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Research Article

Proteomics of MIH and DMH Affected Permanent and Primary Molars

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Abstract

MIH (Molar Incisor Hypomineralization), a qualitative reduction of enamel mineralization, became a world wide burden in pediatric dentistry. The prevalence of the phenomenom increased during the last decades from 3% to 13.5% worldwide. In Israel the prevalence is almost 18% and an increase of 2% was observed between the young and older group. In hypomineralized molars a significant reduction in mineral content was observed. The aim of the present study was to analyze the proteomics of permanent and deciduous molars affected by hypomineralization. A sample of 5 permanent molars affected by MIH was compared to 5 healthy teeth ans another sample of 5 deciduous molars affected by dmh was compared to 5 healthy teeth. The results showed that almost 370 proteins were detected and 37 of them showed a significant statistical difference between the affected and the healthy groups. PCA analysis showed distinct grouping between affected molars and healthy teeth in both permanent and deciduous molars. This study shows that there is a significant difference between the proteins content in MIH and DMH enamel in comparison with healthy enamel.

Keywords: MIH; DMH; Enamel; Proteomics

Introduction

Enamel is the hardest calcified matrix of the body. The cells that are responsible for formation of the enamel, the ameloblast, are lost as the tooth erupts in the oral cavity, and hence enamel cannot renew itself. To compensate for this inherent limitation, enamel has acquired a complex structural organization and a high degree of mineralization rendered possible by the almost total absence of organic matrix in its mature state. Fully formed enamel consist of approximately 96% mineral and 4% organic material and water. The inorganic content of enamel is a crystalline calcium phosphate (hydroxyapatite) substituted with carbonate ions. Various ions-strontium, magnesium, lead and fluoride- if present during enamel formation, may be incorporated into the crystals [1]. Amelogenesis is a two step process. Ameloblasts secrete matrix proteins and are responsible for creating and maintaining an extracellular environment favorable to mineral deposition. The organic matrix of enamel is made from noncollagenous proteins consisting of several enamel proteins and enzymes. 90% of the proteins are the amelogenins, while the remaining 10% consist of nonamelogenins such as enamelin, ameloblastin, amelotin and tuftelin [1]. Amelogenin is not required for the initiation of mineralization, but essential for elongation of enamel crystals and achievement of proper enamel thickness. Ameloblastin is considered critical for enamel formation since obvious pathology in the ameloblast layer and defects in the junctional epithelium were noted. In Enam null-mice where enamelin protein is not expressed, a true enamel layer is not formed. Amelotin is the most recently discovered enamel glycoprotein, and its function is not yet clear [2]. Members of at least two general families of proteinases are involved in the extracellular processing and degradation of enamel proteins. Enamelysin (MMP20) is involved in the short-term processing of newly secreted matrix proteins. Another enzyme, kallikrein4 (KLK4) function as a bulk digestive enzyme during the maturation stage [1]. Enamelysin (MMP20) is expressed from the onset of enamel matrix secretion through the

early maturation stage, while KlLK4 is expressed from the beginning of transition stage and throughout maturation. MMP20 processes amelogenin, enamelin, and ameloblastin into stable intermediate products, while KLK4 functions to completely degrade the extracellular matrix proteins [2]. The mode of progressive mineralization of enamel is completely different between the matrix formation and maturation stages. During the former stage, the enamel matrix is slightly mineralized, up tp 30%. During the latter stage, which takes a much longer period than the previous stage, the increase in the secondary mineralization takes place first slightly, from the surface toward the inner layer, and then heavily, from the inner layer toward the surface. The narrow outer layer mineralizes very slowly during the middle and late stages of maturation, but finally achieves the highest mineralization of the entire enamel layer. The very narrow innermost layer mineralizes slowly without expanding its width. The former three processes seem to be under the direct control of the ameloblasts [3]. At eruption the enamel mineral content is approximately 85% and the final mineralization occurs in the mouth.

Molar incisor hypomineralization (MIH) defines a qualitative enamel defect affecting one or more first permanent molars with or without the involvement of permanent incisors [4]. The prevalence of MIH ranges between 0.5-40% in different continents and countries [5]. Demarkated hypomineralized enamel lesions are caused by the process of amelogenesis being altered or interrupted, or by reduced degradation of the enamel proteins due to overall imbalance in required level of proteases [6,7].

