ΕΘΝΙΚΟΝ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟΝ
ΠΑΝΕΠΙΣΤΗΜΙΟΝ ΑΘΗΝΩΝ
ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ
ΤΜΗΜΑ ΟΔΟΝΤΙΑΤΡΙΚΗΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ
ΣΤΗΝ ΟΔΟΝΤΙΑΤΡΙΚΗ ΕΠΙΣΤΗΜΗ

«Μεταβολές των ποιοτικών και βιοχημικών χαρακτηριστικών του σάλιου σε συσχέτιση με τη μεταβολική ρύθμιση σε παιδιά και εφήβους με διαβήτη τύπου 1»

“Salivary alterations in relation to metabolic control in children and adolescents with type 1 diabetes”

ΕΥΤΥΧΙΑ ΠΑΠΠΑ

ΑΘΗΝΑ 2019

ΤΡΙΜΕΛΗΣ ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

1. Ελένη Βασταρδή, Επίκουρη Καθηγήτρια, Επιβλέπουσα
2. Ευθυμία Κιτράκη, Καθηγήτρια
3. Κωνσταντίνος Βουγάς, Ερευνητής Β' Ιδρύματος Ιατροβιολογικών Ερευνών Ακαδημίας Αθηνών

ΕΠΤΑΜΕΛΗΣ ΕΞΕΤΑΣΤΙΚΗ ΕΠΙΤΡΟΠΗ

1. Ελένη Βασταρδή, Επίκουρη Καθηγήτρια, Επιβλέπουσα
2. Κωνσταντίνος Βουγάς, Ερευνητής Β' Ιδρύματος Ιατροβιολογικών Ερευνών Ακαδημίας Αθηνών
3. Ευθυμία Κιτράκη, Καθηγήτρια
4. Αντωνία Βλάχου, Ειδική Λειτουργική Επιστήμονας Α' Ιδρύματος Ιατροβιολογικών Ερευνών Ακαδημίας Αθηνών
5. Αθανάσιος Αρμακόλας, Αναπληρωτής Καθηγητής Ιατρικής ΕΚΠΑ
6. Αντώνης Σταματάκης, Αναπληρωτής Καθηγητής Νοσηλευτικής ΕΚΠΑ
7. Χρίστος Ραχιώτης, Αναπληρωτής Καθηγητής

Note for Scholarship

This study was funded by IKY fellowships of Excellence for postgraduate studies in Greece- Siemens Program.

The author additionally acknowledges the support of Onassis Foundation during the final year of her studies.

Στον Παναγιώτη και στον Νικόλα

Στους γονείς μου, Σπύρο και Ελένη

CONTENTS

Abstract

This thesis focuses on the salivary characteristics of children and adolescents with type 1 diabetes in relation to the level of glycemic control, with the aim to investigate their oral health status and at a further level of analysis, explore the salivary proteome of this study group.

The thesis consists of two separate parts

Part I: The aim of this cross-sectional study was to investigate the possible association between salivary dysfunction, xerostomia prevalence and incidence of caries, in relation to the level of metabolic control, in children and adolescents with type 1 diabetes. For the purpose of this study, a total of 150 children and adolescents (10-18 years old) were examined and allocated among 3 groups: 50 patients poorly-controlled (HbA1c≥7.5%), 50 well-controlled (HbA1c<7.5%) and 50 age- and sex-matched healthy controls. The study was approved by the Research Ethics Committee of the National and Kapodistrian University of Athens and the parents signed written informed consent. All subjects were examined for dental caries, oral hygiene and salivary factors. Assessments of salivary characteristics included self-reported xerostomia, quantification of resting and stimulated whole saliva flow rates, pH values, buffering capacity and saliva's viscosity. A questionnaire and a chair-side saliva testing kit were used for the evaluation of salivary function. Caries incidence was recorded using DMFT index. Plaque index and gingival index were additionally evaluated. Data were analysed by Chi-square and Kruskal-Wallis tests. Higher caries levels, higher prevalence of xerostomia and a decreased unstimulated salivary flow rate were recorded in poorly-controlled diabetics. The average caries indexes were $DMFT_{(poor\ c)}$ 3.6, $DMFT_{(well\ c)}$1.2, $DMFT_{(healthy)}$ 1.5, $p < 0.05$). Salivary status and caries index were not found to be significantly different between well-controlled patients and healthy controls. The results of this study indicated that chair-side salivary tests provide the practitioners with an easy-to use and quick method for the evaluation of salivary function and caries risk assessment in young patients with diabetes.

Part II: In this part of our study we investigated the proteomic profile of whole saliva by high resolution mass spectrometry in type 1 diabetic patients. The aim of this research was to characterize the salivary proteome of type 1 diabetes patients in order to identify differentially expressed proteins compared to control subjects, infer deregulated biological pathways, and evaluate the relevance of the findings in the context of diabetes pathophysiology. We analyzed by high resolution mass spectrometry approaches saliva samples collected from 32 children and adolescents: 12 with poorly controlled type 1 diabetes (G1) (HbA1c≥7.5%), 12 with well controlled type 1 diabetes (G2) (HbA1c<7.5%) and 12 healthy controls (Ctrl). According to the results of this study, the composition of the salivary proteome is affected by pathological conditions. The list of more than 2000 high confidence protein identifications constitutes a comprehensive characterization of the salivary proteome. Patients with good glycemic regulation and healthy individuals have comparable proteomic profiles. In contrast, a significant number of differentially

expressed proteins were identified in the saliva of patients with poor glycemic regulation compared to patients with good glycemic control and healthy children. These proteins are involved in biological processes relevant to diabetic pathology such as endothelial damage and inflammation. Moreover, a putative preventive therapeutic approach was identified based on bioinformatic analysis of the deregulated salivary proteins. Thus, thorough characterization of saliva proteins in diabetic pediatric patients established a connection between molecular changes and disease pathology. This proteomic and bioinformatic approach highlights the potential of salivary diagnostics in diabetes pathology and opens the way for preventive treatment of the disease.

Περίληψη

Ο Σακχαρώδης Διαβήτης (ΣΔ) είναι η πιο κοινή νόσος των ενδοκρινών αδένων στο γενικό πληθυσμό. Ο Ινσουλινοεξαρτώμενος ή τύπου 1 ΣΔ προκαλείται από την επίδραση διαφόρων περιβαλλοντικών παραγόντων που ενεργοποιούν τον αυτοάνοσο μηχανισμό καταστροφής των β-κυττάρων του παγκρέατος σε γενετικά προδιατεθειμένο άτομο. Η οδοντοστοματολογική υγεία των ατόμων με ΣΔ έχει αποτελέσει αντικείμενο πολλών μελετών που αναφέρονται στην εμφάνιση τερηδόνας και περιοδοντικής νόσου, εξετάζοντας διάφορους παράγοντες που πιθανόν να συμβάλλουν ή όχι στην εμφάνιση και εξέλιξη των νόσων αυτών.

Ο πρώτος σκοπός της παρούσας κλινικής μελέτης ήταν να διερευνηθεί η συσχέτιση του μεταβολικού ελέγχου με ποιοτικά και ποσοτικά χαρακτηριστικά του σάλιου (ροή, pH, σύσταση, ρυθμιστική ικανότητα) σε παιδιά και εφήβους με Τύπου 1 ΣΔ (Τ1ΣΔ) καθώς και με την εμφάνιση τερηδόνας στους ασθενείς αυτούς. Ο δεύτερος σκοπός της μελέτης ήταν η διερεύνηση του πρωτεωμικού προφίλ των παιδιών με Τ1ΣΔ για την ανίχνευση πιθανών σιαλικών βιομορίων που θα μπορούσαν να αποτελέσουν στο μέλλον διαγνωστικά εργαλεία για τον εντοπισμό αλλά και την παρακολούθηση της μεταβολικής ρύθμισης ασθενών με Τ1ΣΔ.

Για τους σκοπούς αυτούς, η μελέτη χωρίστηκε σε δύο μέρη.

Κατά το πρώτο μέρος, εξετάστηκαν 150 παιδιά και έφηβοι (10-18), εκ των οποίων 50 εμφάνιζαν αρρύθμιστο Τ1ΣΔ (HbA1c≥7.5%), 50 ρυθμισμένο Τ1ΣΔ (HbA1c<7.5%) και 50 ήταν υγιείς μάρτυρες. Το δείγμα της μελέτης προήλθε από τους ασθενείς που παρακολουθούνται στο Διαβητολογικό Κέντρο του Νοσοκομείου Παίδων «Π & Α Κυριακού» και η συλλογή του δείγματος γινόταν κατά τη διάρκεια της τριμηνιαίας παρακολούθησής τους.

Οι 3 ομάδες μελετήθηκαν ως προς τα επιμέρους χαρακτηριστικά του σάλιου και την επίπτωση της τερηδόνας, χρησιμοποιώντας το δείκτη DMFT. Για τις 2 ομάδες των ασθενών με Τ1ΣΔ μετρήθηκε επιπρόσθετα η γλυκοζυλιωμένη αιμοσφαιρίνη (HbA1c) για να υπολογιστεί το επίπεδο ρύθμισης του ΣΔ και καταγράφηκε ο χρόνος διάγνωσης του ΣΔ για να υπολογιστεί η διάρκεια της νόσου. Κοινωνικοοικονομικές παράμετροι, επίπεδο στοματικής υγιεινής, συχνότητα επίσκεψης στον οδοντίατρο και διατροφικές συνήθειες διερευνήθηκαν κατά την επιλογή του δείγματος ώστε οι

συμμετέχοντες να παρουσιάζουν μια κατά το δυνατόν ομοιογενή εικόνα ως προς αυτές τις παραμέτρους.

Μεταξύ των κριτηρίων συμμετοχής των ασθενών στη μελέτη ήταν: η απουσία από το ιατρικό ιστορικό άλλων νοσημάτων που επηρεάζουν τη ροή του σάλιου, η μη λήψη φαρμάκων που επηρεάζουν τη ροή του σάλιου, η μη λήψη αντιβιοτικών και αντιμικροβιακών φαρμάκων για 15 μέρες πριν την κλινική εξέταση, η μη χρήση μέσων στοματικής υγιεινής με αντιμικροβιακές ουσίες όπως χλωρεξιδίνη.

Η αξιολόγηση των ποιοτικών και ποσοτικών χαρακτηριστικών του σάλιου έγινε με βάση το σκεύασμα GC Saliva Check Buffer (3M ESPE). Στα χαρακτηριστικά που μελετήθηκαν περιλαμβάνονται η σύσταση, το pH σάλιου ηρεμίας και διέγερσης, η ροή σάλιου σε κατάσταση ηρεμίας και σε κατάσταση διέγερσης καθώς και η ρυθμιστική ικανότητα του σάλιου. Η σύσταση του σάλιου αξιολογήθηκε στις βαθμίδες ορώδες σάλιο χαμηλού ιξώδους, φυσαλιδώδες σάλιο αυξημένου ιξώδους και κολλώδες σάλιο αυξημένου ιξώδους. Ακολούθως, το κάτω χείλος στεγνώθηκε με γάζα και παρατηρήθηκε η δημιουργία σταγονιδίων σάλιου στα στόμια των ελασσόνων σιελογόνων αδένων του χείλους για να αξιολογηθεί η ροή σε κατάσταση ηρεμίας. Αξιολογήθηκαν στη συνέχεια το pH του σάλιου ηρεμίας, η ροή του σάλιου διέγερσης μετά από μάσηση κύβου παραφίνης και το pH και ρυθμιστική ικανότητα του σάλιου διέγερσης. Καταγράφηκαν επιπρόσθετα ο δείκτης πλάκας, ο δείκτης τερηδόνας DMFT καθώς και η υποκειμενική αίσθηση ξηρότητας του στόματος με τη συμπλήρωση ερωτηματολογίου αξιολόγησης της ξηροστομίας. Η στατιστική ανάλυση των δεδομένων έγινε με τις δοκιμασίες χ2 και Kruskal-Wallis σε επίπεδο στατιστικής σημαντικότητας $p<0.05$.

Η ροή και το pH σάλιου ηρεμίας, η σύσταση του σάλιου και η τερηδονική κατάσταση των ασθενών με μη ρυθμισμένο Τ1ΣΔ βρέθηκαν να διαφέρουν σε στατιστικά σημαντικό βαθμό ($p<0.05$) σε σύγκριση με τις τιμές των ασθενών με ρυθμισμένο Τ1ΣΔ, που παρουσίαζαν χαρακτηριστικά παρόμοια με αυτά των υγιών μαρτύρων. Παρά το ότι η ροή στο σάλιο ηρεμίας και διέγερσης δε φάνηκε να διαφέρει μεταξύ ρυθμισμένων και υγιών μαρτύρων, οι μεταβολικά ρυθμισμένοι ασθενείς με Τ1ΣΔ ανέφεραν ξηροστομία σε μεγαλύτερη συχνότητα από τους υγιείς, όπως και οι αρρύθμιστοι ασθενείς. Συμπερασματικά, η μεταβολική ρύθμιση των παιδιών και εφήβων με Τ1ΣΔ φάνηκε να επηρεάζει σημαντικά τα ποιοτικά και ποσοτικά χαρακτηριστικά του σάλιου και είναι απαραίτητη για τη διατήρηση της οδοντοστοματικής τους υγείας. Η αξιολόγηση των χαρακτηριστικών αυτών με τη βοήθεια ενός εύχρηστου Kit δίνει τη δυνατότητα στους κλινικούς κάθε ειδικότητας, χωρίς να απαιτείται ειδικός εξοπλισμός ή οδοντιατρικές γνώσεις, να εντοπίσουν εύκολα, γρήγορα και έγκαιρα παιδιά με αυξημένο κίνδυνο για εμφάνιση τερηδόνας και αντίστοιχα να τα ενεργοποιήσουν προς την κατεύθυνση της οδοντιατρικής φροντίδας και πρόληψης.

Το δεύτερο μέρος της μελέτης περιελάμβανε την ανάλυση της γονιδιακής έκφρασης του σάλιου, προκειμένου να εντοπιστούν τυχόν διαφορές στην πρωτεϊνική έκφραση μεταξύ υγιών και πασχόντων, και πραγματοποιήθηκε με την εφαρμογή των

δοκιμασιών υγρής χρωματογραφίας/ φασματογραφίας μάζας σε σειρά (liquid chromatography/tandem mass spectrometry) ώστε να διερευνηθεί το «πρωτεωμικό» προφίλ του σάλιου μεταξύ των διαφορετικών ομάδων ασθενών. Η δοκιμασία MRM εφαρμόστηκε στη συνέχεια για να επιβεβαιώσει τη διαφορετική έκφραση συγκεκριμένων πρωτεϊνικών μορίων, με βάση τα αποτελέσματα της χρωματογραφίας. 32 παιδιά και έφηβοι συμμετείχαν σε αυτό το τμήμα της μελέτης: 12 με αρρύθμιστο Τ1ΣΔ (G1) (HbA1c≥7.5%), 12 με καλή ρύθμιση του Τ1ΣΔ (G2) (HbA1c<7.5%) και 12 υγιείς μάρτυρες (Ctrl). Συνολικά, ταυτοποιήθηκαν και ποσοτικοποιήθηκαν 4877 πρωτεΐνες, με τη χρήση του Trans Proteomic Pipeline, λογισμικού ανάλυσης και επεξεργασίας των αποτελεσμάτων της φασματομετρίας. Η ταυτοποίηση των πρωτεϊνών έγινε με στάθμη εμπιστοσύνης 95%. 2031 πρωτεΐνες ήταν παρούσες σε ποσοστό μεγαλύτερο ή ίσο του 70% στο σύνολο της κάθε ομάδας. Για τη στατιστική ανάλυση των αποτελεσμάτων χρησιμοποιήθηκε το λογισμικό R και ο έλεγχος κανονικότητας κατανομής έγινε με το Kolmogorov-Smirnov Test. Διπλό κριτήριο στατιστικής σημαντικότητας εφαρμόστηκε κατά την ανάλυση αυτή: t test p-value και Log2ratio p-value <0.05. 33 πρωτεΐνες βρέθηκαν με διαφορετική έκφραση μεταξύ των ομάδων G1-Ctrl, 37 πρωτεΐνες μεταξύ των G2-Ctrl, και 61 πρωτεΐνες μεταξύ των G1-G2. Με βάση τα αποτελέσματα της μελέτης, το πρωτεωμικό προφίλ των συμμετεχόντων φάνηκε ικανό να μπορεί να διαχωρίσει τους ασθενείς ανάλογα με τη μεταβολική τους ρύθμιση. Το πρωτεωμικό προφίλ των ρυθμισμένων ασθενών βρέθηκε παρόμοιο με αυτό των υγιών και στατιστικά σημαντικά διαφορετικό από αυτό των αρρύθμιστων. Παράλληλα, βιολογικά μονοπάτια επιπλοκών που παρουσιάζονται στην ενήλικη ζωή εντοπίστηκαν ενεργοποιημένα ήδη από την παιδική ηλικία στους αρρύθμιστους ασθενείς. Τέλος, με την εφαρμογή ειδικού λογισμικού βιοπληροφορικής ανάλυσης στις πρωτεΐνες που βρέθηκαν να έχουν διαφορετική έκφραση μεταξύ των ομάδων, προτείνεται μια πιθανή προληπτική προσέγγιση που θα μπορούσε να αποτελέσει πεδίο μελλοντικής διερεύνησης.

Acknowledgements

This work is the end result of the collaboration among the department of Restorative Dentistry of Dental School of Athens (NKU), the Diabetic Centre of P&A Kyriakou Children's Hospital and the Biomedical Research Foundation of the Academy of Athens. During my Phd thesis, I have met many people without whom my experience would not have been the same. My acknowledgements to them pay tribute to their fundamental contribution to the completion of this doctoral study.

First of all, I am deeply indebted to Professor Afrodite Kakaboura for her support during this journey. Her valuable guidance, all these years, has inspired me both academically and personally.

I would like to extend my sincere gratitude to Dr Konstantinos Vougas for his time, his undivided attention and for expanding my knowledge in the field of Oral Biology. He has been my mentor during this demanding and challenging project and for that I am filled with appreciation.

I am also grateful to Professor Efthimia Kitraki and Professor Antonis Stamatakis for their insightful comments throughout my doctoral study. Similarly, I would like to thank the members of the Examining Committee, Professor Athanassios Armakolas and Dr Antonia Vlahou, as well as Dr Andriani Vazaiou-Gerasimidi, for their input to my PhD Thesis. Finally, I am grateful to Professor Christos Rachiotis for dedicating time and effort, whenever I sought for his advice and to Dr Jerome Zoidakis for his priceless contribution in the preparation of the manuscript for publication.

I address my gratitude to my supervisor, Professor Heleni Vastardis, for being my driving force during this demanding period. I could not have hoped for a more positive, supportive and meaningful guidance.

My family's continuous sustenance has been fundamental for every step I have taken. Especially, thank you Panagiotis for inspiring me to dream big.

This study was financially supported by "IKY Fellowships of Excellence for Postgraduate Studies in Greece – Siemens Programme". I additionally acknowledge the financial support from Onassis Foundation during the last year of my doctoral studies.

Figure 1: An overview of proteomics technologies

Figure 2: High-throughput proteomic techniques applied for various analyses

Figure 3: Schematic representation of the advanced proteomic pipeline

Figure 4: Salivary diagnostic applications in paediatrics

Figure 5: The Salivette (Sarstedt, Newton, NC) collector. The system contains a cotton pad that is placed in the mouth, the saliva saturated pad is returned to its carrier. The saliva sample is then recovered from the pad by centrifugation

Figure 6: Saliva-Check Buffer (GC) for chair-side evaluation of quality and quantity of saliva

Figure 7: The graphical abstract outlines the proteomic and bioinformatic analysis of saliva samples

Figure 8: a) Functional classification of these proteins revealed that Enzymes and Cytokines were the main functional groups of the salivary proteome. b) Clustering indicated that total proteomic profile is capable of distinguishing poorly controlled subjects from well controlled and healthy subjects. The latter ones present similarities, as expected

Figure 9: Comparisons among 3 groups and statistical analysis a) Among possible comparisons, G1vsCtrl and G1vsG2 yield proteins with high fold change and low p-values. Volcano plots present that the variance in G2vsCtrl is smaller than in the other two comparisons, indicating higher similarity between G2 and Ctrl subjects. b) All possible comparisons were performed among the 3 groups: (G1-Ctrl, G2-Ctrl, G1-G2). 33 proteins were found to be differentially expressed between G1-Ctrl, 37 between G2-Ctrl and 61 between G1-G2. c) Multiple Reaction Monitoring (MRM) was utilized in order to validate the relative quantitation obtained by the iTRAQ technology. For each group comparison, we selected the most relevant proteins, based on differential expression and clinical relevance. The proteins selected presented low p-value, high fold-change and were the most relevant to clinical pathways. In G1vsG2, 9 out of 12 proteins presented positive correlation between the iTRAQ and MRM quantitation. 9 out of 12 proteins presented positive correlation in iTRAQ and MRM quantitation in comparison G1vsCtrl as well, whereas in G2vsCtrl, 10 out of 15 presented positive correlation

Figure 10: PPI networks generated by STRING for the differentially expressed proteins identified in the three comparisons

Figure 11: The effect of diabetes on fibrin clot formation is presented with annotated differentially expressed proteins. In green downregulated proteins are shown. These proteins are inhibitors of fibrin clot formation. Thus, fibrin clot formation is activated in diabetes. (http://www.wikipathways.org/index.php/Pathway:WP558)

List of Publications

1. Saliva Proteomics Analysis Offers Insights on Type 1 Diabetes Pathology in a Pediatric Population.

Pappa E, Vastardis H, Mermelekas G, Gerasimidi-Vazeou A, Zoidakis J, Vougas K.

Front Physiol. 2018 Apr 26;9:444. doi: 10.3389/fphys.2018.00444.

2. Saliva in the "Omics" era: A promising tool in paediatrics.

Pappa E, Kousvelari E, Vastardis H.

Oral Dis. 2018 May 11. doi: 10.1111/odi.12886.

List of Presentations

1. “Chair-side saliva diagnostic tests: an evaluation tool for xerostomia and caries risk assessment of adolescents with type 1 diabetes.” 8th International Conference on Advanced Technologies & Treatments for Diabetes, February 2015, Paris, France

2. “Xerostomia And Caries Incidence In Relation To Metabolic Control In Children And Adolescents With Type 1 Diabetes.”, ICDM 2015: 17th International Conference on Diabetes and Metabolism, May 2015, Rome, Italy

3. “Cardiovascular Complications in Children with Type I Diabetes. A Proteomic Analysis” 4th International Conference on Prehypertension, Hypertension and Cardiometabolic Syndrome, 3-6 March 2016, Venice, Italy

4. “Association between metabolic control, salivary status and caries in children and adolescents with type 1 diabetes.” ORCA, July 2016, Athens, Greece

5. «Μεταβολές των χαρακτηριστικών του σάλιου σε συσχέτιση με τη μεταβολική ρύθμιση σε εφήβους με Τ1ΣΔ», 20ο Πανελλήνιο Συνέδριο Εσωτερικής Παθολογίας, Δεκέμβριος 2014, Αθήνα

6. «Το σάλιο ως καθρέφτης της γενικής υγείας του οργανισμού», 36^{ο} Πανελλήνιο Οδοντιατρικό Συνέδριο, Νοέμβριος 2016, Αθήνα

Chapter 1

Introduction

Diabetes mellitus currently constitutes a global epidemic, with type 1 diabetes being one of the most common chronic diseases of childhood. It has been a decade since a report in the Lancet had accurately foreseen that "if present trends continue, doubling of new cases of type 1 diabetes in European children younger than 5 years is predicted between 2005 and 2020, and prevalent cases younger than 15 years will rise by 70%. Adequate health-care resources to meet these children's needs should be available". (Patterson, Dahlquist, Gyurus, Green, & Soltesz, 2009) The need to improve the quality and efficacy of pediatric diabetes care and intervention is still urgent today. During the last 30 years in our country, diabetes has quadrupled and it is estimated that 8% - 9% of the population (800-900.000) suffers from the disease. (Kyriazis, Rekleiti, Beliotis, & Saridi, 2013) About 3-4 % does not know that they suffer from diabetes and as far as pediatric population is concerned, type 1 diabetes is growing by 3 % per year in children and adolescents. (Kyriazis, et al., 2013)

Proper regulation of type 1 diabetes is found to be directly related to the complications of the disease. (Harding, Pavkov, Magliano, Shaw, & Gregg, 2019) This is of outmost importance especially during adolescence when clinical data clearly show the difficulty in reaching and maintaining optimal glycemic control. (Sauder et al., 2019) (Kordonouri et al., 2014) From pre-puberty to young adulthood, less than 15% of the young patients manage to keep constant or reach HbA1c levels below 8%. (Dabadghao, Vidmar, & Cameron, 2001) This significant percentage of patients with prolonged hyperglycemia has direct clinical significance, since, contrary to earlier belief, puberty years do not protect against the risk of developing microvascular complications later in life. (Donaghue, Chiarelli, Trotta, Allgrove, & Dahl-Jorgensen, 2009) (Virk et al., 2016) Studies actually show that the sufficient glycemic control during adolescence lowers the risk of developing complications related to diabetes later in life, even if the level of control is not maintained afterwards. It appears that adolescence is a crucial period for a proper "programming" and for the establishment of lifelong positive health-related behaviors. (Demirel, Tepe, Kara, & Esen, 2013) (Donaghue et al., 2018)

Saliva, a biological fluid with popular diagnostic potential due to the exponential technological advances, offers an attractive alternative to blood samples, particularly in children and adolescents, where blood sample collection often reduces compliance to follow-up (Lima, Diniz, Moimaz, Sumida, & Okamoto, 2010; Yeh et al., 2010). Moreover, salivary diagnostics nowadays provide a cost-effective tool in monitoring oral and systemic health and disease in large populations, especially when repeated sampling is necessary (Lima, et al., 2010; Samaranayake, 2007; Yeh, et al., 2010).

This thesis focuses on the salivary characteristics of children and adolescents with type 1 diabetes in relation to the level of glycemic control, with the aim to investigate their oral health status and at a further level of analysis, explore the salivary proteome of this study group.

Diabetes mellitus

Definition, description and classification

“Diabetes is a group of metabolic diseases characterised by hyperglycemia resulting from defects in insulin secretion, insulin action, or both”. ("Diagnosis and classification of diabetes mellitus," 2014) The chronic hyperglycemia results to default in metabolism of fat, protein and carbohydrate and can long term lead to damage and failure of multiple organs ("Definition, diagnosis and classification od diabetes mellitus and its complications.," 1999). Eyes, kidneys, nerves, heart and blood vessels are affected when the disease is poorly controlled, and as a consequence, they present a number of severe complications.

The development of diabetes is the result of several pathogenic processes, which frequently coexist in the same patient and it is often unclear which one, if either alone, is the primary cause of hyperglycemia. These processes range from impairment of insulin secretion to defects and resistance in insulin action. The resulted hyperglycemia is associated with early symptoms such as polyuria, polydipsia, weight loss and frequently polyphagia and blurred vision. Growth deficiency and susceptibility to various infections may also be present as symptoms of the disease. When left uncontrolled, hyperglycemia may lead to acute, life-threatening events of ketoacidosis, or the nonketotic hyperosmolar syndrome. (Kerner & Bruckel, 2014)

According to recent dada from the WHO, 347 million people worldwide suffer from diabetes (Danaei G, 2011) and it is projected that by the year 2030 diabetes will be the 7th leading cause of death ("Global status report on noncommunicable diseases 2010. ," 2011). The most frequent medical implications of diabetes are retinopathy, neuropathy, peripheral neuropathy with high risk of foot ulcers and amputations, autonomic neuropathy, which can cause gastrointestinal, genitourinary, cardiovascular symptoms and also sexual dysfunction ("Report of the Expert Committee on the Diagnosis and Classification of Diabetes," 1997; Report of the Expert Committee on the Diagnosis and Classification of Diabetes," 2003).

The American Diabetes Association (ADA) classified diabetes mellitus into four general categories based on the aetiopathogenesis of the disease, as follows ("Diagnosis and classification of diabetes mellitus," 2014) (Table 1):

- Type 1 diabetes mellitus (previously known as insulin-dependent diabetes mellitus)
- Type 2 diabetes mellitus (previously known as non-insulin dependent diabetes mellitus)
- Other specific types of diabetes
- Gestational diabetes mellitus

Type 1 diabetes and type 2 diabetes are the most common categories to which the vast majority of cases of diabetes are comprised. Type 1 diabetes is caused by the absolute deficiency of insulin secretion while type 2 is the result of resistance to insulin action combined with an inadequate compensatory insulin secretory response. The later category, which is a much more prevalent one, is characterized by an asymptomatic period, during which a degree of hyperglycemia may be present for a long period of time without any clinical symptoms and long before the diabetes is detected.

Table 1: Aetiologic classification of diabetes ("Diagnosis and classification of diabetes mellitus," 2014)

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)
   A. Immune mediated
   B. Idiopathic

II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)

III. Other specific types
   A. Genetic defects of β-cell function
      1. MODY 3 (Chromosome 12, HNF-1α)
      2. MODY 1 (Chromosome 20, HNF-4α)
      3. MODY 2 (Chromosome 7, glucokinase)
      4. Other very rare forms of MODY (e.g., MODY 4: Chromosome 13, insulin promoter factor-1; MODY 6: Chromosome 2, *NeuroD1*; MODY 7: Chromosome 9, carboxyl ester lipase)
      5. Transient neonatal diabetes (most commonly ZAC/HYAMI imprinting defect on 6q24)
      6. Permanent neonatal diabetes (most commonly KCNJ11 gene encoding Kir6.2 subunit of β-cell K_{ATP} channel)
      7. Mitochondrial DNA
      8. Others
   B. Genetic defects in insulin action
      1. Type A insulin resistance
      2. Leprechaunism
      3. Rabson-Mendenhall syndrome
      4. Lipoatrophic diabetes
      5. Others
   C. Diseases of the exocrine pancreas
      1. Pancreatitis
      2. Trauma/pancreatectomy
      3. Neoplasia
      4. Cystic fibrosis
      5. Hemochromatosis
      6. Fibrocalculous pancreatopathy
      7. Others
   D. Endocrinopathies
      1. Acromegaly
      2. Cushing's syndrome
      3. Glucagonoma
      4. Pheochromocytoma
      5. Hyperthyroidism
      6. Somatostatinoma
      7. Aldosteronoma
      8. Others
   E. Drug or chemical induced
      1. Vacor
      2. Pentamidine
      3. Nicotinic acid
      4. Glucocorticoids
      5. Thyroid hormone
      6. Diazoxide
      7. β-Adrenergic agonists
      8. Thiazides
      9. Dilantin
      10. γ-Interferon
      11. Others
   F. Infections
      1. Congenital rubella
      2. Cytomegalovirus
      3. Others
   G. Uncommon forms of immune-mediated diabetes
      1. Stiff-man syndrome
      2. Anti-insulin receptor antibodies
      3. Others
   H. Other genetic syndromes sometimes associated with diabetes
      1. Down syndrome
      2. Klinefelter syndrome
      3. Turner syndrome
      4. Wolfram syndrome
      5. Friedreich ataxia
      6. Huntington chorea
      7. Laurence-Moon-Biedl syndrome
      8. Myotonic dystrophy
      9. Porphyria
      10. Prader-Willi syndrome
      11. Others

IV. Gestational diabetes mellitus

Patients with any form of diabetes may require insulin treatment at some stage of their disease. Such use of insulin does not, of itself, classify the patient.

Type 1 Diabetes

This category of diabetes, which accounts for only 5-10% of those with diabetes, was previously described by the terms insulin-dependent or juvenile-onset diabetes and is precipitated by an immune-mediated destruction of β-cells of the pancreas. It is frequently diagnosed in childhood, but the disease can also develop during adolescence and in adulthood. The immune destruction of pancreatic β-cells located in the islets of Langerhans, is characterized by markers such as islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD65) and autoantibodies to the tyrosine phosphatases IA-2 and IA-2β. More than 90% of individuals with newly diagnosed type 1 diabetes have one or more of these autoantibodies at disease onset. They are present months to years before the symptomatic onset of the disease which, apart from the diagnostic value, renders them capable to identify people with increased risk for developing type 1 diabetes. (Atkinson, Eisenbarth, & Michels, 2014)

The cause of type 1 diabetes still remains unknown. Based on the observation of specific autoantibodies present, type 1 diabetes is considered to be an autoimmune disease. The β-cell destruction occurs in individuals at genetic risk, a process which is believed to be triggered by one or several environmental factors. (Canivell & Gomis, 2014) Studies have well documented that heredity is causally linked with T1D, particularly the HLA class II genes, of which the haplotype HLA-DR3 and DR4 present the greatest risk. (Concannon, Rich, & Nepom, 2009) Moreover, genome studies have associated more than 40 genes with high type 1 diabetes risk. (Concannon et al., 2009; Rich et al., 2009) These can be subdivided in three main categories: immune function, insulin expression and β-cell function.

