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ORIGINAL ARTICLE

Saliva proteomic profile of early childhood caries and caries-free children

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ABSTRACT

Objective: Saliva plays an important antimicrobial role and it is related to the pathogenesis of early childhood caries (ECC). The aim of this study was to compare the proteomic profile of unstimulated saliva of children aged 3–5 years who had ECC and caries-free (CF) children.

Materials and methods: After the saliva collection from 20 children (ECC: $n = 10$; CF: $n = 10$), the samples were processed for proteomic analysis on a mass spectrometer.

Results: 1638 proteins were identified, of which 355 were present in both groups. A total of 579 proteins were exclusively identified in the CF group and included Leucine-rich alpha-2-glycoprotein, Protein S100-A5, Protein S100-A8 and Mucin-2. Moreover, 704 proteins were exclusively identified in the ECC group, including Enamelin. The differential expression analysis revealed that 112 proteins were up-regulated in the CF group. Among these proteins, we highlighted Hemoglobin subunit gamma-1 (343-fold increase), gamma-2 (336-fold increase) and alpha (40-fold increase).

Conclusions: The proteomic profile of the saliva varied substantially between the groups. Hemoglobin subunit gamma-1, gamma-2 and alpha may play a protective role in children with ECC. These proteins should be evaluated in future studies, because they may be possible good candidates to be included in anti-caries dental products.

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Saliva; proteomics; early childhood caries; prevention

Introduction

Early childhood caries (ECC) affects greater than 600 million children globally and it is characterized as one of the most frequent biofilm-dependent infections in children [1]. The pathogenesis of ECC and the formation of cariogenic biofilm are the result of complex interactions that occur on the dental surface between oral microorganisms, dietary carbohydrates and biological factors [1]. Among the host factors identified, saliva has received particular consideration regarding caries.

Human saliva contains approximately 2290 proteins and several of them, such as Mucins and Proline-rich proteins (PRPs), are important precursors for the formation of acquired enamel pellicle (AEP) which forms a protective layer on the dental surface [2]. Salivary proteins play a role in the dynamic equilibrium between demineralization and remineralization, which may alter resistance to caries [3]. The components of saliva and the non-invasive and simple nature of its collection, combined with proteomic studies, make it an excellent sample type to assist in the diagnosis of some diseases.

If ECC is not diagnosed and treated in the initial stages, it may cause severe tooth destruction, resulting in local, psychological and systemic consequences that affect the quality of life of children and their families [1]. Therefore, early diagnosis and prevention are of great clinical significance and the search for salivary biomarkers that may be associated with caries has increased.

Wang et al. [4] and Guedes et al. [5] used advanced proteomic methods to identify salivary biomarkers for caries in children. Both methods showed promising results; however, due to the use of different methodologies and samples, the identification of salivary biomarkers and proteins with potential protector roles associated with ECC remains uncertain. Therefore, this study aimed to compare the salivary proteomic profile of 3-to-5-year-old children who had ECC with that of caries-free (CF) children. The null hypothesis was that there was no difference in the protein profile of saliva in children diagnosed with ECC compared with CF children.

Materials and methods

Ethical considerations

The research protocol was conducted in accordance with the 1964 Helsinki Declaration and it was approved by the local Human Research and Ethics Committee (CAAE 85974218.3.0000.5347). The study was only initiated once this approval was obtained. Regarding the recruitment of

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subjects and the collection of samples, written informed consent was obtained from the parents or guardians and written assent was signed by the participants. The personal information of the patients was kept confidential.

Subject recruitment and study design

The study participants consisted of children who had received dental care at the Paediatric Dentistry Clinic at the Faculty of Dentistry, Universidade Federal Do Rio Grande Do Sul (UFRGS). From a waiting list of children aged between 3 and 5 years, 80 were scheduled for an initial screening, 54 attended the consultation and 20 were included in the study. The sample size was estimated based on previous studies of quantitative proteomic analysis of saliva [6–12]. The sample size was calculated through MSstats [13] using data from our previous experiment [14–16], where $\alpha = 0.05$ and $1-\beta = 0.8$. The effect size (difference in protein abundance) was considered as 1.5. The estimated number of samples was three per group. Considering the low amount of proteins typically recovered from the saliva *in vivo*, it was decided to include 10 volunteers to constitute three pools (biological triplicates). To be included in the study, children could not be receiving dental care, could not have had any teeth restoration and they must have complied with the inclusion criteria for either of the two study groups (which are described in the subsequent sections). Children who had respiratory infections, had been using antibiotics during the previous month, had a medical history of congenital diseases and whose parents did not sign the written informed consent were not included in the study.

All children were subjected to an examination of the oral mucosa and a clinical examination to detect carious lesions. These exams were performed by two experienced dentists in the Paediatric Clinic of the Faculty of Dentistry, UFRGS and included artificial light, air compressor, suction, clinical mirror, probe and relative isolation. Dental prophylaxis was performed prior to the exams. No alterations were detected in the examination of the oral mucosa. The decayed, missing, filled teeth (DMFT) and the decayed, missing, filled surface (DMFS) indices were used to score the children's caries experience [17].

In addition to a dental caries examination, the 20 children included in the study were also examined regarding the visible plaque index (VPI) and the gingival bleeding index (GBI), and their guardians answered questionnaires that included data such as the frequency of tooth brushing and a 24-h dietary interview to evaluate the frequency of sucrose intake.

The participants were divided into two groups as follows:

- ECC group ($n = 10$):

Caries-active children with ECC who were diagnosed according to the age when one or more of the following characteristics were observed: (a) DMFT ≥ 1 in anterior teeth in children aged 3–5 years or (b) DMFT >4 in children aged 3 years, DMFT >5 in children aged 4 years and DMFT >6 in children aged 5 years [18]. After the collection of saliva, the children received the indicated dental treatment as well as oral hygiene and preventive dental care instructions.

- CF group ($n = 10$):

Children in the CF group showed no signs of cavitated caries or non-cavitated dental caries (DMFT = 0). After the collection of saliva, the children and their guardians received oral hygiene and preventive dental care instructions.

Collection of saliva samples and preparation for proteomic analysis

Unstimulated saliva was collected from the children for 10 min in pre-weighed and sterilized 50 ml plastic containers, with the aid of pipettes. At least 10 ml should have been collected. All collections were performed in the morning, between 10 a.m. and 11 a.m. Two hours before the collection of the saliva, the volunteers received dental prophylaxis with a pumice and rubber cup and did not ingest food or liquids until the time of collection [19].