In first molars affected by MIH, the mineral content was significantly lower. The reduction of calcium was by 35% and of phosphate by 60%. The oxygen and carbon content was increased by more than 30%. In primary molars affected by hypomineralization, the reduction in minerals was by 10% of phosphate and 40% of calcium on demarcated opacities and by 64% phosphate and 72% calcium at the border of breakdown of enamel [8]. Proteomic studies on MIH-affected teeth have revealed higher content of non-specific enamel proteins, mainly non-amelogenins like albumin, Hb, C-3 alpha-1-anti-trypsin, type I collagen, and nearly normal levels of amelogenins [7,9,10]. In saliva samples, the proteomic that analysis showed 88 proteins were identified exclusively in MIH patients and 16 proteins in healthy controls only, suggesting that the protaneous composition of saliva is affected in MIH patients [11].

The aim of this study was to compare the proteomics of permanent molars affected by MIH to control teeth and of deciduous molars affected by DMH to control deciduous molars.

Materials and Methods

Ten permanent molars (five with MIH and five healthy), and 10 deciduous molars (five with DMH and five healthy) were examined. The teeth were extracted during routine dental treatment and the parents gave their consent to leave them at the clinic. On each tooth, using a 330-carbide bur under copious amount of water, a square of 2X2 mm enamel was removed from the area affected by MIH or DMH and from similar area on the healthy teeth (Figure 1).

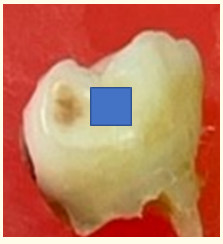


Figure 1: The area of enamel removed from a deciduous molar affected by DMH.

The dentin was removed from the internal surface with a round bur mounted on low speed hand piece and the internal surface was observed under a light microscope at X20 enlargement, in order to make sure that all the dentin was removed. The enamel was grounded and the enamel dust was sent to department of physiology and cell biology, Faculty of Cell Sciences, Ben Gurion University of the Negev for protein extraction. For every sample three tubes were prepared with 0.01gr enamel powder each. To each tube 20% trichloroacetic with protease inhibitors and metalloprotease inhibitor were added to total volume of 1ml per tube. The tubes were incubated over night at room temperature. The next day the tubes were centrifuged at 13,000g for 20 minutes at 4 degrees celsius. The sup was discarded and the pallet was saved. Every pellet was washed for three times with 500µl acetone and the tube was left open in a chemical hood to evaporate the acetone. Every third tube were resuspended with total 100µl sodium dodecyl sulphate 5% supllemented with protease and metalloprotease inhibitors. The samples were kept at -80 degrees celsius until sent for proteomics analysis on dry ice. For each sample one tube was sent.

The samples were sent to the Nancy and Stephen Grand Israel National Center for Personalised Medicine at Weizmann Institute of Science for analyses.

Sample preparation**,** liquid chromatography mass spectrometry and data processing: The samples were subjected to tryptic digestion using an S-trap. The resulting peptides were analyzed

using nanoflow liquid chromatography (nanoAcquity) coupled to high resolution, high mass accuracy mass spectrometry (Q Exactive HF). Each sample was analyzed on the instrument separately in a random order in discovery mode. Raw data was processed with Meta Morpheusversion 0.0.320. The data was searched against the human proteome database (xml version) appended with common lab protein contaminants and the following modifications: carbamidomethylation of C as a fixed modification and oxidation of M as a variable one. Quantification was performed using the embedded FlashLFQ and protein inference algorithms. The processing included the unique G-PTM-D method for identification of dozens of PTMs. The protein intensities were calculated and used for further calculations using Perseus v1.6.2.3. Decoy hits were filtered out. The protein intensities were log transformed and only proteins that had at least 3 valid values in at least one experimental group were kept. The remaining missing values were imputed.

Results

In total ~370 proteins were identified and quantified. The leading proteins that had the highest number of identifications were different collagens. Also, many oxidations of different amino acids (predominantly proline and lysine) were detected. In general, quantification of proteins identified and quantified by a single peptide only is less accurate and should be taken with caution. As a rule of thumb we usually consider significant differences to be at least two peptide per protein, >2 or <0.5 fold change, and p-value < 0.05. Table 1 shows the proteins with significant statistical differences in deciduous molars. 37 different proteins were detected.