Genetic susceptibility might also influence responses to environmental stimuli or physiological pathways. Contrary to previous belief, genetic predisposition is a clear but not the sole prerequisite for developing type 1 diabetes. In fact, numerous environmental influences have been suggested to affect the incidence and epidemiological characteristics of type 1 diabetes. Infant and adolescent diet (early introduction of bovine milk proteins into the diet, daily intake of nitrate and sources of drinking water), vitamin D depletion and vitamin D pathway components as well as gut microbiome receive the most focus in an attempt to clarify the pathogenetic mechanisms of the disease. (Atkinson, et al., 2014) Moreover, several studies have investigated the role of viruses as causal mediators in type 1 diabetes. (Hyoty, 2016) In this direction, enterovirus infection has been found to be significantly associated with the occurrence of islet autoantibodies and the onset of clinical type 1 diabetes. (Yeung, Rawlinson, & Craig, 2011)

Living with type 1 diabetes remains a challenge, especially for a child and the whole family, even when updated treatment protocols and continuous medical monitoring are applied. (Hagger, Hendrieckx, Sturt, Skinner, & Speight, 2016) No interventions so far have proven to be capable of ceasing the development of the disease, thus patients are in a lifelong need for exogenous insulin therapy with close medical

supervision, unless the beta cells are replaced by islet or pancreas transplantation. In order to keep blood glucose levels into normal ranges, patients with type 1 diabetes depend on multiple daily insulin injections or an insulin pump (continuous subcutaneous insulin infusion). (Martinez et al., 2018) They also need to self-monitor their blood glucose levels several times daily and at the same time keep a balance between their energy intake and physical activity. Even when optimum treatment strategies are applied, the mortality of patients with type 1 diabetes is three to four times higher than among the general population (Gagnum et al., 2015)

Epidemiology of type 1 diabetes

Diabetes mellitus is one of the largest global emergencies of the 21st century. 425 million people have diabetes in the world and more than 58 million people in Europe; by 2045 this number is expected to rise to 66.7 million. According to the International Diabetes Federation, there were 578.300 cases of diabetes in Greece in 2017. ("IDF Diabetes Atlas, 8th edition," 2017) A recent nation-wide real-world data analysis on medication prescribed diabetes showed that the current prevalence in Greece is 7.0 %, ranging from 0.08% in children and adolescents to 8.2% in adults and 30.3% in those over 75 years old. (Liatis et al., 2016)

Type 1 diabetes presents a peak in presentation between 5-7 years of age or near puberty. The incidence of T1D is also characterized by seasonal changes; more cases are diagnosed in autumn and winter while those who are born in the spring have higher chances of having T1D. (Kahn et al., 2009) This seasonal synchronization could support the theoretical concept of the environmental influence on the genetic predisposition of the T1D patients. (Atkinson, et al., 2014)

Every year, more and more people are diagnosed with this disease and are confronted with life-threatening complications. Type 1 diabetes may be less common than type 2 diabetes, however the newly diagnosed patients are increasing by 3% every year, which is continually posing a significant economic burden in the country' s undermined economy. Prevalence of type 1 diabetes in Greece reaches 0.24% and more than half cases are diagnosed after 14 years of age. In children <15 years of age, the prevalence of type 1 diabetes was 0.08%, while in those ≥15 years it was 0.27%. (Liatis, et al., 2016) Whereas most autoimmune disorders disproportionately affect women, type 1 diabetes is slightly more common in boys and men. ("Diagnosis and classification of diabetes mellitus," 2014) If incidence rates continue to increase with such pace, global incidence is predicted to be double over the next decade. Alarmingly, in Europe the most significant increases are observed in children younger than 5 years of age. (Harjutsalo, Sjoberg, & Tuomilehto, 2008)

Diagnostic criteria for diabetes mellitus

Historically, the diagnosis of diabetes has been based on glucose calculation and has included fasting blood glucose higher than 126 mg/dL (7 mmol/L), any blood glucose of 200 mg/dL (11.1 mmol/L) or higher accompanied with symptoms of hyperglycaemia or an abnormal 2-h oral glucose-tolerance test. ("Standards of

medical care in diabetes--2012," 2012) Since 2009, the guidelines of the American Diabetes Association have been modified to additionally include glycated haemoglobin (HbA1c) of 6.5% or higher (Table 2). HbA1c is a widely used marker of chronic glycemia and is used as a reflection of the average blood glycose levels over a 2- to 3-month period of time. The measurement of HbA1c plays a significant role in the management of the patient with diabetes; it is found to have a close correlation with both microvascular and macrovascular complications and is commonly considered the standard biomarker for the evaluation of glycemic control in these patients. The diagnostic cut point of 6.5% was established after consideration of epidemiological evidence by an international expert committee and ADA furthermore affirmed that decision. ("Diagnosis and classification of diabetes mellitus," 2014) For glycemic control, a target HbA1c level below 7.5% is recommended in pediatric diabetes care. (M. Rewers et al., 2009)

Table 2: Criteria for the diagnosis of diabetes ("Diagnosis and classification of diabetes mellitus," 2014)

A1C ≥6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
FPG ≥126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
Two-hour plasma glucose ≥200 mg/dL (11.1 mmol/L) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L).

*In the absence of unequivocal hyperglycemia, criteria 1–3 should be confirmed by repeat testing.

Management of type 1 diabetes

The elements of chronic care model for the management of diabetes include decision support, clinical information systems, self-management education and delivery system redesign. A multifaceted approach is a prerequisite for optimized outcomes, when managing such a complex disease. Despite the alarming reports, the major obstacle to the implementation of effective interventions globally is the lack of supportive health-care systems. (Lebovitz et al., 2006)

Diabetes management in modern countries often includes use of insulin analogues and mechanical technologies, such as insulin pumps and continuous glucose monitors for improved treatment of type 1 disease. (Hirsch, 2009) Technological advancements are used in order to achieve optimum glycemic control and fewer

hypoglycaemic incidences. Point-of-care HbA1c measurements, self-monitoring blood-glucose reports and real-time continuous glucose monitors are used for that purpose. (Atkinson, et al., 2014)

Diabetes complications and hyperglycemia

Chronic hyperglycemia is the critical factor for the development and progression of diabetic complications. Cardiovascular disease (CVD), chronic inflammation, nephropathy, retinopathy and peripheral neuropathy are the most common complications of the disease. The risk of death from CVD in adults with poorly-controlled type 1 diabetes is ten times greater than in the general population (Katz, Giani, & Laffel, 2015). While optimal glycemic control is crucial for the reduction of CVD risk, adolescents and young adults demonstrate higher Hb1Ac levels compared to other age groups, thus they are at high risk for early complications (Katz, et al., 2015). According to the Epidemiology of Diabetes Interventions and Complications, intensive diabetes treatment reduced the risk of cardiovascular events by 42% in patients with type 1 diabetes compared with conventional treatment. (Nathan et al., 2005)

One of the most important mechanisms in cardiovascular complications is endothelial dysfunction, which is associated with the onset of diabetes. It is still unclear whether endothelial dysfunction is causally linked with diabetes or with other factors related to the disease. Various mechanisms could explain the diabetes related endothelial dysfunction, as follows:

- Hyperglycemia results in an increase of intracellular glucose concentration within endothelial cells (ECs) causing structural changes in them, in the form of increased deposition of collagen and fibronectin. It also decreases endothelial proliferation, NO production and increased apoptosis. (Baumgartner-Parzer et al., 1995) (Salt, Morrow, Brandie, Connell, & Petrie, 2003)

- Hyperglycemia alters EC function indirectly by the alteration of growth and vascular factors in other cells. (Kofler, Nickel, & Weis, 2005)

- Metabolic alterations such as dyslipidaemia, hypertension. Inflammation, may also cause endothelial dysfunction. (Cacicedo, Yagihashi, Keaney, Ruderman, & Ido, 2004)

Hyperglycaemia, through various biological pathways as stated above, leads to endothelial and vascular dysfunction. Accordingly, the risk for microvascular complications, including retinopathy, nephropathy, and neuropathy, decreases with intensive insulin therapy. Several clinical trials have advanced the prediction and prevention of microvascular complications, which include retinopathy, nephropathy, and neuropathy (Table 3) (Atkinson, et al., 2014) The molecular mechanisms influencing the severity of diabetic complications are still not fully understood in the early stages of the disease. It is shown that the severity of complications is modified by

genetic factors, as many diabetic patients do not develop complications even when their glycaemic control is not optimal. (Rosenstock et al., 1998)

Table 3 : Studies on prediction and prevention of complications associated with type 1 diabetes (Atkinson, et al., 2014)

	Complications assessed	**Main findings**
Diabetes Control and Complications Trial (DCCT)/Pittsburgh Epidemiology of Diabetes Complications study (2009)	Cardiovascular disease, nephropathy, retinopathy	The frequencies of serious complications in patients with type 1 diabetes, especially when treated intensively, are lower than those reported historically
Finnish Diabetic Nephropathy (FinnDiane) Study (2009)	Cardiovascular disease, nephropathy	In patients with type 1 diabetes, variations in glycated haemoglobin concentration predicted the incidence of microalbuminuria and progression to renal disease, and incidence of cardiovascular disease
DCCT/ Epidemiology of Diabetes Interventions and Complications (EDIC) study (2011)	Nephropathy	In patients with type 1 diabetes and persistent microalbuminuria, intensive glycaemic control, blood pressure control, and favourable lipid panels lead to fewer long-term renal Complications
FinnDiane (2009)	Nephropathy	An independent and graded association exists between the presence and severity of kidney disease and premature mortality in type 1 diabetes
Genetics of Diabetes in Kidney Collection (2009)	Nephropathy	Identifi ed genes associated with susceptibility to diabetic nephropathy, near the *FRMD3* and *CARS* loci
Swedish Renal Registry (2010)	Nephropathy	Substantial differences in risk for nephropathy in male versus female patients with type 1 diabetes, with age at diagnosis an important factor (early diagnosis lowers risk)
DCCT/EDIC (2009)	Autonomic neuropathy	Patients given intensive insulin therapy had less cardiac autonomic neuropathy than those who received conventional treatment
Acetyl-L-carnitine Clinical Trials (2009)	Neuropathy	Raised triglycerides correlate with progression of diabetic neuropathy
DCCT/EDIC (2008)	Retinopathy	Intensive insulin therapy (*vs* conventional therapy) reduces development and progression of diabetic retinopathy, with a treatment-related difference (metabolic memory) continuing for at least 10 years
DIabetic REtinopathy Candesartan Trials (DIRECT; 2008)	Retinopathy	The angiotensin receptor blocker, candesartan, reduces retinopathy development but does not stop retinopathy progression

Inflammation, AGEs and diabetes

Inflammation is the complex biological response of vascular tissues to harmful stimuli, such as pathogens or irritants. It is a protective mechanism by the organism to eliminate the injurious stimuli and begin the healing process of the tissue.

There are two fundamental types of inflammation: acute and chronic inflammation. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. A cascade of biochemical events spreads and develops the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells which are present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. (Libby, 2007)

Chemical mediators play a significant role during an inflammatory process. These inflammatory mediators come from plasma proteins or cells including mast cells, platelets, neutrophils and monocytes/macrophages, B and T cell lymphocytes. They are triggered by bacterial products or host proteins. Chemical mediators bind to specific receptors on target cells and can increase vascular permeability and neutrophil chemotaxis, stimulate smooth muscle contraction, have direct enzymatic activity, induce pain or mediate oxidative damage. Examples of chemical mediators include prostaglandins and cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins (IL). (Libby, 2007)

The basic inflammatory response favors a catabolic state and suppresses anabolic pathways, such as the highly conserved and powerful insulin signalling pathway. (Bayes et al., 2005) In 1998, a hypothesis was proposed according to which long-term innate immune system activation, resulting in chronic inflammation, elicited disease instead of repair, leading to the development of type 2 diabetes (Pickup & Crook, 1998). Lately, numerous studies have shown that low-grade inflammation is associated with the risk of developing type 2 diabetes. (Duncan & Schmidt, 2006; van Greevenbroek, Schalkwijk, & Stehouwer, 2013) Chronic subclinical inflammation is nowadays considered to be a part of the insulin resistance syndrome and is strongly related to features of the metabolic syndrome. (Festa et al., 2000) The mechanisms by which chronic inflammation can evoke type 2 diabetes are not clear. However, adipose tissue is known to be able to synthesize and release the main pro-inflammatory cytokines, tumour necrosis factor-alpha (TNF-a), interleukin-1 (IL-1) and interleukin-6 (IL-6), and that inflammatory markers are associated with body fat mass. (Juhan-Vague, Alessi, Mavri, & Morange, 2003) Pro-inflammatory cytokines and acute phase reactants are involved in multiple metabolic pathways relevant to insulin resistance, including insulin regulation, reactive oxygen species, lipoprotein lipase action and adipocyte function. Therefore, activated innate immunity and inflammation are relevant factors in the pathogenesis of diabetes, with numerous data suggesting that type 2 diabetes and inflammation are significantly associated. (Crook, 2004) (Pickup & Crook, 1998)

In type 1 diabetes, complications are evidently associated with chronic inflammatory processes. Deregulation of pro-inflammatory pathways and distinct elevation of inflammation patterns are observed in T1D patients. Lately, it is also suggested that in addition to autoimmune destruction of insulin-producing cells, there might also be inflammation-induced insulin resistance, as a pathogenetic process of the disease. (Koulmanda et al., 2007; Purohit et al., 2018) In fact, according to Bending et al., the pathological process in this complex T cell-mediated autoimmune disease is also regulated by inflammation. The β-cell's response to stress and inflammation is actually the critical factor in predicting disease outcome and that, immunologically, creates a delicate balance between regulation and inflammation at the site of islet infiltration. (Bending, Zaccone, & Cooke, 2012)

Diabetes lowers the host's resistance to infections and to impair wound healing. Insulin is essential for the entry of glycose into cells. It also provides a source of energy for the uptake of amino acids to synthesise proteins and for the inhibition of adipose tissue lipolysis. Insulin deficiency disturbs the basic cell functions of the body. For example, first-line host defence against microbes is severely affected; PMN (polymorphonuclear) cell function is impaired with abnormalities of adherence, chemotaxis, phagocytosis and intracellular killing. The main component of extracellular matrix, collagen, undergoes changes such as decreased synthesis, increased degradation of newly synthesised and decreased solubility of mature collagen. Additionally, hyperglycaemia is known to increase blood viscosity, reduce erythrocyte deformability and increase platelet aggregation, causing blood flow abnormalities and the release of serotonin and lysosomal enzymes. (Casqueiro & Alves, 2012; K. Karjalainen, 2000)

Lately, non-enzymatic glycosylation has been on the spotlight as a critical pathophysiologic event behind all these hyperglycaemia-related modifications and in the pathophysiology of diabetic complications. Proteins and lipids exposed to sugars go through reactions which are not enzyme-dependent, and generation of reversible Schiff bases or Amadori products take place. Later, through further molecular processes, irreversible advanced glycosylation end products (AGEs) are produced. This mechanism is also present during normal ageing, but in diabetes their formation is accelerated to an extent related to the level and duration of hyperglycaemia. (Vlassara, 1997)

The potential pathophysiological significance of AGEs is associated with their accumulation in plasma, cells and tissues and their contribution to the formation of cross-links, generation of reactive oxygen intermediates and interactions with particular receptors on cellular surfaces. (Vlassara, 1996) AGEs have direct effects on the host response by affecting tissue structures, for example by increasing collagen cross-links, which is followed by alterations in collagen solubility and turnover (Monnier, Glomb, Elgawish, & Sell, 1996). Thickening of basement membranes is partly due to glycosylation of membrane proteins or entrapment of glycosylated serum proteins into basement membranes. Specific cell-surface receptors for the recognition of AGEs were first found on mononuclear phagocytes, and AGEs were observed to attract and retain mononuclear phagocytes. (Schmidt et al., 1993) These receptors have also been identified on lymphocytes, endothelial cells and smooth muscle cells as well as on other cellular systems that participate in both normal tissue remodelling and tissue damage. AGEs are bound to the specific cell surface receptors for AGEs, within which family of receptors RAGE is well defined and resembles macrophage scavenger receptors. This interaction causes oxidant stress of the target cells, stimulating production of different patterns of cytokines and growth factors, depending on the type of cells involved. This extra formation of growth factors and cytokines plays a key role in both micro- and macrovascular modifications. (Hocine et al., 2015)

AGEs appear to induce reactive oxygen intermediates and the interaction between AGE and RAGE further generates production of intra- and extracellular oxidants.

Oxidative modifications of lipoproteins, in turn, accelerate atherogenesis. (Witztum & Horkko, 1997) Free oxygen radicals cause tissue destruction directly and exaggerate the inflammation related tissue destruction because activated monocytes produce pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α. (Schmidt, et al., 1993) Conclusively, it is clear that AGEs can interact with cell functions, tissue remodelling and inflammatory reactions in several different ways. Hyperglycaemia, either directly or through AGE formation, causes various structural and functional modifications of cells as well as quantitative and qualitative alterations of the extracellular matrix, which may all alter tissue homeostasis and modify the host response even in periodontal and other oral tissues.

References

Atkinson, M. A., Eisenbarth, G. S., & Michels, A. W. (2014). Type 1 diabetes. *Lancet, 383*(9911), 69-82. doi: S0140-6736(13)60591-7 [pii]
10.1016/S0140-6736(13)60591-7

Baumgartner-Parzer, S. M., Wagner, L., Pettermann, M., Grillari, J., Gessl, A., & Waldhausl, W. (1995). High-glucose--triggered apoptosis in cultured endothelial cells. *Diabetes, 44*(11), 1323-1327.

Bayes, B., Granada, M. L., Lauzurica, R., Pastor, M. C., Navarro, M., Bonet, J., et al. (2005). Effect of low doses of atorvastatin on adiponectin, glucose homeostasis, and clinical inflammatory markers in kidney transplant recipients. *Transplant Proc, 37*(9), 3808-3812. doi: S0041-1345(05)00909-7 [pii]
10.1016/j.transproceed.2005.08.064

Bending, D., Zaccone, P., & Cooke, A. (2012). Inflammation and type one diabetes. *Int Immunol, 24*(6), 339-346. doi: dxs049 [pii]
10.1093/intimm/dxs049

Cacicedo, J. M., Yagihashi, N., Keaney, J. F., Jr., Ruderman, N. B., & Ido, Y. (2004). AMPK inhibits fatty acid-induced increases in NF-kappaB transactivation in cultured human umbilical vein endothelial cells. *Biochem Biophys Res Commun, 324*(4), 1204-1209. doi: S0006-291X(04)02245-4 [pii]
10.1016/j.bbrc.2004.09.177

Canivell, S., & Gomis, R. (2014). Diagnosis and classification of autoimmune diabetes mellitus. *Autoimmun Rev, 13*(4-5), 403-407. doi: S1568-9972(14)00032-9 [pii]
10.1016/j.autrev.2014.01.020

Casqueiro, J., & Alves, C. (2012). Infections in patients with diabetes mellitus: A review of pathogenesis. *Indian J Endocrinol Metab, 16 Suppl 1*, S27-36. doi: 10.4103/2230-8210.94253
IJEM-16-27 [pii]

Concannon, P., Chen, W. M., Julier, C., Morahan, G., Akolkar, B., Erlich, H. A., et al. (2009). Genome-wide scan for linkage to type 1 diabetes in 2,496 multiplex families from the Type 1 Diabetes Genetics Consortium. *Diabetes, 58*(4), 1018-1022. doi: db08-1551 [pii]
10.2337/db08-1551

Concannon, P., Rich, S. S., & Nepom, G. T. (2009). Genetics of type 1A diabetes. *N Engl J Med, 360*(16), 1646-1654. doi: 360/16/1646 [pii]
10.1056/NEJMra0808284
Crook, M. (2004). Type 2 diabetes mellitus: a disease of the innate immune system? An update. *Diabet Med, 21*(3), 203-207. doi: 1030 [pii]
Dabadghao, P., Vidmar, S., & Cameron, F. J. (2001). Deteriorating diabetic control through adolescence-do the origins lie in childhood? *Diabet Med, 18*(11), 889-894. doi: 593 [pii]
Danaei G, F. M., Lu Y, Singh GM, Cowan MJ, Paciorek CJ et al. (2011). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet, 378*(9785), 31-40.
Definition, diagnosis and classification od diabetes mellitus and its complications. (1999). *(WHO/NCD/NCS/99.2).*
Demirel, F., Tepe, D., Kara, O., & Esen, I. (2013). Microvascular complications in adolescents with type 1 diabetes mellitus. *J Clin Res Pediatr Endocrinol, 5*(3), 145-149. doi: 10.4274/Jcrpe.994
Diagnosis and classification of diabetes mellitus. (2014). *Diabetes Care, 37 Suppl 1*, S81-90. doi: 37/Supplement_1/S81 [pii]
10.2337/dc14-S081
Donaghue, K. C., Chiarelli, F., Trotta, D., Allgrove, J., & Dahl-Jorgensen, K. (2009). Microvascular and macrovascular complications associated with diabetes in children and adolescents. *Pediatr Diabetes, 10 Suppl 12*, 195-203. doi: PDI576 [pii]
10.1111/j.1399-5448.2009.00576.x
Donaghue, K. C., Marcovecchio, M. L., Wadwa, R. P., Chew, E. Y., Wong, T. Y., Calliari, L. E., et al. (2018). ISPAD Clinical Practice Consensus Guidelines 2018: Microvascular and macrovascular complications in children and adolescents. *Pediatr Diabetes, 19 Suppl 27*, 262-274. doi: 10.1111/pedi.12742
Duncan, B. B., & Schmidt, M. I. (2006). The epidemiology of low-grade chronic systemic inflammation and type 2 diabetes. *Diabetes Technol Ther, 8*(1), 7-17. doi: 10.1089/dia.2006.8.7
Festa, A., D'Agostino, R., Jr., Howard, G., Mykkanen, L., Tracy, R. P., & Haffner, S. M. (2000). Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). *Circulation, 102*(1), 42-47.
Gagnum, V., Stene, L. C., Sandvik, L., Fagerland, M. W., Njolstad, P. R., Joner, G., et al. (2015). All-cause mortality in a nationwide cohort of childhood-onset diabetes in Norway 1973-2013. *Diabetologia, 58*(8), 1779-1786. doi: 10.1007/s00125-015-3623-7
Global status report on noncommunicable diseases 2010. . (2011).
Hagger, V., Hendrieckx, C., Sturt, J., Skinner, T. C., & Speight, J. (2016). Diabetes Distress Among Adolescents with Type 1 Diabetes: a Systematic Review. *Curr Diab Rep, 16*(1), 9. doi: 10.1007/s11892-015-0694-2
10.1007/s11892-015-0694-2 [pii]

Harding, J. L., Pavkov, M. E., Magliano, D. J., Shaw, J. E., & Gregg, E. W. (2019). Global trends in diabetes complications: a review of current evidence. *Diabetologia, 62*(1), 3-16. doi: 10.1007/s00125-018-4711-2
10.1007/s00125-018-4711-2 [pii]
Harjutsalo, V., Sjoberg, L., & Tuomilehto, J. (2008). Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. *Lancet, 371*(9626), 1777-1782. doi: S0140-6736(08)60765-5 [pii]
10.1016/S0140-6736(08)60765-5
Hirsch, I. B. (2009). Clinical review: Realistic expectations and practical use of continuous glucose monitoring for the endocrinologist. *J Clin Endocrinol Metab, 94*(7), 2232-2238. doi: jc.2008-2625 [pii]
10.1210/jc.2008-2625
Hocine, A., Belmokhtar, K., Bauley, K., Jaisson, S., Gaha, K., Oubaya, N., et al. (2015). Serum and Tissue Accumulation of Advanced Glycation End-Products Correlates with Vascular Changes. *Perit Dial Int, 35*(5), 592-594. doi: 35/5/592 [pii]
10.3747/pdi.2013.00338
Hyoty, H. (2016). Viruses in type 1 diabetes. *Pediatr Diabetes, 17 Suppl 22*, 56-64. doi: 10.1111/pedi.12370
IDF Diabetes Atlas, 8th edition. (2017).
Juhan-Vague, I., Alessi, M. C., Mavri, A., & Morange, P. E. (2003). Plasminogen activator inhibitor-1, inflammation, obesity, insulin resistance and vascular risk. *J Thromb Haemost, 1*(7), 1575-1579. doi: 279 [pii]
Kahn, H. S., Morgan, T. M., Case, L. D., Dabelea, D., Mayer-Davis, E. J., Lawrence, J. M., et al. (2009). Association of type 1 diabetes with month of birth among U.S. youth: The SEARCH for Diabetes in Youth Study. *Diabetes Care, 32*(11), 2010-2015. doi: dc09-0891 [pii]
10.2337/dc09-0891
Karjalainen, K. (2000). Periodontal diseases, dental caries, and saliva in relation to
clinical characteristics of type 1 diabetes. *Acta Univ. Oul. D*(Academic Dissertation).
Karjalainen, K. M., Knuuttila, M. L., & Kaar, M. L. (1996). Salivary factors in children and adolescents with insulin-dependent diabetes mellitus. *Pediatr Dent, 18*(4), 306-311.
Katz, M., Giani, E., & Laffel, L. (2015). Challenges and Opportunities in the Management of Cardiovascular Risk Factors in Youth With Type 1 Diabetes: Lifestyle and Beyond. *Curr Diab Rep, 15*(12), 119. doi: 10.1007/s11892-015-0692-4
10.1007/s11892-015-0692-4 [pii]
Kerner, W., & Bruckel, J. (2014). Definition, classification and diagnosis of diabetes mellitus. *Exp Clin Endocrinol Diabetes, 122*(7), 384-386. doi: 10.1055/s-0034-1366278
Kofler, S., Nickel, T., & Weis, M. (2005). Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation. *Clin Sci (Lond), 108*(3), 205-213. doi: CS20040174 [pii]
10.1042/CS20040174
Kordonouri, O., Klingensmith, G., Knip, M., Holl, R. W., Aanstoot, H. J., Menon, P. S., et al. (2014). ISPAD Clinical Practice Consensus Guidelines 2014. Other

complications and diabetes-associated conditions in children and adolescents. *Pediatr Diabetes, 15 Suppl 20*, 270-278. doi: 10.1111/pedi.12183

Koulmanda, M., Budo, E., Bonner-Weir, S., Qipo, A., Putheti, P., Degauque, N., et al. (2007). Modification of adverse inflammation is required to cure new-onset type 1 diabetic hosts. *Proc Natl Acad Sci U S A, 104*(32), 13074-13079. doi: 0705863104 [pii]

10.1073/pnas.0705863104

Kyriazis, I., Rekleiti, M., Beliotis, E., & Saridi, M. (2013). Diabetes in Greece- A recent approach. *Ελληνικό Περιοδικό της Νοσηλευτικής Επιστήμης, 6*(2), 60-63.

Lebovitz, H. E., Austin, M. M., Blonde, L., Davidson, J. A., Del Prato, S., Gavin, J. R., 3rd, et al. (2006). ACE/AACE consensus conference on the implementation of outpatient management of diabetes mellitus: consensus conference recommendations. *Endocr Pract, 12 Suppl 1*, 6-12. doi: 1FHJ9E5J6JND69FJ [pii]

10.4158/EP.12.S1.6

Liatis, S., Dafoulas, G. E., Kani, C., Politi, A., Litsa, P., Sfikakis, P. P., et al. (2016). The prevalence and treatment patterns of diabetes in the Greek population based on real-world data from the nation-wide prescription database. *Diabetes Res Clin Pract, 118*, 162-167. doi: S0168-8227(16)30168-1 [pii]

10.1016/j.diabres.2016.06.018

Libby, P. (2007). Inflammatory mechanisms: the molecular basis of inflammation and disease. *Nutr Rev, 65*(12 Pt 2), S140-146.

Lima, D. P., Diniz, D. G., Moimaz, S. A., Sumida, D. H., & Okamoto, A. C. (2010). Saliva: reflection of the body. *Int J Infect Dis, 14*(3), e184-188. doi: S1201-9712(09)00203-3 [pii]

10.1016/j.ijid.2009.04.022

Martinez, K., Frazer, S. F., Dempster, M., Hamill, A., Fleming, H., & McCorry, N. K. (2018). Psychological factors associated with diabetes self-management among adolescents with Type 1 diabetes: A systematic review. *J Health Psychol, 23*(13), 1749-1765. doi: 1359105316669580 [pii]

10.1177/1359105316669580

Monnier, V. M., Glomb, M., Elgawish, A., & Sell, D. R. (1996). The mechanism of collagen cross-linking in diabetes: a puzzle nearing resolution. *Diabetes, 45 Suppl 3*, S67-72.

Nathan, D. M., Cleary, P. A., Backlund, J. Y., Genuth, S. M., Lachin, J. M., Orchard, T. J., et al. (2005). Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *N Engl J Med, 353*(25), 2643-2653. doi: 353/25/2643 [pii]

10.1056/NEJMoa052187

Patterson, C. C., Dahlquist, G. G., Gyurus, E., Green, A., & Soltesz, G. (2009). Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. *Lancet, 373*(9680), 2027-2033. doi: S0140-6736(09)60568-7 [pii]

10.1016/S0140-6736(09)60568-7

Pickup, J. C., & Crook, M. A. (1998). Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia, 41*(10), 1241-1248. doi: 10.1007/s001250051058

Purohit, S., Sharma, A., Zhi, W., Bai, S., Hopkins, D., Steed, L., et al. (2018). Proteins of TNF-alpha and IL6 Pathways Are Elevated in Serum of Type-1 Diabetes Patients with Microalbuminuria. *Front Immunol, 9*, 154. doi: 10.3389/fimmu.2018.00154

Report of the Expert Committee on the Diagnosis and Classification of Diabetes. (1997). *Diabetes Care* (20), 1183-1197.

Report of the Expert Committee on the Diagnosis and Classification of Diabetes. (2003). *Diabetes Care* (26), 3160-3167.

Rewers, M., Pihoker, C., Donaghue, K., Hanas, R., Swift, P., & Klingensmith, G. J. (2009). Assessment and monitoring of glycemic control in children and adolescents with diabetes. *Pediatr Diabetes, 10 Suppl 12*, 71-81. doi: PDI582 [pii]

10.1111/j.1399-5448.2009.00582.x

Rich, S. S., Akolkar, B., Concannon, P., Erlich, H., Hilner, J. E., Julier, C., et al. (2009). Overview of the Type I Diabetes Genetics Consortium. *Genes Immun, 10 Suppl 1*, S1-4. doi: gene200984 [pii]

10.1038/gene.2009.84

Rosenstock, J., Brown, A., Fischer, J., Jain, A., Littlejohn, T., Nadeau, D., et al. (1998). Efficacy and safety of acarbose in metformin-treated patients with type 2 diabetes. *Diabetes Care, 21*(12), 2050-2055.

Salt, I. P., Morrow, V. A., Brandie, F. M., Connell, J. M., & Petrie, J. R. (2003). High glucose inhibits insulin-stimulated nitric oxide production without reducing endothelial nitric-oxide synthase Ser1177 phosphorylation in human aortic endothelial cells. *J Biol Chem, 278*(21), 18791-18797. doi: 10.1074/jbc.M210618200

M210618200 [pii]

Samaranayake, L. (2007). Saliva as a diagnostic fluid. *Int Dent J, 57*(5), 295-299.

Sauder, K. A., Stafford, J. M., Mayer-Davis, E. J., Jensen, E. T., Saydah, S., Mottl, A., et al. (2019). Co-occurrence of early diabetes-related complications in adolescents and young adults with type 1 diabetes: an observational cohort study. *Lancet Child Adolesc Health, 3*(1), 35-43. doi: S2352-4642(18)30309-2 [pii]

10.1016/S2352-4642(18)30309-2

Schmidt, A. M., Yan, S. D., Brett, J., Mora, R., Nowygrod, R., & Stern, D. (1993). Regulation of human mononuclear phagocyte migration by cell surface-binding proteins for advanced glycation end products. *J Clin Invest, 91*(5), 2155-2168. doi: 10.1172/JCI116442

Standards of medical care in diabetes--2012. (2012). *Diabetes Care, 35 Suppl 1*, S11-63. doi: 35/Supplement_1/S11 [pii]

10.2337/dc12-s011

van Greevenbroek, M. M., Schalkwijk, C. G., & Stehouwer, C. D. (2013). Obesity-associated low-grade inflammation in type 2 diabetes mellitus: causes and consequences. *Neth J Med, 71*(4), 174-187.

Virk, S. A., Donaghue, K. C., Cho, Y. H., Benitez-Aguirre, P., Hing, S., Pryke, A., et al. (2016). Association Between HbA1c Variability and Risk of Microvascular Complications in Adolescents With Type 1 Diabetes. *J Clin Endocrinol Metab, 101*(9), 3257-3263. doi: 10.1210/jc.2015-3604

Vlassara, H. (1996). Advanced glycation end-products and atherosclerosis. *Ann Med, 28*(5), 419-426.

Vlassara, H. (1997). Recent progress in advanced glycation end products and diabetic complications. *Diabetes, 46 Suppl 2*, S19-25.

Witztum, J. L., & Horkko, S. (1997). The role of oxidized LDL in atherogenesis: immunological response and anti-phospholipid antibodies. Ann N Y Acad Sci, 811, 88-96; discussion 96-89.

Yeh, C. K., Christodoulides, N. J., Floriano, P. N., Miller, C. S., Ebersole, J. L., Weigum, S. E., et al. (2010). Current development of saliva/oral fluid-based diagnostics. Tex Dent J, 127(7), 651-661.