The saliva samples were stored on dry ice immediately after collection and were subsequently frozen at −80 °C. Next, the samples were centrifuged at 4500 × *g* for 15 min at 4 °C to remove all possible debris. After centrifugation, the supernatant of each sample was removed and frozen at −80 °C until proteomic analysis was performed [12].

For the proteomics analysis, the samples were individually analyzed, as described by Ventura et al. [12]. The samples were thawed and 1000 µl of saliva from each individual was inserted into each tube. Saliva proteins were extracted using an equal volume (1:1) of extraction solution containing 6 M urea and 2 M thiourea in 50 mM NH_4HCO_3 (pH 7.8). Next, the samples were vortexed for 10 min at 4 °C, sonicated for 5 min and centrifuged at 20,817 × *g* for 10 min at 4 °C. These steps were then repeated once more.

After extraction, the samples were placed in 15 ml Falcon tubes and one and a half parts of 50 mM NH_4HCO_3 were added to the tubes to dilute the urea and thiourea, which can influence the action of trypsin. Next, the samples were transferred to Falcon Amicon Ultra-4 10k tubes (Merck Millipore, Ireland) and placed in a centrifuge at 4500 × *g* and 4 °C until they were concentrated to 150 µl in approximately 3 h. Subsequently, the samples were transferred to new Eppendorf tubes with a low residue concentration (suitable for nanoLC-ESI-MS/MS).

For the reduction process, 5 mM dithiothreitol (DTT) (Bio-Rad Laboratories) was added to the samples which were then incubated at 37 °C for 40 min. After this period, the samples were alkylated with 10 mM iodoacetamide (IAA) (GE Healthcare) for 30 min in the dark. After incubation, 100 µl of 50 mM NH_4HCO_3 was added to the samples which were then digested with 2% trypsin (Promega, Madison, WI, USA) for 14 h at 37 °C.

After 14 h of incubation, 10 µl of blocking solution (5% formic acid) was added to the samples and desalination and purification were performed using a C18 Spin column (Thermo Scientific®, Rockford, IL, USA) according to the

manufacturer's instructions. After the desalination, an aliquot of 1 µl was removed from each sample for a total quantification of proteins using the Bradford method (Bio-Rad Bradford Assays). Next, the samples were dried in a Speed Vac, resuspended in 3% acetonitrile and 0.1% formic acid and placed in proper glasses (Recovery – Vial Kit) to be processed in the nanoLC-ESI- MS/MS. All the aforementioned steps were performed according to Ventura et al. [12].

NanoLC-ESI-MS/MS acquisition

For the analysis of the peptides, a Xevo G2 mass spectrometer (Waters) coupled to a nanoACQUITY system (Waters) was used. The nanoACQUITY UPLC system was equipped with a Trap Column (100 Å, 5 µm, 180 µm × 200 mm) that was previously equilibrated with 99.9% phase A (0.1% formic acid in water) at a flow rate of 5 µl/min and an HSS T3 M-Class type column (analytical column; Acquity UPLC HSS T3 M-Class column, 75 µm × 150 mm; 1.8 µm) (Waters, Milford, MA) that was previously equilibrated with 93% mobile phase A and mobile phase B (0.1% formic acid in ACN). The peptides were separated using a linear gradient of 7–85% mobile phase B for 70 min with a 0.35 µl/min flow rate; the column temperature was maintained at 45 °C. The Xevo G2 Q-TOF mass spectrometer was operated in positive nano-electrospray ion mode and the data were collected using the MSE method in elevated energy (19–45 V). The source optimal conditions included capillary voltage, 2.8 kV; sample cone, 40 V; extraction cone, 3.0 V and source temperature 100 °C. However, source conditions may have varied due to detector and lockspray voltage setups. The data acquisition occurred over 70 min and the scan range was 50–1500 Da. The lockspray was run with a [Glu1] fibrinopeptide solution (1 pmol/µl) at a flow rate of 0.3 µl/min as a reference ion in positive mode at *m/z* 785.8427. The raw data were processed using the ProteinLynx Global Server (PLGS) version 3.0.3. The data were extracted, aligned and searched against in the UniProt human proteomic database (version 2020-01) and appended with the enolase (Saccharomyces cerevisiae) sequence. The ion accounting algorithm used was described previously by Li et al. [20]. PLGS utilizes the drift time of mobility-separated peptides to increase the specificity of the alignment or association for precursor and product ions. PLGS also assigns peptide identifications to proteins through an iterative matching process [20].

Label-free quantitative proteome analysis

For label-free quantitative proteome analysis, 10 raw MS files from each group were analyzed. PLGS software (PLGs, v 3.0, Waters Co., Manchester, UK) was used. All proteins identified with a score greater than 95% were included in the quantitative analysis. The identical peptides from each sample were grouped according to mass accuracy (<10 ppm) and retention time tolerance <0.25 min, using the clustering software included in PLGS. The difference in expression between the groups was analyzed by *t*-test ($p < .05$). Peptide analyses and groupings were performed without knowing which group the sample belonged to (blinding).

Bioinformatics analysis

The analyses of the processes that were most affected based on the Gene Ontology (GO) resource were performed using Cytoscape® 3.8.2 Software with ClueGo® plugins. The functional distribution of the proteins identified using the differential expression in the CF vs ECC was performed. The protein categories were based on GO annotation of the broad Biological Process, Molecular Function, Immune System Process and Cell Component. The terms of significance (Kappa = 0.04) and distribution were applied according to the percentage of the number of associated genes. The number of access to the proteins was provided by UniProt.

Results

In total, 20 children were included in the study and all completed saliva collections. Table 1 shows the characteristics of the volunteers according to each allocated group.

As shown in the Venn diagram (Figure 1), a total of 1638 proteins were identified. Among them, 355 were identified in both groups (CF and ECC). The protein profile varied considerably between the groups tested. Among the proteins identified, 579 were exclusively identified in the CF group and included Leucine-rich alpha-2-glycoprotein, Protein S100-A5, Protein S100-A8 and Mucin-2. In the ECC group, 704 proteins were exclusively identified, including Enamelin (Table S1).

Table 1. Characteristics of 3- to 5-year-old children according to the ECC or CF groups.

