEM and EDX analyses, one mm-thick sections of the control and HELIX third molars were prepared with a saw equipped with a diamond disk (Struers, Champigny-sur-Marne, France) under a continuous water spray. After thorough polishing, surfaces were cleaned with 5% sodium hypochlorite under ultrasonic activation for 2 min, rinsed twice with distilled water, etched with 36 % orthophosphoric acid (DeTrey® Conditioner 36, Dentsply Sirona, York, PA) for 12 s, and then thoroughly rinsed with distilled water.

### Study of the microanatomy of dental tissues

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3 The HELIX crown section was mounted on a glass slide for observation and analysis. The section  
4 was analyzed using incident light with a stereomicroscope Leica M8 and transmitted light with a  
5 Zeiss Universal photo-microscope (#4 objective). The Zeiss microscope was fitted with an Idea  
6 camera connected to a computer using Spot software (Version 5.4). The images were processed  
7 with Nikon ViewN2 and their analysis was performed with ImageJ.  
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12 Analysis of the dental microanatomy allows the study of the daily secretion rate (DSR) of the  
13 enamel and the formation time of the crown thanks to the presence of periodical growth lines in  
14 the enamel, the cross-striations and the striae of Retzius. Cross-striations reflect the circadian  
15 variation of the enamel secretion, their spacing is indicative of the amount of enamel formed per  
16 day and yields the DSR <sup>36</sup>. The striae of Retzius, which correspond to longer successive steps of  
17 enamel formation, are formed at regular intervals. Their periodicity is determined by counting the  
18 number of cross-striations in between two successive striae. This periodicity is assumed to be  
19 constant during the entire crown formation time of a given tooth. According to the arrangement of  
20 Retzius' striae in the enamel, the dental crown can be divided into a cuspal part, located at the  
21 occlusal third of the tooth, in which striae are arranged in successive arches around the dentin horn  
22 and a lateral part, which is formed subsequently and until crown completion at the cervix <sup>37</sup>. In the  
23 lateral enamel, striae of Retzius crop out and terminate at the surface of the enamel rather than  
24 arch over the dentin horn.  
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35 The DSR was obtained in the cuspal portion of the crown near the apparent dentin horn. A line  
36 running along the direction of an enamel prism between the enamel-dentin junction (EDJ) and the  
37 outer enamel surface was then divided into 100  $\mu\text{m}$ -thick zones to calculate DSR changes during  
38 the course of crown formation <sup>38,39</sup>. In each zone, the average spacing between cross striations was  
39 measured. This was performed several times in each zone, always across a minimum number of  
40 three cross-striations, in order to obtain an average DSR for each zone, and finally to calculate an  
41 overall average DSR for the cuspal enamel. The total cuspal enamel formation time is equal to the  
42 thickness of cuspal enamel divided by the average daily cross-striations spacing.  
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49 To describe the development of the lateral enamel, the height of the crown, taken between the cusp  
50 tip and the enamel cervix, was divided into deciles of crown height <sup>40</sup>. Noticeably, in the first two  
51 deciles, striae were difficult to distinguish so that the formation time was estimated by dividing  
52 the length of the prism between the limit cuspal-lateral enamel and the first striae of the third decile  
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3 by the DSR of this area. The number of striae of Retzius was counted within each decile and  
4 multiplied by their periodicity. Periodicity was obtained in three locations. The lateral enamel  
5 formation time is equal to the total count of Retzius lines multiplied by their periodicity, i.e. 8  
6 days. The crown formation time was obtained by the sum of cuspal and lateral enamel formation  
7 times.  
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### 15 **SEM imaging and EDX analyses**

16 For SEM imaging, thick sections of the HELIX and control third molars were coated with a thin  
17 gold layer (~30 nm) using a CRESSINGTON 108 AUTO gold sputter coater. For EDX  
18 measurements, the thick sections were coated with a thin layer of carbon (ca. 20 nm) by  
19 evaporation using a CRESSINGTON 208C carbon coater. Imaging and analysis were performed  
20 at 15 kV at different magnifications using a Hitachi SU-70 microscope equipped with a Field  
21 Emission Gun. All the samples were evaluated for Ca, P, Mg content (% atom) in the outer and  
22 inner layers of enamel, and at least three measurements were performed for each layer.  
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### 31 **Raman micro-spectroscopy**

32 Raman analyses were performed using a Senterra Raman micro-spectrometer (Bruker Optics),  
33 using a laser emitting at 785 nm settled to provide a laser power at the sample of about 25 mW.  
34 Data collection was controlled by the OPUS 7.5 software (Bruker Optics). Measurements were  
35 collected across one spectral window (450–1800  $\text{cm}^{-1}$ ) at a spectral resolution of about 3  $\text{cm}^{-1}$ ,  
36 and each analysis was the coaddition of 2 spectra accumulated at up to 30 s of exposure time for  
37 each. Analyses were performed using a 50 $\times$  objective (Olympus, Tokyo, Japan), giving an  
38 analytical spot size of approximately 12  $\mu\text{m}$  in diameter, with the used excitation wavelength.  
39 Analyses were directly performed on polished surfaces of the thin sections. For mapping, the  
40 sample was moved by a computer-controlled stage. Each surface was scanned by moving the  
41 sample by about 18  $\mu\text{m}$  steps. Maps were performed on a 700  $\times$  2150  $\mu\text{m}$  surface for the control  
42 sample and 1000  $\times$  1850  $\mu\text{m}$  surface for the HELIX sample. Chemical maps were generated by  
43 integrating the area of the band centered at 1070  $\text{cm}^{-1}$  ( $I_{1070}$ ) and the band centered at 960  $\text{cm}^{-1}$   
44 ( $I_{960}$ ), attributed to the vibration of carbonate ( $\nu(\text{CO}_3^{2-})$ ) and phosphate ( $\nu(\text{PO}_4^{3-})$ ) groups in the  
45 mineral phase, respectively <sup>41</sup>. Ratios between both maps were done to monitor the chemical  
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3 distribution. Maps have been depicted using the same color scheme. Full width at half maxima  
4 (FWHM) with imposed fitted position was also determined. Maps based on the  $\nu(\text{PO}_4^{3-})$  full width  
5 at half maxima (FWHM<sub>960</sub>) for both samples were also presented with a color scale going from 10  
6 to 16  $\text{cm}^{-1}$ . Baseline subtractions, fitting and map generation were managed by the OPUS 8.7  
7 software (Bruker Optics).  
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### 14 **Synchrotron X-Ray Fluorescence (SXRF) data acquisition and processing**