Yeung, W. C., Rawlinson, W. D., & Craig, M. E. (2011). Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. BMJ, 342, d35. doi: 10.1136/bmj.d35

Chapter 2- Saliva

Composition and Function

Whole saliva is an aqueous fluid containing suspended bacteria, desquamated cells and food debris. It is a transparent biological fluid, containing a complex mixture produced by secretions from the three major salivary glands combined with the secretions from minor salivary glands, the gingival crevicular fluid, in addition to the cellular and food debris, bacteria of the oral cavity and their metabolites, as well as upper airway transudate. (Humphrey & Williamson, 2001) (Amerongen & Veerman, 2002)

This complex oral fluid delivers numerous physiological functions. It is responsible for maintaining the homeostasis of the oral cavity, the health and function of the upper gastrointestinal tract through its functions i.e. oral digestion, food swallowing and tasting, tissue protection and lubrication, maintenance of tooth integrity, and antibacterial and antiviral protection. (Humphrey & Williamson, 2001)

As an aqueous solution, saliva mainly consists of water, which accounts for 99% of its composition. The daily average flow of total saliva in healthy individuals varies between 500 and 1500 mL and the mean volume of saliva in the oral cavity is approximately 1mL (Nagler, Hershkovich, Lischinsky, Diamond, & Reznick, 2002). The flow, total volume and composition of saliva vary significantly and are under neural and hormonal control. This variation provides important information about local and systemic health conditions and is significantly affected by the circadian rhythm, age, dehydration, physical exercise, medication, oral hygiene and food consumption. (Amerongen & Veerman, 2002) (Helmerhorst, Dawes, & Oppenheim, 2018)

The major glands include paired parotid, submandibular, and sublingual glands. Minor salivary glands are located throughout the oral cavity. It is estimated that parotid glands contribute about 20% of unstimulated whole saliva, and about 50% under stimulation. The submandibular glands contribute about 65% of unstimulated whole saliva. The salivary glands are composed primarily of ductal and acinar cells. All fluid movement occurs through the acinar cell component of the salivary tissue. The acinar cells of the parotid gland consist mostly of serous cells, resulting in a more watery saliva, while the acinar cells of the sublingual glands are mostly mucous. The submandibular glands contain both serous and mucous acinar cells. The minor salivary glands are almost solely mucous and produce a viscous saliva that is rich in immunoglobulins and mucins. The ductal cells form a branching network for the transport of the isotonic primary saliva from the acinar endpiece to the oral cavity. The salivary composition is modified as it moves from the acinar region to the oral cavity. Proteins are produced and added to the saliva from both acinar and ductal cells. In the ductal region, sodium and chloride are absorbed while potassium is secreted, which results in a final hypotonic, protein-rich saliva as it enters the oral cavity. Saliva's pH lies between 6.0-7.0. Though being isotonic at first, it becomes

hypotonic as it passes through the network of ducts. Normal resting salivary secretion is hypotonic and slightly alkaline, rich in potassium, whereas stimulated saliva is less hypotonic and has a higher concentration of sodium and chloride, with a lower concentration of potassium. (von Bültzingslowen et al., 2007)

As mentioned above, organic and inorganic molecules are dissolved in the aqueous medium and the rate of salivary flow is considered to be the main factor which affects its composition(Humphrey & Williamson, 2001). Sodium, chloride, calcium as well as potassium, magnesium, bicarbonate, sulphate, thiocyanate, phosphate and fluoride, are saliva's inorganic ions which are accountable for osmotic balance, buffering capacity and dental remineralisation. Bicarbonate, phosphate and urea act as pH modulators and are responsible for saliva's buffering capacity (Lima, et al., 2010).

Immunoglobulins, proteins, enzymes, mucins and nitrogen products such as urea and ammonia represent saliva's main organic components. A group of salivary proteins, with amylase, lipase, proteases and mucins being the most characteristic, are responsible for the digestive process while lactoferrin, lysozyme, lactoperoxidase and histatins present antibacterial properties respondible for hydrolysis of cellular membranes and interference with the binding of microorganisms. (Chiappin, Antonelli, Gatti, & De Palo, 2007; Nagler, et al., 2002) Saliva, via passive and active transportation, diffusion, and/or ultrafiltration, is enriched with a number of molecules from blood. Hence, it becomes a "mirror" of the body's health or disease. (Greabu et al., 2009; Kaczor-Urbanowicz et al., 2017; Williamson, Munro, Pickler, Grap, & Elswick, 2012; D. T. Wong, 2006) Evaluation of salivary characteristics is widely used as a method to monitor and assess caries risk and periodontal diseases and it is also a biological tool for the detailed study of numerous systemic diseases that affect the function of salivary glands and saliva composition, such as Sjogren's syndrome, cystic fibrosis, sarcoidosis, alcoholic cirrhosis, diabetes mellitus and adrenal cortex diseases (Lee & Wong, 2009).

Oral complications in patients with diabetes

The implications of diabetes pathology in the oral health of patients have been extensively studied. (Albert et al., 2012; Busato, Ignacio, Brancher, Moyses, & Azevedo-Alanis, 2012; Cinar, Freeman, & Schou, 2018; Diabetes and oral health. Abstracts of the 19th Annual International Conference of the University Diabetes Outreach Programme of the University of the West Indies and the University of Technology, Jamaica in collaboration with the Association of Public Dental Surgeons. March 21-23, 2013. Ocho Rios, Jamaica," 2013; Ismail, McGrath, & Yiu, 2017; Kanjirath, Kim, & Rohr Inglehart, 2011; K. M. Karjalainen, Knuuttila, & Kaar, 1996; Lamster, 2012; Pussinen & Salomaa, 2018) Various oral and dental manifestations of diabetes mellitus have been reported. Xerostomia, gingivitis and periodontitis, lesions of the oral mucosa and the tongue with an important incidence of candidiasis, increased incidence of dental caries and also poor wound healing are the most frequent of them (Galili, Findler, & Garfunkel, 1994; Mattson & Cerutis, 2001; Murrah, 1985). Importantly in periodontal disease, the bidirectional relationship of

this chronic inflammatory disease and diabetes is well proven and now periodontal disease is considered as a complication of diabetes. (Casanova, Hughes, & Preshaw, 2014)

Xerostomia is a very common symptom in patients with diabetes; it is uncomfortable for the patient and can become a challenge for the dentist due to its difficult treatment and its harmful effect on the oral cavity. Xerostomia is defined as the subjective feeling of the dryness of the mouth and it usually is the result of the salivary gland hypofunction(Cooke, Ahmedzai, & Mayberry, 1996). The salivary gland function and saliva secretion are controlled by the sympathetic and parasympathetic components of the autoimmune nervous system. In the review of von Bultzingslowen at al. about the systematic diseases that affect the salivary gland function in diabetic patients, the microvascular disease and the neuropathy are reported to result in endothelial dysfunction and deterioration of microcirculation, conditions that may impair the saliva secretion and composition (von Bültzingslowen, et al., 2007). Salivary alterations, often observed in diabetic patients, may well be a response to inadequate control of the diabetes and/or a consequence of the diabetes pathological effect on the secretions of salivary glands. Whether xerostomia and actual salivary gland dysfunction exist as a consequence of the diabetes still remains undetermined and further studies are needed to unravel the mechanisms associated with the observed salivary changes in these patients. (Mark, 2016; Poudel et al., 2018; Pussinen & Salomaa, 2018; Rai, Hegde, Kamath, & Shetty, 2011)

Diabetic complications and metabolic control

Diabetes' complications are closely related to the degree of metabolic regulation of the disease. Hyperglycaemia is considered to be etiologically associated with the incidence of complications in these patients. Levels of glycated haemoglobin (HbA1c) are a sensitive indicator of the blood sugar levels in a period of 2-3 months and they are generally used to monitor the patient's progress and mostly to determine the metabolic control of the disease. According to the latest guidelines the target goals of the Hba1c levels for the diabetic adults range from 6.5%-7.5%. More specifically according to the American Diabetes Association the optimal levels of Hba1c is <7% ("Implications of the United Kingdom Prospective Diabetes Study. ," 2003), while according to the American Association of Clinical Endocrinologist this level should be <6.5% ("The AACE system of intensive diabetes self-management—2002 update," 2002). However, it is well accepted that lower levels of Hba1c is related with lower risk of diabetes implications (Saudek, Derr, & R., 2006). Regarding the Hba1c targets-guidelines in children and young adults, things are more complicated as children, and especially those under the 6 years of age, are at greater risk of serious neurologic implications due to severe hypoglycemia. Greater attention should be given in this population where optimum regulation is more difficult to be accomplished (M Rewers et al., 2009). Generally, a target goal <7.5 for all age groups should be achieved. Whilst, each patient should have these targets personalized so as to be as close to normal as they can be, and at the same time avoid severe or moderate

hypoglycemia and hyperglycemia. ("Standards of medical care in diabetes--2012," 2012)

Salivary characteristics in patients with diabetes

The following chapter will present an overview of the literature regarding the differentiations on salivary characteristics (flow, buffering capacity, glucose concentration, pH) in patients with type 1 and type 2 diabetes, in relation to the metabolic control of their disease.

- *Salivary flow*

Table 4: Studies presenting alterations on salivary flow in patients with diabetes
Wc:well controlled, pc:poorly controlled, c:healthy control
DM: diabetes mellitus, DM1: type 1 diabetes, DM2: type 2 diabetes

Authors	Year	Type of DM	Results	
			DM vs c	wc vs pc
Harrison et al (R. Harrison & W. H. Bowen, 1987)	1987	1	DM<c (p>0.05)	wc>pc (p>0.05)
Ben Aryeh et al(Ben-Aryeh, Serouya, Kanter, Szargel, & Laufer, 1993)	1993	1,2	No difference	-
Dodds et al (Dodds & Dodds, 1997)	1997	2	No difference	-
Belazi et al (Belazi, Galli-Tsinopoulou, Drakoulakos, Fleva, & Papanayiotou, 1998)	1998	1	DM<c (p>0.05)	-
Collin et al(Collin et al., 1998)	1998	2	DM<c (p=0.003)	No difference
Dodds et al(Dodds, Yeh, & Johnson, 2000)	2000	2	DM<c (p<0.05)	-
Moore et al(Moore, Guggenheimer, Etzel, Weyant, & Orchard, 2001)	2001	1	No difference	wc>pc (p>0.05)
Edblad et al(Edblad, Lundin,	2001	1	DM<c (p>0.05)	wc>pc (p>0.05)

Sjodin, & Aman, 2001)				
Chavez et al(Chavez, Borrell, Taylor, & Ship, 2001)	2001	2	DM<c ($p<0.05$)	wc>pc ($p<0.05$)
Twetman et al(Twetman, Johansson, Birkhed, & Nederfors, 2002)	2002	1	DM<c	wc>pc
Aren et al(Aren et al., 2003)	2003	1	No difference	-
Lopez et al(Lopez et al., 2003)	2003	1	DM<c	-
Siudikiene et al(Siudikiene, Maciulskiene, & Nedzelskiene, 2005)	2005	1	DM<c ($p<0.05$)	No difference
Bernardi et al(Bernardi et al., 2007)	2007	2	DM<c ($p<0.001$)	wc>pc ($p>0.05$)
Siudikiene et al(Siudikiene, Machiulskiene, Nyvad, Tenovuo, & Nedzelskiene, 2008)	2008	1	DM<c ($p<0.05$)	-
Moreira et al(Moreira, Passos, Sampaio, Soares, & Oliveira, 2009)	2009	1	DM<c ($p<0.01$)	-
Javed et al(Javed, Sundin, Altamash, Klinge, & Engstrom, 2009)	2009	1	DM<c ($p<0.01$)	No difference
Prathibha et al(K, Johnson, Ganesh, & Subhashini, 2013)	2013	2	DM<c ($p<0.05$)	-

The findings of the literature suggest that the diabetic patients, either with type 1 or 2 diabetes mellitus, show a statistically significant lower salivary flow rate than the control group. On the contrary there is no statistically significant difference between the well and poorly controlled diabetic patients, although the findings suggest a tendency towards a higher salivary flow rate in the well-controlled group. Viscosity

and foam are altered in the saliva of these patients, as a result of the diminished flow rate.

- *Buffering capacity*

Table 5: Studies presenting alterations on saliva's buffering capacity in patients with diabetes

Authors	Year	Type of DM	Results
Edblad et al(Edblad, et al., 2001)	2001	1	no difference (wc,pc,c)
Siudikiene et al(Siudikiene, et al., 2005)	2005	1	DM1<c ($p<0.001$)
Bernardi et al(Bernardi, et al., 2007)	2007	2	no difference (wc,pc,c)

The findings of the literature are inconclusive about the effect of DM on the buffering capacity of the saliva. Buffering capacity is the ability to resist pH changes. The buffering capacity of stimulated saliva is related to the mineral content. Stimulated saliva normally contains higher levels of bicarbonate, which may be lacking in patients with low buffering capacity. Furthermore, overall mineral availability is affected by low salivary flow. (Amerongen & Veerman, 2002) Despite diminished salivary flow rates in diabetic patients, the buffering capacity in this population is not reported to be significantly lower. (Bernardi, et al., 2007; Edblad, et al., 2001)

- *Salivary glucose levels*

Table 6: Studies presenting alterations on saliva's glucose levels in patients with diabetes

Authors	Year	Type of DM	Results
Belazi et al(Belazi, et al., 1998)	1998	1	DM1 > c ($p<0.05$)
Twetman et al (Twetman, et al., 2002)	2002	1	pc> wc ($p<0.05$)

Lopez et al(Lopez, et al., 2003)	2003	1	DM1 > c
Bernardi et al(Bernardi, et al., 2007)	2007	2	wc,pc: no difference, DM2> c ($p<0.001$)
Siudikiene et al(Siudikiene, et al., 2008)	2008	1	DM1 > c
Prathibha et al(K, et al., 2013)	2013	2	DM 2> c ($p<0.05$)

The findings of the literature show statistically significant higher levels of salivary glucose in the diabetic patients, both for type 1 and type 2 patients, but the results among well and poorly controlled patients are in conflict. It has been previously reported that increased glucose levels in the saliva of patients with diabetes are the result of increased blood glucose, but a strong correlation between these levels is yet to be found. Salivary glands act as filters of blood glucose, which could explain the differences in the respective concentrations. (Gupta et al., 2017; R. Harrison & W. Bowen, 1987; K. M. Karjalainen, et al., 1996; Shahbaz et al., 2014).

- *Salivary pH*

Table 7: Studies presenting alterations on salivary pH in patients with diabetes

Authors	Year	Type of DM	Results
Lopez et al(Lopez, et al., 2003)	2003	1	DM1< c ($p>0.05$)
Bernardi et al(Bernardi, et al., 2007)	2007	2	wc,pc,c no difference
Moreira et al(Moreira, et al., 2009)	2009	1	DM1 < c ($p<0.05$)

The findings of the literature are inconclusive regarding the alterations in the salivary pH of the diabetic patients. It is reported though that type 1 and 2 patients show a lower pH when compared to healthy controls.(Lopez, et al., 2003; Moreira, et al., 2009) The acid pH in diabetic patients may be associated either to microbial activity or to a decrease of bicarbonate with salivary flow rate. (Lopez, et al., 2003)

The implications of diabetes mellitus affect most of the tissues of the human body and therefore have been thoroughly examined. However, fewer investigations have examined the complications of the disease on the salivary glands, the saliva composition and flow rate, and generally on the oral health of the diabetic patients. The results of these studies as previously presented are contradictory. The crucial role of the saliva for the maintenance of the oral health is commonly accepted. Saliva also contains enzymes, proteins, antibodies etc. which have antibacterial, antiviral and antifungal activity. Due to its buffering capacity and the mineral salts it contains, saliva plays a significant role in the incidence of dental caries.

The prevalence of dry mouth is a common complaint in diabetic patients and it seems to be associated with a poor metabolic control of the disease. Accordingly, a significant number of studies present a decrease in saliva flow rate in patients with type 1 diabetes, as if the overall dehydration could cause irreversible changes of the salivary glands. (Belazi, et al., 1998; Ben-Aryeh, Cohen, Kanter, Szargel, & Laufer, 1988; Bernardi, et al., 2007; Moreira, et al., 2009) The thirst and dry mouth of diabetic patients might be related to poor control of the disease with increased diuresis and fluid loss; accordingly, salivary flow rates may be improved when the disease is well controlled. (Ben-Aryeh, et al., 1993)

The possible explanations for diabetes-related changes in the salivary flow rate or glucose levels may, in the short term, include the effect of absolute or relative insulin deficiency, which impairs the function of salivary gland cells. This has been supported by experimental animal data, since the initiation of insulin treatment has been shown to normalise salivary gland function, i.e. salivary flow rates increased to the level seen in control animals (L. C. Anderson, 1987; Reuterving, 1986). Hyperglycemia-related overall dehydration should not be forgotten as a reason for a reduced salivary flow rate. Along with the longer duration of diabetes, long-term alterations in salivary glands, such as histologically evident lipid accumulation and degenerative changes (Hand & Weiss, 1984) (J. E. Anderson & Thliveris, 1986), may relate to salivary alterations. Neuropathic changes, evident as altered reactivity to stimulation, and histologically evident neuroaxonal abnormalities (L. C. Anderson, Garrett, Thulin, & Proctor, 1989), have also been demonstrated. According to Newrick et al., (Newrick et al., 1991), the lower salivary flow rates in subjects with diabetes compared to non diabetic controls were more obvious in patients with than without neuropathy. Basement membrane alterations could also contribute to salivary changes. Some authors have hypothesized that basement membrane alterations in the salivary glands are the reason for higher salivary glucose levels, because leakage of glucose through salivary gland ductal cells increases due to basement membrane damage (R. Harrison & W. H. Bowen, 1987; Sharon et al., 1985)

Differences in saliva's pH are reported in diabetic patients compared to healthy controls, with a tendency towards lower values, especially when the Hba1c was >8. The decrease of bicarbonate with flow rate as well as the microbial metabolism and activity could be a mechanism that explains the acid pH observed in this group. (Lopez, et al., 2003)

Normal glucose levels in saliva are 0.5-1.00 mg/100 ml, and do not significantly affect oral health or support the growth of microorganisms. However, higher salivary glucose levels favor the proliferation of microorganisms and enhance their colonization on teeth and oral mucous membranes. Glucose serves as a nutrient for candida microorganisms and suppresses the killing capacity of neutrophils, which further accentuates colonization and likely consequences can be proposed as a result of these elevated salivary glucose levels in diabetes. Oral diseases that may be ascribed to the elevated salivary glucose levels include candidiasis, dental caries, gingivitis, periodontal disease, increased risk of infection, burning mouth, fungal infections, taste impairment and poor wound healing. Prolonged xerostomia may also be a contributing factor to these conditions. (Borgnakke, 2010; Ivanovski et al., 2012) The elevated salivary glucose level in diabetes also confirms the effects of diabetic membranopathy, which leads to raised percolation of glucose from blood to saliva, thus altering the salivary composition in diabetes mellitus.(Al-Maskari, Al-Maskari, & Al-Sudairy, 2011; Nazir et al., 2018)

Conclusively,

- Diabetic patients, either with type 1 or 2 diabetes mellitus, show a statistically significant lower salivary flow rate than the control group.
- Statistically significant higher levels of salivary glucose and a tendency to a more acidic pH are observed in diabetic patients.
- There is not enough evidence regarding the alterations in buffering capacity in patients with type 1 and 2 diabetes.

References

The AACE system of intensive diabetes self-management—2002 update. (2002). *Endocrine Practice,, 8*, 40-82.

Al-Maskari, A. Y., Al-Maskari, M. Y., & Al-Sudairy, S. (2011). Oral Manifestations and Complications of Diabetes Mellitus: A review. *Sultan Qaboos Univ Med J, 11*(2), 179-186.

Albert, D. A., Ward, A., Allweiss, P., Graves, D. T., Knowler, W. C., Kunzel, C., et al. (2012). Diabetes and oral disease: implications for health professionals. *Ann N Y Acad Sci, 1255*, 1-15. doi: 10.1111/j.1749-6632.2011.06460.x

Amerongen, A. V., & Veerman, E. C. (2002). Saliva--the defender of the oral cavity. *Oral Dis, 8*(1), 12-22.

Anderson, J. E., & Thliveris, J. A. (1986). Testicular histology in streptozotocin-induced diabetes. *Anat Rec, 214*(4), 378-382. doi: 10.1002/ar.1092140407

Anderson, L. C. (1987). Parotid gland function in streptozotocin-diabetic rats. *J Dent Res, 66*(2), 425-429. doi: 10.1177/00220345870660020701

Anderson, L. C., Garrett, J. R., Thulin, A., & Proctor, G. B. (1989). Effects of streptozocin-induced diabetes on sympathetic and parasympathetic stimulation of parotid salivary gland function in rats. *Diabetes, 38*(11), 1381-1389.

Aren, G., Sepet, E., Ozdemir, D., Dinccag, N., Guvener, B., & Firatli, E. (2003). Periodontal health, salivary status, and metabolic control in children with type 1 diabetes mellitus. *J Periodontol, 74*(12), 1789-1795. doi: 10.1902/jop.2003.74.12.1789

Belazi, M. A., Galli-Tsinopoulou, A., Drakoulakos, D., Fleva, A., & Papanayiotou, P. H. (1998). Salivary alterations in insulin-dependent diabetes mellitus. *Int J Paediatr Dent, 8*(1), 29-33.

Ben-Aryeh, H., Cohen, M., Kanter, Y., Szargel, R., & Laufer, D. (1988). Salivary composition in diabetic patients. *J Diabet Complications, 2*(2), 96-99.

Ben-Aryeh, H., Serouya, R., Kanter, Y., Szargel, R., & Laufer, D. (1993). Oral health and salivary composition in diabetic patients. *J Diabetes Complications, 7*(1), 57-62. doi: 1056-8727(93)90025-T [pii]

Bernardi, M. J., Reis, A., Loguercio, A. D., Kehrig, R., Leite, M. F., & Nicolau, J. (2007). Study of the buffering capacity, pH and salivary flow rate in type 2 well-controlled and poorly controlled diabetic patients. *Oral Health Prev Dent, 5*(1), 73-78.

Borgnakke, W. S. (2010). Salivary glucose levels are unable to predict oral candidiasis or monitor diabetes. *J Evid Based Dent Pract, 10*(4), 237-240. doi: S1532-3382(10)00188-0 [pii]

10.1016/j.jebdp.2010.09.018

Busato, I. M., Ignacio, S. A., Brancher, J. A., Moyses, S. T., & Azevedo-Alanis, L. R. (2012). Impact of clinical status and salivary conditions on xerostomia and oral health-related quality of life of adolescents with type 1 diabetes mellitus. *Community Dent Oral Epidemiol, 40*(1), 62-69. doi: 10.1111/j.1600-0528.2011.00635.x

Casanova, L., Hughes, F. J., & Preshaw, P. M. (2014). Diabetes and periodontal disease: a two-way relationship. *Br Dent J, 217*(8), 433-437. doi: sj.bdj.2014.907 [pii]

10.1038/sj.bdj.2014.907

Chavez, E. M., Borrell, L. N., Taylor, G. W., & Ship, J. A. (2001). A longitudinal analysis of salivary flow in control subjects and older adults with type 2 diabetes. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 91*(2), 166-173. doi: S1079-2104(01)92705-2 [pii]

10.1067/moe.2001.112054

Chiappin, S., Antonelli, G., Gatti, R., & De Palo, E. F. (2007). Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta, 383*(1-2), 30-40. doi: S0009-8981(07)00213-6 [pii]

10.1016/j.cca.2007.04.011
Cinar, A. B., Freeman, R., & Schou, L. (2018). A new complementary approach for oral health and diabetes management: health coaching. *Int Dent J, 68*(1), 54-64. doi: 10.1111/idj.12334
Collin, H. L., Uusitupa, M., Niskanen, L., Koivisto, A. M., Markkanen, H., & Meurman, J. H. (1998). Caries in patients with non-insulin-dependent diabetes mellitus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 85*(6), 680-685. doi: S1079-2104(98)90035-X [pii]
Cooke, C., Ahmedzai, S., & Mayberry, J. (1996). Xerostomia--a review. *Palliat Med. 1996 Oct;10(4):284-92., 10*(4), 284-292.
Diabetes and oral health. Abstracts of the 19th Annual International Conference of the University Diabetes Outreach Programme of the University of the West Indies and the University of Technology, Jamaica in collaboration with the Association of Public Dental Surgeons. March 21-23, 2013. Ocho Rios, Jamaica. (2013). *West Indian Med J, 62 Suppl 1*, 20-36.
Dodds, M. W., & Dodds, A. P. (1997). Effects of glycemic control on saliva flow rates and protein composition in non-insulin-dependent diabetes mellitus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 83*(4), 465-470. doi: S1079-2104(97)90147-5 [pii]
Dodds, M. W., Yeh, C. K., & Johnson, D. A. (2000). Salivary alterations in type 2 (non-insulin-dependent) diabetes mellitus and hypertension. *Community Dent Oral Epidemiol, 28*(5), 373-381.
Edblad, E., Lundin, S. A., Sjodin, B., & Aman, J. (2001). Caries and salivary status in young adults with type 1 diabetes. *Swed Dent J, 25*(2), 53-60.
Galili, D., Findler, M., & Garfunkel, A. (1994). Oral and dental complications associated with diabetes and their treatment. *Compendium. 1994 Apr;15(4):496, 498, 500-9., 15*(4), 496-509.
Greabu, M., Battino, M., Mohora, M., Totan, A., Didilescu, A., Spinu, T., et al. (2009). Saliva--a diagnostic window to the body, both in health and in disease. *J Med Life, 2*(2), 124-132.
Gupta, S., Nayak, M. T., Sunitha, J. D., Dawar, G., Sinha, N., & Rallan, N. S. (2017). Correlation of salivary glucose level with blood glucose level in diabetes mellitus. *J Oral Maxillofac Pathol, 21*(3), 334-339. doi: 10.4103/jomfp.JOMFP_222_15
JOMFP-21-334 [pii]
Hand, A. R., & Weiss, R. E. (1984). Effects of streptozotocin-induced diabetes on the rat parotid gland. *Lab Invest, 51*(4), 429-440.
Harrison, R., & Bowen, W. (1987). Flow rate and organic constituents of whole saliva in insulin dependent diabetic children and adolescents. *Pediatric Dent*(9), 287-291.
Harrison, R., & Bowen, W. H. (1987). Flow rate and organic constituents of whole saliva in insulin-dependent diabetic children and adolescents. *Pediatr Dent, 9*(4), 287-291.
Helmerhorst, E. J., Dawes, C., & Oppenheim, F. G. (2018). The complexity of oral physiology and its impact on salivary diagnostics. *Oral Dis, 24*(3), 363-371. doi: 10.1111/odi.12780

Humphrey, S. P., & Williamson, R. T. (2001). A review of saliva: normal composition, flow, and function. *J Prosthet Dent, 85*(2), 162-169. doi: S0022-3913(01)54032-9 [pii]
10.1067/mpr.2001.113778
Implications of the United Kingdom Prospective Diabetes Study. . (2003). *Diabetes Care, 26*, 28-32.
Ismail, A. F., McGrath, C. P., & Yiu, C. K. Y. (2017). Oral health status of children with type 1 diabetes: a comparative study. *J Pediatr Endocrinol Metab, 30*(11), 1155-1159. doi: 10.1515/jpem-2017-0053
/j/jpem.ahead-of-print/jpem-2017-0053/jpem-2017-0053.xml [pii]
Ivanovski, K., Naumovski, V., Kostadinova, M., Pesevska, S., Drijanska, K., & Filipce, V. (2012). Xerostomia and salivary levels of glucose and urea in patients with diabetes. *Prilozi, 33*(2), 219-229.
Javed, F., Sundin, U., Altamash, M., Klinge, B., & Engstrom, P. E. (2009). Self-perceived oral health and salivary proteins in children with type 1 diabetes. *J Oral Rehabil, 36*(1), 39-44. doi: JOR1895 [pii]
10.1111/j.1365-2842.2008.01895.x
K, M. P., Johnson, P., Ganesh, M., & Subhashini, A. S. (2013). Evaluation of Salivary Profile among Adult Type 2 Diabetes Mellitus Patients in South India. *J Clin Diagn Res, 7*(8), 1592-1595. doi: 10.7860/JCDR/2013/5749.3232
Kaczor-Urbanowicz, K. E., Martin Carreras-Presas, C., Aro, K., Tu, M., Garcia-Godoy, F., & Wong, D. T. (2017). Saliva diagnostics - Current views and directions. *Exp Biol Med (Maywood), 242*(5), 459-472. doi: 1535370216681550 [pii]
10.1177/1535370216681550
Kanjirath, P. P., Kim, S. E., & Rohr Inglehart, M. (2011). Diabetes and oral health: the importance of oral health-related behavior. *J Dent Hyg, 85*(4), 264-272.
Karjalainen, K. M., Knuuttila, M. L., & Kaar, M. L. (1996). Salivary factors in children and adolescents with insulin-dependent diabetes mellitus. *Pediatr Dent, 18*(4), 306-311.
Lamster, I. B. (2012). Diabetes and oral health. What's their relationship? *Diabetes Self Manag, 29*(3), 30, 32-34.
Lee, Y. H., & Wong, D. T. (2009). Saliva: an emerging biofluid for early detection of diseases. *Am J Dent, 22*(4), 241-248.
Lima, D. P., Diniz, D. G., Moimaz, S. A., Sumida, D. H., & Okamoto, A. C. (2010). Saliva: reflection of the body. *Int J Infect Dis, 14*(3), e184-188. doi: S1201-9712(09)00203-3 [pii]
10.1016/j.ijid.2009.04.022
Lopez, M. E., Colloca, M. E., Paez, R. G., Schallmach, J. N., Koss, M. A., & Chervonagura, A. (2003). Salivary characteristics of diabetic children. *Braz Dent J, 14*(1), 26-31. doi: S0103-64402003000100005 [pii]
Mark, A. M. (2016). Diabetes and oral health. *J Am Dent Assoc, 147*(10), 852. doi: S0002-8177(16)30610-9 [pii]
10.1016/j.adaj.2016.07.010
Mattson, J., & Cerutis, D. (2001). Diabetes mellitus: a review of the literature and dental implications. *Compend Contin Educ Dent. , 22*(9), 757-764.
Moore, P. A., Guggenheimer, J., Etzel, K. R., Weyant, R. J., & Orchard, T. (2001). Type 1 diabetes mellitus, xerostomia, and salivary flow rates. *Oral Surg Oral Med*

Oral Pathol Oral Radiol Endod, 92(3), 281-291. doi: S1079-2104(01)55937-5 [pii]
10.1067/moe.2001.117815
Moreira, A. R., Passos, I. A., Sampaio, F. C., Soares, M. S., & Oliveira, R. J. (2009). Flow rate, pH and calcium concentration of saliva of children and adolescents with type 1 diabetes mellitus. *Braz J Med Biol Res, 42*(8), 707-711. doi: S0100-879X2009005000006 [pii]
Murrah, V. (1985). Diabetes mellitus and associated oral manifestations: a review. *J Oral Pathol., 14*(4), 271-281.
Nagler, R. M., Hershkovich, O., Lischinsky, S., Diamond, E., & Reznick, A. Z. (2002). Saliva analysis in the clinical setting: revisiting an underused diagnostic tool. *J Investig Med, 50*(3), 214-225. doi: 10.2310/6650.2002.33436
Nazir, M. A., AlGhamdi, L., AlKadi, M., AlBeajan, N., AlRashoudi, L., & AlHussan, M. (2018). The burden of Diabetes, Its Oral Complications and Their Prevention and Management. *Open Access Maced J Med Sci, 6*(8), 1545-1553. doi: 10.3889/oamjms.2018.294
OAMJMS-6-1545 [pii]
Newrick, P. G., Bowman, C., Green, D., O'Brien, I. A., Porter, S. R., Scully, C., et al. (1991). Parotid salivary secretion in diabetic autonomic neuropathy. *J Diabet Complications, 5*(1), 35-37.
Poudel, P., Griffiths, R., Wong, V. W., Arora, A., Flack, J. R., Khoo, C. L., et al. (2018). Oral health knowledge, attitudes and care practices of people with diabetes: a systematic review. *BMC Public Health, 18*(1), 577. doi: 10.1186/s12889-018-5485-7
10.1186/s12889-018-5485-7 [pii]
Pussinen, P. J., & Salomaa, V. (2018). Oral health: a neglected aspect of diabetes care. *Lancet Diabetes Endocrinol, 6*(12), 917-919. doi: S2213-8587(18)30032-9 [pii]
10.1016/S2213-8587(18)30032-9
Rai, K., Hegde, A. M., Kamath, A., & Shetty, S. (2011). Dental caries and salivary alterations in Type I Diabetes. *J Clin Pediatr Dent, 36*(2), 181-184.
Reuterving, C. O. (1986). Pilocarpine-stimulated salivary flow rate and salivary glucose concentration in alloxan diabetic rats. Influence of severity and duration of diabetes. *Acta Physiol Scand, 126*(4), 511-515. doi: 10.1111/j.1748-1716.1986.tb07849.x
Rewers, M., Pihokerb, C., Donaghuec, K., Hanasd, R., P, S., & G, K. (2009). ISPAD Clinical Practice Consensus Guidelines 2009 Compendium.Assessment and monitoring of glycemic control in children and adolescents with diabetes. *Pediatric Diabetes 2009, 10*(12), 71-81.
Saudek, C., Derr, R., & R., K. (2006). Assessing Glycemia in Diabetes Using Self-monitoring Blood Glucose and Hemoglobin A1c. *JAMA, 295*, 1688-1697.
Shahbaz, S., Katti, G., Ghali, S. R., Katti, C., Diwakar, D. D., & Guduba, V. (2014). Salivary alterations in type 1 diabetes mellitus patients: Salivary glucose could be noninvasive tool for monitoring diabetes mellitus. *Indian J Dent Res, 25*(4), 420-424. doi: IndianJDentRes_2014_25_4_420_142512 [pii]
10.4103/0970-9290.142512

Sharon, A., Ben-Aryeh, H., Itzhak, B., Yoram, K., Szargel, R., & Gutman, D. (1985). Salivary composition in diabetic patients. *J Oral Med, 40*(1), 23-26.

Siudikiene, J., Machiulskiene, V., Nyvad, B., Tenovuo, J., & Nedzelskiene, I. (2008). Dental caries increments and related factors in children with type 1 diabetes mellitus. *Caries Res, 42*(5), 354-362. doi: 000151582 [pii]
10.1159/000151582

Siudikiene, J., Maciulskiene, V., & Nedzelskiene, I. (2005). Dietary and oral hygiene habits in children with type I diabetes mellitus related to dental caries. *Stomatologija, 7*(2), 58-62. doi: 052-06 [pii]

Standards of medical care in diabetes--2012. (2012). *Diabetes Care, 35 Suppl 1*, S11-63. doi: 35/Supplement_1/S11 [pii]
10.2337/dc12-s011

Twetman, S., Johansson, I., Birkhed, D., & Nederfors, T. (2002). Caries incidence in young type 1 diabetes mellitus patients in relation to metabolic control and caries-associated risk factors. *Caries Res, 36*(1), 31-35. doi: 57587 [pii]
10.1159/000057587

von Bültzingslowen, I., Sollecito, T., P, F., Daniels, T., Jonsson, R., & Lockhart, P. e. a. (2007). Salivary dysfunction associated with systemic diseases: systematic review and clinical management recommendations. *Oral Pathol Oral Radiol Endod 103*(1), S57.e51-S57.e15.