Characteristics	ECC	CF
Sex [*n* (%)]		
Male	5 (50.0)	7 (70.0)
Female	5 (50.0)	3 (30.0)
Age (mean ± SD)	4.2 ± 0.91	4.0 ± 0.66
dmf-t (mean ± SD)	8.0 ± 2.49	0
dmf-s (mean ± SD)	18.0 ± 7.31	0
VPI (% mean ± SD)	24.4 ± 10.72	17.6 ± 9.08
GBI (% mean ± SD)	10.0 ± 7.21	4.9 ± 4.80
Frequency of toothbrushing (mean ± SD)	1.0 ± 1.07	3.0 ± 0.67
Frequency of sucrose intake (mean ± SD)	3.0 ± 1.28	3.0 ± 1.03
Total [*n* (%)]	10 (100.0)	10 (100.0)

ECC: early childhood caries; CF: caries-free; VPI: visible plaque index; GBI: gingival bleeding index. $n = 20$.

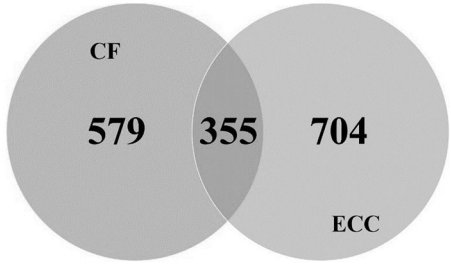

Figure 1. Venn diagram showing the relationships of the proteins identified in common among the groups, as well as the number of proteins exclusively found in one of the groups in each comparison.

When the groups were quantitatively compared using an analysis of differential expression, 189 proteins were identified that were expressed between the groups (Table S2). A total of 112 proteins were up-regulated in the CF group compared to the ECC group. Among these were Basic salivary proline-rich protein 2, Salivary acidic proline-rich phosphoprotein 1/2, Proline-rich protein 4, Submaxillary gland androgen-regulated protein 3B, Cystatin-S, SN, SA, B (2-fold increase), Lipocalin-1, two isoforms of Alpha-Amylase (1 and 2B), Pancreatic alpha-amylase, Neutrophil defensin 1 and 3, Mucin-7, Carbonic anhydrase 6, four isoforms of Immunoglobulin lambda constant (1, 2, 3 and 6) and Immunoglobulin heavy constant gamma 3 and 4, Histatin-1, BPI fold-containing family A member 2, Antileukoproteinase, Beta-2-microglobulin and six isoforms of Hemoglobin (subunit epsilon (5-fold increase), subunit beta (16-fold increase), subunit delta (26-fold increase), subunit alpha (40-fold increase), subunit gamma-1 (343-fold increase) and subunit gamma-2 (336-fold increase). However, 77 proteins were down-regulated in the CF group compared with the ECC group. Among these proteins were Statherin, Basic salivary proline-rich protein 1, Histatin-3, Lysozyme, Lysozyme C, 15 isoforms of Histones, Prolactin-inducible protein, Cystatin-D, Immunoglobulin J chain, Immunoglobulin heavy constant alpha 1 and alpha 2. Table 2 shows the relative quantification of proteins increased or decreased more than 2-fold identified in the saliva collected from the groups ECC and CF.

Figure 2 shows the results of a functional analysis according to the biological process by GO with the most significant terms for a comparison between CF and ECC groups. The category with the highest percentage of genes in the biological process was a defense response to the bacterium (21.3%). The results of a functional analysis according to the immune system process by GO with the most significant terms for a comparison between CF and ECC groups showed that the categories with the highest percentages of genes were a defense response to the bacterium (40.3%), an antimicrobial humoral response (26.9%) and an antibacterial humoral response (14.9%) (Figure 3). The results of functional analysis according to molecular function process by GO with the most significant terms for a comparison between the CF and ECC groups showed that the categories with the highest percentages of genes were cysteine-type endopeptidase inhibitor activity (21.9%), calcium-release channel activity (9.4%) and alpha-amylase activity (9.4%) (Figure 4). Finally, the results of a functional analysis according to the cell component process by GO with the most significant terms showed that the category with the highest percentages of genes in the cellular component was immunoglobulin complex, circulating (22.8%) (Figure 5).

Discussion

Peptidomic and proteomic studies that evaluated the saliva of children with ECC and CF children identified 91 proteins [21] and 306 proteins [5]. In the present study, we obtained an expressive number of identified proteins (1638) and the use of a human database excluded the identification of bacterial proteins that could be present in saliva. This expressive number may be attributed to the collection of unstimulated saliva, in which there is no dilution of the total protein concentration [14]. Analysis of individual samples was also advantageous because the biological variation of samples was accounted for. Moreover, when samples are pooled, certain characteristics of an individual may contribute more to the results than others [12].

The proteomic analyses revealed profound changes in the salivary proteomic profiles of both groups. From a practical point of view, proteins that increased or were uniquely identified in the CF group may play a protective role against caries and are potential candidates to be included in anti-caries dental products. Among proteins uniquely identified in the CF group were Leucine-rich alpha-2-glycoprotein, Protein S100-A8, Protein S100-A5 and Mucin-2. Leucine-rich alpha-2-glycoprotein has shown a response to bacteria and it was also identified exclusively in the CF group in a previous study on Chinese children [22,23]. Protein S100-A8 is a Ca^{2+} binding protein and plays a critical role in modulating the inflammatory response, stimulating leukocyte recruitment and inducing cytokine secretion. It is considered a candidate biomarker for the diagnosis and monitoring of diseases associated with inflammation [24]. In a previous study conducted on young adults and elderly individuals, this protein was up-regulated in the CF group, suggesting a potential protective effect against caries [25]. However, this is the first time that this protein was identified in CF children and further studies are important to understand how protein S100-A8 protects specifically against caries. This is similar to Protein S100-A5 (another Ca^{2+} binding protein) and Mucin-2. Mucins are the first line of oral health defense and may interact with oral microorganisms, promoting their agglutination, as an important antimicrobial agent [26].

PRPs neutralize the acids present in dental biofilm and participate in the AEP formation [27]. In the present study, Basic salivary proline-rich protein 2 and Salivary acidic proline-rich phosphoprotein 1/2 were up-regulated in the CF group. Wang et al. reported similar results [4]. It is important to note that Proline-rich protein 4 and Submaxillary gland androgen-regulated protein 3B (a proline-rich protein typically found in AEP) that most likely influence the formation of oral biofilms [22] were also up-regulated in the CF group in the present study.