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17 The Synchrotron X-ray Fluorescence (SXRF) analysis was performed on the P06 Beamline<sup>42, 43</sup>,  
18 Petra III, at DESY (Deutsches Elektronen-Synchrotron, Hamburg, Germany), a member of the  
19 Helmholtz Association HGF. Both the HELIX third molar and the control third molar were  
20 scanned. The thin section of the HELIX molar was left mounted on a glass slide support, while the  
21 control molar was mounted on suspended kapton foil. The storage ring was operated in 480-bunch  
22 mode in top-up filling mode with an average current of  $120 \text{ mA} \pm 0.5 \text{ mA}$ . The primary X-ray beam  
23 was monochromatized to 16.6 keV using a double crystal Si111 monochromator and focused using  
24 a Kirkpatrick-Baez (KB) mirror system (JTEC, Japan) to approximately  $500 \times 500 \text{ nm}^2$ . The  
25 experimental configuration consisted of two Vortex EM silicon drift detectors (Hitachi High-Tech  
26 Science America, Inc.), the second of which was collimated. Both detectors were positioned  
27 symmetrically at scattering angles of 135 degrees at a distance of 9 mm from the focal point at the  
28 sample surface. The use of dual-detector “backscatter” geometry maximizes the solid angle during  
29 analysis of thin polished samples ( $\sim 110 \mu\text{m}$ -thick on average in this study), and allows large area  
30 to be scanned with micrometric resolution using millisecond dwell times<sup>44</sup>. This setup allowed  
31 capturing  $K\alpha$  emission lines from Si to Sr, with varying detection efficiency.  
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44 Spectral peak deconvolution and integration was performed using the core of PyMca 5.5.0<sup>45</sup>.  
45 Image analysis was performed in HDIP v-1.3.3.1073 (Teledyne CETAC Technologies, Bozeman,  
46 MT, USA). The X-ray yield calculations were performed using an in-house script assuming a  
47 hydroxyapatite matrix ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ) with density  $2.85 \text{ g/cm}^3$  for the enamel phase and  $1.6$   
48  $\text{g/cm}^3$  for the dentin phase<sup>46</sup>. Elemental mass fractions were determined by calculating an areal  
49 density sensitivity from measurements standard Ti, Fe, and Cu foils with areal density of 59.0,  
50 55.0, and  $47.9 \mu\text{g/cm}^2$ , respectively (Micromatter Technologies Inc. Canada), and measured  
51 thickness of the samples. Tooth section thickness was measured throughout the whole surface of  
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3 the specimens in four positions for HELIX and five positions for the control tooth. The average  
4 tooth section thickness (160  $\mu\text{m}$  and 61  $\mu\text{m}$ , respectively) was also taken into account in the X-ray  
5 mass attenuation coefficients of the hydroxyapatite phase during attenuation correction<sup>47</sup>. Glass  
6 slides and kapton foil substrates were included in the overall sample model as appropriate (i.e.,  
7 background subtraction). Normalization to the incoming X-ray flux was applied. In the calibrated  
8 data, SXRF concentrations are reported by mass fraction ( $\mu\text{g}\cdot\text{g}^{-1}$ , i.e., ppm), and/or areal density  
9 ( $\text{g}\cdot\text{cm}^{-3}$ ).

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11 A multiscale scanning strategy was used to optimize efficiency. First, a fast overview scan was  
12 acquired at 100  $\mu\text{m}$  (dwell time: 10 ms) to check that the tooth section is well-centered in the field  
13 of view, and assess the overall signal of the dental tissues. Then, a middle resolution (MR)  
14 overview scan allowed visualizing the elemental variation within the entire tooth section (i.e.,  
15 enamel, and dentin). The HELIX third molar section was scanned at 10  $\mu\text{m}$  with 3 ms dwell time  
16 ( $X = 6.31 \text{ mm} \times Y = 6.44 \text{ mm}$ ,  $t = 33 \text{ minutes}$ ). The control third molar section was scanned at  
17 10  $\mu\text{m}$ , with 3 ms dwell time ( $X = 18.35 \text{ mm} \times Y = 7.99 \text{ mm}$ ,  $t = 1.3 \text{ h}$ ). Finally, based on prior  
18 observations of the HELIX tooth section under the microscope and the MR scans, two small  
19 regions of interest (ROI) were selected for acquiring high resolution (HR) scans: at 1.5  $\mu\text{m}$  (dwell  
20 time = 4 ms): (1) across the lateral enamel just below the end of the cuspal enamel (ROI:  $X = 1.45$   
21  $\text{mm} \times Y = 0.37 \text{ mm}$ ,  $t = 18 \text{ mins}$ ), and (2) across the enamel-dentin junction (EDJ) roughly under  
22 the center of the occlusal basin (ROI:  $X = 1.21 \text{ mm} \times Y = 0.68 \text{ mm}$ ,  $t = 28 \text{ mins}$ ). No HR scan was  
23 acquired on the control molar, due to a normal and monotonous signal in the MR scan.  
24 Visualization and analysis of the SXRF data were performed in HDIP. The color-coded elemental  
25 maps were saved as 32-bit tiffs allowing for a fine-tuning of the contrast and brightness in ImageJ  
26<sup>48, 49</sup> to better reveal the stress pattern. To note that to denoise the images, a 2D Gauss filter was  
27 applied with a kernel size of  $0.8 \times 0.8$ .

### 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 **Variations in Sr content**

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50 Since the chronology of the crown formation was established, any variation in Sr concentration  
51 could be given a time relative to the initiation of crown formation. Each significant variation (i.e.,  
52 enrichment or depletion) in Sr content was allocated a letter, their distance from the EDJ was  
53 measured and their chronological order of formation calculated. The timing of the changes in Sr  
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3 content between the EDJ and the enamel surface along a transect was quantified using the same  
4 methodology as used in the cuspal enamel, that is, the cumulative length of the prism between the  
5 two reference points was divided by the average DSR of the concerned area.  
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## 10 RESULTS

### 11 **Physio-pathological condition and phenotype of the HELIX patient**

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13 The HELIX patient is a 19-year-old French female born at full term from consanguineous parents  
14 (patient A-IV-2), and was raised in France <sup>32</sup>. She displayed xerosis of the skin with keratosis  
15 pilaris of cheeks, arms, thighs with a slight palmo-plantar keratoderma and xerostomia. As  
16 presented in Hadj-Rabia et al.<sup>32</sup>, she had normal serum calcium, high serum magnesium, low serum  
17 potassium concentrations, and hypocalciuria. The patient underwent an orthodontic treatment  
18 between 12 and 14 years of age and, at the time of dental examination, exhibited a metallic  
19 orthodontic retainer at the lower jaw from canine to canine. As shown by the clinical examination,  
20 the orthopantomogram <sup>32</sup> and the CBCT examination (Fig. 1A-C) of the right lower jaw, the patient  
21 exhibited severe enamel wear on all of her erupted teeth (blue arrows, Fig.1A-B). The crown of  
22 the third lower molar was fully formed although retained in a submucosal position and partially  
23 erupted into the oral cavity and displayed a normal morphology (red arrow, Fig.1A-B). In spite of  
24 the relatively low resolution of the CBCT scan (200  $\mu\text{m}$ ), the enamel thickness indices of the  
25 HELIX molar could be calculated on a virtual 2D section taken through the mesial cusps in the  
26 developmental plane (Fig.1C and Supplementary information). These indices were within the  
27 range of the published values for modern human permanent third molars <sup>50, 51</sup>, suggesting that the  
28 volume of formed enamel was not disturbed by claudin-10 deficiency. Furthermore, CBCT  
29 showed that this third molar displayed an almost completed root formation and exhibited an  
30 enamel carious lesion located in the mesial fissure of the occlusal aspect (red arrow, Fig.1A).  
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### 47 **Daily Secretion Rate and crown formation time in the HELIX molar**