Figure 2 shows the PCA and heat map of the first 20 molars examined: 10 permanent and 10 deciduous molars.

Figure 4 shows the PCA of the 10 deciduous molars. Two of the

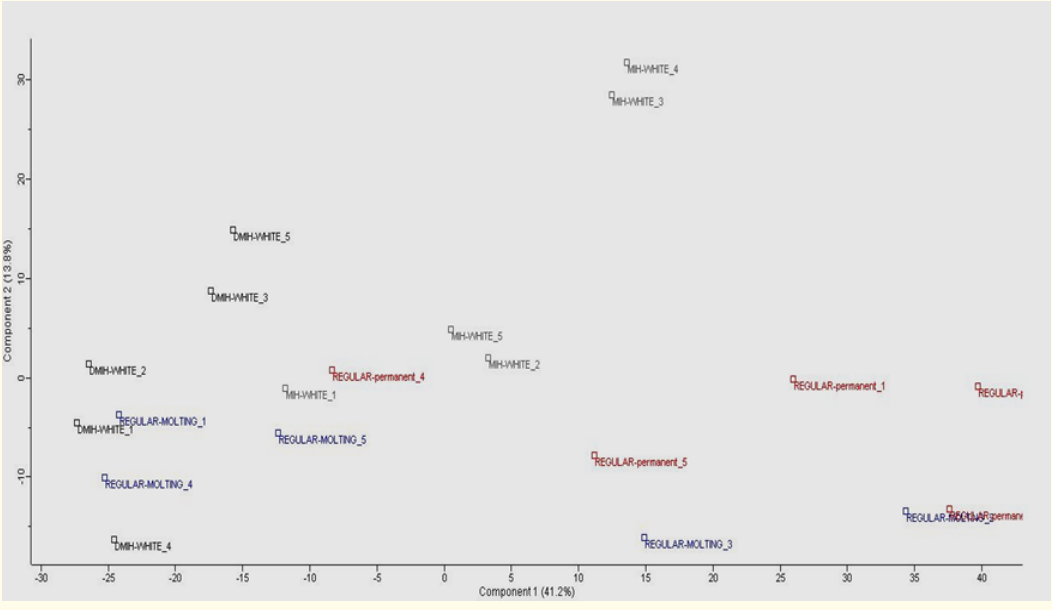
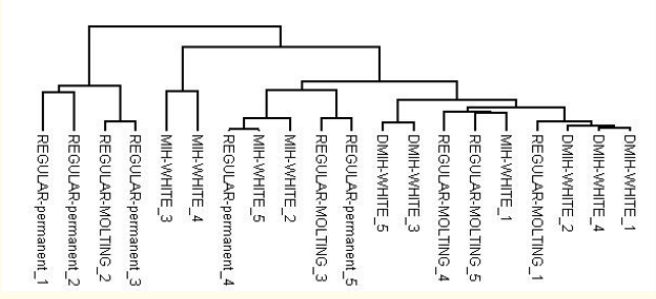


Figure 2: PCA of all teeth - 10 permanent first molars and 10 deciduous molars.

Note: MIH-White (gray)= prmanent molars affected by MIH, Regular-permanent (red)= healthy permanent molars, DMIH-white (black)= deciduous molars affected by DMH, Regular-molting (blue)= healthy deciduous molars.



