P 0646

Identification of possible determinants in periodontal status of type 1 diabetic children

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Introduction and aim

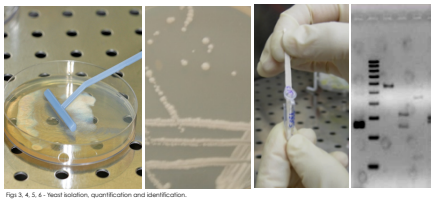
Diabetes mellitus is a group of metabolic disorders of carbohydrate metabolism in which glucose is underutilized and overproduced, causing hyperglycemia. Its current worldwide prevalence is estimated to be approximately 250 x 10^6, and it is expected to reach 380 x 10^6 by 2025. The disease is classified into several categories. **Type 1 diabetes mellitus** (DM1), once known as insulin-dependent diabetes mellitus or juvenile-onset diabetes mellitus, is usually caused by **autoimmune** destruction of the pancreatic islet β-cells, rendering the pancreas unable to synthesize and secrete insulin. Throughout this process there seems to be a marked influence of **T lymphocytes** (CD4+, particularly), besides changes in the secretion of various inflammatory mediators, as exemplified by IL-17, recently considered a potential "marker" of DM1. The number of children with DM1 is continually increasing and they are known to have **additional risk** for developing **early periodontal disturbances**.

The **aim** of this work was to **assess the periodontal condition** (gingival bleeding/calculus) and potential related factors, namely age, gender, metabolic control/duration of the disease, salivary features (flow rate, buffer capacity, yeast colonization, expression of T-cell subsets, metabolites concentration), malocclusion and hygiene habits of a sample of DM1 children and healthy controls.

Material and methods

Figs 1, 2 - Saliva's collection procedure and periodontal status assessment.

Population: 133 DM1 children and 72 healthy controls (5-15 years-old) attending the the Paediatric Hospital of Coimbra and the Dentistry Department (Faculty of Medicine, University of Coimbra). The study was approved by the ethical committee and the informed consent from each legally authorized representative was obtained. Children wearing orthodontic devices or under antibiotic, steroid therapy or antiseptic mouthwash (prior 3 weeks) were excluded. Data on important diabetes-related variables and metabolic control based on the HbA1c records were registered. **Saliva´s collection procedure and periodontal status assessment**: 1) Instructions to not to eat or drink 2h preceding sample collection by the morning; 2) Mouth rinse with water + swab from cheek mucosa; 3) Paraffin wax pellet chewing (3-5 min), stimulated saliva collection, measurement and evaluation on its buffer capacity - CRT® Buffer/CRT® Bacteria (Ivoclar-Vivadent, Liechtenstein). Clinical examination was performed by a calibrated examiner together with periodontal condition assessment noting the presence/absence of gingival bleeding in specific locations (4 points of the buccal and lingual surfaces - mesiobuccal, distobuccal, mesiolingual, distolingual of 11, 21, 31, 41, 16, 26, 36, 46), when present and fully erupted. A periodontal probe was gently inserted (≈0.2N/mm^2) in the gingival sulcus (not exceeding 2 mm). It was also recorded the presence/absence of supragingival calculus visible in direct clinical examination in the same tooth surfaces. The presence of periodontal pockets was not evaluated.



Figs 3, 4, 5, 6 - Yeast isolation, quantification and identification.

Yeast isolation, quantification and identification: Upon the arrival to the laboratory, samples were: 1) spread onto Petri dishes with Sabouraud-cloranfenicol media; 2) incubated (30ºC, 48 h); 3) quantified (CFU/ml-saliva; CFU/swab-mucosal samples); and 4) identified using the sequence of D1/D2 fragment of the 26 S gene6.

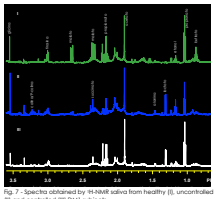
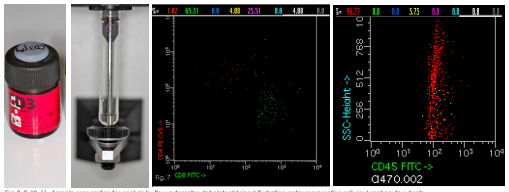


Fig. 7 - Spectra obtained by 1H-NMR saliva from healthy (I), uncontrolled (II) and controlled (III) DM1 subjects.

^{1}H NMR: NMR spectroscopy (mostly ^{1}H-NMR) exploits the behavior of molecules when placed in a magnetic field, allowing the identification of different nuclei based on their resonant frequency. The basic workflow was: quenching/ extraction of metabolites → data collection → data processing/analysis, as reported by Bertram et al., 2009.

Expression of T-cell subpopulations: Salivary samples were incubated with monoclonal antibodies: CD3, CD4, CD8, CD14 and CD45, conjugated with FITC, PE, PE-cy5, PerCP and APC fluorochromes according to manufacturer instructions. Flow cytometry was performed using a *BD FACSCalibur*™ (BD Biosciences, San José, CA, USA) - 10.000 events/acquisition on BD *CellQuest*™ (BD Biosciences, San José, CA, USA) software. Different populations were identified by light scattering and fluorescent properties using the *Paint-A- Gate*™ (BD Biosciences, San José, CA, USA).



Figs 8, 9, 10, 11 - Sample preparation for analysis by flow cytometry; dot plots obtained illustrating gates representing salivary lymphocyte subsets.

Statistics: Data was analysed with IBM® SPSS® 19 and Mann-Whitney/OR/ X^2 tests ($p<0.05$, 95%).