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49 The average DSR for each 100- $\mu\text{m}$  area in the HELIX molar are presented in Supplementary Table  
50 S1. As shown in Fig. 1D, the DSR increased from 1.96  $\mu\text{m}$  in the inner zone of enamel near the  
51 EDJ to 4.62  $\mu\text{m}$  in the outer zone of enamel near the enamel surface. The values and the pattern of  
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3 the DSR for this HELIX third molar were similar to those reported for any normal human tooth <sup>52-</sup>  
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7 The total crown formation time of the HELIX tooth was then established (Fig.1E). For this tooth,  
8 we determined that the formation time of the cuspal enamel was 564 days. The periodicity of the  
9 striae of Retzius was 8 days. The number of striae in each decile of the lateral enamel are given in  
10 Figure 1E. The first two deciles corresponded to 52 days, while decile 3 to decile 10 contains 89  
11 striae of Retzius formed over 712 days. The crown formation time was therefore 1328 days (3.64  
12 years). The number of striae increased towards the cervix, from three striae in decile 3 (cusp tip)  
13 to 20 striae in decile 10 (cervix), indicating that the number of days within each decile increased  
14 towards the cervix (Fig.1E). In other words, the rate of crown lengthening slowed down from cusp  
15 tip to cervix. The pattern of striae of Retzius spacing among each of the deciles, as well as the  
16 formation time within each decile and the overall crown formation time found in the HELIX third-  
17 molar were similar to those already established for healthy modern human third molars <sup>40</sup>.

### 28 29 **Enamel microstructure of the HELIX tooth**

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31 We next investigated whether the microstructural characteristics of the enamel were affected by  
32 the HELIX syndrome. SEM showed no difference between the control and HELIX samples  
33 concerning the microstructure of the enamel (Fig.2A-B). The enamel rods were perfectly formed  
34 and aligned in both cases (Fig.2C-F). Of note, the cross-striations, which correspond to the  
35 circadian variation in enamel apposition <sup>36</sup>, were perfectly distinguishable in the HELIX enamel  
36 rods (Fig.2E, black arrow-head), indicating a normal secretion process.

### 43 44 45 **Crystallinity and carbonatation of the mineral phase**

46  
47 We then explored the mineral phase composition and structure of the HELIX dental tissues by  
48 Raman microscopy. Control and HELIX enamel samples were analyzed by recording Raman  
49 mappings based on the FWHM of the  $\nu(\text{PO}_4^{3-})$  band (Fig.3), indicative of the crystallinity of the  
50 apatite component of the tooth, and on the ratio between the integrated area of the  $\nu(\text{CO}_3^{2-})$  and  
51  $\nu(\text{PO}_4^{3-})$  vibration bands, indicative of the carbonatation rate of this apatite component <sup>55</sup>. The

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3 selected area extended from the outer enamel layer to the dentin core (Fig.3A, D). For the control  
4 sample, largest FWHM<sub>960</sub> were found in the dentin area and, after an intermediate layer, the enamel  
5 part was characterized by a quite homogeneous, smaller FWHM<sub>960</sub> value (Fig.3B). Similarly, the  
6 I<sub>1070</sub>/I<sub>960</sub> ratio decreased from dentin to enamel but the area with intermediate values extended  
7 much significantly in the latter tissue (Fig.3C). These results are consistent with the fact that the  
8 mineral phase of enamel is a highly crystalline hydroxyapatite with low carbonatation rate whereas  
9 the mineral phase of dentin has a higher degree of substitution and lower crystallinity<sup>56</sup>. No  
10 significant difference in the evolution of both FWHM<sub>960</sub> (Fig.3E) and I<sub>1070</sub>/I<sub>960</sub> (Fig.3F) values  
11 from dentin to enamel could be evidenced in the HELIX sample compared to the control,  
12 suggesting the absence of modification of the crystallinity and carbonatation degree of the mineral  
13 phase in these two tissues.  
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## 24 **Chemical analyses**

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27 An investigation on the chemical composition of the enamel was performed to determine if it was  
28 altered, which would indicate an abnormal maturation and contribute to explain the rapid enamel  
29 wear observed in all the patients with the HELIX syndrome<sup>32</sup>. EDX analysis revealed that the Ca  
30 /P ratio was slightly lower in the HELIX enamel, especially in the outer layer, when compared to  
31 the control enamel (outer enamel layer: 1.54 *versus* 1.58; inner layer: 1.59 *versus* 1.60,  
32 respectively), and this was mainly due to a slightly lower Ca content (Table 1; Supplementary Fig.  
33 S1 and S2). As most of the patients with HELIX syndrome have been reported to have  
34 hypermagnesemia<sup>30,32</sup>, the (Ca + Mg) / P ratio was also calculated, revealing a similar pattern in  
35 both enamel samples (Table 1). No differences were found for the dentin by either SEM  
36 (Supplementary Fig.S3) or EDX (data not shown).  
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41 Next, a multi-element analysis of the samples was performed using SXRF to investigate further  
42 possible modifications of the chemical composition of enamel induced by the HELIX syndrome  
43 (Fig. 4).  
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### 46 Calcium:

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49 In the HELIX tooth, the SXRF levels of Ca in enamel and dentin are uniform and reach on average  
50 4.0 × 10<sup>5</sup> ppm and 3.4 × 10<sup>5</sup> ppm, respectively (Fig. 4, left upper panel). In the control tooth  
51 (permanent third molar), Ca levels range between 3.6 × 10<sup>5</sup> and 3.85 × 10<sup>5</sup> ppm in enamel, and  
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3 between  $2.0 \times 10^5$  and  $2.8 \times 10^5$  ppm in dentin (Fig. 4, right upper panel). These Ca values are  
4 compatible with the published values of enamel and dentin imaged by SXRF, ranging from  
5  $3.8 \times 10^5$  to  $5.0 \times 10^5$  and from  $3.1 \times 10^5$  to  $3.7 \times 10^5$  ppm, respectively<sup>57-60</sup>. The present results  
6 show that *CLDN10* deficiency did not alter significantly the process of tooth tissue mineralization.  
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#### 10 Zinc:

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12 Zn levels imaged by SXRF in the first-formed inner enamel are relatively low. They rise in a steep  
13 gradient towards the outer enamel surface (OES) where Zn levels are greatly enriched. In HELIX  
14 tooth, the OES at the occlusal basin reaches  $1.8 \times 10^3$  ppm while at the lateral enamel surface it  
15 peaks at  $1.2 \times 10^3$  ppm (Fig. 4, left middle panel). The middle and inner enamel is at  $\sim 100$  ppm  
16 while the dentin is at  $\sim 220$  ppm. In the control sample, although the enamel cap is not fully  
17 preserved, Zn values at the OES peak at  $\sim 1.0 \times 10^3$  ppm on the occlusal aspect of the cusps, while  
18 it is slightly less on the surface of the lateral enamel (600-800 ppm). The inner and middle zones  
19 of enamel contain  $\sim 60$  to  $\sim 100$  ppm of Zn, while the dentin is at  $\sim 200$  ppm. Zinc enrichment at the  
20 outer enamel surface has been previously described as a normal feature, potentially related to the  
21 processes of enamel mineralization and maturation<sup>57,61,62</sup>. SXRF values in human deciduous teeth  
22 show peak values ranging from 400 to 500 ppm at the OES<sup>57</sup>, while in *Pongo*, the outer layer of  
23 enamel concentrates  $1.5 \times 10^3$  to  $2.0 \times 10^3$  ppm of Zn<sup>63</sup>. Rautray et al.<sup>64</sup> reported Zn values on  
24 human healthy enamel yielding an average of 172.2 ppm, which is within the same order of  
25 magnitude as the present middle and inner values measured in HELIX and control.  
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#### 38 Strontium:

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40 In the control tooth, the Sr distribution (Fig. 4, right lower panel) followed previously published  
41 observations<sup>57</sup>. On average, Sr levels in enamel ranged from 50 to 130 ppm (outer vs inner enamel,  
42 respectively), which is compatible with an average of 174.76 ppm calculated after<sup>64</sup>. In dentin, Sr  
43 values in the control tooth ranged between 40-80 ppm, with an accentuated event at 150 ppm. In  
44 the HELIX sample (Fig. 4, left lower panel), a strong pattern of variation in Sr concentrations was  
45 visible using SXRF in both the enamel and dentin, from the earliest stages of formation of the third  
46 molar until crown completion and beyond into the root dentin. An alternation of strong depletions  
47 and enrichments occurred with a high frequency. Both HR SXRF scans at  $1.5 \mu\text{m}$  confirmed that  
48 these accentuated Sr markings occurred simultaneously in enamel and dentin (Supplementary Fig.  
49 S4). As the abrupt changes in the content of Sr followed the direction of the striae of Retzius (Fig.  
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3 5A), further investigation was performed to determine whether these events take place at specific  
4 periods during the crown formation, with no overprinting from subsequent Sr ingestions<sup>63</sup>. Within  
5 the ~1330  $\mu\text{m}$  of cuspal enamel, these several episodes of Sr variation were each calculated to last  
6 from 33 to 121 days, (Fig. 5B, Supplementary Table S2). Within enamel, these bands of Sr  
7 variations peaked at 860 ppm (direct measurement in HDIP v-1.3.3.1073, outside of the blue  
8 transect in Fig. 5A) in the middle of the cuspal enamel, while the strongest depletions dropped  
9 down to ~300-350 ppm, especially at the outer enamel surface (Fig. 4 left lower panel). In dentin,  
10 values were lower with peaks at 600-650 ppm and troughed at 280-340 ppm (Fig. left lower panel).  
11 The variation in Sr concentration as well as their timing in days did not suggest that these changes  
12 followed any regular and periodical pattern.  
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### 20 21 Other elements:

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23 Other elements with  $K\alpha$  lines in the detectable energy range, which include Cu, Fe, Mn, Ti, Cr, S,  
24 Cl, Ar and Rb, did not yield any significant data (Supplementary Fig. S4).  
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## 30 **DISCUSSION**

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32 The HELIX syndrome is a very rare disorder (OMIM 617671; Prevalence:  $<1 / 1,000,000$ ), which  
33 manifests as abnormalities in renal ion homeostasis resulting in hypermagnesemia, hypocalciuria,  
34 and hypokalemia, in epidermal integrity and in homeostasis of the ectodermal glands including  
35 salivary glands. We previously reported that all patients with HELIX syndrome displayed early  
36 and severe enamel wear<sup>32</sup>, compromising their oral health and particularly their chewing capacity.  
37 The present study is based on the observation of a third molar from a patient with HELIX  
38 syndrome, which was collected prior to full eruption of the tooth and therefore before the crown  
39 was fully exposed to the challenges of the environment of the oral cavity<sup>65-67</sup>, and shows that the  
40 enamel was normally formed and displayed normal maturation.  
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48 The general pattern of enamel formation appears to be consistent with that described for normal  
49 human permanent molars<sup>52</sup>. Specifically, the DSR gradient increase from the EDJ to the surface  
50 in the cuspal enamel matches that in normal third permanent molars. The time taken to form lateral  
51 enamel is also within the ranges reported for human third permanent molars<sup>40</sup>, and the total enamel  
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3 formation time within the ranges reported in the literature <sup>40, 50, 68, 69</sup>. It seems unlikely, therefore,  
4 that the HELIX syndrome has any impact on the timing of enamel formation.  
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7 The structure of enamel in both HELIX and control samples was studied both at the microscale by  
8 SEM and Raman spectroscopy. Although these techniques were previously shown to clearly  
9 characterize other dental disorders such as *Amelogenesis Imperfecta* <sup>70, 71</sup> or X-linked  
10 hypophosphatemia <sup>72</sup>, no significant difference could be found in the present study between the  
11 HELIX and the control molars.  
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16 Similarly, the EDX determination of the Ca, P and Mg content led to similar values for both  
17 samples. However, this technique has a rather high detection limit (~ 1000 ppm), especially for  
18 light elements. In contrast, SXRF allows for quantification below 1 ppm and was already applied  
19 for multi-element analysis of trace elements in many biological tissues <sup>73</sup>, including teeth <sup>57, 63</sup>.  
20 Here, among the many collected elements, Sr stood out as the only investigated element showing  
21 a clear difference in amount and distribution between the two samples. Not only its local  
22 concentration could be six times higher in the enamel of the HELIX tooth compared to the control  
23 but it also formed well-defined zones of enrichment and depletion parallel to the striae of Retzius,  
24 attesting that this chemical signal has been integrated to the dental tissues during development.  
25 However, in contrast to these periodical growth markings, no periodicity could be found in the  
26 occurrence of these Sr bands, suggesting that Sr incorporation is not linked to a specific, regular  
27 step of amelogenesis. In fact, it is notable that Sr bands are also present synchronously in the  
28 dentin, suggesting that Sr presence is related to an overall mineral homeostasis disorder rather than  
29 to a local disturbance of amelogenesis.  
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41 Dental hard tissues contain trace elements of both dietary and environmental origin<sup>74</sup>. Among  
42 them, strontium ions are divalent cations and thus can easily substitute for Ca in the hydroxyapatite  
43 structure or interact with the mineral phase <sup>75</sup>. For instance, it was found that by oral treatment of  
44 increasing Sr dose in rats, it was possible to obtain Sr/(Sr+Ca) molar ratio > 0.01 in femur without  
45 altering the bone structure <sup>76</sup>. As a comparison, the Sr / (Sr+Ca) molar ratio here was ~ 0.002,  
46 which could explain the absence of a clear difference in the enamel structure between HELIX and  
47 control in this study. A possible explanation of this high and random adsorption of Sr within  
48 enamel and dentin might be an excess of strontium consumption in the patient's diet or by  
49 swallowing toothpaste <sup>77</sup>. However, our HELIX patient, who was born and raised in France, did  
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3 not report either a specific diet or using, at any time of her growth including the period  
4 corresponding to the third molar formation, any specific toothpaste enriched in strontium ions to  
5 prevent tooth hypersensitivity.  
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9 The renal clearance of Sr can be computed to 2-3 ml/min in subjects with normal glomerular  
10 filtration rate (GFR), close to that of calcium and magnesium, and much lower than GFR,  
11 indicating that Sr, as Ca and Mg, is extensively reabsorbed by the renal tubule<sup>78,79</sup>. Patients with  
12 HELIX syndrome have very low urinary calcium and magnesium excretion in urine, despite  
13 normal serum Ca and high serum Mg levels, indicative of increased tubular Ca and Mg  
14 reabsorption<sup>30</sup>. That Sr is also excessively reabsorbed across the renal tubular epithelium is a  
15 sound hypothesis, albeit not documented. Subsequently, patients with HELIX syndrome may have  
16 a higher than normal serum Sr concentration, all the more so serum Sr level increases when GFR  
17 decreases<sup>78</sup>. Then, the higher serum Sr level may promote deposition of Sr in bone and tooth.  
18 Future studies are needed to investigate whether abnormal levels of Sr are also identified in bones  
19 of HELIX syndrome patients and, more broadly, how the kidney dysfunction associated to the  
20 HELIX syndrome impacts plasma and urine Sr concentrations.  
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23 One of the main function of the enamel organ during the maturation stage is to transport very large  
24 amounts of mineral ions, especially calcium and phosphate, from the blood vessels to the enamel  
25 matrix. This critical process is finely controlled by a great number of ion transporters and  
26 exchangers, mainly to neutralize the excess of protons generated by hydroxyapatite crystal  
27 formation<sup>10, 80-82</sup>. In the HELIX syndrome, one of the consequences of the renal dysfunction is  
28 hypocalciuria<sup>30</sup>.  
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31 The precise role of Mg<sup>2+</sup> in amelogenesis remains not fully understood, yet the expression of  
32 CNNM4 in the basolateral membrane of ameloblasts at the maturation stage suggests that Mg<sup>2+</sup>  
33 needs to be removed from the enamel matrix to promote mineralization. It has indeed been  
34 consistently reported that Mg<sup>2+</sup> content of the enamel is inversely correlated with the extent of  
35 mineralization<sup>83, 84</sup>. Here, the magnesium content was found to be normal in the HELIX enamel,  
36 suggesting that the hypermagnesemia found in the HELIX patients<sup>30</sup> may not have direct  
37 consequence on enamel mineralization.  
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41 Hypokalemia is a frequent feature of the HELIX patients<sup>30</sup>. Potassium ions have been shown to  
42 be important for normal enamel formation as several ion exchangers or cotransporters are K<sup>+</sup>-  
43 dependent<sup>10, 80, 82, 85</sup>. For example, the alteration of the K<sup>+</sup>-dependent Na<sup>+</sup>/Ca<sup>2+</sup> exchanger isoform  
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3 4 (SLC24A4) into rat maturation ameloblasts by an excess of fluorides in the drinking water was  
4 shown to impair amelogenesis<sup>85</sup>. Furthermore, patients with syndromes associated with  
5 hypokalemia such as the Bartter's syndrome were reported to display *Amelogenesis Imperfecta*<sup>86</sup>,  
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Taken together, our data show that enamel formation is not significantly impaired by *CLDN10* deficiency, rather designating xerostomia as the main culprit of the enamel wear found in HELIX patients. However, the abnormal concentrations of Sr measured in the dental mineralized tissues suggest that the tooth mineral content may reflect repeated episodes of worsening of renal dysfunction. These events urge the need to investigate more teeth from HELIX patients. Serum strontium concentration should also be monitored. Nevertheless, the study of a murine model of the HELIX syndrome may provide further insights on the direct role of claudin-10 in amelogenesis.