Cystatin-S, Cystatin-SN, Cystatin-SA and Cystatin-B (2-fold increase) were up-regulated in the CF group. Cystatin-S and Cystatin-SN were also correlated with the absence of caries in previous studies of saliva [4], as well as Lipocalin-1, which was also up-regulated in the CF group. Cystatin-S, Cystatin-SN and Lipocalin-1 may indirectly provide tooth protection by inhibiting proteolytic events on other salivary proteins. Additionally, Cystatin-B is an intracellular thiol proteinase inhibitor and has cysteine-type endopeptidase inhibitor activity [22]. Interestingly, the results of a functional analysis of the most affected processes in the molecular function, when comparing the CF vs ECC groups, showed that 21.9% was

Table 2. Relative quantification of proteins increased or decreased more than 2-fold identified in the saliva collected from the groups ECC and CF.

[†]Access number	Protein name	PLGS Score	[‡]Ratio, CF:ECC
O95714	E3 ubiquitin-protein ligase HERC2	138	343.78
P69891	Hemoglobin subunit gamma-1	1481	343.78
P69892	Hemoglobin subunit gamma-2	1481	336.97
P69905	Hemoglobin subunit alpha	21,716	40.45
Q9UHD8	Septin-9	350	27.94
Q9HC52	Chromobox protein homolog 8	263	27.11
P02042	Hemoglobin subunit delta	5903	26.58
O43766	Lipoyl synthase_ mitochondrial	227	25.53
G3V1N2	HCG1745306_ isoform CRA_a	17,661	22.87
Q9UK13	Zinc finger protein 221	255	22.42
K7EIF9	Signal transducer and activator of transcription 5A (Fragment)	302	22.20
O94772	Lymphocyte antigen 6H	303	21.76
Q12901	Zinc finger protein 155	178	18.92
Q96R06	Sperm-associated antigen 5	708	17.12
P68871	Hemoglobin subunit beta	21,726	16.44
Q86XA9	HEAT repeat-containing protein 5A	168	13.46
P46940	Ras GTPase-activating-like protein IQGAP1	60	13.33
I6L9I8	EPN3 protein	509	11.82
Q14839	Chromodomain-helicase-DNA-binding protein 4	140	11.36
O00763	Acetyl-CoA carboxylase 2	244	11.02
Q9BY12	S phase cyclin A-associated protein in the endoplasmic reticulum	131	10.59
Q9H201	Epsin-3	509	10.49
P00450	Ceruloplasmin	215	8.85
Q86Z02	Homeodomain-interacting protein kinase 1	136	8.41
P80188	Neutrophil gelatinase-associated lipocalin	3356	8.41
Q68CZ1	Protein fantom	252	7.39
Q5JSZ5	Protein PRRC2B	344	7.39
Q8NFC6	Biorientation of chromosomes in cell division protein 1-like 1	133	7.32
I3L3J8	Sodium/potassium-transporting ATPase subunit beta (Fragment)	131	7.17
Q8IXW7	FMR1 protein	179	6.89
Q92793	CREB-binding protein	228	6.42
P08670	Vimentin	303	6.42
P02100	Hemoglobin subunit epsilon	1481	5.64
P02812	Basic salivary proline-rich protein 2	5366	5.42
Q99250	Sodium channel protein type 2 subunit alpha	117	5.37
Q8NAV2	Uncharacterized protein C8orf58	374	5.37
A8K0R7	Zinc finger protein 839	241	5.05
Q5VW36	Focadhesin	393	4.66
P07202	Thyroid peroxidase	172	4.48
P14384	Carboxypeptidase M	313	4.44
Q12996	Cleavage stimulation factor subunit 3	439	4.22
Q9UMS6	Synaptopodin-2	321	4.22
Q8NEU8	DCC-interacting protein 13-beta	182	4.18
P52209	6-phosphogluconate dehydrogenase_ decarboxylating	351	3.97
B4E258	cDNA FLJ52650_ moderately similar to Synaptopodin-2	311	3.86
P13929	Beta-enolase	197	3.78
P07602	Prosaposin	245	3.74
A0A075B6K4	Immunoglobulin lambda variable 3–10	292	3.71
A8K2U0	Alpha-2-macroglobulin-like protein 1	1020	3.39
P59666	Neutrophil defensin 3	702	3.19
P59665	Neutrophil defensin 1	702	3.13
Q16378	Proline-rich protein 4	699	3.06
A6NMZ7	Collagen alpha-6(VI) chain	138	2.97
Q09428	ATP-binding cassette sub-family C member 8	192	2.94
Q15811	Intersectin-1	77	2.94
P06744	Glucose-6-phosphate isomerase	178	2.89
Q5W0V3	Protein FAM160B1	293	2.89
P10909	Clusterin	336	2.83
Q9Y6R7	IgGFc-binding protein	32	2.77
Q86TI0	TBC1 domain family member 1	249	2.75
P60174	Triosephosphate isomerase	792	2.75
P23280	Carbonic anhydrase 6	1384	2.69
P01857	Immunoglobulin heavy constant gamma 1	258	2.61
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1	247	2.59
Q9UBG3	Cornulin	1688	2.51
P10070	Zinc finger protein GLI2	72	2.41
P03973	Antileukoproteinase	383	2.32
P04080	Cystatin-B	3215	2.12
Q6ZN06	Zinc finger protein 813	177	0.49
Q9H972	Uncharacterized protein C14orf93	488	0.48
P61626	Lysozyme C	663	0.45
P58876	Histone H2B type 1-D	748	0.45
Q99880	Histone H2B type 1-L	748	0.45
P06899	Histone H2B type 1-J	748	0.44

(*continued*)

Table 2. Continued.