Williamson, S., Munro, C., Pickler, R., Grap, M. J., & Elswick, R. K., Jr. (2012). Comparison of biomarkers in blood and saliva in healthy adults. *Nurs Res Pract, 2012*, 246178. doi: 10.1155/2012/246178

Wong, D. T. (2006). Towards a simple, saliva-based test for the detection of oral cancer 'oral fluid (saliva), which is the mirror of the body, is a perfect medium to be explored for health and disease surveillance'. *Expert Rev Mol Diagn, 6*(3), 267-272. doi: 10.1586/14737159.6.3.267

Chapter 3- Proteomics

The Human Proteome

The concept of the proteome was introduced by Marc Wilkins in 1994 at a conference on "2D Electrophoresis: from protein maps to genomes" in Siena, Italy, where he first coined the term, to define protein-based gene expression analysis. Part of his PhD thesis appeared in print in 1996, where it was shown that proteins can be separated and identified by two-dimensional (2-D) electrophoresis allowing for protein-based gene expression analysis. (M. R. Wilkins, Pasquali, et al., 1996) The term “proteome” derives from a combination of words and describes “the entire PROTein complement expressed by a genOME”, or by a cell or tissue type. (M. R. Wilkins, Pasquali, et al., 1996)

Most of the functional information of genes is characterized by the proteome. Accordingly, the proteome is a dynamic and relatively complex complement. While there is only one definitive genome of an organism, the proteome represents the assortment of proteins produced at a specific time. (Vidova & Spacil, 2017) While the genome is characterized by its stability, the proteome dynamically changes in response to various factors, including the organism’s developmental stage and various internal and external conditions. (M. Wilkins, 2009; M. R. Wilkins, Sanchez, et al., 1996) As such, it is considered a snapshot-in-time of the biochemical system studied; a reflection of a particular set of biochemical conditions. Due to this variability, combined with the different patterns of post-translational modifications of proteins, the proteome represents a much more complex group than either the genome or the transcriptome. (Thul & Lindskog, 2018)

Proteomics refers to the large-scale experimental analysis of proteins and proteomes and has enabled the identification and quantification of ever increasing number of proteins in tissues, cells and organisms. It is an interdisciplinary domain which has benefitted greatly from the genetic information of various genome projects and covers the exploration of proteomes from the overall level of protein composition, expression, structure, functions, interactions and modifications.(Thul & Lindskog, 2018) (Vidova & Spacil, 2017) Structural proteomics analyze protein structures and can help identify the functions of newly discovered genes. (Artigues et al., 2016) Expression proteomics identify the main proteins found in a particular sample and proteins differentially expressed in related samples, (Boersema, Kahraman, & Picotti, 2015) while interaction proteomics study the characterization of protein-protein interactions in order to determine protein functions and analyze how proteins assemble in larger complexes. (Havugimana, Hu, & Emili, 2017)

Proteomics-based technologies are applied in different research settings for the detection of various diagnostic markers, for vaccine production, for understanding pathogenetic mechanisms, for the exploration of the expression patterns in response to different signals and for the interpretation of functional protein pathways in different diseases for prevention, diagnosis and monitoring. (Aslam, Basit, Nisar, Khurshid, & Rasool, 2017)

Figure 1: An overview of proteomics technologies (Aslam, et al., 2017)

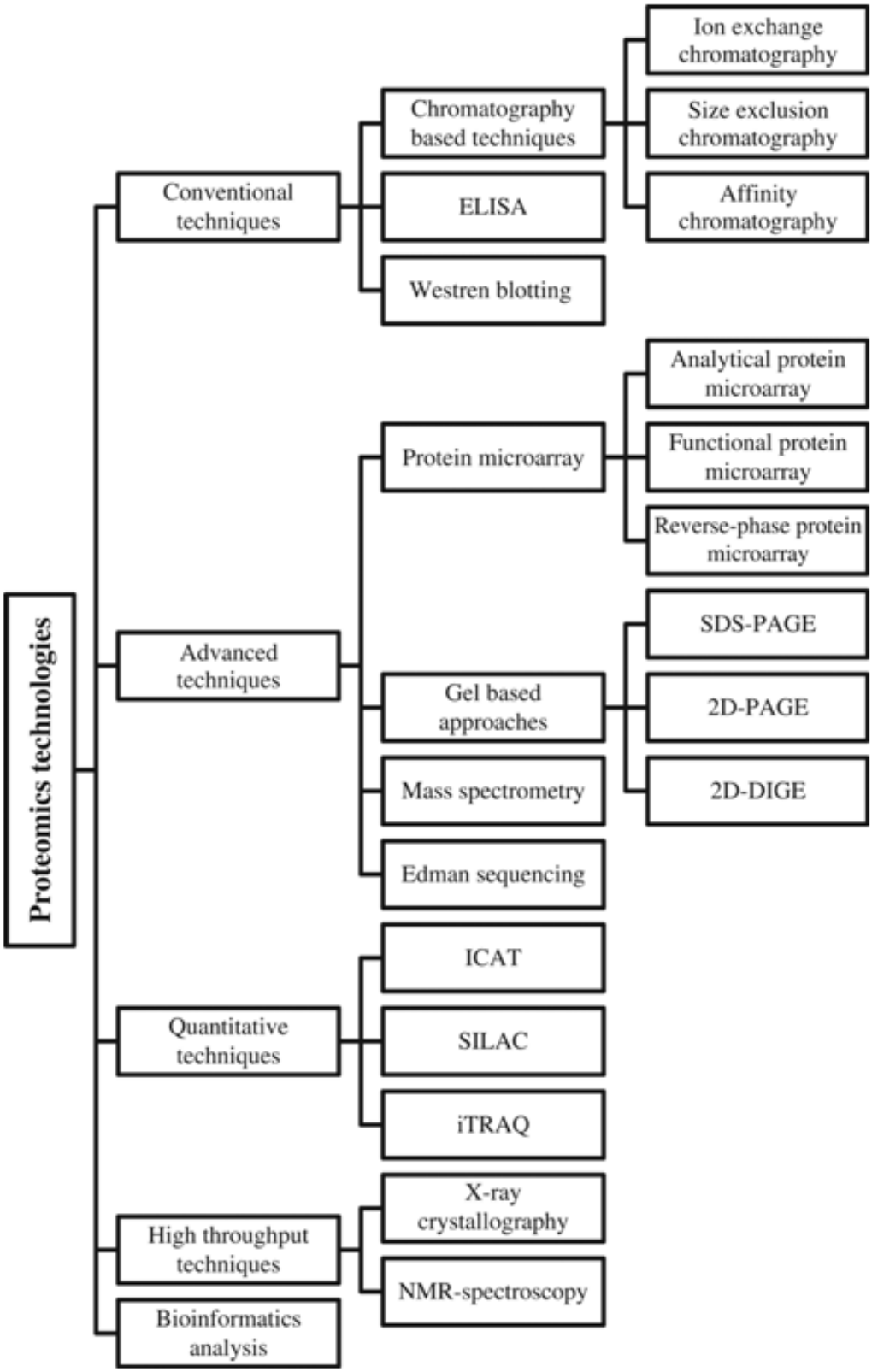
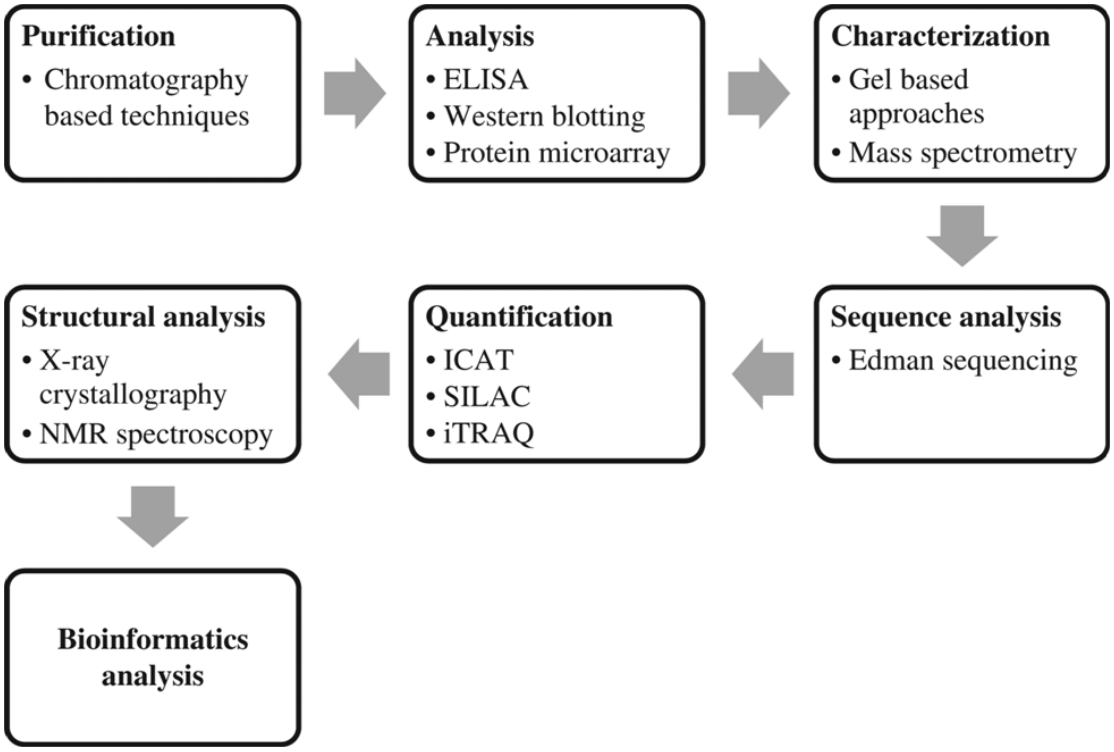


Figure 2: High-throughput proteomic techniques applied for various analyses (Aslam, et al., 2017)



Advanced Proteomic Technologies

Overall, methodologies employed in proteomics are presented in Figure 1. Rather than a tool, "proteomic technologies" refer to a combination of innovations and advances in separation techniques, mass spectrometry and bioinformatics for data analysis and integration. They may be classified into two large categories: bottom-up and top-down types. (F. M. Amado, Ferreira, & Vitorino, 2013) Bottom up proteomics are also termed shotgun and include liquid chromatography separation of peptides derived from tryptic digestion of complex protein solutions, followed by mass-spectrometry (MS) analysis. (Manadas, Mendes, English, & Dunn, 2010) The top-down method is the process in which intact proteins and not peptides are analysed via MS. Therein, proteins are analyzed without proteolytic digestion and are subjected to gel-phase separation, fragmentation, fragment separation and interpretation of mass spectrometric data which yield both the molecular weight of the intact protein and the protein fragmentation ladders. (Capriotti, Cavaliere, Foglia, Samperi, & Lagana, 2011)

The purification of proteins is performed with chromatography based, conventional techniques such as ion exchange chromatography (IEC), size exclusion chromatography (SEC) and affinity chromatography. For analysis of selective proteins, enzyme-linked immunosorbent assay (ELISA) and western blotting can be used. These techniques are restricted to analysis of only a few individual proteins and they are also incapable to define protein expression level. For separation of complex protein samples, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2D) and two-dimensional differential gel electrophoresis (2D-DIGE) techniques are applied. (Aslam, et al., 2017) (Figure 2)

Protein microarrays or chips, that have been established for rapid expression analyses, are unable to explore the function of a complete genome. Thus, high-throughput proteomics approaches such as mass spectrometry have developed to analyze the complex protein mixtures with higher sensitivity. Isotope-coded affinity tag (ICAT) labeling, stable isotope labeling with amino acids in cell culture (SILAC) and isobaric tag for relative and absolute quantitation (iTRAQ) techniques have recently developed for quantitative proteomic analyses. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are two major advanced techniques that provide three-dimensional (3D) structure of proteins that might be helpful to understand their biological function. (Aslam, et al., 2017) (Figure 2)

As mentioned above, gel-based (one-dimensional (1D) gel electrophoresis, two-dimensional polyacrylamide gel electrophoresis, 2D difference in-gel electrophoresis (2D-DIGE)) and gel-free (liquid chromatography (LC), capillary electrophoresis) approaches have been developed and utilized in a variety of combinations in order to separate proteins prior to mass spectrometric analysis. The typical proteomics experiment requires four steps: (Figure3- (Lippolis & De Angelis, 2016))

1. proteins isolated from body fluid or tissues were separated by both gel based and gel free methods

2. proteins were enzymatically digested to peptides

3. peptides were analyzed by mass-spectrometer

4. mass spectra of the peptides were matched against protein sequence databases for protein identification.

Gel-based approaches for proteome characterization

Since it was first introduced in 1975 (O'Farrell, 1975), 2-DE has evolved at different levels and became the standard of protein separation and the method of choice for differential protein expression analysis. Proteins first undergo isoelectric focusing (IEF) based on their net charge at different pH values and in the orthogonal second dimension further separation is performed based on the molecular weight (MW). Initial studies on proteomic analysis relied on the application of 2-DE for protein separation and quantification, followed by MS for identification. (Ghafouri, Tagesson, & Lindahl, 2003) Lately, 2-DE has lost its popularity due to its known limitations and also due to the development of alternative MS-based approaches. Some of the reasons behind this trend include issues related to reproducibility, poor representation of low abundant proteins, highly acidic/basic proteins, or proteins with extreme size or hydrophobicity, and difficulties in automation of the gel-based techniques. Moreover, the comigration of multiple proteins in a single spot renders comparative quantification rather inaccurate. (Abdallah, Dumas-Gaudot, Renaut, & Sergeant, 2012) Nevertheless, this approach presents advantages in the characterization of saliva proteome post-translational modifications (PTM), as well as the distinction between intact proteins and their fragments. (Rabilloud, Chevallet, Luche, & Lelong, 2010)

One dimensional SDS-PAGE, also termed GeLC-MS/MS, has been recently used for protein fractionation as a result of its simplicity and reproducibility. In this approach, proteins are typically in-gel digested and the resulting peptides are fractionated in a reversed-phase-HPLC coupled to a mass spectrometer for protein identification. Because of that, substances (e.g. salts) or protein complexes which interfere with downstream mass spectrometric acquisition are eliminated/disrupted resulting in the identification of more than 200 proteins. However, this approach does not allow the identification of the abundant low molecular weight salivary proteins due to their small size or to the non-existence of cleavage sites for trypsin digestion. (F. M. Amado, et al., 2013)

Gel-free based approaches for proteome characterization

Gel-free approaches are characterized by the analysis of proteome in a wider dynamic range and broader proteome coverage. Lately, these strategies have provided complementary valuable information to 2-DE since they helped to overcome its disadvantages such as the laborious procedure involved, the large amount of sample required, the limited dynamic range, the difficulties in resolving low abundant proteins and the ones with extreme isoelectric point, molecular weights and hydrophobicity. In shotgun proteomics, a mixture of proteins is digested into peptides that are loaded onto at least a two-dimensional chromatography based separation system. Peptides are then eluted into a tandem mass spectrometer, in an automated fashion, and the resulting tandem mass spectrometry data is analyzed by powerful computational systems. (F. M. Amado, et al., 2013; Sun & Markey, 2011)

The analysis of global proteome, in which hundreds or thousands of proteins can be present, represents always a challenge, especially if PTMs analysis is included. As previously referred, PTMs overview is easily obtained using gel-based approaches with specific stains. However, the knowledge of the exact modification and in which amino acid residues occur is crucial to the understanding of proteins' physiological roles as well as the molecular pathways in which they participate. Thus, gel-free based approaches involving chemical or protein affinity for the capture of modified proteins have been widely used in the characterization of the most abundant PTMs (glycosylation and phosphorylation) especially in saliva. (F. M. Amado, et al., 2013; Fang & Zhang, 2008)

Mass spectrometry for identification

Protein separation and peptide fractionation, via electrophoresis and chromatography, is followed by identification; this is performed by mass spectrometry. MS is a technique that allows the detection of compounds by separating ions by their unique mass (mass-to-charge ratios) using a mass spectrometer. The method relies on the fact that every compound has a unique fragmentation pattern (mass spectrum). It basically consists of ionizing a compound and evaluating the ion mass/charge (m/z) ratio. The equipment comprises a ionization source, one or two mass analyzers and a detector. The first component is used to generate peptide or protein ions, usually transferring protons (H+) to the molecules without modifying their chemical structure. The ion is accelerated by an electric field and separated by m/z in a mass analyzer, or it is selected according to a previously determined m/z, being fragmented in a tandem process (MS2 or MS/MS). Finally, the ions pass through the detector, which is connected to a computer with data analysis software.

Ionization methods

Currently, two main ionization methods are available and used in proteomics, Matrix-Assisted Laser Desorption/Ionization (MALDI) and Electrospray Ionization (ESI), with the former being employed for solid state samples and the latter for liquid state samples. In MALDI, peptides are co-crystallized with an organic matrix, usually alpha-cyano-4-hydroxycinnamic acid. After laser bombardment, the matrix sublimates and its ions transfer the charge to analytes, resulting in peptide ion formation. One MALDI variant termed Surface-Enhanced Laser Desorption/Ionization (SELDI) is usually employed to analyze a low-molecular-weight proteome and uses several matrices or chips that explore the chromatographic and biophysical characteristics of different proteins. These chips can exhibit hydrophobic surfaces; ion exchange surfaces or surfaces with immobilized metallic ions; or even antibodies, receptors, enzymes, and ligands with high affinity for specific proteins. Thus, after washing out unbound compounds, a matrix is added to the chip surface and spectra are acquired through laser ionization. Another MALDI variant is Imaging Mass Spectrometry (IMS), allowing peptide and protein mass data to be obtained directly

from biological tissue sections. This method offers important advantages over immunohistochemical analysis, including speed and independence from antibody use. (Barbosa et al., 2012) In contrast with MALDI, in ESI an aqueous solution with the analyte is forced to pass through a capillary needle undergoing high voltage. The solution is ejected as a spray with highly charged droplets that generate analyte ionized forms after the solvent is evaporated by a heated inert gas flow. (Barbosa, et al., 2012)

Mass analyzers

For proteomics research, four types of mass analyzers are commonly used: quadrupole (Q), ion trap (quadrupole ion trap, QIT; linear ion trap, LIT or LTQ), time-of-flight (TOF) mass analyzer, and Fourier-transform ion cyclotron resonance (FTICR) mass analyzer. They vary in their physical principles and analytical performance.

In TOF analyzers, the ions resulting from the first step are accelerated by a potential between two electrodes and pass through a vacuum tube at a speed that is inversely related to their mass. When the ions reach the detector, the time elapsed from the ionization up to the detection is used to derive the m/z value. The detector converts the signal of the ion passage into an analog signal, which is read and interpreted by a workstation. The final result is a plot of m/z versus intensity (ion count), usually referred to as MS spectrum. The generated signals are compared with information available in databases, in order to identify the protein of interest. (Barbosa, et al., 2012)

The IT analyzers filter and entrap ions of interest in a tridimensional electric field and these are gradually released in an m/z ascending order. Fourier Transform Ion Cyclotron Resonances (FT-ICRs) are ion traps with an additional magnetic field forcing ions to exhibit a circular movement with high frequency cycles. The analyzer determines the m/z ratio from the cyclotronic movement frequency by using the Fourier transform. Orbitrap is another type of IT analyzer wherein ions oscillate along and around a single spiral electrode. (Zubarev & Makarov, 2013) This oscillation frequency is directly related to the square root of the m/z ratio and can be determined with high accuracy. This technology has migrated towards hybrid systems with two independent mass spectrometers that combine, for example, an ion trap and an orbitrap, or an ion trap and a FT-ICR. (Barbosa, et al., 2012; Zubarev & Makarov, 2013)

Quantitative proteomics

Although mass spectrometry is often only used to demonstrate the presence of a protein or PTM within a sample, it can also be used to measure dynamic changes in protein and PTM abundances. Quantification strategies make use of stable isotopes (^{2}H, ^{13}C, ^{15}N, and ^{18}O) for sample labeling although label-free methods have also been proposed. The rationale behind stable isotope labeling is to create a mass shift

that distinguishes identical peptides from different samples within a single MS analysis. (Han, Aslanian, & Yates, 2008; Negishi et al., 2009)

The introduction of stable isotopes by metabolic labeling occurs during protein synthesis. Metabolic labeling was first applied to proteomic analysis by mass spectrometry using ^{15}N. The use of stable isotope labeling of amino acids in cell culture (SILAC) has emerged as a popular alternative in which only select amino acids are labeled, typically arginine and lysine. Following cleavage with trypsin, all peptides contain at least one labeled amino acid. SILAC has proven useful in measuring the output of signaling networks and discerning true protein interactions.

Post-biosynthetic labeling strategies can be applied to any set of samples, including primary cell culture and human, because they are performed post-lysis. However, sample-processing discrepancies can lead to the introduction of error with these methods. Accurately distinguishing the small mass difference produced by enzymatic labeling requires an instrument with high mass accuracy and high mass resolution. Chemical labeling targets reactive groups on the side-chains of amino acids or peptide termini. Consequently, comprehensive stable isotope incorporation is difficult to achieve because labeling is sequence-dependent. Side reactions are also a common problem. Improving on the use of isotope-coded affinity tags (ICAT), isobaric mass tags are all the same mass and it is only upon fragmentation that the different mass tags are observed. The isobaric mass tag consists of an amine-specific reactive group, a balancer group and a reporter mass group. The peptide amino-termini and lysine side-chains are targeted by the amine-specific reactive group. A popular version of isobaric mass tags is the iTRAQ reagent, which has recently expanded to incorporate up to eight reporter mass ions. Consequently, the amount of run time required to analyze multiple samples can be reduced. This is of particular relevance to biological experiments in which multiple conditions or multiple time-points are being evaluated such as signaling networks.(Han, et al., 2008; Serpa et al., 2012)

Metabolic and post-biosynthetic labeling both provide relative quantification of proteins. One advantage provided by the use of stable isotope-labeled synthetic peptides is that absolute measurement of protein abundance can be achieved. This is possible because a defined amount labeled synthetic peptide is added to the sample. Absolute quantification of proteins uses a synthetic stable isotope-labeled peptide at a known concentration. The choice of peptide is based upon previous sampling results. The use of synthetic peptides for protein quantification by MS is typically performed using selective reaction monitoring (SRM) or multiple reaction monitoring (MRM) in which the intact peptide mass and specific fragment ions are monitored during the course of the run. (Han, et al., 2008)

Figure 3: Schematic representation of the advanced proteomic pipeline (Lippolis & De Angelis, 2016)

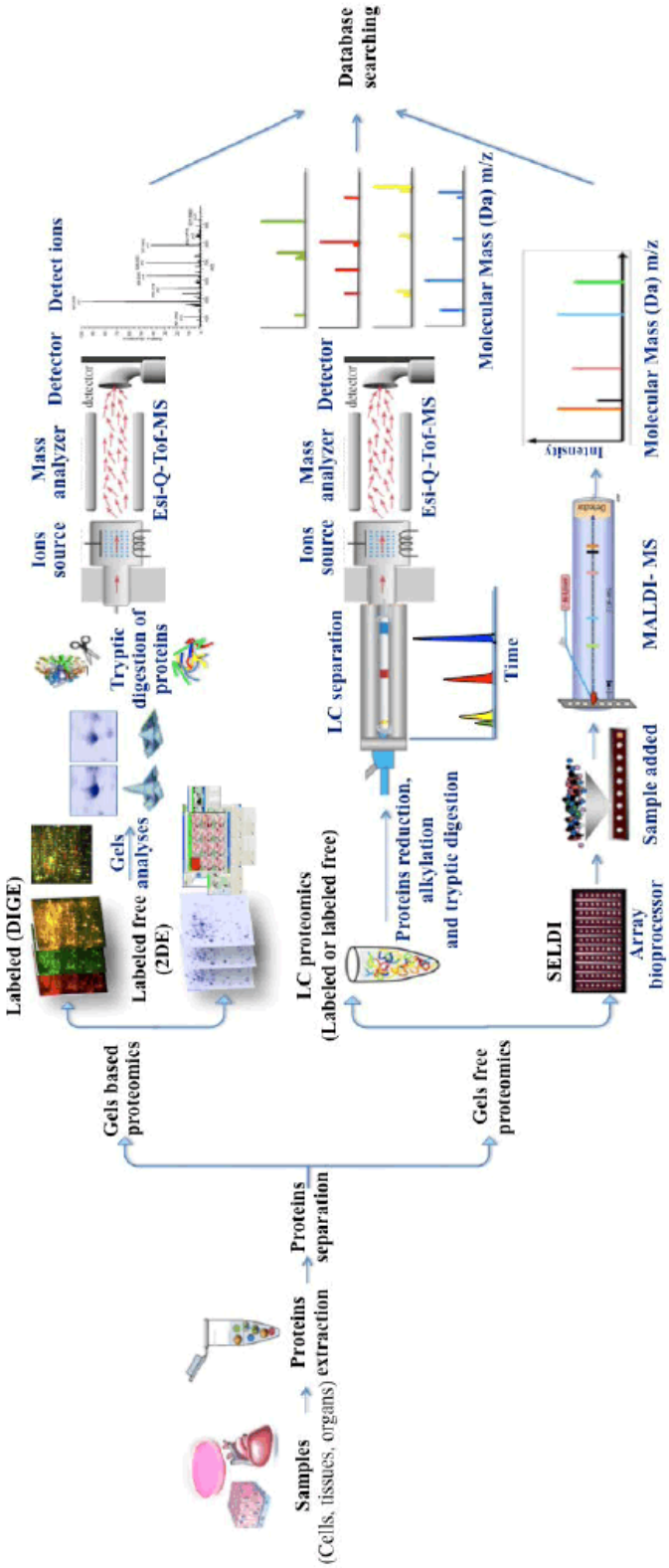


Table 8: Biomarkers in saliva (whole, unstimulated) associated with various conditions and disorders (modified from (J. Zhang, Zhang, Ma, Lin, & Chen, 2013)

Disease	Sample (patients & control)	Method	Biomarkers	Validation
Head and Neck Squamous Cellcarcinoma (Dowling et al., 2008)	8&8	2D-DIGE	Beta fibrin S100 calcium-binding protein transferrin cofilin-1	Western Blotting
		LC/MS		
Cleft lip and palate (Szabo et al., 2012)	31&20	MALDI-TOF/MS	Arpc3 Dermokine	
Gingivitis (Goncalves Lda et al., 2011)	10&10	LC/MS MALDI-TOF/ MS	Albumin Hemoglobin Immunoglobulin Keratins	
Oral Squamous Cellcarcinoma (de Jong et al., 2010)	4&4		Myosin Actin	Western Blotting
Chronic periodontitis (Goncalves Lda et al., 2010)	10&10	2D-DIGE MALDI-TOF/MS nLC-MS/MS	Immunoglobulin Hemoglobin Albumin Cystatin	
Dental caries (Vitorino et al., 2006)	16&16	2DE MALDI-TOF/MS	Amylase IgA Lactoferrin	
Sjögren's syndrome (Ryu, Atkinson, Hoehn, Illei, & Hart, 2006)	8&8	2DE ESI-MS/MS	Keratin Albumin 2 actin isoforms	
Oral Squamous Cellcarcinoma (Brinkmann et al., 2011)	35&51		IL-1B IL-8 M2BP	ELISA
Gastric cancer (Z. Z. Wu, Wang, & Zhang, 2009)	23&18	MALDI-TOF/MS	4 proteins (1472.78 Da, 2936.49 Da, 6556.81 Da, and 7081.17 Da)	
Breast cancer (L. Zhang et al., 2010)	10&10	2D-DIGE	CA6	Immunoblotting
Rheumatoid arthritis (Giusti et al., 2010)	20&20	2DE MALDI-TOF/MS	2 S100A Apolipoprotein a-1	Immunoblotting
Type I diabetes (Cabras et al., 2010)	31&31	LC-ESI-MS	S100A9 PRP-1/PRP-3	
Type I diabetes (Caseiro et al., 2013; Caseiro et al., 2012)	15&5	iTRAQ MALDI-TOF/MS LC-MS/MS	HbA1c BPI MMP-9	
Type II diabetes (Rao et al., 2009)	40&10	LC-MS/MS	5 metabolism proteins	ELISA

Applications in the study of human diseases

The advancements in proteomic technologies have remarkable applications in several clinical research areas such as diagnosis, therapy response monitoring, risk determination, prediction, disease subtype classification, characterization of metabolic pathways and therapeutic target generation. Due to the exponential interest in this scientific field, the literature on the benefits of proteomic studies for human disease management is extensive (reviewed by (Lippolis & De Angelis, 2016).
Of the tissues and body fluids accessible for proteomic analysis, saliva presents the most attractive diagnostic potential and is extensively reviewed in Chapter 4. Table 8 presents an overview of the different methods that studies have applied in salivary proteomics lately and the respective fields of applications. (J. Zhang, et al., 2013)
What is important to be mentioned is that despite the technological achievements, there still are significant technical challenges in the use of the biological tissues and fluids as diagnostic media, that need to be surpassed. The complexity and the dynamic characteristics of protein composition combined with the need for large sample sizes (in order to eliminate intra- and inter- individual variability) and the requirement for standardization of protocols, render the field of proteomic analysis for biomarkers discovery demanding and challenging. (Al-Tarawneh, Border, Dibble, & Bencharit, 2011)

References

Abdallah, C., Dumas-Gaudot, E., Renaut, J., & Sergeant, K. (2012). Gel-based and gel-free quantitative proteomics approaches at a glance. *Int J Plant Genomics, 2012*, 494572. doi: 10.1155/2012/494572

Al-Tarawneh, S. K., Border, M. B., Dibble, C. F., & Bencharit, S. (2011). Defining salivary biomarkers using mass spectrometry-based proteomics: a systematic review. *OMICS, 15*(6), 353-361. doi: 10.1089/omi.2010.0134

Amado, F. M., Ferreira, R. P., & Vitorino, R. (2013). One decade of salivary proteomics: current approaches and outstanding challenges. *Clin Biochem, 46*(6), 506-517. doi: S0009-9120(12)00579-6 [pii]
10.1016/j.clinbiochem.2012.10.024

Artigues, A., Nadeau, O. W., Rimmer, M. A., Villar, M. T., Du, X., Fenton, A. W., et al. (2016). Protein Structural Analysis via Mass Spectrometry-Based Proteomics. *Adv Exp Med Biol, 919*, 397-431. doi: 10.1007/978-3-319-41448-5_19

Aslam, B., Basit, M., Nisar, M. A., Khurshid, M., & Rasool, M. H. (2017). Proteomics: Technologies and Their Applications. *J Chromatogr Sci, 55*(2), 182-196. doi: bmw167 [pii]
10.1093/chromsci/bmw167

Barbosa, E. B., Vidotto, A., Polachini, G. M., Henrique, T., Marqui, A. B., & Tajara, E. H. (2012). Proteomics: methodologies and applications to the study of human diseases. *Rev Assoc Med Bras (1992), 58*(3), 366-375. doi: S0104-42302012000300019 [pii]

Boersema, P. J., Kahraman, A., & Picotti, P. (2015). Proteomics beyond large-scale protein expression analysis. *Curr Opin Biotechnol, 34*, 162-170. doi: S0958-1669(15)00014-2 [pii]
10.1016/j.copbio.2015.01.005

Brinkmann, O., Kastratovic, D. A., Dimitrijevic, M. V., Konstantinovic, V. S., Jelovac, D. B., Antic, J., et al. (2011). Oral squamous cell carcinoma detection by salivary biomarkers in a Serbian population. *Oral Oncol, 47*(1), 51-55. doi: S1368-8375(10)00325-8 [pii]
10.1016/j.oraloncology.2010.10.009

Cabras, T., Pisano, E., Mastinu, A., Denotti, G., Pusceddu, P. P., Inzitari, R., et al. (2010). Alterations of the salivary secretory peptidome profile in children affected by type 1 diabetes. *Mol Cell Proteomics, 9*(10), 2099-2108. doi: M110.001057 [pii]
10.1074/mcp.M110.001057

Capriotti, A. L., Cavaliere, C., Foglia, P., Samperi, R., & Lagana, A. (2011). Intact protein separation by chromatographic and/or electrophoretic techniques for top-down proteomics. *J Chromatogr A, 1218*(49), 8760-8776. doi: S0021-9673(11)00785-0 [pii]
10.1016/j.chroma.2011.05.094

Caseiro, A., Ferreira, R., Padrao, A., Quintaneiro, C., Pereira, A., Marinheiro, R., et al. (2013). Salivary proteome and peptidome profiling in type 1 diabetes mellitus using a quantitative approach. *J Proteome Res, 12*(4), 1700-1709. doi: 10.1021/pr3010343

Caseiro, A., Vitorino, R., Barros, A. S., Ferreira, R., Calheiros-Lobo, M. J., Carvalho, D., et al. (2012). Salivary peptidome in type 1 diabetes mellitus. *Biomed Chromatogr, 26*(5), 571-582. doi: 10.1002/bmc.1677

de Jong, E. P., Xie, H., Onsongo, G., Stone, M. D., Chen, X. B., Kooren, J. A., et al. (2010). Quantitative proteomics reveals myosin and actin as promising saliva biomarkers for distinguishing pre-malignant and malignant oral lesions. *PLoS One, 5*(6), e11148. doi: 10.1371/journal.pone.0011148

Dowling, P., Wormald, R., Meleady, P., Henry, M., Curran, A., & Clynes, M. (2008). Analysis of the saliva proteome from patients with head and neck squamous cell carcinoma reveals differences in abundance levels of proteins associated with tumour progression and metastasis. *J Proteomics, 71*(2), 168-175. doi: S1874-3919(08)00066-3 [pii]

10.1016/j.jprot.2008.04.004

Fang, X., & Zhang, W. W. (2008). Affinity separation and enrichment methods in proteomic analysis. *J Proteomics, 71*(3), 284-303. doi: S1874-3919(08)00104-8 [pii]

10.1016/j.jprot.2008.06.011

Ghafouri, B., Tagesson, C., & Lindahl, M. (2003). Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. *Proteomics, 3*(6), 1003-1015. doi: 10.1002/pmic.200300426

Giusti, L., Baldini, C., Ciregia, F., Giannaccini, G., Giacomelli, C., De Feo, F., et al. (2010). Is GRP78/BiP a potential salivary biomarker in patients with rheumatoid arthritis? *Proteomics Clin Appl, 4*(3), 315-324. doi: 10.1002/prca.200900082

Goncalves Lda, R., Soares, M. R., Nogueira, F. C., Garcia, C., Camisasca, D. R., Domont, G., et al. (2010). Comparative proteomic analysis of whole saliva from chronic periodontitis patients. *J Proteomics, 73*(7), 1334-1341. doi: S1874-3919(10)00070-9 [pii]

10.1016/j.jprot.2010.02.018

Goncalves Lda, R., Soares, M. R., Nogueira, F. C., Garcia, C. H., Camisasca, D. R., Domont, G., et al. (2011). Analysis of the salivary proteome in gingivitis patients. *J Periodontal Res, 46*(5), 599-606. doi: 10.1111/j.1600-0765.2011.01378.x

Han, X., Aslanian, A., & Yates, J. R., 3rd. (2008). Mass spectrometry for proteomics. *Curr Opin Chem Biol, 12*(5), 483-490. doi: S1367-5931(08)00117-8 [pii]

10.1016/j.cbpa.2008.07.024

Havugimana, P. C., Hu, P., & Emili, A. (2017). Protein complexes, big data, machine learning and integrative proteomics: lessons learned over a decade of systematic analysis of protein interaction networks. *Expert Rev Proteomics, 14*(10), 845-855. doi: 10.1080/14789450.2017.1374179

Lippolis, R., & De Angelis, M. (2016). Proteomics and Human Diseases. *J Proteomics Bioinform, 9*, 63-74.