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### Authors contribution:

CC, FRR, TC, and TB conceived and designed the study. FRR, NO, TNNG and EG prepared the tooth thin-sections. JG, ALC, MCD, and SVM acquired the SXRF data. SVM and JG post-

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3 processed the SXRF data. AP and CP recorded the Raman data. AP, CP and TC interpreted the  
4 Raman data. CC, NO and TC interpreted the SEM and EDX data. ALC, FRR and MCD analyzed  
5 and interpreted the SXRF data. CC, NO, ALC, CB, TC, PH, SHR, FRR drafted the manuscript,  
6 and with revisions and final approval of the submitted version by all co-authors.  
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## Legends to Figures

### Figure 1: Anatomy and microanatomy of the HELIX molar

**A-B:** Cross section of CBCT image (**A**) and 3D reconstruction (**B**) of the right lower jaw of the female HELIX patient. The partially erupted third molar (red arrow) shows an unworn and normally formed enamel, whereas the erupted molars display occlusal wear (blue arrows).

**C:** 3D reconstructions of the third molar crowns, showing that the HELIX enamel has a comparable 3D enamel thickness distribution to the control.

**D:** Determination of the daily secretion rate (DSR) in the HELIX enamel. The DSR ( $\mu\text{m}/\text{day}$ ) increases from the enamel dentin junction (EDJ) to the outer enamel surface (OES).

**E:** Quantification of the HELIX crown formation time. The cuspal enamel took 564 days (1.55 years) to form. The number of striae of Retzius and corresponding days of formation (Days) are given for each decile of the lateral enamel. The total crown formation time is 1328 days (3.64 years).

### Figure 2: Characteristics of the HELIX enamel by SEM.

**A-F:** Representative aspects of the HELIX and control enamel microstructure imaged by SEM at various magnifications (**A, B:**  $\times 25\text{-}30$ , **C, D:**  $\times 300$ , **E, F:**  $\times 1000$ , respectively). There is no difference between the HELIX enamel and the control enamel. Note that in the HELIX enamel cross-striations are particularly well-distinguishable (**E**, black arrow-head).

EDJ: enamel dentin junction

### Figure 3: Characterization of the HELIX tooth using Raman micro-spectroscopy.

**A, D:** Optical microscopy of the imaged section of control and HELIX samples. The dashed area corresponds to the surface mapped by Raman spectroscopy.

**B,E:** Raman mapping of the control and HELIX samples based on the full width at half maxima (FWHM) of the  $\nu(\text{PO}_4^{3-})$  vibration band at  $960\text{ cm}^{-1}$ .

**C,F:** Raman mapping of control and HELIX samples based on the ratio between the integrated area of the  $\nu(\text{CO}_3^{2-})$  and  $\nu(\text{PO}_4^{3-})$  vibration bands at  $1070\text{ cm}^{-1}$  and  $960\text{ cm}^{-1}$ , respectively.

E: enamel; D: dentin

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3 **Figure 4: SXRF characterization of the HELIX molar.**  
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5 SXRF characterization of the HELIX (left) and control (right) teeth for mapping Ca (upper panel),  
6 Zn (middle panel) and Sr (lower panel) (SXRF overviews at 10  $\mu\text{m}$ ). In both the HELIX and  
7 control molar crowns, Ca and Zn have a similar distribution with comparable ranges of  
8 concentration (in ppm). HELIX shows Sr levels that are six times higher than in the control, with  
9 marked patterns of alternating Sr enrichments and depletions in both the enamel and the dentin.  
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17 **Figure 5: Variations in Sr content in the cuspal enamel of the HELIX tooth.**  
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19 **A:** Sr SXRF map of the HELIX tooth showing the variation in Sr content in enamel and dentine.  
20 Datapoints were collected along the blue transect to plot Sr concentrations in function of distance  
21 (see B) from the enamel-dentin junction (EDJ) to the outer enamel surface (OES). Marked Sr  
22 variations following the direction of the enamel growth lines were attributed letters (from A to H;  
23 black arrows). There is no clear temporal periodicity in the occurrence of these Sr variations.  
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31 **Table 1:** Atomic quantification of elements in HELIX and Control in the inner and outer layers of  
32 enamel by Energy Dispersive X-ray (EDX) analyses.  
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## Supplemental material

**Table S1:** Daily secretion rate (DSR) from the enamel-dentin junction (EDJ) to the outer enamel surface (OES).

**Table S2:** Cumulative formation time and distance from the outer enamel surface (OES) to the enamel dentin junction (EDJ) of the major Strontium (Sr) variations evidenced by Synchrotron X-Ray Fluorescence (SXRF).