[†]Access number	Protein name	PLGS Score	[‡]Ratio, CF:ECC
Q6P5S2	Protein LEG1 homolog	1038	0.44
Q8IVL0	Neuron navigator 3	103	0.44
Q99879	Histone H2B type 1-M	748	0.44
Q99877	Histone H2B type 1-N	748	0.43
Q93079	Histone H2B type 1-H	748	0.43
Q5QNW6	Histone H2B type 2-F	748	0.43
P33778	Histone H2B type 1-B	748	0.42
P62807	Histone H2B type 1-C/E/F/G/I	748	0.42
O60814	Histone H2B type 1-K	748	0.42
Q8N257	Histone H2B type 3-B	748	0.42
U3KQK0	Histone H2B	748	0.42
P23527	Histone H2B type 1-O	748	0.41
P57053	Histone H2B type F-S	748	0.41
Q96DT5	Dynein heavy chain 11_ axonemal	30	0.37
Q9UJZ1	Stomatin-like protein 2_ mitochondrial	90	0.35
P25311	Zinc-alpha-2-glycoprotein	1134	0.35
Q13464	Rho-associated protein kinase 1	80	0.33
P02763	Alpha-1-acid glycoprotein 1	156	0.32
P19013	Keratin_ type II cytoskeletal 4	2238	0.32
Q8NHU2	Cilia- and flagella-associated protein 61	607	0.32
O75116	Rho-associated protein kinase 2	96	0.30
Q9BZL6	Serine/threonine-protein kinase D2	389	0.29
P49792	E3 SUMO-protein ligase RanBP2	75	0.29
O15460	Prolyl 4-hydroxylase subunit alpha-2	208	0.27
Q03112	Histone-lysine N-methyltransferase MECOM	229	0.25
P15516	Histatin-3	2074	0.25
B7ZMI3	ITPR1 protein	111	0.23
P35900	Keratin_ type I cytoskeletal 20	162	0.22
Q99456	Keratin_ type I cytoskeletal 12	135	0.21
O60308	Centrosomal protein of 104 kDa	220	0.20
P35609	Alpha-actinin-2	267	0.19
P55286	Cadherin-8	190	0.18
C9J296	Laminin subunit beta-1 (Fragment)	198	0.18
Q9H2A2	2-aminomuconic semialdehyde dehydrogenase	191	0.16
Q04695	Keratin_ type I cytoskeletal 17	162	0.16
P13646	Keratin_ type I cytoskeletal 13	3873	0.15
Q8N1A0	Keratin-like protein KRT222	174	0.15
P08779	Keratin_ type I cytoskeletal 16	481	0.14
P04259	Keratin_ type II cytoskeletal 6B	276	0.14
P02533	Keratin_ type I cytoskeletal 14	299	0.13
P48668	Keratin_ type II cytoskeletal 6C	289	0.13
Q9Y618	Nuclear receptor corepressor 2	67	0.12
Q3V6T2	Girdin	128	0.11
P13942	Collagen alpha-2(XI) chain	518	0.11
Q8IWG1	WD repeat-containing protein 63	211	0.11
A0A0C4DFS1	COL11A2	517	0.10
P19012	Keratin_ type I cytoskeletal 15	415	0.09
Q14643	Inositol 1_4_5-trisphosphate receptor type 1	116	0.08
Q86Y46	Keratin_ type II cytoskeletal 73	1037	0.06
P08727	Keratin_ type I cytoskeletal 19	193	0.04
P02538	Keratin_ type II cytoskeletal 6A	289	0.04
P13647	Keratin_ type II cytoskeletal 5	121	0.03
O95678	Keratin_ type II cytoskeletal 75	113	0.03
Q5XKE5	Keratin_ type II cytoskeletal 79	128	0.03

[†]Identification is based on protein ID from UniProt protein database, reviewed only (http://wwwwww.uniprot.org/).
[‡]Proteins with expression significantly altered are organized according to the ratio.

involved in cysteine-type endopeptidase inhibitor activity (Figure 4).

Salivary alpha-amylase can modulate bacterial colonization by binding to membranes of *Streptococcus mutans* and *Lactobacilli*, promoting their removal from the oral cavity and decreasing the risk of caries [28]. It was previously reported in a longitudinal study that Alpha-amylase activity was significantly higher in the saliva of CF children compared to children with ECC, suggesting that the low salivary activity of this enzyme may be an ECC risk predictor [29]. In the present study, Alpha-amylase 1 and 2b were up-regulated in the CF group, suggesting the likely protective potential of them in young children. Additionally, the functional analysis demonstrated that 9.4% of the processes most affected were involved with alpha-amylase activity in the molecular function (Figure 4). However, a recent study that investigated the activity of pellicle enzymes and their distribution and fluorescence pattern in children revealed that activities of amylase were not associated with caries status [30].

Beta-2-microglobulin and BPI fold-containing family A member 2 have potent antibacterial properties and Antileukoproteinase down-regulates responses to bacterial lipopolysaccharide (LPS) [22]. These proteins were also up-regulated in the CF group. These findings corroborate the results of the present study regarding the analysis of the most affected activities in the biological process, with 21.3%