Results

		Total n (%)	Control n (%)	Diabetics n (%)
Number		205	72	133
Gender	Female	88 (42.9)	24 (33.3)	64 (48.1)
	Male	117 (57.1)	48 (66.7)	69 (51.9)
Age groups (years)	5-12	133 (64.9)	55 (76.4)	78 (58.6)
	13-15	72 (35.1)	17 (23.6)	55 (41.4)
Metabolic control (% HbA_{1c})	≤ 7.5	–	–	45
	7.6 – 8.5	–	–	50
	> 8.5	–	–	38
Salivary flow rate (ml/min)	≥1	170 (83.7)	61 (85.9)	109 (82.5)
	<1	33 (16.2)	10 (14.0)	23 (17.4)
Saliva buffer capacity	High	139 (68.4)	43 (61.0)	96 (72.7)
	Medium	47 (23.2)	22 (31.0)	25 (18.9)
	Low	17 (8.4)	6 (8.5)	11 (8.3)
Oral hygiene (daily frequency)	1	99	41	58
	2	79	27	52
	>2	11	1	10
	No hygiene	16	3	13
Periodontal status	Healthy	34 (47,2)	12 (26,7)	19(21,6)
	Bleeding/ Calculus	36 (50,0)	29 (64,4)	64(72,7)
	Excluded	2 (2,8)	4 (8,9)	5 (5,7)
Malocclusion	Yes	48 (66,7)	18 (40,0)	50 (56,8)
	No	24 (33,3)	27 (60,0)	38 (43,2)

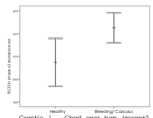
Table 1 - Global characterization of the study population.

		Control	Diabetic		
			Metabolic control (% HbA1c)		
			≤ 7,5 %	7,6 – 8,5 %	> 8,5 %
No yeasts	**Mucosa**	39	31	32	21
	Saliva	31	20	18	15
CFU (mean± STD)	**Mucosa** CFU/swab	123,8±403,5	206,7±380,8	253,7±404,2	276,0±327,2
	Saliva CFU/ml	197,5±316,3	203,9±250,1	341,5± 381,9	247,1±330,6

Table 2 - Oral yeast carriage (mucosa/saliva) in DM1 and control subjects.

		Control	HbA_{1c} ≤ 7,5	HbA_{1c} > 7,5
CD	**45+**	31	32	21
	14+	20	18	15
	3+	55,04 ± 22,44	64,56 ± 15,88	59,64 ± 18,58
	4+	206,7±380,8	253,7±404,2	276,0±327,2
	8+	203,9±250,1	341,5± 381,9	247,1±330,6

Table 3 - Characterization of salivary components of the inflammatory response in % (CD14 +, CD3 +, CD4 + and CD8 +) and number of cells event/10.000 events (CD45 +).



Graphic 1 – Chart error bars (mean±2 standard error) reflecting the % of CD3+ in "Healthy" and "Bleeding/calculus" groups

	Control	HbA_{1c} ≤ 7,5	HbA_{1c} > 7,5
Propionate	0.5930 ± 0.9256	0.4272 ± 0.4202	0.8819 ± 2.3521
Lactate	0.0988 ± 0.5831	0.0829 ± 0.2177	0.1064 ± 0.3069
Alanine	0.0342 ± 0.0698	0.0211 ± 0.0155	0.0247 ± 0.0178
Malate	0.9418 ± 2.1900	0.1800 ± 0.3741	0.4669 ± 1.7485
Butyrate	0.0994 ± 0.1717	0.0413 ± 0.0511	0.1360 ± 0.4622
Acetate	3.3165 ± 4.7462	2.2155 ± 1.3213	3.5820 ± 6.5282
Formate	0.6133 ± 3.6408	0.1613 ± 0.2184	0.3125 ± 0.7555
Ethanol	0.2782 ± 0.4997	0.1480 ± 0.1264	0.1822 ± 0.2603

Table 4 - Concentration (in µ mol) of different salivary metabolites according to the condition: healthy (controls) , controlled or uncontrolled DM1(HbA1c ≤ 7.5 or HbA1c> 7.5)

Statistical results showed **significant differences** only between periodontal condition and:

a) being a DM1/healthy children (X^2, p=0,001); OR=2,832, IC95% (1,524;5,263)
- DM1 children showed apparently worse periodontal condition

b) being a controlled DM1/healthy (X^2, p=0,023); OR=2,361, IC95% (1,120;4,976)
- even with good metabolic control, DM1 children showed apparently worse periodontal condition than healthy children; surprisingly, the same result could not be verified in uncontrolled DM1 group

c) age (X^2, p=0,002); OR=2,630, IC95% (1,413;4,894)
- older children showing apparently worse periodontal condition

d) frequency of tooth brushing (X^2, p=0,017)
- children exhibiting less frequent brushing habits showing apparently worse periodontal condition

e) malocclusion (X^2, p=0,004); OR=2,398, IC95% (1,304;4,405)
- children with malocclusion showing apparently worse periodontal condition

f) salivary subset of CD3+ T cells (Mann-Whitney, p=0,014)
- the highest % in "Bleeding/ Calculus" group

Conclusion

The percentage of **children with gingival alterations** was higher in the **diabetic group** meaning that, hypothetically, the condition disease could, by itself, represent a significantly higher risk factor. The **relevance assumed by oral hygiene** in periodontal status can warn for prevention of further periodontal complications in DM1 children. Once the host response is believed to have protective as well as a destructive role both in periodontal disease and DM1, the differences found in the salivary **CD3+ T-cells subset** may reflect differences in the **immunopathology of clinical lesions**. It should also be following underlined that, because CD3 is required for T-cell activation, drugs (usually monoclonal antibodies) that targeted it are being investigated as immunosuppressant therapies for DM1 and other autoimmune diseases. The salivary immune response analysis must be, for these same reasons, taken into account in global assessment of these children with **potential purpose**, both at **preventive** or in the **diagnosis** and **treatment stages**.

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