### Supplementary Fig. S1: Analysis of the HELIX enamel layers by EDX

EDX atomic mass spectra of the outer (B) and inner (C) enamel. Area of interest are shown (A) (yellow: outer, inner: green)

### Supplementary Fig. S2: Analysis of the Control enamel layers by EDX

EDX atomic mass spectra of the outer (B) and inner (C) enamel layers. Area of interest are shown (A) (yellow: outer, inner: green).

### Supplementary Fig. S3: Characteristics of the HELIX dentin by SEM

Representative aspects of the HELIX (A) and control (B) dentin microstructure imaged by SEM at x300 magnification. There is no difference between the HELIX dentin and the control dentin.

### Supplementary Fig. S4: SXRF characterization of the HELIX molar

SXRF characterization of the HELIX and control teeth for mapping Fe, Cu, Mn, Ti, Cr, Br, Ar, S, Rb, Cl (SXRF overviews at 10  $\mu\text{m}$ ). In both the HELIX and control molar crowns, all these elements have a similar distribution with comparable ranges of concentration (in ppm).

### Supplementary information: Quantification of enamel thickness

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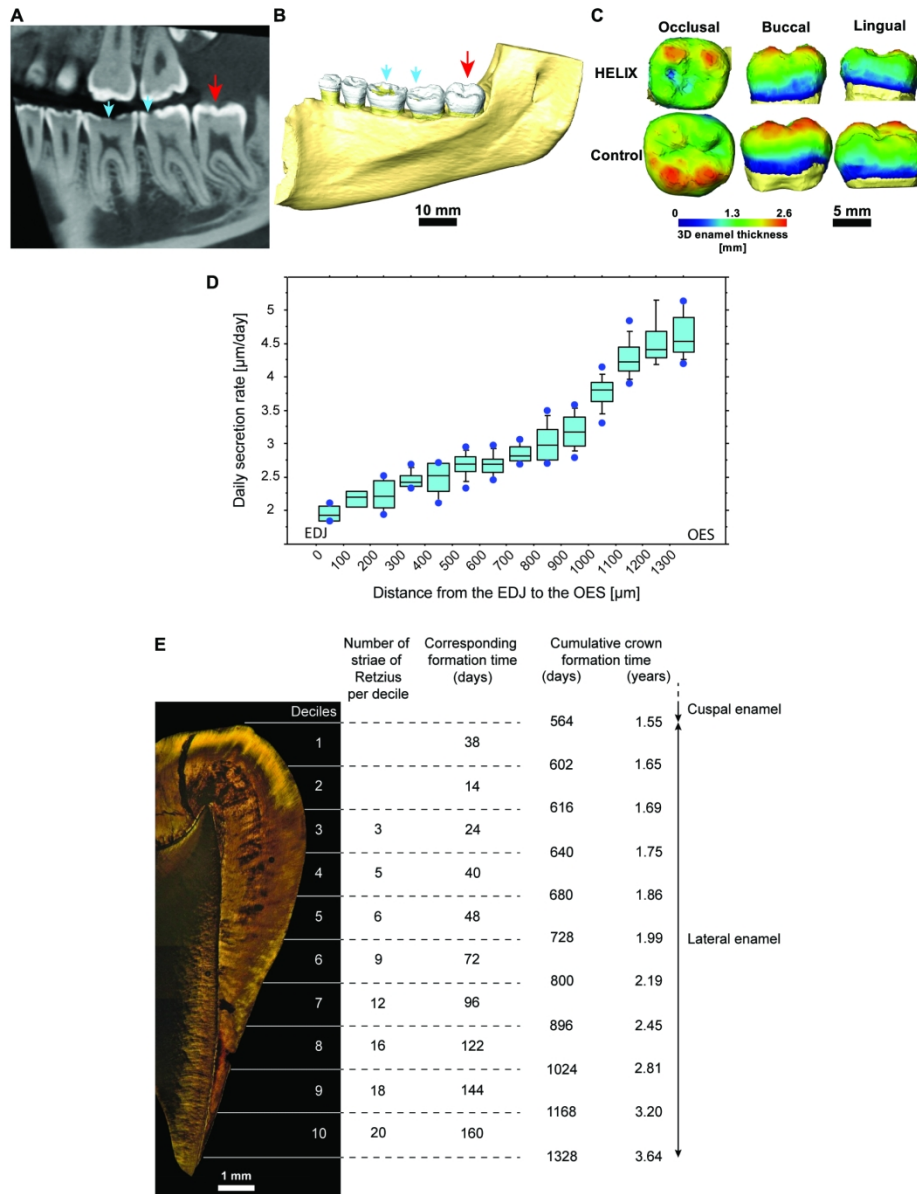


Figure 1: Anatomy and microanatomy of the HELIX molar

A-B: Cross section of CBCT image (A) and 3D reconstruction (B) of the right lower jaw of the female HELIX patient. The partially erupted third molar (red arrow) shows an unworn and normally formed enamel, whereas the erupted molars display occlusal wear (blue arrows).

C: 3D reconstructions of the third molar crowns, showing that the HELIX enamel has a comparable 3D enamel thickness distribution to the control.

D: Determination of the daily secretion rate (DSR) in the HELIX enamel. The DSR ( $\mu\text{m}/\text{day}$ ) increases from the enamel dentin junction (EDJ) to the outer enamel surface (OES).

E: Quantification of the HELIX crown formation time. The cuspal enamel took 564 days (1.55 years) to form. The number of striae of Retzius and corresponding days of formation (Days) are given for each decile of the lateral enamel. The total crown formation time is 1328 days (3.64 years).