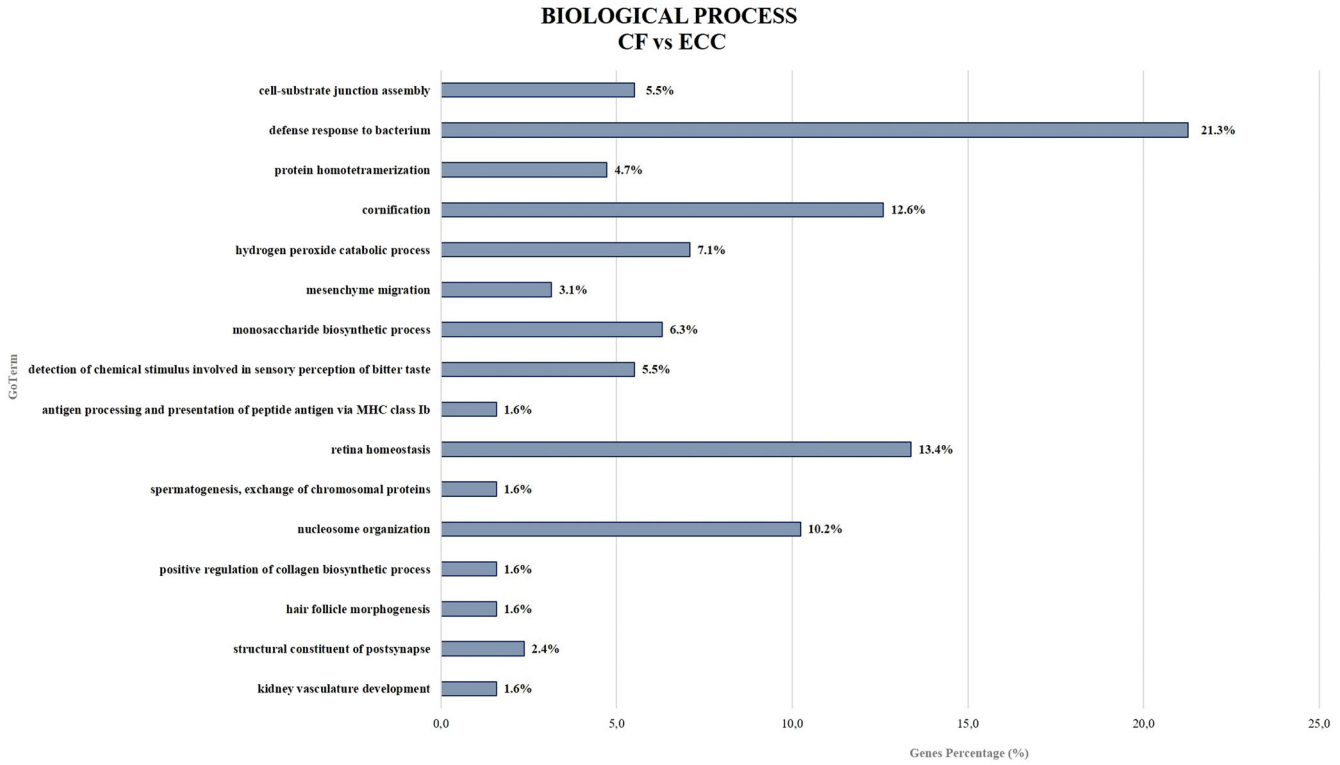

Figure 2. Graphs of the functional distribution of proteins identified using differential expression in the CF vs. ECC groups. The protein categories are based on Gene Ontology (GO) annotation of the broad Biological Process. The terms of significance (Kappa = 0.04) and distribution were applied according to the percentage of the number of associated genes. The number of access to the proteins was provided by UniProt. The gene ontology was evaluated according to the ClueGo® plugins of the software Cytoscape® 3.8.2.

being involved in the defense response to the bacterium (Figure 2). Furthermore, in the most affected activities of the immune system process, 40.3, 26.9, and 14.9% were involved in the defense response to the bacterium, an antimicrobial humoral response and an antibacterial humoral response, respectively (Figure 3). To the best of our knowledge, this is the first time that these proteins have been reported in a proteomic study conducted on children with ECC and CF children. The up-regulation of these proteins in the CF group indicates increased antibacterial activity in these children when compared to the ECC group.

Previous studies reported that Neutrophil defensins 1 and 3 have antimicrobial activity against a wide spectrum of microorganisms [31]. This corroborates the results of a study that reported the presence of unique lipoteichoic acid-binding proteins (Sm.LTA-BPs), including Neutrophil defensin 1, in the saliva of CF subjects. These proteins were associated with the host's antimicrobial defense [32] and were up-regulated in the CF group.

It is important to note that we identified several isoforms of Hemoglobin that were up-regulated in the CF group with an expressive increase of 343-, 336- and 40-fold for subunit gamma-1, gamma-2 and alpha, respectively. Hemoglobin synergistically regulates the immunostimulatory activity of lipoteichoic acids of various gram-positive bacteria and it was considered to be acid-resistant at increased levels in the AEP and saliva in patients with gastroesophageal reflux who did not show erosive tooth wear [33]. Therefore, the results of the present study suggest that Hemoglobin may play a protective role for ECC.

Proteins involved in innate immunity and inflammatory proteins may show an increase in saliva samples of patients with active caries compared with CF and are thought to protect an individual from virulence factors. This may be a plausible explanation for controversies in the literature involving dental caries biomarkers, i.e. proteins are considered to be protective in certain studies and in others they have been found exclusively or at increased levels in groups of patients with the disease. In the present study, Mucin-7 was up-regulated in the CF group, suggesting a protective role. Mucin-7 is capable of directly binding to *S. mutans* and it is an important AEP component. However, some studies did not report a significant correlation between Mucin-7 levels and ECC and identified Mucin-7 as being up-regulated in the caries children group [4]. The potential for this protein as a biomarker for caries remains controversial.

Carbonic anhydrase 6 (CAH-6) maintains pH homeostasis in human body tissues and low CAH-6 levels were reported to be associated with increased caries prevalence in young men [34]. The results of the present study revealed that CAH-6 was up-regulated in the CF group, like in a previous study conducted on 6- to 8-year-old children [23]. Glucose-6-phosphate isomerase (G6PI) was up-regulated in the CF group in the present study and a recent proteomic study with stimulated saliva of children with ECC and CF children reported similar results [5].

Immunoglobulins have also been discussed in the search for dental caries biomarkers. These proteins may directly influence the colonization of *S. mutans* on the dental surface [35]. Numerous previous studies have reported a correlation