Manadas, B., Mendes, V. M., English, J., & Dunn, M. J. (2010). Peptide fractionation in proteomics approaches. *Expert Rev Proteomics, 7*(5), 655-663. doi: 10.1586/epr.10.46

Negishi, A., Ono, M., Handa, Y., Kato, H., Yamashita, K., Honda, K., et al. (2009). Large-scale quantitative clinical proteomics by label-free liquid

chromatography and mass spectrometry. *Cancer Sci, 100*(3), 514-519. doi: CAS1055 [pii]
10.1111/j.1349-7006.2008.01055.x
O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem, 250*(10), 4007-4021.
Rabilloud, T., Chevallet, M., Luche, S., & Lelong, C. (2010). Two-dimensional gel electrophoresis in proteomics: Past, present and future. *J Proteomics, 73*(11), 2064-2077. doi: S1874-3919(10)00175-2 [pii]
10.1016/j.jprot.2010.05.016
Rao, P. V., Reddy, A. P., Lu, X., Dasari, S., Krishnaprasad, A., Biggs, E., et al. (2009). Proteomic identification of salivary biomarkers of type-2 diabetes. *J Proteome Res, 8*(1), 239-245. doi: 10.1021/pr8003776
10.1021/pr8003776 [pii]
Ryu, O. H., Atkinson, J. C., Hoehn, G. T., Illei, G. G., & Hart, T. C. (2006). Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis. *Rheumatology (Oxford), 45*(9), 1077-1086. doi: kei212 [pii]
10.1093/rheumatology/kei212
Serpa, J. J., Parker, C. E., Petrotchenko, E. V., Han, J., Pan, J., & Borchers, C. H. (2012). Mass spectrometry-based structural proteomics. *Eur J Mass Spectrom (Chichester), 18*(2), 251-267. doi: 10.1255/ejms.1178
Sun, C. S., & Markey, M. K. (2011). Recent advances in computational analysis of mass spectrometry for proteomic profiling. *J Mass Spectrom, 46*(5), 443-456. doi: 10.1002/jms.1909
Szabo, G. T., Tihanyi, R., Csulak, F., Jambor, E., Bona, A., Szabo, G., et al. (2012). Comparative salivary proteomics of cleft palate patients. *Cleft Palate Craniofac J, 49*(5), 519-523. doi: 10.1597/10-135
Thul, P. J., & Lindskog, C. (2018). The human protein atlas: A spatial map of the human proteome. *Protein Sci, 27*(1), 233-244. doi: 10.1002/pro.3307
Vidova, V., & Spacil, Z. (2017). A review on mass spectrometry-based quantitative proteomics: Targeted and data independent acquisition. *Anal Chim Acta, 964*, 7-23. doi: S0003-2670(17)30150-2 [pii]
10.1016/j.aca.2017.01.059
Vitorino, R., de Morais Guedes, S., Ferreira, R., Lobo, M. J., Duarte, J., Ferrer-Correia, A. J., et al. (2006). Two-dimensional electrophoresis study of in vitro pellicle formation and dental caries susceptibility. *Eur J Oral Sci, 114*(2), 147-153. doi: EOS328 [pii]
10.1111/j.1600-0722.2006.00328.x
Wilkins, M. (2009). Proteomics data mining. *Expert Rev Proteomics, 6*(6), 599-603. doi: 10.1586/epr.09.81
Wilkins, M. R., Pasquali, C., Appel, R. D., Ou, K., Golaz, O., Sanchez, J. C., et al. (1996). From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Biotechnology (N Y), 14*(1), 61-65.
Wilkins, M. R., Sanchez, J. C., Gooley, A. A., Appel, R. D., Humphery-Smith, I., Hochstrasser, D. F., et al. (1996). Progress with proteome projects: why all

proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev, 13*, 19-50.

Wu, Z. Z., Wang, J. G., & Zhang, X. L. (2009). Diagnostic model of saliva protein finger print analysis of patients with gastric cancer. *World J Gastroenterol, 15*(7), 865-870.

Zhang, J., Zhang, X., Ma, Q., Lin, J., & Chen, F. (2013). Salivary Peptidomic Analysis-The Extension of Proteomics. *J Mol Biomark Diagn, 4*(2).

Zhang, L., Xiao, H., Karlan, S., Zhou, H., Gross, J., Elashoff, D., et al. (2010). Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. *PLoS One, 5*(12), e15573. doi: 10.1371/journal.pone.0015573

Zubarev, R. A., & Makarov, A. (2013). Orbitrap mass spectrometry. *Anal Chem, 85*(11), 5288-5296. doi: 10.1021/ac4001223

Chapter 4- Salivary Diagnostics

Saliva as a Diagnostic Tool

Nowadays, the combination of biotechnologies and salivary diagnostics has extended the diagnostic potential of this biofluid. A large number of significant analytes in saliva are thoroughly examined and represent biomarkers for different diseases including cancer(L. Zhang, et al., 2010), autoimmune(Hu et al., 2010), bacterial(Al Kawas, Rahim, & Ferguson, 2012), cardiovascular diseases(Arredouani et al., 2016; Javaid, Ahmed, Durand, & Tran, 2016) and metabolic such as diabetes mellitus(Rao, et al., 2009). This progress has expanded the salivary diagnostic approach from the oral cavity to the whole physiological system, and appears promising for the application of these advances in the purposes of personalized medicine(Xiao & Wong, 2011).

Properties and Limitations of Saliva as a Diagnostic Fluid

Saliva is identified as functional equivalent to serum, reflecting the physiological state of the body, as well as hormonal, emotional, nutritional and metabolic alterations(Lee & Wong, 2009). Saliva's collection is an easy, non invasive, simple, chair-side procedure which does not require any special equipment. It ensures patients' compliance, diminishing the discomfort which is often associated with blood and urine collection. It is an ideal diagnostic tool for studies conducted on special populations such as children, anxious, handicapped or elderly patients. It does not clot compared to blood, it is cheaper to store and ship, it facilitates repeated and voluminous sampling in short intervals of time and at the same time it is safer for both the operator and the patient. Methods for collecting saliva may vary depending on the time of day, the use of stimuli, the mode of unstimulated collection (draining, spitting or suction methods) and prior cleaning of the oral cavity with water rinse(Al Kawas, et al., 2012).

Despite the aforementioned properties, saliva as a biofluid presents limitations which restrict its diagnostic potential. Saliva may be considered a mirror of oral and systemic health but levels of certain biomolecules are not always consistent with the levels of these markers in serum. Salivary composition may vary depending on the method and time of collection, the technique used and the degree of stimulation of salivary flow. These alterations, combined with changes in salivary pH and the variability of salivary flow in the same individual throughout the day, may considerably affect the concentration of salivary markers. Salivary gland function is additionally affected by a number of systemic disorders, numerous medications and radiation therapy. Moreover, proteolytic enzymes which derive from the host and oral microorganisms in whole saliva, disturb the stability and concentration of certain biomarkers(Castagnola, Cabras, Vitali, Sanna, & Messana, 2011; Nagler, et al., 2002).

Advances in Saliva Analysis Technology

Salivary diagnostic technology has been developed to monitor periodontal diseases(Spielmann & Wong, 2011), to assess caries risk(Cunha-Cruz et al., 2013), to unveil valuable biomarkers for systemic diseases such as cancer, autoimmune diseases(Hu, et al., 2010), viral, bacterial and cardiovascular diseases (da Silva Modesto et al., 2015; Javaid, et al., 2016; Shahar, Pollack, Kedem, Hassoun, & Nagler, 2008). Saliva' s principle limitation as a diagnostic fluid is that important biomolecules are found at low abundance as opposed to serum. Emerging technologies in the last decade are surpassing these limitations, particularly for the analysis of proteins and nucleic acids. Proteome, genome and transcriptome-based approaches have been applied in order to analyse saliva as a source of disease markers(Castagnola, et al., 2011; Choi, 2010; Schafer et al., 2014; Zhou et al., 2016).

The analysis of salivary genome allows identification of the presence of pathogens and unveils profiles of transcription that reflect pathological genetic processes such as cancer. The salivary genome consists of DNAs of the individual's genome and of the oral microbiota. The quality and yield of DNA obtained from saliva can be used for genotyping, amplification or sequencing and can be stored for a long time without significant degradation(Zimmermann, Park, & Wong, 2007). However, the salivary DNA cannot provide information about upregulation or downregulation of gene expression and is limited in reflecting the presence or absence of specific genes or mutations(Schafer, et al., 2014; Zhou, et al., 2016).

Salivary transcriptomics devoted to identify alterations in the transcription of particular miRNAs and mRNAs. Currently, simple methods of stabilization of mRNA in saliva samples have been developed; however the microarray technology continues to be the gold standard for the identification of salivary transcripts, by means of qPCR(Park, Li, Yu, Brinkman, & Wong, 2006).

Proteomic analysis of body fluids is a mirror of the life and function, disease and death of cells, organs and thus the organism. The proteome, the total amount of proteins expressed in the organism at the time of sample collection, provides a direct representation of cellular function since expression of proteins is controlled in both the transcription and the translation level. The human salivary proteome has been well characterized and different classes of salivary biomarkers are reported as significant for diagnosis. Whole saliva contains specific proteins produced by the salivary glands whereas crevicular fluid, mucosal tissue, bacteria, viruses and fungi also contribute to the composition of the saliva proteome (Dodds, Johnson, & Yeh, 2005). The acinar cells of the salivary glands are responsible for the secretion of more than 85% of salivary proteins, and the glandular duct cells secrete proteins with vital biological functions such as growth factors and immunoglobulins (Vitorino et al., 2004). Approximately 40–50% of the salivary proteome consists of small proteins and peptides(F. Amado, Lobo, Domingues, Duarte, & Vitorino, 2010) derived by proteolysis in the oral cavity(Vitorino et al., 2010). Several studies indicate the effect of systemic diseases on salivary variables and outline their

importance in understanding the pathogenesis of the disease(Cho, Ko, Kim, & Kho, 2010; Javaid, et al., 2016; Lopez-Pintor et al., 2016; Tishler, Yaron, Shirazi, & Yaron, 1997). Over two thousands of proteins are detected in human saliva proteome nowadays via advanced applications of mass spectrometry(F. M. Amado, et al., 2013).

Recently, researchers demonstrated the utility of salivary metabolomic biomarkers for the detection of oral cancer, breast cancer, pancreatic cancer and periodontal disease(Ai, Smith, & Wong, 2012; Schafer, et al., 2014). The metabolome constitutes the totality of metabolic intermediates, signalling molecules and secondary metabolites. Just as the transcriptome and the proteome, it offers a dynamic reflection of gene and protein expression as well as environmental effects(A. Zhang, Sun, Wang, & Wang, 2013).

Lately, the human oral microbe identification microarray has been used for the profiling and monitoring of changes in the oral microbiota. Pancreatic cancer, oral cancer, lung cancer, colonic and extracolonic malignancy, cardiovascular and celebrovascular disease and preterm birth are examples of disorders which have been found to be associated with alterations in the bacterial profile of the oral cavity(Spielmann & Wong, 2011).

Oral Disease Detection

Caries

Many studies have demonstrated the role of S. mutans in initiating dental caries, while Lactobacilli have a role in the progression of carious lesions. High salivary levels of both pathogens as detected by a commercially available test (CRT bacteria®, Ivoclar-Vivadent) have shown a positive association with the presence of caries in children and adults. On the other hand, saliva is known to play a protective role against caries since it contains several antibacterial agents, it can mechanically clear the pathogens and has a buffering capacity to decrease the acid concentration on tooth surfaces. Therefore, changes in quantity and composition of the saliva can also provide potential tools to detect and monitor caries. However, no single salivary test has shown consistent accuracy in detecting caries. What has been suggested is rather a combination of known risk factors to predict individuals at risk for caries. However, none of the risk assessment programs proposed to date has shown consistent validity. This can be explained by the involvement of multiple local and systemic risk factors in the caries development process (Abbate, Borghi, Passi, & Levrini, 2014; Bagherian & Asadikaram, 2012; Cunha-Cruz, et al., 2013; Deepa & Thirrunavukkarasu, 2010).

Periodontal Diseases

Various salivary biomarkers have been studied for the diagnosis and prognosis of periodontal diseases. These include inflammatory mediators, enzymes, epithelial keratins, immunoglobulins, salivary ions and hormones. Both whole saliva and

gingival crevicular fluid (GCF) have been used in periodontics to detect these potential biomarkers. More specifically, the presence of matrix metalloproteinase-8 (MMP-8, an enzyme responsible for tissue destruction) in GCF has been positively associated with periodontitis progression. An immunochromatographic chair-side dip-stick test became commercially available to detect the presence or absence of MMP-8 in the GCF with similar precision as conventional laboratory assays(Giannobile et al., 2009). Recently, it has been reported that salivary soluble toll-like receptor-2 and interleukin-4 correlate positively with periodontal disease process(Swaminathan, Prakasam, Puri, & Srinivasan, 2013). Periodontitis is a multifactorial disease; as such, it is proposed that host derived factors as well as oral pathogens should be analysed for risk prediction purposes. Indeed, investigators have detected higher salivary levels of Porphyromonas gingivalis, Tannerella forsythia and Prevotella intermedia in individuals with progressive periodontitis. This finding has also been verified recently, denoting that the combination of salivary P. gingivalis levels with host specific pathogen response would be useful in diagnosing periodontitis with high accuracy (Liljestrand et al., 2014).

Systemic Disease Detection

Oral Cancer

Oral squamous cell carcinoma (OSCC) is the most common form of oral cancer. The key to decrease OSCC mortality and morbidity is early detection. Several research groups have found that salivary levels of specific proteins are increased in whole saliva of patients with OSCC. Elevated levels of salivary defencine-1 were found to be indicative of the presence of OSCC. Additionally, CD44 (a cell surface glycoprotein involved in cell-to-cell interaction), Cyfra 21-1 (a fragment of cytokeratin 19), tissue polypeptide antigen (TPS), and Cancer antigen 125 (CA-125) have been proposed as oral cancer biomarkers. The increase in salivary levels of IL-8 and subcutaneous adipose tissue (SAT) demonstrated the highest levels of sensitivity and specificity to detect OSCC. Another significant biomarker for OSCC is the presence of human papillomavirus (HPV) (Bahar, Feinmesser, Shpitzer, Popovtzer, & Nagler, 2007; J. Y. Wu et al., 2010)

Autoimmune disorders

For the diagnosis of Sjogren's syndrome, a chronic disease affecting the lacrimal and salivary glands, invasive and expensive approaches such as sialography, salivary scintiography, biopsies and serological tests are essential. Analysis of proteins in saliva of patients with Sjogren's syndrome showed increased level of beta 2 macroglobulin, lysozyme C, lactoferrin, and cystatin C, while levels of salivary amylase and carbonic anhydrase were found to be reduced(Malamud, 2011).

In multiple sclerosis, an inflammatory disease characterized by loss of myelin and scarring, a reduction in levels of IgA was found in saliva analysis of the patients. However, such findings are inconclusive for diagnosis and further analysis in the salivary proteome is required in this field(Karlik et al., 2015).

Lymph nodes, lungs, eyes, skin, and other tissues are affected in sarcoidosis, another autoimmune and inflammatory disease to which salivary diagnostics are applied. It is demonstrated that a decreased amount of saliva secretion in these patients is associated with reduced activity of the enzyme alpha-amylase and kallikrein, although this correlation is weak and insufficient to place diagnosis(Carleo, Bennett, & Rottoli, 2016).

Infectious diseases

Today, with the use of salivary analyses, it is possible to identify the herpes virus associated with Kaposi's sarcoma and the presence of bacteria such as *Helicobactrer pylori,* which is associated with gastritis, peptic ulcers and stomach cancer. Besides the usual microorganisms of the oral cavity, saliva may contain viruses and/or bacteria responsible for systemic diseases. PCR is the method of choice in such cases while it is also possible to diagnose infectious diseases through monitoring the presence of antibodies to the organisms.

Studies on patients diagnosed with rubella showed 96% specificity in detecting immunoglobulin M (IgM) in their saliva when compared to blood serum, which indicated that the use of saliva for epidemiological surveillance and control of this virus can be valid(de Oliveira et al., 2000).

Detection of hepatitis A antigen and hepatitis B surface antigen in saliva has been used in epidemiological studies of both types of hepatitis. Analysis of saliva provided a highly sensitive and specific method for the diagnosis of viral hepatitis B and hepatitis C, with commercial kits which offer 100% sensitive and specific results. PCR is also applied for the diagnosis of CMV, HPV 6,7,8, EBV and human forms of rabies(Javaid, et al., 2016).

The use of saliva for the diagnosis of Acquired Immunodeficiency Syndrome (AIDS) is currently under spotlight. Studies have shown that tests based on specific salivary antibodies are equivalent in reliability as compared to those in the serum and are nowadays useful in clinical practice, epidemiological studies, not only for diagnostic purposes but also for monitoring the effectiveness of antiretroviral therapy and the progression of the syndrome(Basham, Richardson, Sutcliffe, & Haas, 2009).

Genetic Disorders

Cystic Fibrosis leads to marked changes in salivary composition, due to a disturbance in salivary glands secretions. Cystic fibrosis affects a gene in chromosome 7, which is responsible for a protein that regulates the passage of sodium and chloride through the cell membranes, thus an elevation of electrolytes like sodium, chloride, calcium and phosphorous is observed. Contrary to this, trace elements vanadium, chromium, selenium, arsenic have lower levels in patients with Cystic Fibrosis (Deepa & Thirrunavukkarasu, 2010).

Stress

Salivary flow and composition is severely affected in the presence of xerostomia, anxiety, depression, Burning mouth syndrome and aphthous stomatitis. Cortisol levels in saliva are considered to be an important marker of stress. A stressful situation induces alterations in salivary characteristics, with an increase in pH, protein levels and especially salivary cortisol concentration. The salivary alpha-amylase is additionally an important biomolecule in the psychobiology of stress. Its levels in humans are regulated by the sympathetic autonomic nervous system and are increased significantly before any other clinical sign. Both cortisol and alpha-amylase are considered effective biomarkers for the evaluation of psychological and metabolic stress. Additionally they have the comparative advantage of non-invasiveness which reassures patients' compliance (Naumova et al., 2014; V. Wong, Yan, Donald, & McLean, 2004).

Malignancies

Salivary genomics, proteomics and transcriptomics provide an outstanding source of rich genetic information. Biomarkers are nowadays used as prognostic indicators, early cancer detectors, as well as tools for more accurate tumour staging, patients' selection for the implementation of specific therapies, and post treatment surveillance(Javaid, et al., 2016).

Analysis of saliva in women diagnosed with breast cancer showed that the soluble fragment of the oncogene c-erbB-2 as well as the antigen for cancer were significantly higher when compared to a control group(Streckfus, Bigler, Tucci, & Thigpen, 2000). The levels of c-erbB-2 may be also used for monitoring patients undergoing chemotherapy to assess the effectiveness of the applied therapy (Streckfus et al., 2000; Streckfus, Bigler, Tucci, et al., 2000).

The use of the salivary transcriptome for detecting early stage resectable pancreatic cancer is another significant step in salivary diagnostics. The salivary transcriptome profile for pancreatic cancer has been found to outerperform currently used blood-based tests in sensitivity and specificity (A. Zhang, et al., 2013; Z. Zhang et al., 2010).

Sixteen candidate lung cancer biomarkers were discovered in salivary proteomic analysis of patients, with three of those markers achieving 88.5% sensitivity and 92.3% specificity in further verification procedures(An et al., 2010; Xiao et al., 2012).

Cardiovascular Disorders

Cardiovascular disease is a major cause of death worldwide, and atherosclerosis, the leading etiological factor, is triggered by the presence of inflammation, which results in deposition of lipids in the arterial walls and progressive narrowing of the arterial lumen. This condition might then culminate in acute myocardial infarction (AMI), a common lethal cardiovascular complication. A significant number of patients

suffering from heart disease lack known risk factors such as family history, hypertension and increased lipid profiles. Similarly, unlike subjects with high serum cholesterol levels, people with increased C-reactive protein (CRP) are more likely to be unaware of their susceptibility to develop cardiovascular disease. CRP is an inflammatory mediator that is produced in response to acute injury or infection and can mediate an inflammatory response by triggering the complement cascade. It can contribute to atherogenesis and its presence has been demonstrated in arterial plaque. Importantly, salivary CRP levels were found to correlate with plasma CRP levels obtained from blood samples of a population at risk for cardiovascular complications. It is also possible to detect in saliva the cardiac troponin (cTn), a biomarker for the detection of AMI that is released in response to cardiac cell necrosis. Salivary cTn levels were shown to be a monitoring/diagnosis tool as sensitive as its serum levels in patients suffering from AMI. There is little doubt that salivary tests will progressively replace blood samples to isolate several biomarkers associated with cardiovascular diseases(Mirzaii-Dizgah, Hejazi, Riahi, & Salehi, 2012; Mirzaii-Dizgah & Riahi, 2013; Wilson, Ryan, & Boyle, 2006).

Salivary-based diagnostic techniques could potentially allow screening of an entire population for a specific disease marker in a cost effective way. Based on their accuracy, efficacy, ease of use and cost effectiveness, salivary diagnostic tests are already their applications in clinical and basic research and it has been suggested that salivary tests will pave the way for chair-side diagnosis of various oral and systemic diseases. However, there is still research to be done in order to incorporate saliva-based diagnostics into daily clinical practice. Salivary collection methods and biomarkers need to be standardized and further validated. Additional studies are needed to establish their accuracy and effectiveness. It is expected that the advent of sensitive and specific salivary diagnostic tools and the establishment of defined guidelines will make salivary diagnostics a reality in the near future.

Nagler, R. M., Hershkovich, O., Lischinsky, S., Diamond, E. & Reznick, A. Z. Saliva analysis in the clinical setting: revisiting an underused diagnostic tool. *J Investig Med* **50**, 214-225, doi:10.2310/6650.2002.33436 (2002).

Humphrey, S. P. & Williamson, R. T. A review of saliva: normal composition, flow, and function. *J Prosthet Dent* **85**, 162-169, doi:S0022-3913(01)54032-9 [pii]
10.1067/mpr.2001.113778 (2001).

Lima, D. P., Diniz, D. G., Moimaz, S. A., Sumida, D. H. & Okamoto, A. C. Saliva: reflection of the body. *Int J Infect Dis* **14**, e184-188, doi:S1201-9712(09)00203-3 [pii]
10.1016/j.ijid.2009.04.022 (2010).

Chiappin, S., Antonelli, G., Gatti, R. & De Palo, E. F. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta* **383**, 30-40, doi:S0009-8981(07)00213-6 [pii]
10.1016/j.cca.2007.04.011 (2007).

Lee, Y. H. & Wong, D. T. Saliva: an emerging biofluid for early detection of diseases. *Am J Dent* **22**, 241-248 (2009).

Zhang, L. *et al.* Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. *PLoS One* **5**, e15573, doi:10.1371/journal.pone.0015573 (2010).

Hu, S. *et al.* Preclinical validation of salivary biomarkers for primary Sjogren's syndrome. *Arthritis Care Res (Hoboken)* **62**, 1633-1638, doi:10.1002/acr.20289 (2010).

Al Kawas, S., Rahim, Z. H. & Ferguson, D. B. Potential uses of human salivary protein and peptide analysis in the diagnosis of disease. *Arch Oral Biol* **57**, 1-9, doi:S0003-9969(11)00202-0 [pii]

10.1016/j.archoralbio.2011.06.013 (2012).

Arredouani, A. *et al.* Metabolomic Profile of Low Copy-Number Carriers at the Salivary Alpha-Amylase Gene Suggests a Metabolic Shift Towards Lipid-Based Energy Production. *Diabetes*, doi:db16-0315 [pii]

10.2337/db16-0315 (2016).

Javaid, M. A., Ahmed, A. S., Durand, R. & Tran, S. D. Saliva as a diagnostic tool for oral and systemic diseases. *J Oral Biol Craniofac Res* **6**, 66-75, doi:10.1016/j.jobcr.2015.08.006

S2212-4268(15)00089-5 [pii] (2016).

Rao, P. V. *et al.* Proteomic identification of salivary biomarkers of type-2 diabetes. *J Proteome Res* **8**, 239-245, doi:10.1021/pr8003776

10.1021/pr8003776 [pii] (2009).

Xiao, H. & Wong, D. T. Proteomics and its applications for biomarker discovery in human saliva. *Bioinformation* **5**, 294-296 (2011).

Castagnola, M., Cabras, T., Vitali, A., Sanna, M. T. & Messana, I. Biotechnological implications of the salivary proteome. *Trends Biotechnol* **29**, 409-418, doi:S0167-7799(11)00068-0 [pii]

10.1016/j.tibtech.2011.04.002 (2011).

Spielmann, N. & Wong, D. T. Saliva: diagnostics and therapeutic perspectives. *Oral Dis* **17**, 345-354, doi:10.1111/j.1601-0825.2010.01773.x (2011).

Cunha-Cruz, J. *et al.* Salivary characteristics and dental caries: evidence from general dental practices. *J Am Dent Assoc* **144**, e31-40, doi:S0002-8177(14)60499-2 [pii] (2013).

da Silva Modesto, K. B. *et al.* Salivary flow rate and biochemical composition analysis in stimulated whole saliva of children with cystic fibrosis. *Arch Oral Biol* **60**, 1650-1654, doi:S0003-9969(15)30024-8 [pii]

10.1016/j.archoralbio.2015.08.007 (2015).

Shahar, E., Pollack, S., Kedem, E., Hassoun, G. & Nagler, R. Effect of HAART on salivary composition and oxidative profile in HIV-infected patients. *Curr HIV Res* **6**, 447-451 (2008).

Choi, M. Saliva diagnostics integrate dentistry into general and preventive health care. *Int J Prosthodont* **23**, 189 (2010).

Schafer, C. A. *et al.* Saliva diagnostics: utilizing oral fluids to determine health status. *Monogr Oral Sci* **24**, 88-98, doi:000358791 [pii]

10.1159/000358791 (2014).

Zhou, J. *et al.* Exploration of Human Salivary Microbiomes--Insights into the Novel Characteristics of Microbial Community Structure in Caries and Caries-Free Subjects. *PLoS One* **11**, e0147039, doi:10.1371/journal.pone.0147039
PONE-D-15-31932 [pii] (2016).
Zimmermann, B. G., Park, N. J. & Wong, D. T. Genomic targets in saliva. *Ann N Y Acad Sci* **1098**, 184-191, doi:1098/1/184 [pii]
10.1196/annals.1384.002 (2007).
Park, N. J., Li, Y., Yu, T., Brinkman, B. M. & Wong, D. T. Characterization of RNA in saliva. *Clin Chem* **52**, 988-994, doi:clinchem.2005.063206 [pii]
10.1373/clinchem.2005.063206 (2006).
Amado, F. M., Ferreira, R. P. & Vitorino, R. One decade of salivary proteomics: current approaches and outstanding challenges. *Clin Biochem* **46**, 506-517, doi:S0009-9120(12)00579-6 [pii]
10.1016/j.clinbiochem.2012.10.024 (2013).
Ai, J. Y., Smith, B. & Wong, D. T. Bioinformatics advances in saliva diagnostics. *Int J Oral Sci* **4**, 85-87, doi:ijos201226 [pii]
10.1038/ijos.2012.26 (2012).
Zhang, A., Sun, H., Wang, P. & Wang, X. Salivary proteomics in biomedical research. *Clin Chim Acta* **415**, 261-265, doi:S0009-8981(12)00519-0 [pii]
10.1016/j.cca.2012.11.001 (2013).
Abbate, G. M., Borghi, D., Passi, A. & Levrini, L. Correlation between unstimulated salivary flow, pH and streptococcus mutans, analysed with real time PCR, in caries-free and caries-active children. *Eur J Paediatr Dent* **15**, 51-54 (2014).
Bagherian, A. & Asadikaram, G. Comparison of some salivary characteristics between children with and without early childhood caries. *Indian J Dent Res* **23**, 628-632, doi:IndianJDentRes_2012_23_5_628_107380 [pii]
10.4103/0970-9290.107380 (2012).
Deepa, T. & Thirrunavukkarasu, N. Saliva as a potential diagnostic tool. *Indian J Med Sci* **64**, 293-306, doi:IndianJMedSci_2010_64_7_293_99854 [pii]
10.4103/0019-5359.99854 (2010).
Giannobile, W. V. *et al.* Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontol 2000* **50**, 52-64, doi:PRD288 [pii]
10.1111/j.1600-0757.2008.00288.x (2009).
Swaminathan, V., Prakasam, S., Puri, V. & Srinivasan, M. Role of salivary epithelial toll-like receptors 2 and 4 in modulating innate immune responses in chronic periodontitis. *J Periodontal Res* **48**, 757-765, doi:10.1111/jre.12066 (2013).
Liljestrand, J. M. *et al.* Combining salivary pathogen and serum antibody levels improves their diagnostic ability in detection of periodontitis. *J Periodontol* **85**, 123-131, doi:10.1902/jop.2013.130030 (2014).
Bahar, G., Feinmesser, R., Shpitzer, T., Popovtzer, A. & Nagler, R. M. Salivary analysis in oral cancer patients: DNA and protein oxidation, reactive nitrogen species, and antioxidant profile. *Cancer* **109**, 54-59, doi:10.1002/cncr.22386 (2007).
Wu, J. Y. *et al.* Potential biomarkers in saliva for oral squamous cell carcinoma. *Oral Oncol* **46**, 226-231, doi:S1368-8375(10)00011-4 [pii]
10.1016/j.oraloncology.2010.01.007 (2010).
Malamud, D. Saliva as a diagnostic fluid. *Dent Clin North Am* **55**, 159-178, doi:S0011-8532(10)00081-9 [pii]

10.1016/j.cden.2010.08.004 (2011).
Karlik, M. *et al.* Markers of oxidative stress in plasma and saliva in patients with multiple sclerosis. *Clin Biochem* **48**, 24-28, doi:S0009-9120(14)00703-6 [pii]
10.1016/j.clinbiochem.2014.09.023 (2015).
Carleo, A., Bennett, D. & Rottoli, P. Biomarkers in sarcoidosis: the contribution of system biology. *Curr Opin Pulm Med* **22**, 509-514, doi:10.1097/MCP.0000000000000306 (2016).
de Oliveira, S. A. *et al.* Diagnosis of rubella infection by detecting specific immunoglobulin M antibodies in saliva samples: a clinic-based study in Niteroi, RJ, Brazil. *Rev Soc Bras Med Trop* **33**, 335-339 (2000).
Basham, R. J., Richardson, D. M., Sutcliffe, C. B. & Haas, D. W. Effect of HIV-1 infection on human DNA yield from saliva. *HIV Clin Trials* **10**, 282-285, doi:Y65W64K36346NK41 [pii]
10.1310/hct1004-282 (2009).
Naumova, E. A. *et al.* Dynamic changes in saliva after acute mental stress. *Sci Rep* **4**, 4884, doi:srep04884 [pii]
10.1038/srep04884 (2014).
Wong, V., Yan, T., Donald, A. & McLean, M. Saliva and bloodspot cortisol: novel sampling methods to assess hydrocortisone replacement therapy in hypoadrenal patients. *Clin Endocrinol (Oxf)* **61**, 131-137, doi:10.1111/j.1365-2265.2004.02062.x
CEN2062 [pii] (2004).
Streckfus, C., Bigler, L., Tucci, M. & Thigpen, J. T. A preliminary study of CA15-3, c-erbB-2, epidermal growth factor receptor, cathepsin-D, and p53 in saliva among women with breast carcinoma. *Cancer Invest* **18**, 101-109 (2000).
Streckfus, C. *et al.* The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin Cancer Res* **6**, 2363-2370 (2000).
Zhang, Z. *et al.* Identification of human gastric carcinoma biomarkers by differential protein expression analysis using 18O labeling and nanoLC-MS/MS coupled with laser capture microdissection. *Med Oncol* **27**, 296-303, doi:10.1007/s12032-009-9208-x (2010).
An, Z. *et al.* Integrated ionization approach for RRLC-MS/MS-based metabonomics: finding potential biomarkers for lung cancer. *J Proteome Res* **9**, 4071-4081, doi:10.1021/pr100265g (2010).
Xiao, H. *et al.* Proteomic analysis of human saliva from lung cancer patients using two-dimensional difference gel electrophoresis and mass spectrometry. *Mol Cell Proteomics* **11**, M111 012112, doi:M111.012112 [pii]
10.1074/mcp.M111.012112 (2012).
Wilson, A. M., Ryan, M. C. & Boyle, A. J. The novel role of C-reactive protein in cardiovascular disease: risk marker or pathogen. *Int J Cardiol* **106**, 291-297, doi:S0167-5273(05)00434-1 [pii]
10.1016/j.ijcard.2005.01.068 (2006).
Mirzaii-Dizgah, I., Hejazi, S. F., Riahi, E. & Salehi, M. M. Saliva-based creatine kinase MB measurement as a potential point-of-care testing for detection of myocardial infarction. *Clin Oral Investig* **16**, 775-779, doi:10.1007/s00784-011-0578-z (2012).