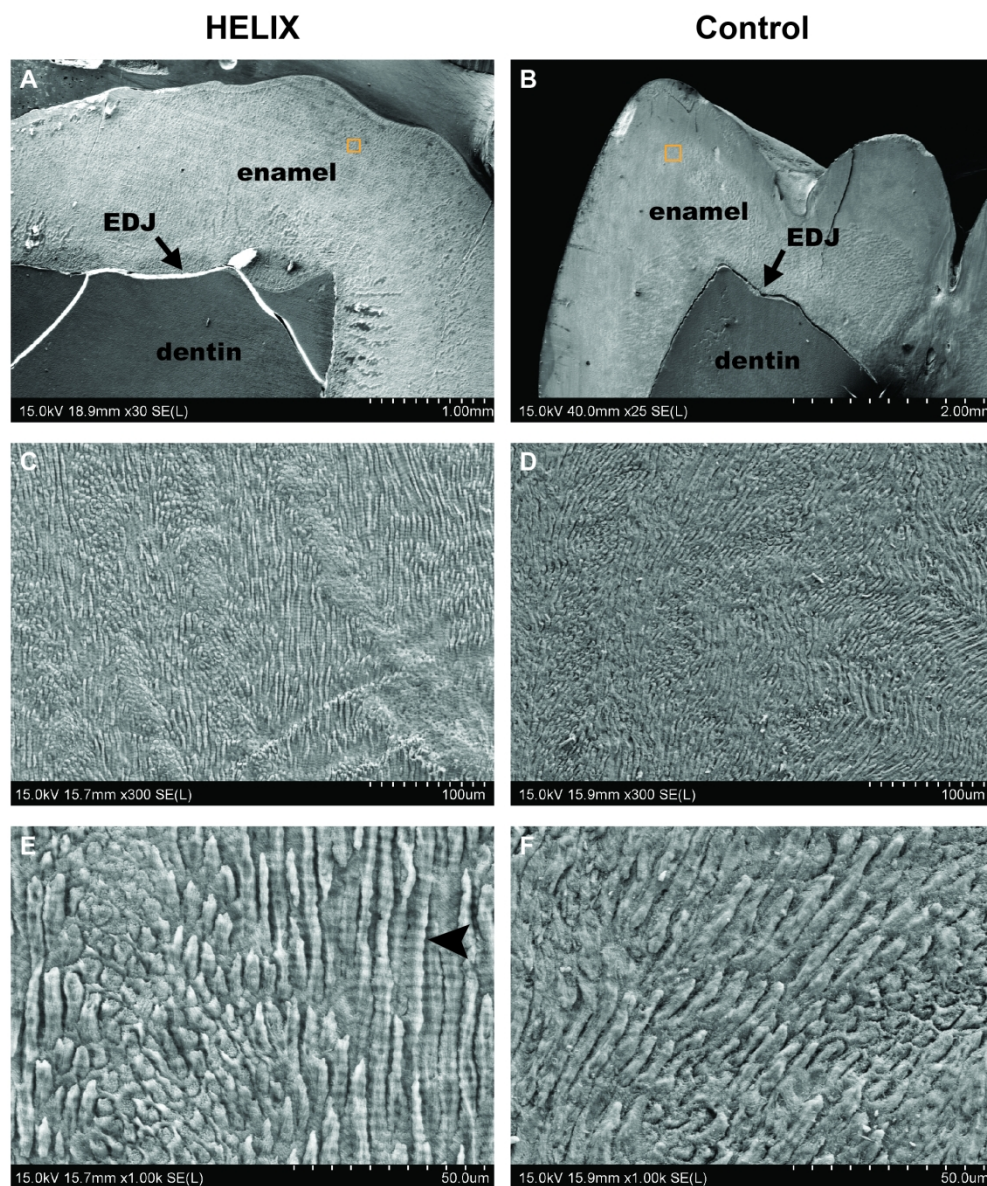


Figure 2: Characteristics of the HELIX enamel by SEM.

A-F: Representative aspects of the HELIX and control enamel microstructure imaged by SEM at various magnifications (A, B:  $\times 25-30$ , C, D:  $\times 300$ , E, F:  $\times 1000$ , respectively). There is no difference between the HELIX enamel and the control enamel. Note that in the HELIX enamel cross-striations are particularly well-distinguishable (E, black arrow-head).

EDJ: enamel dentin junction



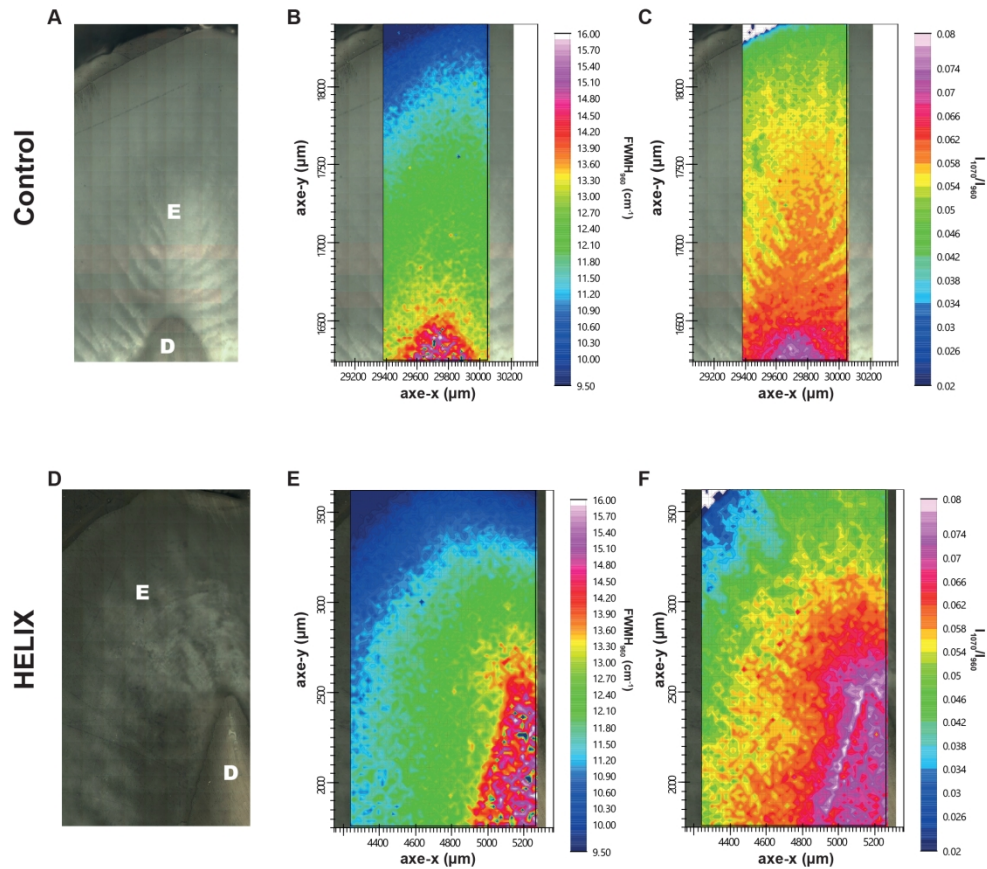


Figure 3: Characterization of the HELIX tooth using Raman micro-spectroscopy.

A, D: Optical microscopy of the imaged section of control and HELIX samples. The dashed area corresponds to the surface mapped by Raman spectroscopy.

B,E: Raman mapping of the control and HELIX samples based on the full width at half maxima (FWHM) of the  $\nu(\text{PO}_4^{3-})$  vibration band at  $960\text{ cm}^{-1}$ .

C,F: Raman mapping of control and HELIX samples based on the ratio between the integrated area of the  $\nu(\text{CO}_3^{2-})$  and  $\nu(\text{PO}_4^{3-})$  vibration bands at  $1070\text{ cm}^{-1}$  and  $960\text{ cm}^{-1}$ , respectively.