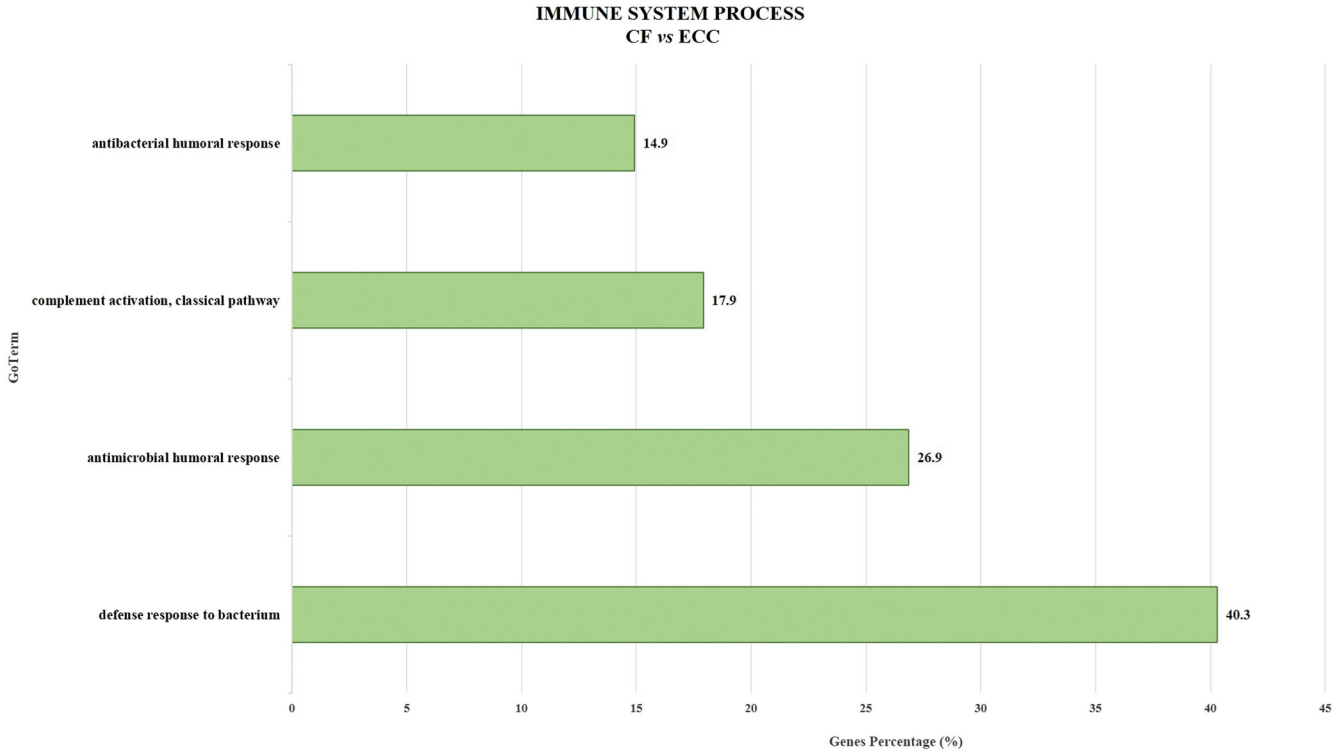

Figure 3. Graphs of the functional distribution of proteins identified using differential expression in the CF vs. ECC groups. The protein categories are based on Gene Ontology (GO) annotation of the broad Immune System Process. The terms of significance (Kappa = 0.04) and distribution were applied according to the percentage of the number of associated genes. The number of access to the proteins was provided by UniProt. The gene ontology was evaluated according to the ClueGo® plugins of the software Cytoscape® 3.8.2.

between Immunoglobulin and a high risk of caries [4,5,23], suggesting an immune system response to aggressive factors. In the present study, isoforms of Immunoglobulin were present in both groups, which may be a confusing factor in the search for biomarkers. However, it is worth noting that the majority of isoforms of Immunoglobulin were up-regulated (Immunoglobulin lambda constant 1, 2, 3, and 6 and Immunoglobulin heavy constant gamma 3 and 4) in the CF group. Additionally, in the cellular component process by GO in the functional analysis comparison of the CF vs ECC groups, the immunoglobulin complex, circulating represented 22.8% of the most affected processes (Figure 5).

Histatin-1, Statherin and Basic salivary proline-rich protein 1 were associated with the absence of caries, indicating the protective role of these proteins. Histatin-1 is an important factor present in dental structure with antibacterial and antifungal activity [4]. Thus, it has been suggested that this protein plays a protective role for caries and the results of the present study agree with these findings. However, Statherin and Basic salivary proline-rich protein 1 were down-regulated in the CF group in the present study, contradicting the results described in a previous study [36].

Lysozyme, Lysozyme C, Histones and Prolactin-inducible protein (PIP) were up-regulated in the ECC group. The higher level of Lysozyme in the ECC group may suggest a compensatory protective mechanism. In specific situations, such as in caries disease or high levels of *S. mutans*, Lysozyme secretion is stimulated [37]. Similar results for Lysozyme C were reported previously [31]. However, a recent study on the activity and distribution of pellicle enzymes, such as Lysozyme, in children did not reveal significant differences in enzyme activity associated with children's caries activity [30]. Although, it is important to emphasize that the analysis of pellicles may not be used as a predictor of metabolite concentrations in saliva and vice versa. The formation of pellicles seems to be a specific process. Not all analytes found in saliva are detectable in the pellicle, and the ratio of analytes also differs in the saliva and in the AEP [38].

Enamelin was among the proteins that were only identified in the ECC group and may be considered a potential biomarker for ECC. This protein is essential for the integrity of the ameloblast and the formation of enamel [39]. The presence of Enamelin in the saliva of ECC children may be associated with enamel dissolution.

The controversies that exist in the literature are important to consider, as they may provide limitations regarding the definition of dental caries biomarkers and concerning an understanding of the protective role of proteins. The opposing findings may also be due to different methodologies that were applied in each study, including sample differences such as age, eating and oral hygiene habits, which influence the type of proteins detected [30]. We suggest that a longitudinal study should be conducted and evaluated in future studies. Additionally, protein-protein interactions should be examined, considering that proteins undergo interactions and it is not realistic to consider a unique isolated salivary factor as potential protection against caries disease. Furthermore, the multifactorial aetiology of caries is also a complicating factor for the establishment of a unique variable as a predictive or protective disease factor.