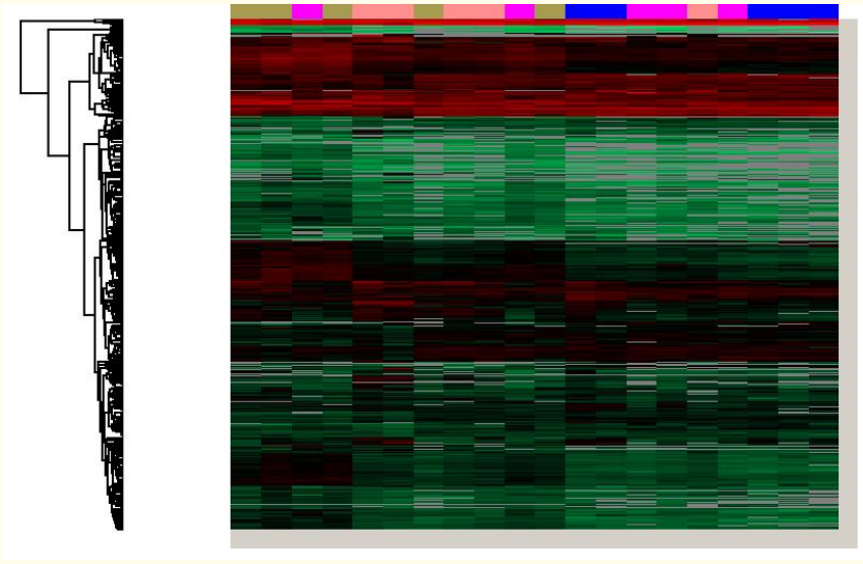


Figure 2a: Clustering and heat map of all 20 molars.

Fig 3 shows the PCA of the permanent molars. There is a distinct clustering of the MIH affected molars vs healthy

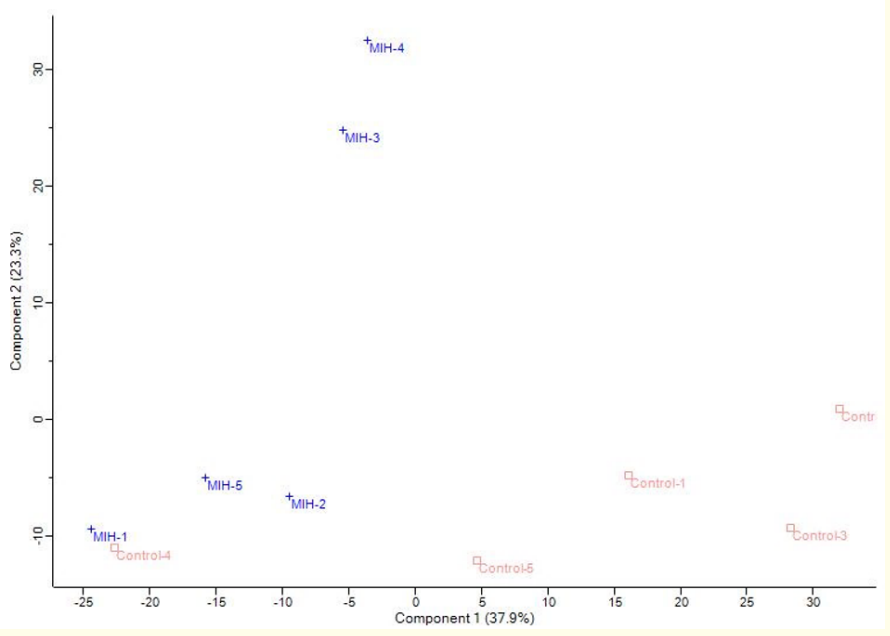


Figure 3b: PCA of the permanent molars. We can observed a distinct clustering between the affected MIH molars and the healthy ones.

Note: MIH (blue)= permanent molars affected by MIH, Control (red)- healthy permanent molars.

control teeth are very far away from the remaining 7 and it may affect the clustering. In order to examine the clustering of the group between -20 to +10 of component 1 we removed the two controls.

Figure 5 shows the PCA of the remaining 7 deciduous molars and here we can observed the clustering of affected deciduous molars in comparison with healthy molars.