E: enamel; D: dentin

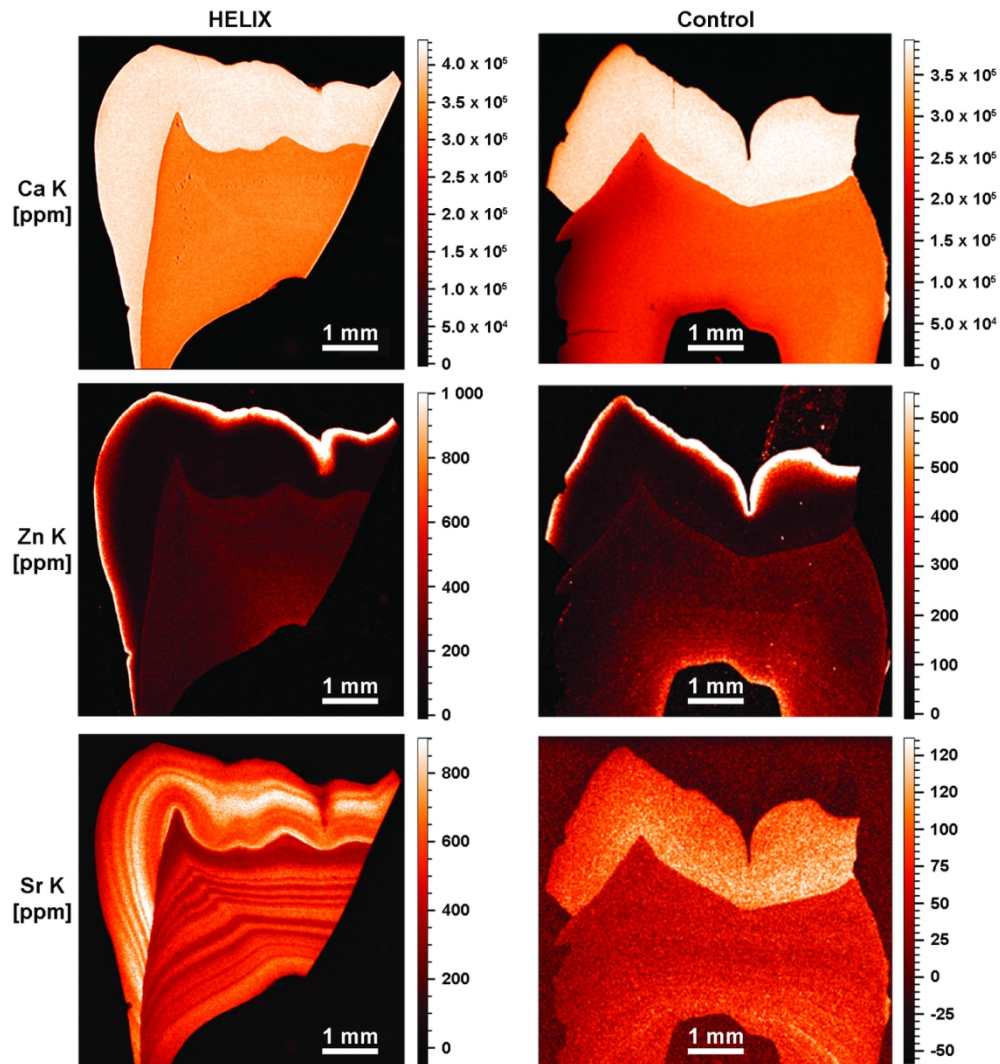


Figure 4: SXRF characterization of the HELIX molar. SXRF characterization of the HELIX (left) and control (right) teeth for mapping Ca (upper panel), Zn (middle panel) and Sr (lower panel) (SXRF overviews at  $10\ \mu\text{m}$ ). In both the HELIX and control molar crowns, Ca and Zn have a similar distribution (SXRF with comparable ranges of concentration (in ppm)). HELIX shows Sr levels that are six times higher than in the control, with marked patterns of alternating Sr enrichments and depletions in both the enamel and the dentin.

131x140mm (300 x 300 DPI)

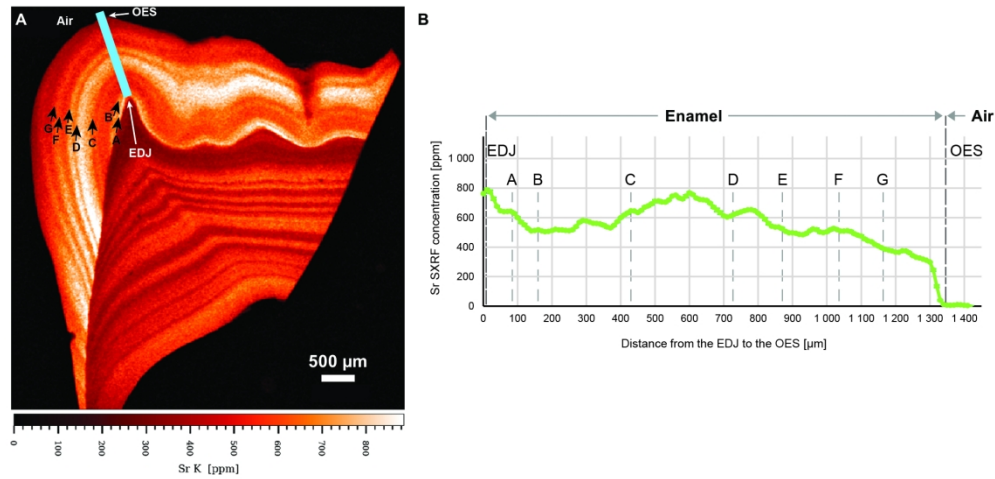


Figure 5: Variations in Sr content in the cuspal enamel of the HELIX tooth.

A: Sr SXRf map of the HELIX tooth showing the variation in Sr content in enamel and dentine. Datapoints were collected along the blue transect to plot Sr concentrations in function of distance (see B) from the enamel-dentin junction (EDJ) to the outer enamel surface (OES). Marked Sr variations following the direction of the enamel growth lines were attributed letters (from A to H; black arrows). There is no clear temporal periodicity in the occurrence of these Sr variations.

**Table 1:** Atomic quantification of elements (in At (%)) in HELIX and Control in the inner and outer layers of enamel by Energy Dispersive X-ray (EDX) analyses

Outer	HELIX			Control			
	Element	P	Ca	Mg	P	Ca	Mg
	Max	13.7	21.0	0.2	13.7	21.6	0.2
	Min	13.5	20.9	0.1	13.5	21.4	0.1
	Average	13.6	21.0	0.2	13.6	21.5	0.2
	Standard Deviation	0.1	0.1	0.0	0.1	0.1	0.0
	Ca/P	1.54			1.58		
	(Ca+Mg)/P	1.55			1.59		

Inner	HELIX			Control			
	Element	P	Ca	Mg	P	Ca	Mg
	Max	13.6	21.4	0.5	14.0	22.4	0.4
	Min	12.7	20.2	0.4	13.8	22.1	0.4
	Average	13.0	20.7	0.4	13.9	22.2	0.4
	Standard Deviation	0.5	0.7	0.1	0.1	0.2	0.0
	Ca/P	1.59			1.60		
	(Ca+Mg)/P	1.62			1.62		