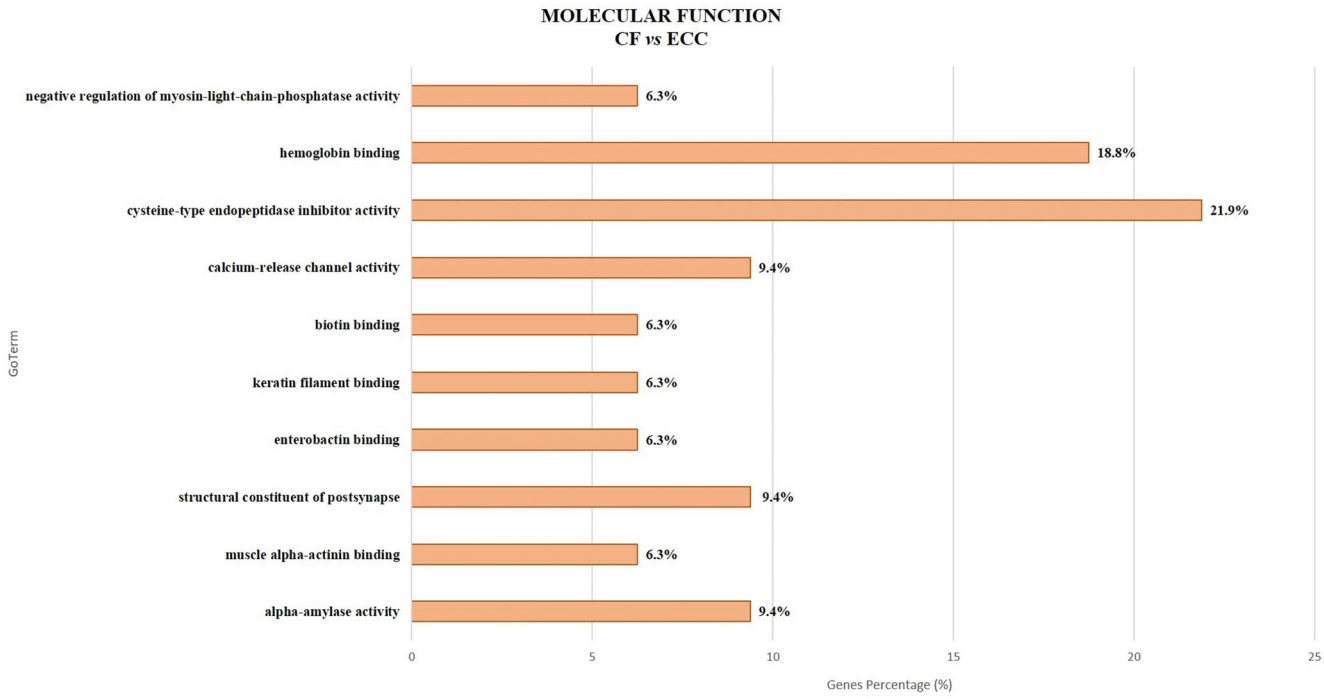

Figure 4. Graphs of the functional distribution of proteins identified using differential expression in the CF vs. ECC groups. The protein categories are based on Gene Ontology (GO) annotation of the broad Molecular Function. The terms of significance (Kappa = 0.04) and distribution were applied according to the percentage of the number of associated genes. The number of access to the proteins was provided by UniProt. The gene ontology was evaluated according to the ClueGo® plugins of the software Cytoscape® 3.8.2.

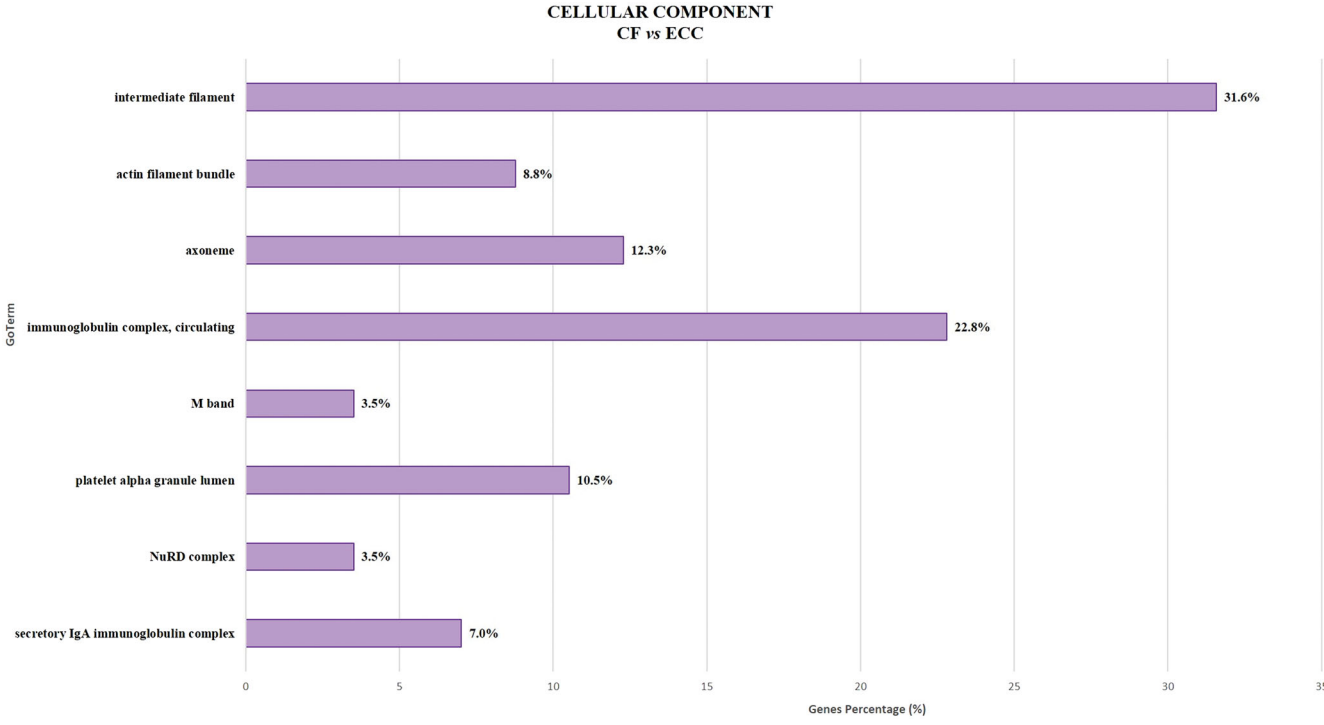

Figure 5. Graphs of the functional distribution of proteins identified using differential expression in the CF vs. ECC groups. The protein categories are based on Gene Ontology (GO) annotation of the broad Cellular Component. The terms of significance (Kappa = 0.04) and distribution were applied according to the percentage of the number of associated genes. The number of access to the proteins was provided by UniProt. The gene ontology was evaluated according to the ClueGo® plugins of the software Cytoscape® 3.8.2.

To the best of our knowledge, this is the first *in vivo* study that compared the proteomic profile of unstimulated saliva of children with ECC with that of CF children. We observed profound differences in the profiles obtained in saliva samples from both groups, and the proteins Hemoglobin subunit gamma-1, Hemoglobin subunit gamma-2 and Hemoglobin subunit alpha may play a protective role in ECC. The results of the present study can be used as a basis for

further studies to investigate the protective effect of the proteins identified. The proteins that showed an increase or were uniquely identified in the CF group may play a protective role against caries and are potential candidates to be included in anti-caries dental products. Conversely, the proteins that showed an increase or were only identified in the ECC group may be evaluated in future studies as potential biomarkers for the early detection and prevention of the disease.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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