Discussion and Conclusion

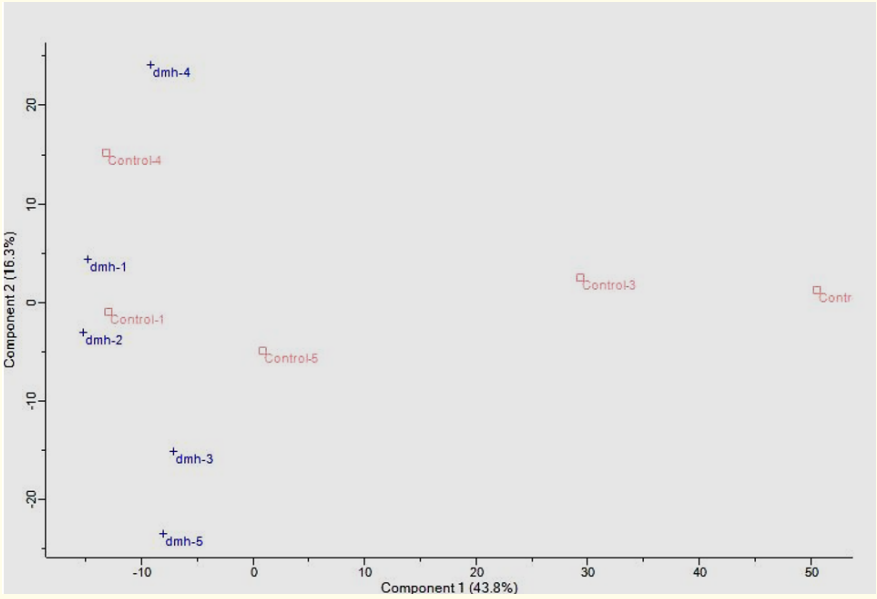


Figure 4: PCA of the deciduous molars. Note: dmh (blue)= deciduous molars affected by DMH, Control (red)= healthy deciduous molars.

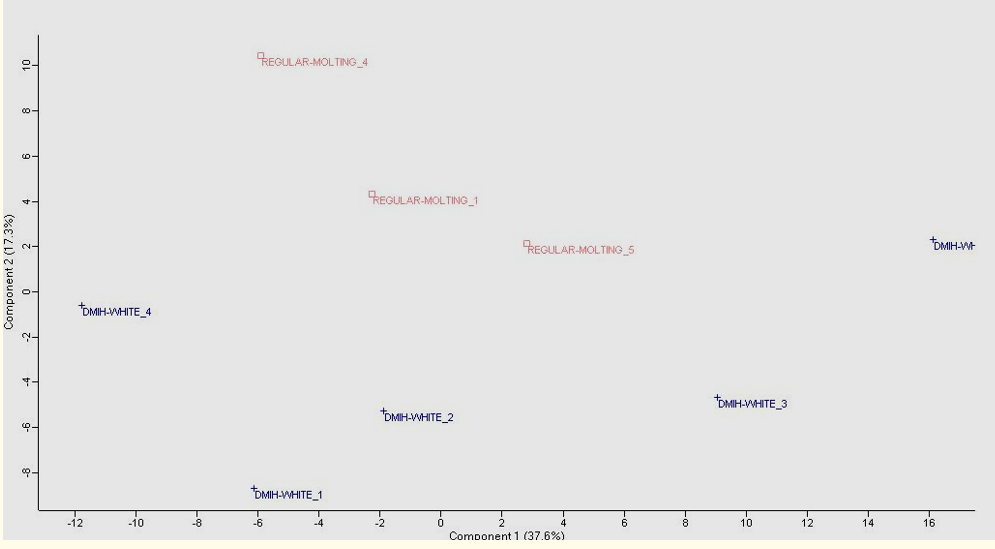


Figure 5: PCA of 5 primary molars affected by DMH compared to only 3 controls. Note: DMIH (blue)= primary molars affected by DMH, Regular molting (red)= healthy primary molars.

Based on a meta analysis of published data, the weighted mean for prevalence of MIH was 12.8% [12], and in Israel the prevalence was 17.9% [13]. A sub analysis of eleven studies revealed strong evidence for an increasing prevalence between the years 1992 (3%) and 2013 (13%) [12]. In Israel there was an increase of 2% between age 6-10 years and age 10-16 years [13], similar to the findings from Brazil [14]. The time line for development of MIH in permanent first molars is between birth, beginning of mineralization to 2-3 years old, mineralization of the occlusal two thirds of the crown. The eruption of the first permanent molars occurs at age 6-7 years and then the clinical examination will reveal the hypomineralization of the enamel. Most publications tried to correlate between the appearance of MIH to peri, and post natal risk factors [15], and gene-environment interaction [16]. Based on the process of mineralization and degradation of enamel proteins, the concentration of MMP20 and KLK4 was analyzed from blood