Mirzaii-Dizgah, I. & Riahi, E. Salivary high-sensitivity cardiac troponin T levels in patients with acute myocardial infarction. *Oral Dis* **19**, 180-184, doi:10.1111/j.1601-0825.2012.01968.x (2013).

Saliva's diagnostic applications in Paediatrics

In the late 1960s, salivary studies in children with cystic fibrosis reported a significant increase in the concentration of calcium in submandibular saliva, paving the way for research on the diagnostic potential of the oral fluid. (Mandel, Kutscher, Denning, Thompson, & Zegarelli, 1967)

In this chapter, the main characteristics of saliva as a diagnostic fluid and the various applications of salivary diagnostics in paediatrics are presented (Figure4).

Figure 4: Salivary diagnostic applications in paediatrics

As noted earlier, saliva, as a diagnostic fluid, presents favorable properties when compared to blood. The easy, simple, painless, non-invasive method of sample collection alleviates patients' discomfort and ensures compliance, especially in vulnerable patient populations such as neonates and children. (Ranger & Grunau, 2014) It is an ideal diagnostic medium and has often been used for studies conducted in paediatric populations (Table 9), a practical biofluid to obtain in community settings, and exceptionally useful for large epidemiological cohort studies. (Hartman et al., 2016) Moreover, saliva allows for repeated sampling, is safer to handle, does not require trained personnel or special equipment and diminishes the risk of anaemia, which is increased during monitoring of at-risk infants and children, due to serial phlebotomy. (Hassaneen & Maron, 2017)

Disease/ Pathogens	Salivary biomarkers
Periodontal disease	*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Campylobacter rectus*, *Treponema denticola*
Dental caries	*Streptococcus mutans*, *Lactobacillus* spp.
Obesity	*Selenomonas noxia*
Metabolic syndrome	Uric acid, pH, lysozyme, CRP, glucose, insulin, TNF-α, IL-6, INF-γ, MIP-18, resistin, leptin
Diabetes type 1	Proinflammatory biomarkers, statherin, proline-rich peptides and histatin 1
Diabetes type 2	Glucose, HbA, IgA, SP lactoferin, MPO, SPO, albumin, MMP-9 and cathepsin D
Cysticfibrosis	Mg, lactate dehydrogenase activity, lipids and proteins, chlorine, sodium, calcium, phosphate
Stress	α-amylase, cortisol
Depression	Cortisol
Autism	Statherin phosphorylation levels, histatin 1, acidic and proline-rich proteins
Cytomegalovirus	DNA
Human herpes virus	DNA
Epstein-Barr virus	DNA
Hepatitis A virus	IgM, IgA, IgG, and RNA
Hepatitis B virus	HbsAg, HbsAb, HbcAb, and DNA
Measles	Measles virus antibodies
Rubella	Antibodies to rubella virus

Table 9: Salivary biomarkers in paediatric diseases

The level of evidence supporting the validity of the salivary biomarkers presented in Table 1 differs significantly. The concept of analyzing saliva for infection or disease may seem straightforward; however, the reality of integrating it into clinical practice has proven difficult. Some of the biomarkers are more powerful than others; salivary cortisol, for example is a well-studied biomarker for stress and is used in clinical studies as a "gold standard", compared to salivary α-amylase for which the literature is still contradictory. (Strahler, Skoluda, Kappert, & Nater, 2017) (Hellhammer, Wust, & Kudielka, 2009) (Bosch, Veerman, de Geus, & Proctor, 2011) Similarly, salivary leptin is a valuable obesity marker compared to salivary uric acid. (Hartman, et al., 2016) For the majority of the reported conditions, a single biomarker would not suffice to define the pathogenesis of a disease. Studies in large cohorts are needed to validate suggested salivary biomarkers and determine which saliva biomarkers are stronger candidates for a particular disease than others. At the same time, clinically-oriented research will essentially improve the level of supporting evidence available. Ultimately, the best diagnostic use of saliva will likely come from a combination of biomarkers for each pathologic condition, with clinical validity and improved accuracy and specificity.

Saliva collection

There is a variety of saliva collection methodologies. At present, saliva collection by passive drool directly into plastic tubes (unstimulated saliva) is the most recommended method, as most analytes may be quantified without any changes. (Kaczor-Urbanowicz, et al., 2017) However, the choice of collection systems should be carefully evaluated, taking into account the analyte to be quantified and the involved populations (elderly, neonates and children). In the case of infants and children, some commercial collection devices such as Salivette® Cortisol (Sarstedt, Newton, NC, USA) shown in figure 5 may be the preferable system.

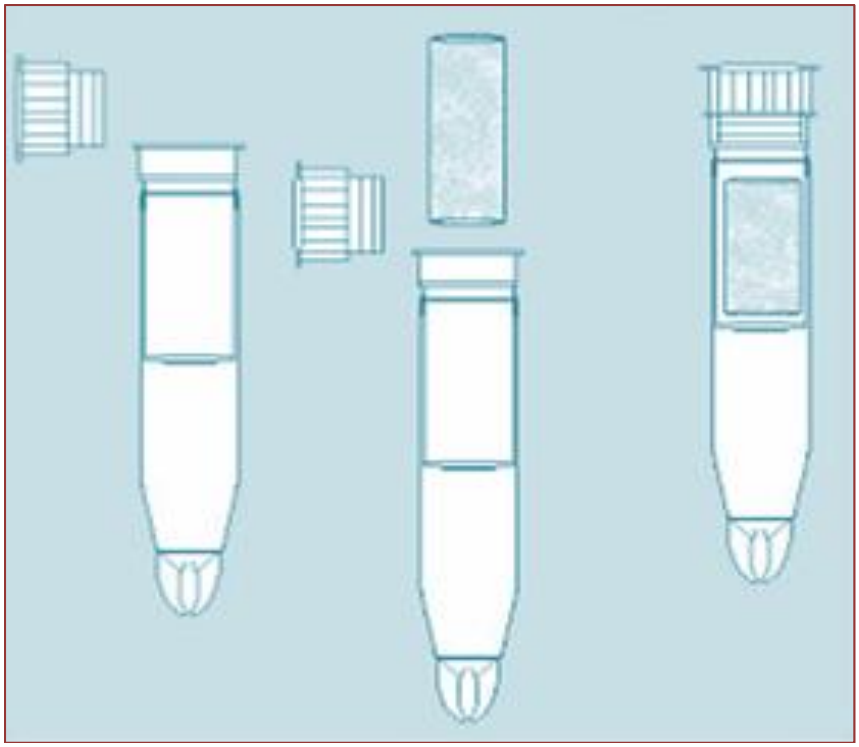


Figure 5: The Salivette® (Sarstedt, Newton, NC) collector. The system contains a cotton pad that is placed in the mouth, the saliva saturated pad is returned to its carrier. The saliva sample is then recovered from the pad by centrifugation.

Regardless of the saliva collection method, standardization is necessary for pre-analytical and analytical variables, such as collection and storage methods, circadian variation, sample recovery, prevention of sample contamination and analytical procedures. The concentration of biomarkers can be additionally affected by the salivary flow rates, age, the physiological status of the patients and their gingival health. This significant range of variables raises concern over the accuracy and reproducibility of diagnoses using salivary biomarkers. (Novy, 2014; Wren, Shirtcliff, & Drury, 2015) Despite the challenges, the use of saliva as a diagnostic or screening medium has advanced exponentially in the last decade. (Kaczor-Urbanowicz, et al., 2017)

In addition to conditions shown in Table 9, applications of salivary diagnostics in a paediatric population include immunological and inflammatory responses to vaccination, as well as cognitive function. In newborns, saliva is used for the enhancement of neonatal diagnosis, assessment of the developmental stage and monitoring of newborns' health in the neonatal intensive care unit. (Romano-Keeler, Wynn, & Maron, 2014)

Age-dependent variations in saliva composition

From early infancy to adolescence, salivary components, enzymes, hormones, electrolytes and inorganic compounds may vary significantly and their concentration reflects both the age and the developmental stage of the young individuals. More

specifically, salivary α-amylase increases with age, reaching its peak in adulthood, while calcium and magnesium levels decrease. (Nater & Rohleder, 2009) Studies on basal salivary α-amylase activity report undetectable concentrations in newborns and levels similar to adult concentrations at adolescence, which might be related to the physiological development of salivary glands. However, divergent results are observed on salivary α-amylase levels after various types of stressors across different age groups. (Bosch, et al., 2011; Nater & Rohleder, 2009) Accordingly, salivary cortisol levels are highly dependent on circadian rhythm, which continues to develop throughout the first 3 years of life. These confounding parameters, in combination with the interactions between hormones during puberty, further complicate comparisons among studies. (Jessop & Turner-Cobb, 2008) As a result, the observed age dependent variations in saliva composition are not reliable enough to be useful in clinical practice. (Bosch, et al., 2011)

In the early oral cavity, microbial colonization alters saliva's composition and is affected by the mode of delivery (caesarean or vaginal), the initiation of feeding and the type of nutrition (breastfeeding or formula). For example, between 3 to 6 months of age, tooth eruption along with the presence of crevicular fluid and the initiation of solid foods result in further variations in oral microbiota and differentiations in salivary profiles. (Maron et al., 2010; Morzel et al., 2011)

Saliva diagnostics of early childhood caries

Early childhood caries (ECC) describes dental caries in children 6 years old or younger. It is one of the most common infectious diseases in preschool children and results in severe carious lesions of the primary dentition, with potentially harmful physical and psychological effects. Early diagnosis, timely detection of risk factors and reinforcement of defence mechanisms of the oral cavity are important in order to protect this vulnerable population from the ECC occurrence. (Hajishengallis, Forrest, & Koo, 2016; Hajishengallis, Parsaei, Klein, & Koo, 2017) Susceptibility to caries is a result of the imbalance between oral microorganisms and the protective properties of saliva which, through specific biomolecules, regulate the defence against cariogenic bacteria and modulate oral microbiota. To date, the available risk assessment programs evaluate a number of parameters such as salivary levels of cariogenic pathogens (mutans streptococci and lactobacilli), salivary flow rate, pH and buffering capacity, previous dental history, dietary parameters, oral hygiene but have yet to show consistent validity. This can be explained by the multifactorial aetiology involved in the caries development process. (Pitts et al., 2017) Current research focuses on surmounting these limitations with the aid of omics technology, for the identification and quantification of salivary microbes that could be utilized as biomarkers for caries risk assessment and the exploration of salivary proteins that could be used to predict caries susceptibility. (Ao et al., 2017; Tian et al., 2017; Wang et al., 2018) Cariogenic bacteria (mutans streptococci, *C. albicans, Prevotella* spp.) and certain salivary proteins such as IgA, IgG immunoglobulins, PRP and histatin peptides, seem to be possibly valuable biomolecules to be used as biomarkers for caries risk prediction in the near future. (Hemadi, Huang, Zhou, & Zou, 2017)

Saliva diagnostics in aggressive periodontitis

Studies on salivary biomarker profile in children with respect to periodontal status report a positive correlation between the severity of inflammation and IgA concentration. (Li, Wong, Sun, Wen, & McGrath, 2015) Treated patients exhibited higher IgA and IgG levels to periodontal pathogens *porphyromonas gingivalis* and *Treponema Denticola*, while an increase in salivary IgG to *Aggregatibacter. Actinomycetemcomitans* (Aa) was observed in patients diagnosed with aggressive periodontitis. (Cuevas-Cordoba & Santiago-Garcia, 2014) IL-1β, IL-6, MMP-8, PGE_2, MIP-1a (Macrophage Inflammatory Protein-1a) and their respective salivary concentrations are widely studied to discriminate health from periodontal disease. MIP-1a in particular, is significantly correlated with children's susceptibility to localized aggressive periodontitis (LAP) and could be used as an early salivary biomarker for LAP. In a longitudinal cohort study on 96 periodontally healthy children, MIP-a was elevated 50-fold in students who developed disease 6 to 9 months prior to radiographic detection of bone loss and was additionally related to increasing probing depth. (Fine et al., 2009) A cross-sectional study in children with LAP reported that specific bacteria, such as *Aa,* play a destructive role in this disease and suggested that other species may be playing a protective role against LAP. (Shaddox et al., 2012) Given its rare occurrence, additional longitudinal studies are required to support the potential of such markers to assess children at risk for LAP.

Saliva diagnostics in conditions related to hypothalamic-pituitary-adrenal (HPA) axis activity.

Focus on salivary cortisol levels with respect to the hypothalamic-pituitary-adrenal (HPA) axis activity in children, are reported to correlate well with the deregulation of HPA axis. (Ryan, Booth, Spathis, Mollart, & Clow, 2016) Accordingly, assessment of cortisol in saliva is routinely measured as a biomarker of psychological stress. Stress-free saliva collection reflects the psychological condition in a real time manner, thus being a unique medium in stress research and psychoneuroendocrinology. (Hellhammer, et al., 2009; Shirtcliff et al., 2015)

HPA axis deregulation characterizes numerous diseases prevalent in childhood. Autism, depression and post traumatic stress disorder are examples of pathologies with reported alterations in salivary cortisol measurements in paediatric populations. (Putnam et al., 2012) In children, salivary cortisol is a significant biomarker for early identification of risk factors related to autoimmune diseases, cardiovascular disease and metabolic syndrome. (Jessop & Turner-Cobb, 2008) (Cozma et al., 2017; Keil, 2012) In paediatric psychophysiology, salivary cortisol is used for the assessment of the response of children to stressors such as mother-child separation events, start of kindergarten and pre-school programs, exposure to marital violence and response to dental treatment. (Bozovic, Racic, & Ivkovic, 2013; Simons, Cillessen, & de Weerth, 2017)

Several assay techniques have been used to measure salivary cortisol, including radio immune assay and more recently liquid chromatography–tandem mass

spectrometry. (Inder, Dimeski, & Russell, 2012) Clinically, the commonest use for salivary cortisol is measuring late-night salivary cortisol as a screening test for Cushing's syndrome. (Doi et al., 2008) A number of studies have shown diagnostic sensitivities and specificities of over 90%, which compares favorably with other screening tests for Cushing's syndrome such as the 24-h urinary-free cortisol and the 1-mg overnight dexamethasone suppression test. Lately, salivary cortisol is used in diagnosing adrenalin sufficiency, particularly in conditions associated with low cortisol–binding globulin levels, and in monitoring of glucocorticoid replacement. (Simons, et al., 2017)

It has been suggested that salivary cortisol can be used as a surrogate of free serum cortisol thus offering some advantages over measurements of serum cortisol. (Estrada & Orlander, 2011) However, age, gender, developmental stage, body mass index, sampling and assay conditions are all variables which should be taken into account in studies that use salivary cortisol as a marker. (Jessop & Turner-Cobb, 2008; Nayak, Bhad Patil, & Doshi, 2014)

Saliva diagnostics of metabolic disease

Salivary biomarkers have been recently explored as potentially useful screening tools in patients diagnosed with metabolic disorders such as obesity or diabetes. (Katsareli & Dedoussis, 2014) (Desai & Mathews, 2014; Hartman, et al., 2016) The urgent need to control the global obesity epidemic brings to attention at risk paediatric population for the application of preventive strategies, which include dietary guidelines and physical activity suggestions. Moderate exercise in children is found to decrease the incidence of infections. Salivary IgA, an immune biomarker also known as the “first-line of defence” against pathogens, was reported to be upregulated in children after moderate exercise, and downregulated following overexertion. (Starzak, Konkol, & McKune, 2016) According to the results of the reported study, after the examination of 132 children, body mass index (BMI) was found to be an independent predictor of salivary IgA secretion rate. (Starzak, et al., 2016) Salivary C-reactive protein (CRP), an inflammatory biomarker of the nonspecific acute phase response, is reported to be elevated in the saliva of obese children. (Cook et al., 2000) (Goodson et al., 2014) (Naidoo, Konkol, Biccard, Dudose, & McKune, 2012) In a study of 170 South African children, salivary CRP concentrations showed that obese children had significantly higher salivary CRP values compared to the normal-weight control group. (Naidoo, et al., 2012) Cook et al. examined 699 children and found that obesity is a major determinant of CRP levels, which were also significantly correlated with several cardiovascular risk factors. (Cook, et al., 2000) Similar results were reported by Goodson et al. who studied the metabolic disease risk in 744 11-year old children. Alterations in levels of salivary CRP, salivary insulin, leptin and adiponectin,which were observed in this large cohort, suggest that salivary biomarkers could be used for identification of vulnerable subjects. (Goodson, et al., 2014) The above reported alterations in salivary biomarkers in children and adolescents could suggest the initiation of

metabolic changes associated with obesity and diabetes. However, due to the complexity of biological pathways interactions, further research and longitudinal studies are necessary before any of these markers could reach an accurate diagnostic value. (Hartman, et al., 2016)

Saliva omics in paediatrics: applications in oral and systemic health and disease

Saliva's principle limitation as a diagnostic fluid is that many important biomolecules are found to be in low concentration when compared to blood. Over the past two decades, advances in high-throughput technologies are surmounting these limitations; nowadays, genome, proteome, transcriptome, metabolome and microbiome-based approaches have been applied in order to comprehensively analyse this oral fluid, discover discriminatory biomarkers and combine them in order to obtain additive and powerful diagnostic information. This cutting edge technology offers clinicians a unique opportunity to discriminate between health and disease and associate specific biomolecules to diagnosis, prognosis and personalized therapeutic strategies. (Kaczor-Urbanowicz, et al., 2017; Yang, Peretz-Soroka, Liu, & Lin, 2016) Portable, user-friendly devices offer a rapid, reliable and sensitive instrument for on-the-spot biomarker discovery and monitoring. (Meagher & Kousvelari, 2018) For example, the detection of sexually transmitted infections, glucose testing, cardiovascular risk assessment, drug detection and quantification of cortisol and α-amylase levels for stress assessment are only few paradigms of such advances. However, saliva based microfluidic devices are not yet in use in clinical setting. (Aro, Wei, Wong, & Tu, 2017)

Saliva is not only a source of cellular DNA but also a provider of cell-free, exosomal DNA, with encoded information regarding cell-to-cell communication. In a recent pilot study, Tu et al. reported oncogenic mutations by using a novel liquid biopsy system in non-small lung cancer patients. (Tu, Chia, Wei, & Wong, 2016) Studies on paediatric populations are yet to be conducted but salivary cell-free DNA assays may, in the near future, have a direct impact on cancer biology applications and offer young patients a non-invasive way to assess and monitor response to therapy.

Salivary proteomics

Diabetes research is an example of the applications of salivary proteomics. Saliva proteome changes are shown in type 1 and type 2 diabetic patients. In a comprehensive proteomic analysis, Rao et al. paved the way for the characterization of the salivary proteome in subjects with type 2 diabetes, identifying a total of 487 proteins. Sixty five of them were found to be differentially expressed in saliva from patients with diabetes versus controls. (Rao, et al., 2009) Moreover, salivary proteomic modifications were identified in children with type 1 diabetes, when compared to healthy controls, indicating down-regulation of peptides involved in oral cavity host defence in these patients. (Cabras, et al., 2010) Statherin, proline-rich peptide PB, salivary acidic proline-rich phosphoprotein 1/2 and histatin 1 were reported to be significantly less abundant, whereas isoforms of S100 calcium-binding protein A9 were more abundant in the saliva of children with type 1 diabetes.

(Cabras, et al., 2010) This study provides evidence for the utility of well identified markers for the diagnosis and monitoring of the disease. Moreover, in a recent salivary proteomics study of our group, poorly controlled diabetic type 1 subjects were distinguished from well controlled and healthy subjects. In turn, the functional analysis of the differentially expressed proteins (among which: S100 calcium-binding protein A7, alpha-2 macroglobulin, alpha-1 antitrypsin, apolipoprotein A1, plasmin, complement component 3) demonstrated the deregulation of biological mechanisms highly relevant to diabetic pathophysiology (inflammation, atherosclerosis signaling, coagulation, etc.) before any clinical complications (retinopathy, microalbuminuria, neuropathy) associated with the disease. The use of proteomics and bioinformatics analyses could allow the assessment of the status of asymptomatic diabetic patients in the future. (Pappa, 2018)

Salivary proteomics are also utilized to identify skeletal growth markers during childhood. Salivary insulin growth factor (IGF-1) was found to be a promising indicator of skeletal maturity. (Nayak, et al., 2014) In a pilot study by Ngounou Wetie et al., a large number of proteins exhibiting differential expression were identified in pooled saliva of 6 children with ASD and their matched controls. (Ngounou Wetie et al., 2015) Specifically, increased levels of prolactin-induced protein, lactotransferrin, annexin A1, neutrophil-defensin 1, lactoperoxidase, and lipocalin-1 were detected in ASD versus controls, whereas, the levels of salivary acidic proline-rich phosphoprotein 1/2, submaxillary gland androgen regulated protein 3B, antileukoproteinase, pleckstrin-homologydomain-containing family H member, and statherin were decreased in ASD versus controls. (Ngounou Wetie, et al., 2015) Ultimately, prospective studies are necessary to validate the above molecules as diagnostic markers of ASD.

Salivary microbiomics

The human salivary microbiome play a role in diseases of the oral cavity and perhaps more broadly, through interactions with microbiomes of other microenvironments of the human body. (Costalonga & Herzberg, 2014) Characterization of the enormous diversity in the human salivary microbiome will aid in the diagnosis of human infectious disease. (Nasidze, Li, Quinque, Tang, & Stoneking, 2009) Saliva is a suitable source for comprehensive genotyping. Genomics, metagenomic and metatranscriptomic analyses have been used to characterize the diversity and community composition of the salivary microbiota. (Belstrom et al., 2017)

Microbial profiles of saliva enable the detection of various oral infectious diseases, as well as upper respiratory infections caused by influenza virus, human bocavirus type 1 and infections by cytomegalovirus (CMV), Epstein-Barr virus, human herpes virus (HHV6, HHV7) and Zika virus. (D. T. Wong, 2012) The development of microfluidic technologies based on molecular diagnosis has enabled early detection of infections, especially in developing countries where infectious diseases are the principal cause of death of children. (Hill, You, Inoue, & Oestergaard, 2012)

Salivary analysis in paediatric populations is used to detect immunological response to mumps, measles, human immunodeficiency virus, hepatitis B virus and herpes simplex virus, and response to vaccination, through measurements of saliva Ig levels. (Lim, Garssen, & Sandalova, 2016; Nokes et al., 2001) Measuring the level of salivary antibodies enables detection of Morbillivirus infection causing measles (with 97% sensitivity and 100% specificity), Paramyxoviridae causing mumps (94% sensitivity and 94% specificity), or Togaviridiae causing rubella (98% sensitivity and 98% specificity). (Kaczor-Urbanowicz, et al., 2017) Oral microbial alterations have also been observed in children with autism spectrum disorder (ASD). (Rosenfeld, 2015) (Qiao et al., 2018) Specifically, Qiao et al., via high-throughput sequencing of 111 oral samples in 32 children with ASD and 27 healthy controls, showed that the salivary and dental microbiota of ASD patients were highly distinct from those of healthy individuals. Pathogens such as Haemophilus in saliva and Streptococcus in plaques showed significantly higher abundance in ASD patients, whereas commensals such as Prevotella, Selenomonas, Actinomyces, Porphyromonas, and Fusobacterium were reduced. The distinguishable bacteria were also correlated with clinical indices, reflecting disease severity and the oral health status (i.e. dental caries). Finally, diagnostic models based on key microbes were constructed, with 96.3% accuracy in saliva. Characterization of the specific profile of the oral microbiota in ASD patients, might help develop novel strategies for the diagnosis of ASD. (Qiao, et al., 2018) In the future, oral microbial profile changes could provide a new approach into the design of novel strategies with increased value for the diagnosis of ASD than the existing empirical DSM method. However, till such time verification of oral microbial changes in large cohorts of ASD patient is required.

Salivary diagnostics in neonates

Integration of the omics technologies with neonatal saliva promises real time evaluation of the health condition in preterm infants. With the aid of transcriptomic technology, Maron et al. unveiled an enormous amount of developmental information encrypted in neonatal saliva. Their group identified 2,186 regulatory genes in as little as 50 µL of saliva of premature infants who were unsuccessful in oral feeding. The panel of identified genes included gene transcripts for hunger signaling, palate development and sensory integration. Clearly, these advancements should pave the way for well-designed cohort studies to exploit the unique potential of the neonatal salivary proteome in enhancing our understanding of developmental biology. (Maron, et al., 2010) A recent pilot study aimed to translate the previous described gene expression panel to a salivary proteomic platform with the goal to timely and accurately assess the oral feeding capability. The developed proteomic platform was used to assess hypothalamic feeding development in neonatal saliva; however, only the proteins involved in hunger signaling were detected in neonatal saliva at measurable levels. (Khanna, Maron, & Walt, 2017)

Diagnosis of congenital CMV infection, a leading cause of hearing loss in children, can quickly and effectively detected by real-time polymerase chain reaction (PCR) assays of newborns' saliva specimens. Boppana et al. have simplified this screening method, with the use of dried specimens and processing that does not require a

DNA-extraction step, without significant loss of sensitivity or specificity. (Boppana et al., 2011) This strategy appears to be an attractive high-throughput assay for large-scale screening to identify newborns with congenital CMV infection in the future. CMV-infected neonates are usually asymptomatic at birth, which highlights the need for rapid assessment and appropriate intervention in such cases. In a large clinical trial, 11715 consecutive newborns were screened for congenital CMV by polymerase chain reaction (PCR) using saliva. Differences were found depending on the socio demographic characteristics of women giving birth to an infected baby after primary and non primary infection. Seronegative, parous women represent the highest risk population for congenital CMV in countries with low to intermediate seroprevalence. (Leruez-Ville et al., 2017) In developed countries, academic medical centres often utilize CMV quantitative and qualitative PCR testing. (Wissel, 2017)

Salivary proteomics studies have recently showed markers with prognostic potential for brochopulmonary dysplasia (BPD), a complex disorder in neonatal populations for which there is not yet an effective treatment. Clearly, reliable markers are of outmost importance for early detection of at risk babies. (Piersigilli & Bhandari, 2016)

Neonatal salivary CRP is readily detectable in the neonate and demonstrates good accuracy at discriminating between clinically relevant serum CRP thresholds. It presents ideal characteristics for closely monitoring infants at risk for post surgical complications thus, eliminating the need of frequent blood draws in newborns. It is considered as a nonspecific marker of inflammation; yet, its negative predictive value reaches 99% of accuracy for identifying non septic neonates. (Keane, Fallon, Riordan, & Shaw, 2015) (Iyengar, Paulus, Gerlanc, & Maron, 2014) The findings of Iyengar et al. highlighted the clinical utility of salivary CRP in predicting serum CRP levels and at the same time, laid the foundation for the development of a non-invasive sepsis screening tool. Despite these advancements, the field has yet to see its integration into clinical practice. (Iyengar & Maron, 2015; Iyengar, et al., 2014)

Saliva is a useful diagnostic blood surrogate. Biomarkers in saliva can be highly informative and discriminatory in early detection of a variety of diseases and conditions in neonates and children. Because of its easy, simple, painless, non-invasive collection, saliva alleviates patients' discomfort and facilitates compliance, especially in neonates and children where repeated blood sampling can be both traumatic and difficult. Complementing this, the exponential growth of miniaturized microfluidic diagnostic technologies utilizing saliva, enable various on-chip chemical and biological assays with reduced reagent and sample consumption. Together with mobile phone image processing tools such platforms offer powerful systems for routine health tests for a number of disease diagnosis at the point-of-care i.e. the doctor's office and even at home. We envision that in the future paediatricians and other caregivers will be able to diagnose and monitor therapeutic strategies in metabolic diseases such diabetes and obesity, inflammation, infection, stress response and development conditions in children at different ages with only a drop of saliva at the point-of-care.

References

Abbate, G. M., Borghi, D., Passi, A., & Levrini, L. (2014). Correlation between unstimulated salivary flow, pH and streptococcus mutans, analysed with real time PCR, in caries-free and caries-active children. *Eur J Paediatr Dent, 15*(1), 51-54.

Ai, J. Y., Smith, B., & Wong, D. T. (2012). Bioinformatics advances in saliva diagnostics. *Int J Oral Sci, 4*(2), 85-87. doi: ijos201226 [pii]
10.1038/ijos.2012.26

Al Kawas, S., Rahim, Z. H., & Ferguson, D. B. (2012). Potential uses of human salivary protein and peptide analysis in the diagnosis of disease. *Arch Oral Biol, 57*(1), 1-9. doi: S0003-9969(11)00202-0 [pii]
10.1016/j.archoralbio.2011.06.013

Amado, F., Lobo, M. J., Domingues, P., Duarte, J. A., & Vitorino, R. (2010). Salivary peptidomics. *Expert Rev Proteomics, 7*(5), 709-721. doi: 10.1586/epr.10.48

Amado, F. M., Ferreira, R. P., & Vitorino, R. (2013). One decade of salivary proteomics: current approaches and outstanding challenges. *Clin Biochem, 46*(6), 506-517. doi: S0009-9120(12)00579-6 [pii]
10.1016/j.clinbiochem.2012.10.024

An, Z., Chen, Y., Zhang, R., Song, Y., Sun, J., He, J., et al. (2010). Integrated ionization approach for RRLC-MS/MS-based metabonomics: finding potential biomarkers for lung cancer. *J Proteome Res, 9*(8), 4071-4081. doi: 10.1021/pr100265g

Ao, S., Sun, X., Shi, X., Huang, X., Chen, F., & Zheng, S. (2017). Longitudinal investigation of salivary proteomic profiles in the development of early childhood caries. *J Dent, 61*, 21-27. doi: S0300-5712(17)30090-8 [pii]
10.1016/j.jdent.2017.04.006

Aro, K., Wei, F., Wong, D. T., & Tu, M. (2017). Saliva Liquid Biopsy for Point-of-Care Applications. *Front Public Health, 5*, 77. doi: 10.3389/fpubh.2017.00077

Arredouani, A., Stocchero, M., Culeddu, N., El-Sayed Moustafa, J., Tichet, J., Balkau, B., et al. (2016). Metabolomic Profile of Low Copy-Number Carriers at the Salivary Alpha-Amylase Gene Suggests a Metabolic Shift Towards Lipid-Based Energy Production. *Diabetes*. doi: db16-0315 [pii]
10.2337/db16-0315

Bagherian, A., & Asadikaram, G. (2012). Comparison of some salivary characteristics between children with and without early childhood caries. *Indian J Dent Res, 23*(5), 628-632. doi: IndianJDentRes_2012_23_5_628_107380 [pii]
10.4103/0970-9290.107380

Bahar, G., Feinmesser, R., Shpitzer, T., Popovtzer, A., & Nagler, R. M. (2007). Salivary analysis in oral cancer patients: DNA and protein oxidation, reactive nitrogen species, and antioxidant profile. *Cancer, 109*(1), 54-59. doi: 10.1002/cncr.22386

Basham, R. J., Richardson, D. M., Sutcliffe, C. B., & Haas, D. W. (2009). Effect of HIV-1 infection on human DNA yield from saliva. *HIV Clin Trials, 10*(4), 282-285. doi: Y65W64K36346NK41 [pii]
10.1310/hct1004-282
Belstrom, D., Constancias, F., Liu, Y., Yang, L., Drautz-Moses, D. I., Schuster, S. C., et al. (2017). Metagenomic and metatranscriptomic analysis of saliva reveals disease-associated microbiota in patients with periodontitis and dental caries. *NPJ Biofilms Microbiomes, 3*, 23. doi: 10.1038/s41522-017-0031-4
31 [pii]
Boppana, S. B., Ross, S. A., Shimamura, M., Palmer, A. L., Ahmed, A., Michaels, M. G., et al. (2011). Saliva polymerase-chain-reaction assay for cytomegalovirus screening in newborns. *N Engl J Med, 364*(22), 2111-2118. doi: 10.1056/NEJMoa1006561
Bosch, J. A., Veerman, E. C., de Geus, E. J., & Proctor, G. B. (2011). alpha-Amylase as a reliable and convenient measure of sympathetic activity: don't start salivating just yet! *Psychoneuroendocrinology, 36*(4), 449-453. doi: S0306-4530(11)00007-2 [pii]
10.1016/j.psyneuen.2010.12.019
Bozovic, D., Racic, M., & Ivkovic, N. (2013). Salivary cortisol levels as a biological marker of stress reaction. *Med Arch, 67*(5), 374-377.
Cabras, T., Pisano, E., Mastinu, A., Denotti, G., Pusceddu, P. P., Inzitari, R., et al. (2010). Alterations of the salivary secretory peptidome profile in children affected by type 1 diabetes. *Mol Cell Proteomics, 9*(10), 2099-2108. doi: M110.001057 [pii]
10.1074/mcp.M110.001057
Carleo, A., Bennett, D., & Rottoli, P. (2016). Biomarkers in sarcoidosis: the contribution of system biology. *Curr Opin Pulm Med, 22*(5), 509-514. doi: 10.1097/MCP.0000000000000306
Castagnola, M., Cabras, T., Vitali, A., Sanna, M. T., & Messana, I. (2011). Biotechnological implications of the salivary proteome. *Trends Biotechnol, 29*(8), 409-418. doi: S0167-7799(11)00068-0 [pii]
10.1016/j.tibtech.2011.04.002
Cho, M. A., Ko, J. Y., Kim, Y. K., & Kho, H. S. (2010). Salivary flow rate and clinical characteristics of patients with xerostomia according to its aetiology. *J Oral Rehabil, 37*(3), 185-193. doi: JOR2037 [pii]
10.1111/j.1365-2842.2009.02037.x
Choi, M. (2010). Saliva diagnostics integrate dentistry into general and preventive health care. *Int J Prosthodont, 23*(3), 189.
Cook, D. G., Mendall, M. A., Whincup, P. H., Carey, I. M., Ballam, L., Morris, J. E., et al. (2000). C-reactive protein concentration in children: relationship to adiposity and other cardiovascular risk factors. *Atherosclerosis, 149*(1), 139-150. doi: S0021-9150(99)00312-3 [pii]
Costalonga, M., & Herzberg, M. C. (2014). The oral microbiome and the immunobiology of periodontal disease and caries. *Immunol Lett, 162*(2 Pt A), 22-38. doi: S0165-2478(14)00187-4 [pii]
10.1016/j.imlet.2014.08.017