Protein Accession	Gene	Organism	Protein Full Name	Number of Peptides	Number of Unique Peptides	Student's T-test p-value DMH vs control
P38646	HSPA9	Homo sapiens	Stress-70 protein, mitochondrial	1	1	0.001507
P34096	RNASE4	Homo sapiens	Ribonuclease 4	1	1	0.002813
P00734	F2	Homo sapiens	Prothrombin	14	10	0.006628
P06576	ATP5F1B	Homo sapiens	ATP synthase subunit beta, mitochondrial	1	1	0.006949
O15347	HMGB3	Homo sapiens	High mobility group protein B3	2	1	0.007503
P02766	TTR	Homo sapiens	Transthyretin	10	10	0.009053
P00740	F9	Homo sapiens	Coagulation factor IX	3	3	0.010465
P25705	ATP5F1A	Homo sapiens	ATP synthase subunit alpha, mitochondrial	1	1	0.010563
Q9NS15	LTBP3	Homo sapiens	Latent-transforming growth factor beta-binding protein 3	2	2	0.013672
Q969Q0	RPL36AL	Homo sapiens	60S ribosomal protein L36a-like	3	2	0.021522
P04259	KRT6B	Homo sapiens	Keratin, type II cytoskeletal 6B	35	2	0.022942
P54725	RAD23A	Homo sapiens	UV excision repair protein RAD23 homolog A	2	1	0.023969
P02788	LTF	Homo sapiens	Lactotransferrin	6	5	0.026332
Q9Y3U8	RPL36	Homo sapiens	60S ribosomal protein L36	4	3	0.026853
P31944	CASP14	Homo sapiens	Caspase-14	4	4	0.028837
P62753	RPS6	Homo sapiens	40S ribosomal protein S6	2	2	0.029469
Q02413	DSG1	Homo sapiens	Desmoglein-1	5	3	0.029735
Q96DA0	ZG16B	Homo sapiens	Zymogen granule protein 16 homolog B	6	6	0.030182
P00441	SOD1	Homo sapiens	Superoxide dismutase [Cu-Zn]	2	1	0.03222
P46779	RPL28	Homo sapiens	60S ribosomal protein L28	7	7	0.032817
Q15323	KRT31	Homo sapiens	Keratin, type I cuticular Ha1	13	0	0.033197
P83731	RPL24	Homo sapiens	60S ribosomal protein L24	3	2	0.034628
P00338	LDHA	Homo sapiens	L-lactate dehydrogenase A chain	4	4	0.034991
P09429	HMGB1	Homo sapiens	High mobility group protein B1	19	3	0.037046
P07996	THBS1	Homo sapiens	Thrombospondin-1	11	11	0.037913
P82979	SARNP	Homo sapiens	SAP domain-containing ribonucleoprotein	2	2	0.040745
P35908	KRT2	Homo sapiens	Keratin, type II cytoskeletal 2 epidermal	48	27	0.041104
P02647	APOA1	Homo sapiens	Apolipoprotein A-I	18	12	0.041327
P02765	AHSG	Homo sapiens	Alpha-2-HS-glycoprotein	11	2	0.041786
P13645	KRT10	Homo sapiens	Keratin, type I cytoskeletal 10	39	12	0.042777
P68871	HBB	Homo sapiens	Hemoglobin subunit beta	9	2	0.043536
P69905	HBA1	Homo sapiens	Hemoglobin subunit alpha	7	0	0.04496
P06748	NPM1	Homo sapiens	Nucleophosmin	2	2	0.045356
Q9NZM1	MYOF	Homo sapiens	Myoferlin	2	2	0.048508
P04264	KRT1	Homo sapiens	Keratin, type II cytoskeletal 1	50	18	0.048556
P04004	VTN	Homo sapiens	Vitronectin	12	10	0.048819

Table 1: The proteins with significant statistical differences and with more than a single peptide in deciduous molars.

the concentration of MMP20 and KLK4 was analyzed from blood samples of children aged 0-5 years and four years later the clinical appearance of MIH was correlated to the concentration of the proteases. A high correlation was found between the concentration of KLK4 at the early age and MIH (P = 0.02) [17].

The proteomics od deciduous and permanent affected molars showed a high number of proteins and only 10% of them showed significant statistical differences between the affected and healthy enamel. The proteins with the significant statistical difference found are not the main proteins that are found during amelogenesis, but it can be explained by the late mineralization after eruption of the teeth and inclusion of proteins into the enamel during this final stage of mineralization.

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