Cozma, S., Dima-Cozma, L. C., Ghiciuc, C. M., Pasquali, V., Saponaro, A., & Patacchioli, F. R. (2017). Salivary cortisol and alpha-amylase: subclinical indicators of stress as cardiometabolic risk. *Braz J Med Biol Res, 50*(2), e5577. doi: S0100-879X2017000200201 [pii]
10.1590/1414-431X20165577
Cuevas-Cordoba, B., & Santiago-Garcia, J. (2014). Saliva: a fluid of study for OMICS. *OMICS, 18*(2), 87-97. doi: 10.1089/omi.2013.0064
Cunha-Cruz, J., Scott, J., Rothen, M., Mancl, L., Lawhorn, T., Brossel, K., et al. (2013). Salivary characteristics and dental caries: evidence from general dental practices. *J Am Dent Assoc, 144*(5), e31-40. doi: S0002-8177(14)60499-2 [pii]
da Silva Modesto, K. B., de Godoi Simoes, J. B., de Souza, A. F., Damaceno, N., Duarte, D. A., Leite, M. F., et al. (2015). Salivary flow rate and biochemical composition analysis in stimulated whole saliva of children with cystic fibrosis. *Arch Oral Biol, 60*(11), 1650-1654. doi: S0003-9969(15)30024-8 [pii]
10.1016/j.archoralbio.2015.08.007
de Oliveira, S. A., Siqueira, M. M., Brown, D. W., Litton, P., Camacho, L. A., Castro, S. T., et al. (2000). Diagnosis of rubella infection by detecting specific immunoglobulin M antibodies in saliva samples: a clinic-based study in Niteroi, RJ, Brazil. *Rev Soc Bras Med Trop, 33*(4), 335-339.
Deepa, T., & Thirrunavukkarasu, N. (2010). Saliva as a potential diagnostic tool. *Indian J Med Sci, 64*(7), 293-306. doi: IndianJMedSci_2010_64_7_293_99854 [pii]
10.4103/0019-5359.99854
Desai, G. S., & Mathews, S. T. (2014). Saliva as a non-invasive diagnostic tool for inflammation and insulin-resistance. *World J Diabetes, 5*(6), 730-738. doi: 10.4239/wjd.v5.i6.730
Dodds, M. W., Johnson, D. A., & Yeh, C. K. (2005). Health benefits of saliva: a review. *J Dent, 33*(3), 223-233. doi: S0300-5712(04)00172-1 [pii]
10.1016/j.jdent.2004.10.009
Doi, M., Sekizawa, N., Tani, Y., Tsuchiya, K., Kouyama, R., Tateno, T., et al. (2008). Late-night salivary cortisol as a screening test for the diagnosis of Cushing's syndrome in Japan. *Endocr J, 55*(1), 121-126. doi: JST.JSTAGE/endocrj/K07E-023 [pii]
Estrada, Y. M. R. M., & Orlander, P. R. (2011). Salivary cortisol can replace free serum cortisol measurements in patients with septic shock. *Chest, 140*(5), 1216-1222. doi: S0012-3692(11)60588-6 [pii]
10.1378/chest.11-0448
Fine, D. H., Markowitz, K., Furgang, D., Fairlie, K., Ferrandiz, J., Nasri, C., et al. (2009). Macrophage inflammatory protein-1alpha: a salivary biomarker of bone loss in a longitudinal cohort study of children at risk for aggressive periodontal disease? *J Periodontol, 80*(1), 106-113. doi: 10.1902/jop.2009.080296
10.1902/jop.2009.080296 [pii]
Giannobile, W. V., Beikler, T., Kinney, J. S., Ramseier, C. A., Morelli, T., & Wong, D. T. (2009). Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontol 2000, 50*, 52-64. doi: PRD288 [pii]
10.1111/j.1600-0757.2008.00288.x

Goodson, J. M., Kantarci, A., Hartman, M. L., Denis, G. V., Stephens, D., Hasturk, H., et al. (2014). Metabolic disease risk in children by salivary biomarker analysis. *PLoS One, 9*(6), e98799. doi: 10.1371/journal.pone.0098799
PONE-D-14-07713 [pii]
Hajishengallis, E., Forrest, C. B., & Koo, H. (2016). Early Childhood Caries: Future Perspectives in Risk Assessment. *JDR Clin Trans Res, 1*(2), 110-111. doi: 10.1177/2380084416637577
10.1177_2380084416637577 [pii]
Hajishengallis, E., Parsaei, Y., Klein, M. I., & Koo, H. (2017). Advances in the microbial etiology and pathogenesis of early childhood caries. *Mol Oral Microbiol, 32*(1), 24-34. doi: 10.1111/omi.12152
Hartman, M. L., Goodson, J. M., Barake, R., Alsmadi, O., Al-Mutawa, S., Ariga, J., et al. (2016). Salivary Biomarkers in Pediatric Metabolic Disease Research. *Pediatr Endocrinol Rev, 13*(3), 602-611.
Hassaneen, M., & Maron, J. L. (2017). Salivary Diagnostics in Pediatrics: Applicability, Translatability, and Limitations. *Front Public Health, 5*, 83. doi: 10.3389/fpubh.2017.00083
Hellhammer, D. H., Wust, S., & Kudielka, B. M. (2009). Salivary cortisol as a biomarker in stress research. *Psychoneuroendocrinology, 34*(2), 163-171. doi: S0306-4530(08)00299-0 [pii]
10.1016/j.psyneuen.2008.10.026
Hemadi, A. S., Huang, R., Zhou, Y., & Zou, J. (2017). Salivary proteins and microbiota as biomarkers for early childhood caries risk assessment. *Int J Oral Sci, 9*(11), e1. doi: ijos201735 [pii]
10.1038/ijos.2017.35
Hill, K., You, D., Inoue, M., & Oestergaard, M. Z. (2012). Child mortality estimation: accelerated progress in reducing global child mortality, 1990-2010. *PLoS Med, 9*(8), e1001303. doi: 10.1371/journal.pmed.1001303
PMEDICINE-D-11-03032 [pii]
Hu, S., Gao, K., Pollard, R., Arellano-Garcia, M., Zhou, H., Zhang, L., et al. (2010). Preclinical validation of salivary biomarkers for primary Sjogren's syndrome. *Arthritis Care Res (Hoboken), 62*(11), 1633-1638. doi: 10.1002/acr.20289
Inder, W. J., Dimeski, G., & Russell, A. (2012). Measurement of salivary cortisol in 2012 - laboratory techniques and clinical indications. *Clin Endocrinol (Oxf), 77*(5), 645-651. doi: 10.1111/j.1365-2265.2012.04508.x
Iyengar, A., & Maron, J. L. (2015). Detecting infection in neonates: promises and challenges of a salivary approach. *Clin Ther, 37*(3), 523-528. doi: S0149-2918(15)00072-7 [pii]
10.1016/j.clinthera.2015.02.006
Iyengar, A., Paulus, J. K., Gerlanc, D. J., & Maron, J. L. (2014). Detection and potential utility of C-reactive protein in saliva of neonates. *Front Pediatr, 2*, 131. doi: 10.3389/fped.2014.00131
Javaid, M. A., Ahmed, A. S., Durand, R., & Tran, S. D. (2016). Saliva as a diagnostic tool for oral and systemic diseases. *J Oral Biol Craniofac Res, 6*(1), 66-75. doi: 10.1016/j.jobcr.2015.08.006
S2212-4268(15)00089-5 [pii]

Jessop, D. S., & Turner-Cobb, J. M. (2008). Measurement and meaning of salivary cortisol: a focus on health and disease in children. *Stress, 11*(1), 1-14. doi: 780648151 [pii]
10.1080/10253890701365527
Kaczor-Urbanowicz, K. E., Martin Carreras-Presas, C., Aro, K., Tu, M., Garcia-Godoy, F., & Wong, D. T. (2017). Saliva diagnostics - Current views and directions. *Exp Biol Med (Maywood), 242*(5), 459-472. doi: 1535370216681550 [pii]
10.1177/1535370216681550
Karlik, M., Valkovic, P., Hancinova, V., Krizova, L., Tothova, L., & Celec, P. (2015). Markers of oxidative stress in plasma and saliva in patients with multiple sclerosis. *Clin Biochem, 48*(1-2), 24-28. doi: S0009-9120(14)00703-6 [pii]
10.1016/j.clinbiochem.2014.09.023
Katsareli, E. A., & Dedoussis, G. V. (2014). Biomarkers in the field of obesity and its related comorbidities. *Expert Opin Ther Targets, 18*(4), 385-401. doi: 10.1517/14728222.2014.882321
Keane, M., Fallon, R., Riordan, A., & Shaw, B. (2015). Markedly raised levels of C-reactive protein are associated with culture-proven sepsis or necrotising enterocolitis in extremely preterm neonates. *Acta Paediatr, 104*(7), e289-293. doi: 10.1111/apa.12978
Keil, M. F. (2012). Salivary cortisol: a tool for biobehavioral research in children. *J Pediatr Nurs, 27*(3), 287-289. doi: S0882-5963(12)00050-4 [pii]
10.1016/j.pedn.2012.02.003
Khanna, P., Maron, J. L., & Walt, D. R. (2017). Development of a Rapid Salivary Proteomic Platform for Oral Feeding Readiness in the Preterm Newborn. *Front Pediatr, 5*, 268. doi: 10.3389/fped.2017.00268
Lee, Y. H., & Wong, D. T. (2009). Saliva: an emerging biofluid for early detection of diseases. *Am J Dent, 22*(4), 241-248.
Leruez-Ville, M., Magny, J. F., Couderc, S., Pichon, C., Parodi, M., Bussieres, L., et al. (2017). Risk Factors for Congenital Cytomegalovirus Infection Following Primary and Nonprimary Maternal Infection: A Prospective Neonatal Screening Study Using Polymerase Chain Reaction in Saliva. *Clin Infect Dis, 65*(3), 398-404. doi: 3737620 [pii]
10.1093/cid/cix337
Li, L. W., Wong, H. M., Sun, L., Wen, Y. F., & McGrath, C. P. (2015). Anthropometric measurements and periodontal diseases in children and adolescents: a systematic review and meta-analysis. *Adv Nutr, 6*(6), 828-841. doi: 6/6/828 [pii]
10.3945/an.115.010017
Liljestrand, J. M., Gursoy, U. K., Hyvarinen, K., Sorsa, T., Suominen, A. L., Kononen, E., et al. (2014). Combining salivary pathogen and serum antibody levels improves their diagnostic ability in detection of periodontitis. *J Periodontol, 85*(1), 123-131. doi: 10.1902/jop.2013.130030
Lim, P. W., Garssen, J., & Sandalova, E. (2016). Potential Use of Salivary Markers for Longitudinal Monitoring of Inflammatory Immune Responses to Vaccination. *Mediators Inflamm, 2016*, 6958293. doi: 10.1155/2016/6958293
Lopez-Pintor, R. M., Casanas, E., Gonzalez-Serrano, J., Serrano, J., Ramirez, L., de Arriba, L., et al. (2016). Xerostomia, Hyposalivation, and Salivary Flow in

Diabetes Patients. *J Diabetes Res, 2016*, 4372852. doi: 10.1155/2016/4372852

Malamud, D. (2011). Saliva as a diagnostic fluid. *Dent Clin North Am, 55*(1), 159-178. doi: S0011-8532(10)00081-9 [pii]
10.1016/j.cden.2010.08.004

Mandel, I. D., Kutscher, A., Denning, C. R., Thompson, R. H., Jr., & Zegarelli, E. V. (1967). Salivary studies in cystic fibrosis. *Am J Dis Child, 113*(4), 431-438.

Maron, J. L., Johnson, K. L., Rocke, D. M., Cohen, M. G., Liley, A. J., & Bianchi, D. W. (2010). Neonatal salivary analysis reveals global developmental gene expression changes in the premature infant. *Clin Chem, 56*(3), 409-416. doi: clinchem.2009.136234 [pii]
10.1373/clinchem.2009.136234

Meagher, R. J., & Kousvelari, E. (2018). Mobile oral heath technologies based on saliva. *Oral Dis, 24*(1-2), 194-197. doi: 10.1111/odi.12775

Mirzaii-Dizgah, I., Hejazi, S. F., Riahi, E., & Salehi, M. M. (2012). Saliva-based creatine kinase MB measurement as a potential point-of-care testing for detection of myocardial infarction. *Clin Oral Investig, 16*(3), 775-779. doi: 10.1007/s00784-011-0578-z

Mirzaii-Dizgah, I., & Riahi, E. (2013). Salivary high-sensitivity cardiac troponin T levels in patients with acute myocardial infarction. *Oral Dis, 19*(2), 180-184. doi: 10.1111/j.1601-0825.2012.01968.x

Morzel, M., Palicki, O., Chabanet, C., Lucchi, G., Ducoroy, P., Chambon, C., et al. (2011). Saliva electrophoretic protein profiles in infants: changes with age and impact of teeth eruption and diet transition. *Arch Oral Biol, 56*(7), 634-642. doi: S0003-9969(11)00004-5 [pii]
10.1016/j.archoralbio.2010.12.015

Nagler, R. M., Hershkovich, O., Lischinsky, S., Diamond, E., & Reznick, A. Z. (2002). Saliva analysis in the clinical setting: revisiting an underused diagnostic tool. *J Investig Med, 50*(3), 214-225. doi: 10.2310/6650.2002.33436

Naidoo, T., Konkol, K., Biccard, B., Dudose, K., & McKune, A. J. (2012). Elevated salivary C-reactive protein predicted by low cardio-respiratory fitness and being overweight in African children. *Cardiovasc J Afr, 23*(9), 501-506. doi: 10.5830/CVJA-2012-058

Nasidze, I., Li, J., Quinque, D., Tang, K., & Stoneking, M. (2009). Global diversity in the human salivary microbiome. *Genome Res, 19*(4), 636-643. doi: gr.084616.108 [pii]
10.1101/gr.084616.108

Nater, U. M., & Rohleder, N. (2009). Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. *Psychoneuroendocrinology, 34*(4), 486-496. doi: S0306-4530(09)00032-8 [pii]
10.1016/j.psyneuen.2009.01.014

Naumova, E. A., Sandulescu, T., Bochnig, C., Al Khatib, P., Lee, W. K., Zimmer, S., et al. (2014). Dynamic changes in saliva after acute mental stress. *Sci Rep, 4*, 4884. doi: srep04884 [pii]
10.1038/srep04884

Nayak, S., Bhad Patil, W. A., & Doshi, U. H. (2014). The relationship between salivary insulin-like growth factor I and quantitative cervical maturational stages of

skeletal maturity. *J Orthod, 41*(3), 170-174. doi: 1465313313Y.0000000091 [pii]
10.1179/1465313313Y.0000000091
Ngounou Wetie, A. G., Wormwood, K. L., Russell, S., Ryan, J. P., Darie, C. C., & Woods, A. G. (2015). A Pilot Proteomic Analysis of Salivary Biomarkers in Autism Spectrum Disorder. *Autism Res, 8*(3), 338-350. doi: 10.1002/aur.1450
Nokes, D. J., Enquselassie, F., Nigatu, W., Vyse, A. J., Cohen, B. J., Brown, D. W., et al. (2001). Has oral fluid the potential to replace serum for the evaluation of population immunity levels? A study of measles, rubella and hepatitis B in rural Ethiopia. *Bull World Health Organ, 79*(7), 588-595. doi: S0042-96862001000700003 [pii]
Novy, B. B. (2014). Saliva and biofilm-based diagnostics: a critical review of the literature concerning sialochemistry. *J Evid Based Dent Pract, 14 Suppl*, 27-32. doi: S1532-3382(14)00068-2 [pii]
10.1016/j.jebdp.2014.04.004
Pappa, E., Vastardis, H., Mermelekas, G., Gerasimidi-Vazeou, A., Zoidakis, J., & Vougas, K. (2018). Saliva proteomics analysis offers insights on type 1 diabetes pathology in a pediatric population. *Frontiers in Physiology, 9, 444.* doi: 10.3389/fphys.2018.00444
Park, N. J., Li, Y., Yu, T., Brinkman, B. M., & Wong, D. T. (2006). Characterization of RNA in saliva. *Clin Chem, 52*(6), 988-994. doi: clinchem.2005.063206 [pii]
10.1373/clinchem.2005.063206
Piersigilli, F., & Bhandari, V. (2016). Biomarkers in neonatology: the new "omics" of bronchopulmonary dysplasia. *J Matern Fetal Neonatal Med, 29*(11), 1758-1764. doi: 10.3109/14767058.2015.1061495
Pitts, N. B., Zero, D. T., Marsh, P. D., Ekstrand, K., Weintraub, J. A., Ramos-Gomez, F., et al. (2017). Dental caries. *Nat Rev Dis Primers, 3*, 17030. doi: nrdp201730 [pii]
10.1038/nrdp.2017.30
Putnam, S. K., Lopata, C., Fox, J. D., Thomeer, M. L., Rodgers, J. D., Volker, M. A., et al. (2012). Comparison of saliva collection methods in children with high-functioning autism spectrum disorders: acceptability and recovery of cortisol. *Child Psychiatry Hum Dev, 43*(4), 560-573. doi: 10.1007/s10578-012-0284-3
Qiao, Y., Wu, M., Feng, Y., Zhou, Z., Chen, L., & Chen, F. (2018). Alterations of oral microbiota distinguish children with autism spectrum disorders from healthy controls. *Sci Rep, 8*(1), 1597. doi: 10.1038/s41598-018-19982-y
10.1038/s41598-018-19982-y [pii]
Ranger, M., & Grunau, R. E. (2014). Early repetitive pain in preterm infants in relation to the developing brain. *Pain Manag, 4*(1), 57-67. doi: 10.2217/pmt.13.61
Rao, P. V., Reddy, A. P., Lu, X., Dasari, S., Krishnaprasad, A., Biggs, E., et al. (2009). Proteomic identification of salivary biomarkers of type-2 diabetes. *J Proteome Res, 8*(1), 239-245. doi: 10.1021/pr8003776
10.1021/pr8003776 [pii]
Romano-Keeler, J., Wynn, J. L., & Maron, J. L. (2014). Great expectorations: the potential of salivary 'omic' approaches in neonatal intensive care. *J Perinatol, 34*(3), 169-173. doi: jp2013170 [pii]
10.1038/jp.2013.170

Rosenfeld, C. S. (2015). Microbiome Disturbances and Autism Spectrum Disorders. *Drug Metab Dispos, 43*(10), 1557-1571. doi: dmd.115.063826 [pii]
10.1124/dmd.115.063826
Ryan, R., Booth, S., Spathis, A., Mollart, S., & Clow, A. (2016). Use of Salivary Diurnal Cortisol as an Outcome Measure in Randomised Controlled Trials: a Systematic Review. *Ann Behav Med, 50*(2), 210-236. doi: 10.1007/s12160-015-9753-9
10.1007/s12160-015-9753-9 [pii]
Schafer, C. A., Schafer, J. J., Yakob, M., Lima, P., Camargo, P., & Wong, D. T. (2014). Saliva diagnostics: utilizing oral fluids to determine health status. *Monogr Oral Sci, 24*, 88-98. doi: 000358791 [pii]
10.1159/000358791
Shaddox, L. M., Huang, H., Lin, T., Hou, W., Harrison, P. L., Aukhil, I., et al. (2012). Microbiological characterization in children with aggressive periodontitis. *J Dent Res, 91*(10), 927-933. doi: 0022034512456039 [pii]
10.1177/0022034512456039
Shahar, E., Pollack, S., Kedem, E., Hassoun, G., & Nagler, R. (2008). Effect of HAART on salivary composition and oxidative profile in HIV-infected patients. *Curr HIV Res, 6*(5), 447-451.
Shirtcliff, E. A., Buck, R. L., Laughlin, M. J., Hart, T., Cole, C. R., & Slowey, P. D. (2015). Salivary cortisol results obtainable within minutes of sample collection correspond with traditional immunoassays. *Clin Ther, 37*(3), 505-514. doi: S0149-2918(15)00084-3 [pii]
10.1016/j.clinthera.2015.02.014
Simons, S. S., Cillessen, A. H., & de Weerth, C. (2017). Cortisol stress responses and children's behavioral functioning at school. *Dev Psychobiol, 59*(2), 217-224. doi: 10.1002/dev.21484
Spielmann, N., & Wong, D. T. (2011). Saliva: diagnostics and therapeutic perspectives. *Oral Dis, 17*(4), 345-354. doi: 10.1111/j.1601-0825.2010.01773.x
Starzak, D. E., Konkol, K. F., & McKune, A. J. (2016). Effects of Cardiorespiratory Fitness and Obesity on Salivary Secretory IgA and Alpha-Amylase in South African Children. *Children (Basel), 3*(3). doi: children3030012 [pii]
10.3390/children3030012
Strahler, J., Skoluda, N., Kappert, M. B., & Nater, U. M. (2017). Simultaneous measurement of salivary cortisol and alpha-amylase: Application and recommendations. *Neurosci Biobehav Rev, 83*, 657-677. doi: S0149-7634(17)30026-X [pii]
10.1016/j.neubiorev.2017.08.015
Streckfus, C., Bigler, L., Dellinger, T., Dai, X., Kingman, A., & Thigpen, J. T. (2000). The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin Cancer Res, 6*(6), 2363-2370.
Streckfus, C., Bigler, L., Tucci, M., & Thigpen, J. T. (2000). A preliminary study of CA15-3, c-erbB-2, epidermal growth factor receptor, cathepsin-D, and p53 in saliva among women with breast carcinoma. *Cancer Invest, 18*(2), 101-109.
Swaminathan, V., Prakasam, S., Puri, V., & Srinivasan, M. (2013). Role of salivary epithelial toll-like receptors 2 and 4 in modulating innate immune responses

in chronic periodontitis. *J Periodontal Res, 48*(6), 757-765. doi: 10.1111/jre.12066

Tian, C., Sun, X., Liu, X., Huang, X., Chen, F., & Zheng, S. (2017). Salivary peptidome profiling analysis for occurrence of new carious lesions in patients with severe early childhood caries. *PLoS One, 12*(8), e0182712. doi: 10.1371/journal.pone.0182712

PONE-D-17-12442 [pii]

Tishler, M., Yaron, I., Shirazi, I., & Yaron, M. (1997). Saliva: an additional diagnostic tool in Sjogren's syndrome. *Semin Arthritis Rheum, 27*(3), 173-179. doi: S0049-0172(97)80017-0 [pii]

Tu, M., Chia, D., Wei, F., & Wong, D. (2016). Liquid biopsy for detection of actionable oncogenic mutations in human cancers and electric field induced release and measurement liquid biopsy (eLB). *Analyst, 141*(2), 393-402. doi: 10.1039/c5an01863c

Vitorino, R., Alves, R., Barros, A., Caseiro, A., Ferreira, R., Lobo, M. C., et al. (2010). Finding new posttranslational modifications in salivary proline-rich proteins. *Proteomics, 10*(20), 3732-3742. doi: 10.1002/pmic.201000261

Vitorino, R., Lobo, M. J., Ferrer-Correira, A. J., Dubin, J. R., Tomer, K. B., Domingues, P. M., et al. (2004). Identification of human whole saliva protein components using proteomics. *Proteomics, 4*(4), 1109-1115. doi: 10.1002/pmic.200300638

Wang, K., Wang, Y., Wang, X., Ren, Q., Han, S., Ding, L., et al. (2018). Comparative salivary proteomics analysis of children with and without dental caries using the iTRAQ/MRM approach. *J Transl Med, 16*(1), 11. doi: 10.1186/s12967-018-1388-8

10.1186/s12967-018-1388-8 [pii]

Wilson, A. M., Ryan, M. C., & Boyle, A. J. (2006). The novel role of C-reactive protein in cardiovascular disease: risk marker or pathogen. *Int J Cardiol, 106*(3), 291-297. doi: S0167-5273(05)00434-1 [pii]

10.1016/j.ijcard.2005.01.068

Wissel, M., Demmler, G., Gandaria. C., Cravens. S., Berg, A., Widen, A., Altrich, M., Kleiboeker, S. . (2017). Detection of Cytomegalovirus (CMV) in Saliva of Congenitally Infected Neonates. *Open Forum Infectious Diseases, 4*. doi: 10.1093/ofid/ofx163.862

Wong, D. T. (2012). Salivary diagnostics. *Oper Dent, 37*(6), 562-570. doi: 10.2341/12-143-BL

Wong, V., Yan, T., Donald, A., & McLean, M. (2004). Saliva and bloodspot cortisol: novel sampling methods to assess hydrocortisone replacement therapy in hypoadrenal patients. *Clin Endocrinol (Oxf), 61*(1), 131-137. doi: 10.1111/j.1365-2265.2004.02062.x

CEN2062 [pii]

Wren, M. E., Shirtcliff, E. A., & Drury, S. S. (2015). Not all biofluids are created equal: chewing over salivary diagnostics and the epigenome. *Clin Ther, 37*(3), 529-539. doi: S0149-2918(15)00092-2 [pii]

10.1016/j.clinthera.2015.02.022

Wu, J. Y., Yi, C., Chung, H. R., Wang, D. J., Chang, W. C., Lee, S. Y., et al. (2010). Potential biomarkers in saliva for oral squamous cell carcinoma. *Oral Oncol, 46*(4), 226-231. doi: S1368-8375(10)00011-4 [pii]
10.1016/j.oraloncology.2010.01.007
Xiao, H., & Wong, D. T. (2011). Proteomics and its applications for biomarker discovery in human saliva. *Bioinformation, 5*(7), 294-296.
Xiao, H., Zhang, L., Zhou, H., Lee, J. M., Garon, E. B., & Wong, D. T. (2012). Proteomic analysis of human saliva from lung cancer patients using two-dimensional difference gel electrophoresis and mass spectrometry. *Mol Cell Proteomics, 11*(2), M111 012112. doi: M111.012112 [pii]
10.1074/mcp.M111.012112
Yang, K., Peretz-Soroka, H., Liu, Y., & Lin, F. (2016). Novel developments in mobile sensing based on the integration of microfluidic devices and smartphones. *Lab Chip, 16*(6), 943-958. doi: 10.1039/c5lc01524c
Zhang, A., Sun, H., Wang, P., & Wang, X. (2013). Salivary proteomics in biomedical research. *Clin Chim Acta, 415*, 261-265. doi: S0009-8981(12)00519-0 [pii]
10.1016/j.cca.2012.11.001
Zhang, L., Xiao, H., Karlan, S., Zhou, H., Gross, J., Elashoff, D., et al. (2010). Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. *PLoS One, 5*(12), e15573. doi: 10.1371/journal.pone.0015573
Zhang, Z., Li, M., Zhang, G., Fang, P., Yao, H., Xiao, Z., et al. (2010). Identification of human gastric carcinoma biomarkers by differential protein expression analysis using 18O labeling and nanoLC-MS/MS coupled with laser capture microdissection. *Med Oncol, 27*(2), 296-303. doi: 10.1007/s12032-009-9208-x
Zhou, J., Jiang, N., Wang, S., Hu, X., Jiao, K., He, X., et al. (2016). Exploration of Human Salivary Microbiomes--Insights into the Novel Characteristics of Microbial Community Structure in Caries and Caries-Free Subjects. *PLoS One, 11*(1), e0147039. doi: 10.1371/journal.pone.0147039
PONE-D-15-31932 [pii]
Zimmermann, B. G., Park, N. J., & Wong, D. T. (2007). Genomic targets in saliva. *Ann N Y Acad Sci, 1098*, 184-191. doi: 1098/1/184 [pii]
10.1196/annals.1384.002

Aim and Hypothesis

This thesis consists of two different parts

Part I: The aim of this cross-sectional study was to examine self reported xerostomia, salivary parameters (flow rate, pH, buffer capacity, viscosity) and incidence of caries in children and adolescents with satisfactory and poor glycemic control of type 1 diabetes.

Part II: In this part of our study we investigated the proteomic profile of whole saliva by high resolution mass spectrometry in type 1 diabetic children and adolescents with satisfactory and poor glycemic control in comparison with sex- and age-matched healthy controls. The aim of the present work was to characterize the salivary proteome of type 1 diabetes patients in order to identify differentially expressed proteins compared to control subjects, infer deregulated biological pathways, and evaluate the relevance of the findings in the context of diabetes pathophysiology.

Materials and Methods

Study design and clinical data

The study protocol and written consent forms were approved by the Medical Ethics Committee of the Faculty of Medicine of the National and Kapodistrian University of Athens (according to the instructions of the Declaration of Helsinki) and all experimental methods were performed in accordance with the relevant guidelines and regulations. The study protocol was explained to both parents and children, and informed written consent to participate in the study was obtained from a parent. Diabetic patients were enrolled at the Diabetic Centre of P&A Kyriakou, Athens, Children's Hospital and controls at the respective Pediatric Department. Subjects were all examined by a group of internal medicine physicians during their regular follow-up.

Part I

A total of 150 children and adolescents (10-18 years old) were examined and allocated among 3 groups: 50 patients poorly-controlled (HbA1c≥7.5%) (58 mmol/mol), 50 well-controlled (HbA1c<7.5%) and 50 age- and sex-matched healthy controls. Subjects' demographics are presented in Table 10.

	Well Control	Poor Control	Healthy
Number of Subjects	**50**	**50**	**50**
Age(yrs),mean (SD)	**13.2 (4.4)**	**11.9 (3.9)**	**12 (2.8)**
Gender, n (M/F)	**20/30**	**20/30**	**20/30**
Time with DM1 (yrs), mean (SD)	**5.3 (2.5)**	**5.8 (3.4)**	**-**
HbA1c,mean (SD)	**6.5 (0.9)**	**11.2 (1.8)**	**-**

Table 10: Subjects' demographics (part I)

Glucose salivary concentration was measured with a glucosimeter (Accu-Chek Advantage, Roche). The upper limit of fasting glucose that was considered as normal was 100mg/dL. The level of metabolic control of diabetes mellitus was determined by the glycosylated haemoglobin, HbA1c, reflecting levels of glycemia over the preceding 6-12 wk. Percentage of hemoglobin Hb1Ac was determined with the use of the HPLC (HA8140) Instrument. HbA1c values ≥7.5% (58 mmol/mol) indicated poor glycemic control for type 1 diabetes.

The control group was established by matching one non-diabetic child, who did not have any systemic disease nor receive any medication, to each of the children with diabetes. The matching criteria were age, gender, city of residence, fluoride exposure, social background (based on parental education level) and oral hygiene routine. (Siudikiene, et al., 2005) A questionnaire analysis determined caries risk factors for all the participants in the study, so as to eliminate differences between groups with regard to dietary habits, oral hygiene, dental visits, fluoride intake and social background.

All subjects who participated in the study were requested to report current or previous medication use. The use of any drugs known to induce xerostomia during the preceding semester was determined as an exclusion criterium. Xerogenic drugs comprised anticholinergics, amphetamines, antidepressants, antihistamines, diuretics and antihypertensive agents. For the pediatric population studied, the antihistamines were the most common reason for exclusion regarding medication use. (Moore, et al., 2001)

All subjects were examined for dental caries and salivary factors. Assessments of salivary function included self-reported xerostomia, quantification of resting and stimulated whole saliva flow rates, pH values, buffering capacity and saliva's viscosity. The clinical dental health status was measured using the Decayed, Missing and Filled Teeth (DMFT) Index for permanent teeth according to the WHO caries diagnostic criteria for epidemiological studies. (WHO, 1997) Plaque index and gingival index were additionally evaluated. PI ≤1 was a prerequisite for the participation in the study.

Caries examinations were performed under standardized conditions: with examination light, using mouth mirrors and dental explorers, by a specialized dentist. Decayed (all stages of lesion formation), filled and missing teeth were recorded (DMFT- WHO caries index).

Standardized sample collection

The composition of saliva varies considerably depending on different conditions(Castagnola et al., 2012). In order to effectively control potential sources of variability, the following protocol was applied:

Prior to the day of the examination, participants were advised not to eat or drink one hour before their scheduled appointment. All saliva samples were collected between 10:00 AM and 12:00 PM, to minimize any inter-individual variation of saliva composition associated with circadian rhythms.

Self-reported Xerostomia

A dental questionnaire, which has been previously reported to assess the subjective experience of dry mouth (xerostomia), was used in order to conduct the self-assessment of salivary function. (Fox, Busch, & Baum, 1987)

The following questionnaire was completed:

a. Do you have to sip liquids to aid in swallowing dry foods?
b. Does your mouth feel dry when eating a meal?
c. Do you have difficulty swallowing dry foods?
d. Does the amount of saliva in your mouth seem to be too little?

An affirmative reply to one or more of these questions was considered positive for xerostomia. Additionally, participants were asked two xerostomia questions that have been particularly studied, as presented by Moore et al. (Moore, et al., 2001):

1. Does your mouth usually feel dry? (Mouth dry?)
2. Do you regularly need to keep your mouth moist? (Mouth moist?)

Chair-side testing of salivary characteristics

The evaluation of saliva's characteristics was performed with the use of chair-side (in-office tests). The kit (Saliva-Check Buffer, GC) comprised of 6 different steps, the first 3 steps involved unstimulated saliva while the last 3 steps involved stimulated saliva.

Figure 6: Saliva-Check Buffer (GC) for chair-side evaluation of quality and quantity of saliva.

Step 1: Visual assessment of the resting flow rate of saliva, after the lower lip labial mucosa had been gently blotted with 2x2 gauze. This procedure was timed to determine whether the patient had a low, normal or high resting flow rate.

Step 2: Assessment of salivary consistency by looking at the saliva in the oral cavity to determine whether it was sticky frothy saliva, frothy bubbly saliva or water clear saliva. This determined whether the patient had residues, increased viscosity or normal viscosity.

Step 3: Assessment of the pH of resting saliva. The one end of the pH strip provided in the package was placed into the buccal mucosa for 10 seconds before comparing it to the colour chart on the Saliva Check Buffer Testing Mat. The highly acidic saliva (pH=5.0 to 5.8) is represented by the red section, moderately acidic (pH=6.0 to 6.6) is represented by yellow and healthy saliva (pH=6.8 to 7.8) is represented by green.

Step 4: Measurement of the quantity of the stimulated saliva by requiring the patient to chew on a piece of wax for 30 seconds and expectorate into the

measuring cup, then continue chewing for a total of 5 minutes and expectorating after every 15 to 20 seconds. The volume of liquid in the cup, excluding froth, was measured and recorded. Volumes less than 3.5 ml was considered very low stimulated saliva production, volumes 3.5 to 5.0 ml was considered low and volumes greater than 5.0 ml were considered normal.

Step 5: Evaluation of the pH of the stimulated saliva by taking the other end of the pH strip and dipping it into the cup of stimulated saliva for 10 seconds and then using the Saliva Check Buffer Mat for comparison to determine the pH.

Step 6: Evaluation of the buffering capacity of the stimulated saliva. The pipette was used to draw up some stimulated saliva from the cup, and 1 drop was dispensed onto the 3 test pads of the buffering strip. The test strip was turned on its side to drain excess saliva using a tissue. After 2 minutes, the colour of the 3 pads was compared to the table on the Saliva Check Buffer Mat, and the 3 scores were totalled to determine the buffering ability category for the patient. Very low buffering ability was 0 to 5, low was 6 to 9 and normal was 10 to 12.

Finally, for the statistical analysis, a modification of the characterization scales was performed, with the use of a two grade evaluation instead of a three grade one, as shown in Table 11.

Salivary Characteristics	Grade	Grade
Resting Flow Rate	Low	Low
	High	High
Stimulated Flow Rate	Low	Low
	Medium	Low
	High	High
Viscosity	Thick	Thick
	Bubbly	Thick
	Watery	Watery
pH	Very Low	Low
	Low	Low
	Normal	Normal
Buffering Capacity	Very Low	Low
	Low	Low
	Normal	Normal

Table 11: Chair side saliva tests, modified in a two grade evaluation.

Part II

The second part of the study comprised of a total of 36 subjects, who were allocated in three groups. Group 1 (G1) consisted of 12 type 1 diabetic patients with poor glycemic control, group 2 (G2) of 12 patients with satisfactory glycemic control while the control group (Ctrl) comprised of 12 healthy subjects sex-and-aged-matched accordingly. HbA1c values ≥7.5% (58 mmol/mol) indicated poor glycemic control for type 1 diabetes.

During examination, the assessment of complications consisted of clinical assessment by the endocrinologist, neurologist and ophthalmologist accordingly. Screening for retinopathy, microalbuminuria, and neuropathy took place during the examination. The presence/diagnosis of any diabetic complication was considered as an exclusion criterium for the participation in the second part of the study. Percentage of hemoglobin Hb1Ac was determined with the use of the HPLC (HA8140) Instrument. BMI, blood pressure and cholesterol values were additionally measured by routine clinical laboratory methods and details on all clinical parameters are shown in Table 12.

As in part I, participants were advised not to eat or drink one hour before their scheduled appointment. All saliva samples were collected between the previously mentioned time interval (10:00-12:00), to minimize any inter-individual variation of saliva composition associated with circadian rhythms. Unstimulated whole saliva was collected from all participants. In case the subject became stressed or began to cry, the sample was discarded.

Gingival index was additionally recorded and subjects with oral inflammation were excluded from this part of the study. During saliva collection, a specialized dentist examined all participants using as exclusion criteria the presence of gingivitis or any clinical signs of oral inflammation. The gingival index (Loe, 1967) was recorded during a clinical examination; a score below 1 was a prerequisite for the subjects of all three groups(Loe, 1967). Whole saliva was collected from the anterior floor of the oral cavity using a soft plastic aspirator for less than one minute and transferred to a plastic tube. Collection tubes were stored on ice at all times during the examination, and 3,6% v/v protease inhibitors (Roche) were used in order to prevent proteolytic degradation of salivary proteins.

Protein digestion and iTRAQ labeling

The protein present in each saliva sample was precipitated through acetone precipitation, hence by mixing 4 volumes of ice-cold acetone with one volume of saliva sample, overnight incubation at -20°C and consequent centrifugation for 20 minutes at 4000g at 4°C. The precipitated protein was re-dissolved in 200 µL dissolution buffer 0.5 M triethylammonium bicarbonate (TEAB) with extensive vortex mixing and pulsed probe sonication for 20 sec. Undissolved material was separated from the protein solution with centrifugation at 13,000 rpm for 10 min. The total of 36 samples were separated in 6 batches, each batch containing 6 samples two from each group (G1, G2 & Ctrl). Each batch was processed separately as described in the following section and the samples of each batch were iTRAQ-labeled as described in S1 Table (see online version for supplementary material).

For each sample a total protein amount of 50 µg was measured with Bradford assay (Bio-Rad Protein Assay) according to manufacturer's instructions and was diluted with the addition of dissolution buffer up to a final volume 20 µL. Cysteine disulfide bonds were reduced with the addition of 2 µL reducing reagent 50 mM tris-2-carboxymethyl phosphine (TCEP) followed by 1 h incubation in heating block at 60 °C. Cysteine residues were blocked by the addition of 1 µL 200 mM methanethiosulfonate (MMTS) in isopropanol and 10 min incubation at room temperature. Samples were diluted with 14 µL ultrapure water and 6 µL of proteomics grade trypsin (Roche Diagnostics) solution 500 ng/µL were added for overnight digestion at 37 °C. A 50 µL volume of isopropanol was added to each iTRAQ-8plex reagent vial and after vortex mixing the content of each iTRAQ vial was transferred to each sample tube. Labeling reaction was completed in 2 h at room temperature, samples were pooled and the whole mixture was dried with a speedvac concentrator. The labeled peptide samples were stored at -20 °C until they were analyzed by high-pH Reversed Phase (RP) Chromatography.

High-pH reverse phase (RP) peptide fractionation

High-pH RP C18 fractionation of the iTRAQ-8plex labelled peptides was performed on the Dionex P680 pump equipped with PDA-100 photodiode array detector using the Waters, XBridge C18 columm (150 x 4.6 mm, 3.5 µm). Mobile phase (A) was composed of 0.05 % v/v ammonium hydroxide aqueous solution and mobile phase (B) was composed of 100 % v/v acetonitrile, 0.05 % v/v ammonium hydroxide. The peptide pellet of each batch was dissolved in 200 µL mobile phase (A) with bath sonication. Sample was centrifuged at 13,000 rpm for 5 min and the supernatant solution was injected through a 200 µL sample loop. The separation method was as follows: for 15 min isocratic 5 % (B), for 10 min gradient up to 35 % (B), for 5 min gradient up to 80 % (B), for 5 min isocratic 80% (B) at a flow rate 0.4 mL/min. Signal was monitored at 280, 254 and 215 nm and the column temperature was set to 30 °C. Eight fractions were collected and were finally dried with speedvac concentrator for 4-5 h and stored at -20 °C until the LC-MS analysis.

LC-MS Analysis

All LC-MS experiments were performed on the Dionex Ultimate 3000 UHPLC system coupled with the high resolution nano-ESI Orbitrap-Elite mass spectrometer (Thermo Scientific). Individual high-pH RP peptide fractions were reconstituted in 30 µL loading solution composed of 2 % acetonitrile, 0.1 % formic acid in ultra pure water. A 5 µL volume was injected and loaded for 8 min on the Acclaim PepMap 100, 100 µm × 2 cm C18, 5 µm, 100 Å trapping column with the ulPickUp Injection mode with the loading pump operating at flow rate 5 µL/min. The peptides were eluted under a 315 minute gradient from 2% (B) to 33%(B). Flow rate was 300 nL/min and column temperature was set at 35 °C. Gaseous phase transition of the separated peptides was achieved with positive ion electrospray ionization applying a voltage of 2.5 kV. For every MS survey scan, the top 10 most abundant multiply charged precursor ions between m/z ratio 300 and 2200 and intensity threshold 500 counts were selected with FT mass resolution of 60,000 and subjected to HCD fragmentation. Tandem mass spectra were acquired with FT resolution of 15,000. Normalized collision energy was set to 33 and already targeted precursors were dynamically excluded for further isolation and activation for 45 sec with 5 ppm mass tolerance.

Database search

The collected HCD tandem mass spectra were submitted to the cited Tandem search engine(Craig & Beavis, 2004) implemented on the Trans Proteomic Pipeline (TPP) software version 4.6 for peptide and protein identifications(Deutsch et al., 2015). All spectra were searched against a UniProt Fasta file containing 20,200 human reviewed entries. The TPP included the following parameters: Precursor Mass Tolerance 10 ppm, Fragment Mass Tolerance 0.05 Da, Oxidation of M (+15.995 Da) was the only Dynamic Modification considered and Static Modifications were iTRAQ8plex at any N-Terminus, K, Y (+304.205 Da) and Methylthio at C (+45.988 Da). The Peptide and Protein Prophet TPP-modules were used for the determination of the confidence level for peptide and protein identifications with decoy database searching controlling the False Discovery Rate (FDR) at 1% and 5% at the peptide and protein levels respectively. The Libra module of the Trans Proteomic Pipeline (Pedrioli, 2010) module was utilised for peptide and protein quantification through the iTRAQ reporter ions. The signal intensity of the individual reporter ions was normalized in LIBRA and further normalization to account for unequal loading was performed in R(Team, 2016). For each iTRAQ batch, each individual iTRAQ ion reporter was normalized according to the following formula:
Normalized iTRAQ reporter ion intensity (i) = iTRAQ reporter ion intensity (i) / Sum of all 6 iTRAQ reporter ion intensities.

Based on the assumptions that a) all samples in each batch were equally loaded and b) the majority of proteins are not differentially expressed across comparisons, we infer that the average normalized iTRAQ reporter intensity for all identified proteins per sample should equal 16,67 % (100/6). If the average of normalized values deviates significantly from the aforementioned value, this is due to unequal loading. All iTRAQ reporter ions in each batch were corrected for an equal loading by setting the average of each iTRAQ reporter ion distribution at 16,67%.

Our analysis was based on high confidence protein identifications (peptide level FDR<1%, protein level FDR<5%, as controlled by TPP) (Kinsinger et al., 2012).

iTRAQ reporter ion intensities meta-analysis

Data analysis and statistical analysis were performed as follows. The normalized iTRAQ reporter ion intensities of all 36 samples were used for the identification of differentially expressed proteins across all the possible comparisons among the three groups, namely G1 vs Ctrl, G2 vs Ctrl and G1 vs G2. The normalized intensities of each protein which were found to be normally distributed according to the Kolmogorov-Smirnov test for normality were tested for differential expression using the t-test. Following that the average expression ratios of each protein for all the aforementioned comparisons were calculated and consequently log-2 transformed and centered. A second differential protein expression calculation was performed based solely on the magnitude of change indicated by the log2ratios, where p-values indicating differential expression were then calculated. For both tests $p<0.05$ was considered significant. The above procedures were carried out in the R language(Team, 2016).

Pathway analysis

The significantly deregulated proteins from the previous procedure were imported into QIAGEN's Ingenuity® Pathway Analysis and were analyzed for biological context against the IPA Knowledge base (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). In IPA analysis differentially expressed proteins were considered those with log2ratio p-value < 0.05. The pathway enrichment analysis was performed by using as reference database not the complete human proteome but only the identified proteins in our analysis. Further biological insight along with suggestions for potential clinical interventions was obtained from the L1000CDS^2 database(Vempati et al., 2014).

Protein-Protein interactions (PPI) network analysis

The STRING bioinformatics tool (https://string-db.org/) was used in order to create protein-protein interaction networks for the differentially expressed proteins identified in the three comparisons (G1 vs G2, G1 vs control, and G2 vs control). Only the protein-protein interactions based on experimental evidence were retained for creating the networks.

Sample preparation for MRM

Multiple reaction monitoring (MRM) was used for quantitation of analytes. 12 different samples were pooled to final 100µg total protein saliva extract for each group (G1,G2,Ctrl) and each pooled sample was analyzed in three technical replicates. Each pooled sample was diluted to a final volume 100 µl with urea buffer (8M urea, 50 mM NH_4HCO_3) followed by reduction (10 mM DTE) and alkylation (40 mM Iodoacetamide). The samples were then diluted in to final volume of 2 ml with 50 mM NH_4HCO_3 in order to dilute urea to final concentration below 1 M. Trypsin was added to enzyme: protein ratio 1:100 and incubated overnight. After trypsinization, the samples are desalted by zip-tip. Finally, the desalted samples were dried (speedVac) and reconstituted in appropriate volume of mobile phase A (water, 0.1 % formic acid) and heavy peptide mix (Final concentration approximately 10 ng/ ul of each peptide) to final protein concentration of 1 µg/µl and measured by LC/MRM as described below.

MRM assay design and method development

Proteotypic peptide selection

The human spectral library was searched using the software Skyline and the peptide atlas repository to identify proteotypic peptides for the 24 proteins selected for validation(Deutsch, 2010). Spectral information of proteotypic peptides for the design of MRM experiments exist for all proteins and 1-3 peptides per protein were selected (S2 Table). The final transition selection (S3 Table) was based on the quality of the MS/MS spectrum of each peptide in the human spectral library, downloaded from NIST (National Institute of Standards and Technology, http://www.nist.gov/), and on the score and number of observations in MS-based proteomics experiments as provided from PeptideAtlas.

LC-MRM set-up

Liquid chromatography was performed using an Agilent 1200 series nano-pump system (Agilent Technologies, Inc., Palo Alto, CA), coupled with a C18 nano-column (150 mm × 75 µm, particle size 3.5 µm) from AB Sciex. Peptide separation and elution was achieved with a 40 min 5-35% ACN/water 0.1 % FA gradient at a flow rate of 300 nl/min. Four microliters of each sample were injected. Each pooled sample was analyzed in triplicates.

Tryptic peptides were analyzed on an AB/MDS Sciex 4000 QTRAP with a nano-electrospray ionization source controlled by Analyst 1.5 software (Sciex). The mass spectrometer was operated in MRM mode, with the first (Q1) and third quadrupole (Q3) at 0.7 unit mass resolution. Three to five transitions were recorded for the

endogenous (light) and heavy peptides. In total 501 transitions for 44 peptides were monitored in two methods. Optimum collision energies for each transition were automatically calculated by the Skyline software.

Data analysis and quantification

Isotope labelled peptides (^{13}C and ^{15}N) identical to the endogenous ones were used in order to determine the specificity of the detection. Data analysis was performed using Skyline software and all chromatograms were manually inspected to ensure high data quality and accurate peak picking. More specifically, two criteria were used to determine high confidence peptide identification: the correlation with the spectral library and the co-elution of isotope labeled and endogenous peptides(Feng & Picotti, 2016). Finally, the sum of peak areas of at least three transitions per endogenous peptide was used for quantification (S3 Table).

Results

Part I

Evaluation of Xerostomia

Evaluation of Xerostomia	Well C DM	Poor C DM	Healthy
1. Mouth dry?	12 % [a]	34 % [b]	6 % [c]
2. Mouth moist?	16 % [a]	46 % [b]	8 % [a]
FOX QUESTIONNAIRE:			
a. Do you have to sip liquids to aid in swallowing dry foods?	12 % [a]	12 % [a]	6 % [b]
b. Does your mouth feel dry when eating a meal?	8 %	10 %	4 %
c. Do you have difficulty swallowing dry foods?	10 % [a]	16 % [b]	4 % [a]
d. Does the amount of saliva in your mouth seem to be too little?	12 % [a]	32 % [a]	8 % [b]
'YES' to any of the above (FOX SUMMARY)	14 % [a]	36% [b]	10 % [a]

Table 12: Evaluation of xerostomia. Statistically significant differences between groups with different letters (a, b, c)

Salivary characteristics and caries incidence

	Thick/ bubbly viscosity*	Low resting flow rate*	Low pH for resting saliva*	Low stimulated flow rate	Low pH stimulated saliva	Low Buffering Capacity	DMFT * (mean/ SD)
Well C DM	18% [a]	24% [a]	20% [a]	6%	6%	10%	1.2 (0.5) [a]
Poor C DM	66% [b]	82% [b]	70% [b]	18%	16%	20%	3.6 (1.2) [b]
Healthy	0% [a]	0% [a]	10% [a]	0%	0%	10%	1.5 (0.6) [a]

Table 13: Salivary characteristics and caries incidence. Statistically significant differences between groups with different letters (a, b, c)
* Variables with statistically significant difference ($p<0.05$)

Subjects with diabetes reported xerostomia more frequently than healthy controls ($p<0.05$).
Unstimulated salivary flow rate and pH values remained significantly lower in subjects with poor control of DM1 compared to well-control patients with DM1 and healthy controls.
Low values of resting salivary flow rate were associated with a higher prevalence of dental caries in children and adolescents with poorly-controlled DM1 ($p<0.05$).

The results indicated higher caries levels and a decreased unstimulated salivary flow rate in poorly-controlled diabetics. The average caries indexes were $DMFT_{(poor\ c)}$ 3.6, $DMFT_{(well\ c)}$ 1.2, $DMFT_{(healthy)}$ 1.5, $p < 0.05$). Salivary status and caries index were not found to be significantly different between well-controlled patients and healthy controls.

Data were analysed by Chi-square and Kruskal-Wallis tests, using SPSS software.

Part II

Salivary proteome

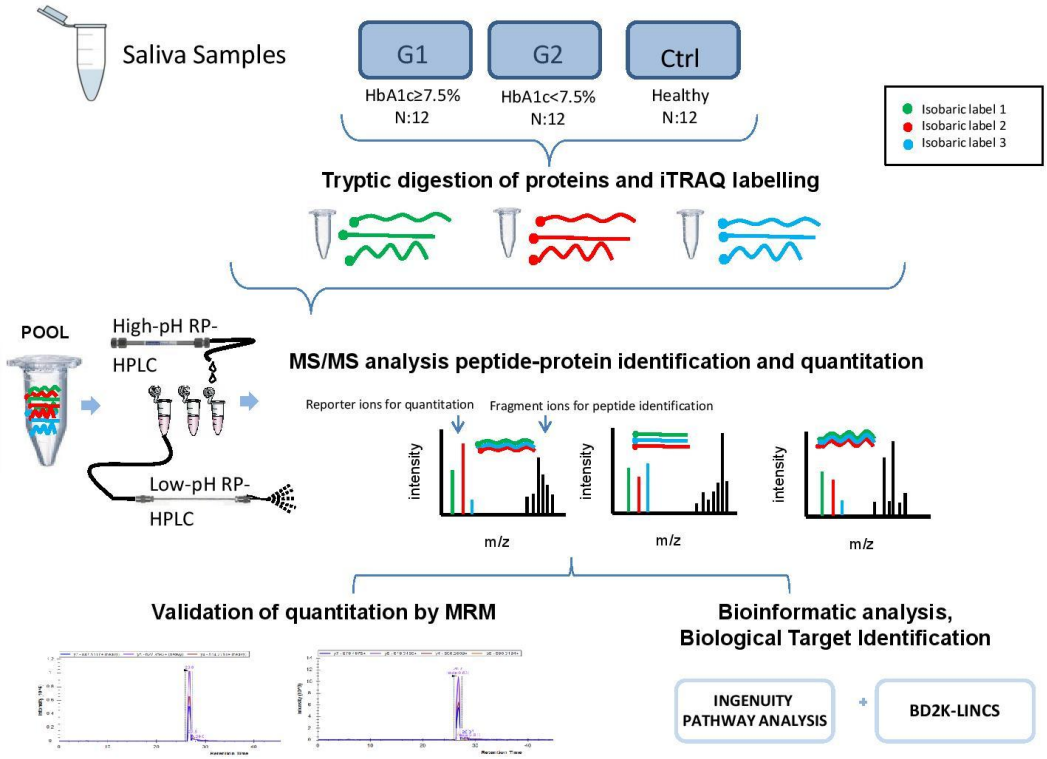
Saliva samples from 36 children and adolescents aged 10-18 were divided in three groups: Group 1 (G1) consisted of 12 with well-regulated type 1 diabetes, group 2 (G2) of 12 with poorly-regulated type 1 diabetes and 12 healthy subjects were the control group (Ctrl). The level of blood glycated hemoglobin (HbA1c) was accessed and presented normal values in controls (below 5.9%) and ranged from 6 to 12% in type 1 diabetic patients. HbA1c values ≥7.5% indicated poor metabolic control for type 1 diabetes while values<7.5% were considered as well control of the disease (Table1). BMI, blood pressure, cholesterol values were additionally measured and details on all clinical parameters are shown in Table 14.

Table 14. Subjects' demographics (*p-value <0.05, t-test) (part II). Mean and standard deviation values are reported.

	G1	G2	Ctrl
Age(yrs), mean (SD)	14.5 ±1.7	14.1 ±1.3	14.9 ±1.8
Gender, n (M/F)	5/7	5/7	5/7
Time with DM1 (yrs),	5.8 ± 1.9	6.4 ± 2.8	-
HbA1c % (mmol/mol)	9.7 ± 0.7 *(83)	6.2 ± 0.4 * (44)	4.2 ± 0.4 * (22)
BMI (kg/m^2)	22.9 ± 4	20.7 ± 5	24.3 ± 3
Blood Pressure (mmHg)	82 ± 5	79 ± 4	85 ± 5
Diastolic Blood Pressure (mmHg)	67 ± 3	63 ± 3	70 ± 4
Systolic Blood Pressure (mmHg)	113 ± 4	109 ± 3	114 ± 3
Total cholesterol (mg/dL)	165 ± 10	160 ± 12	168 ± 15
LDL cholesterol (mg/dL)	92 ± 6	88 ± 5	94 ± 8

The experimental outline for proteomic and bioinformatic analysis of the saliva samples is illustrated in Figure 7.

Figure 7. The graphical abstract outlines the proteomic and bioinformatic analysis of saliva samples.



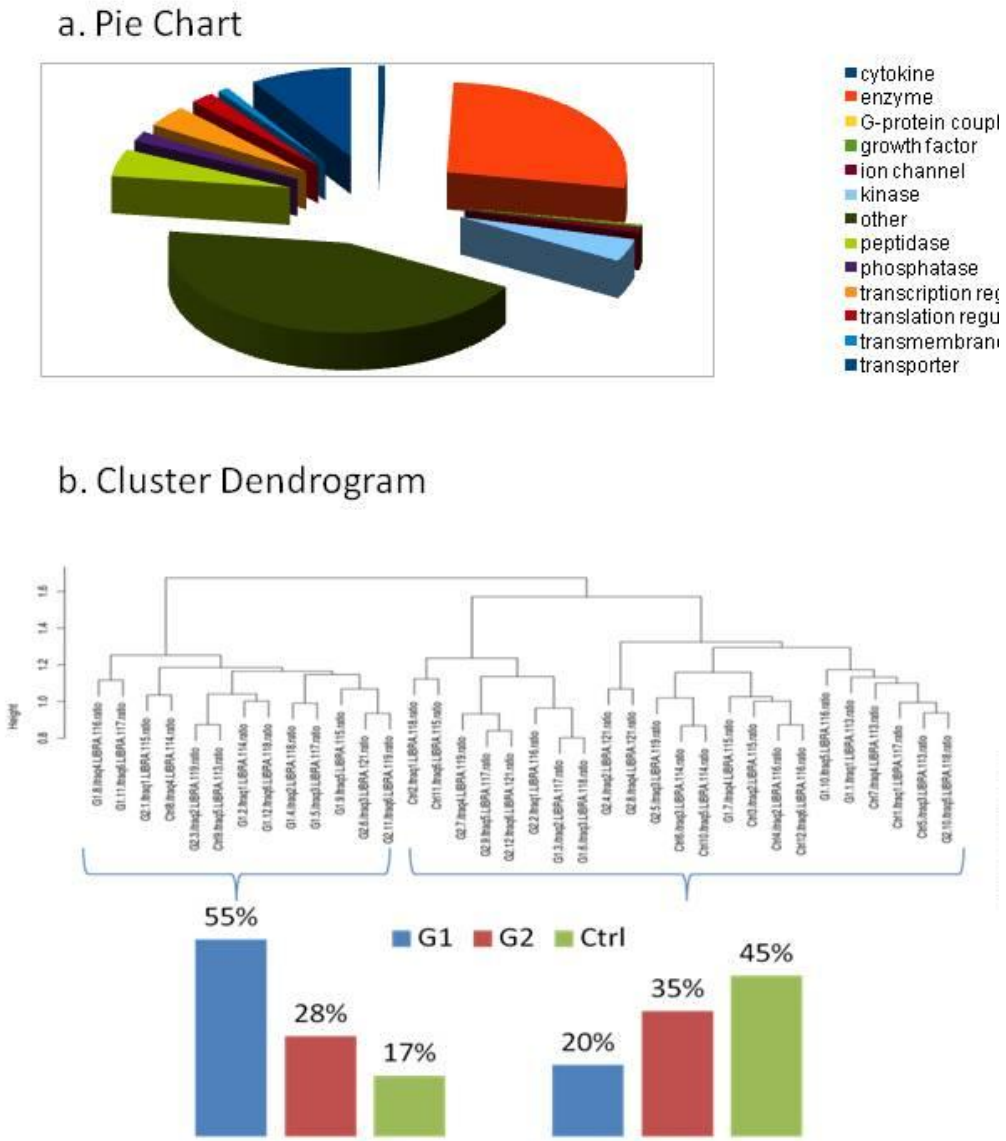
The proteomic analysis yielded 22028 peptides that were confidently identified (FDR < 1%) (S4 Table) and quantified by the iTRAQ reporter ions. These peptides corresponded to 4876 individual confident protein identifications (FDR< 5%)(Kinsinger, et al., 2012) (S5 Table).

For the comparative analysis among groups (poorly-regulated type 1 diabetic patients, well-regulated type 1 diabetic patients & healthy controls), only the proteins being present at a percentage equal or greater than 70% of the samples (9≥12) in each group were selected. The total protein number considered for analysis was reduced to 2031 proteins (S6 Table). Functional classification of these proteins revealed that Enzymes and Cytokines were the main functional groups of the salivary proteome. (Figure 8a, S7 Table-Functional IPA).

Protein identifications for each individual iTRAQ batch and the protein prophet analysis through which the false discovery rate was controlled are presented in S10 Table. Furthermore, S6 Table presents the 2031 proteins for analysis and the protein identification probabilities for each protein across all six iTRAQ batches. Notably, each protein identification across all iTRAQ batches is confirmed by at least one protein identification probability with FDR<1% as calculated from the data presented in S6 Table.

In our experimental design, each batch consisted of 6 reporter ions because we intended to maintain an identical cross batch experimental design (S1 Table); each iTRAQ batch consisted of 2 G1, 2 G2 and 2 Ctrl with iTRAQ reporter ions randomly assigned to eliminate possible reporter ion intensity bias. The identical cross batch design in combination with Libra normalization eliminates the need for an internal standard in each batch, simulating cross group comparison in label free quantitation experiments. Each sample has been analyzed individually on the mass spectrometer and the precursor ion intensities have been normalized against the total ion current. The advantage of our iTRAQ based cross batch comparison is that our analyzed samples present lower interexperimental variability due to the iTRAQ multiplexing

Figure 8. a) Functional classification of these proteins revealed that Enzymes and Cytokines were the main functional groups of the salivary proteome. **b)** Clustering indicated that total proteomic profile is capable of distinguishing poorly controlled subjects from well controlled and healthy subjects. The latter ones present similarities, as expected.



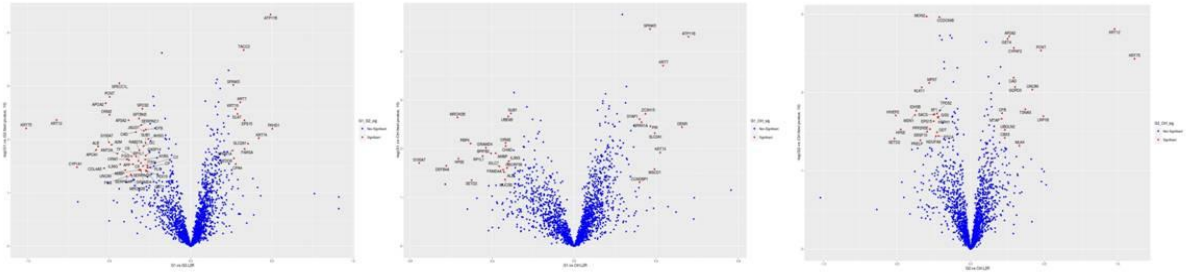
Differential expression

We performed clustering and correlation analysis in order to determine whether quantitation from the total proteome profiling of our samples could produce a meaningful class discrimination (see the relevant Methods section). Hierarchical clustering analysis based on the Euclidean distance of the normalized iTRAQ reporter ions of the total cohort confirms that our experimental design in combination with the applied normalization and processing totally eliminated any batch effect since no batch oriented grouping was detected in any of these analyses. Particularly, clustering indicated that total proteomic profile is capable of distinguishing poorly controlled subjects from well controlled and healthy subjects. The latter ones present similarities, as expected. (Figure 8b).

Selection of differentially expressed proteins for each pair-wise comparison, was performed by applying a double significance criterion. The first one was the t-test between two groups for each individual protein intensity values from iTRAQ reporter ions. The second one was the log2ratio p-value which corresponded solely to the magnitude of change for each protein between two groups (see the relevant Methods section). All possible comparisons were performed among the 3 groups: (G1-Ctrl, G2-Ctrl, G1-G2). 33 proteins were found to be differentially expressed between G1-Ctrl, 37 between G2-Ctrl and 61 between G1-G2 (Figure 9b- S8 Table).

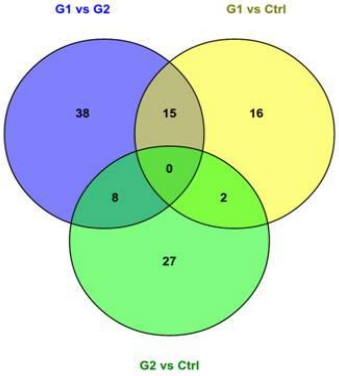
Figure 9. Comparisons among 3 groups and statistical analysis a) Among possible comparisons, G1vsCtrl and G1vsG2 yield proteins with high fold change and low p-values. Volcano plots present that the variance in G2vsCtrl is smaller than in the other two comparisons, indicating higher similarity between G2 and Ctrl subjects. **b)** All possible comparisons were performed among the 3 groups: (G1-Ctrl, G2-Ctrl, G1-G2). 33 proteins were found to be differentially expressed between G1-Ctrl, 37 between G2-Ctrl and 61 between G1-G2. **c)** Multiple Reaction Monitoring (MRM) was utilized in order to validate the relative quantitation obtained by the iTRAQ technology. For each group comparison, we selected the most relevant proteins, based on differential expression and clinical relevance. The proteins selected presented low p-value, high fold-change and were the most relevant to clinical pathways. In G1vsG2, 9 out of 12 proteins presented positive correlation between the iTRAQ and MRM quantitation. 9 out of 12 proteins presented positive correlation in iTRAQ and MRM quantitation in comparison G1vsCtrl as well, whereas in G2vsCtrl, 10 out of 15 presented positive correlation.

a. Volcano Plots



b. Differentially Expressed Proteins

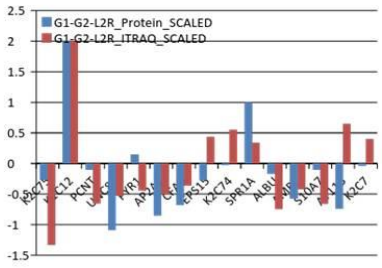
G1 vs G2 Unique	
PKHD1	C9
FARSA	SERPING1
TACC2	RAB27A
DLAT	SPTBN5
CPA4	C6
KRT19	APP
NEDD8	TF
AHSG	SERPINA1
CP	FGG
C3	ORM1
PLG	FGB
PEX19	SPECC1L
CST1	KMT2A
CASP14	A2M
A1BG	APOA2
JMJD7	COL4A5
GC	APOA1
ITIH4	CYP1A1
SERPINC1	SPCS2

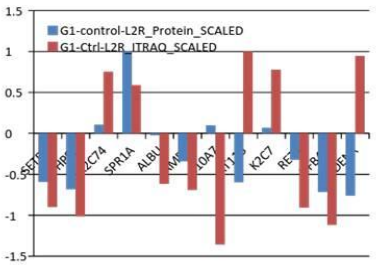


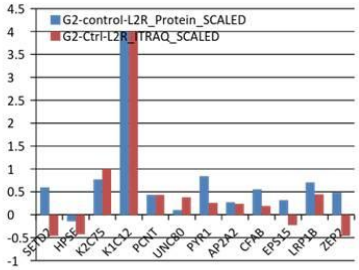
G1 vs Ctrl Unique
DENR
WSCD1
PIR
ZC3H15
SYAP1
CCNDBP1
UBE4B
C6ORF58
CHAD
MUC5B
FRMD4A
IGLC7
BPIFB2
RBP4
DEFB4A

G2 vs Ctrl Unique
LRP1B
TSNAX
MLK4
GDPD3
CYP4F2
GET4
UBQLN2
CBX3
MTAP
CCDC64B
RTCA
GSS
HSPH1
TPD52
DDT
NDUFA9
SF1
SACS
PPP2R5E
MPST
SRSF10
MON2
KLK11
PRELP
IDH3B
MDN1
HIVEP2

c. Mrm







The following results were obtained from the proteomic analysis, for differentially expressed proteins (Table 15)

G1-Ctrl down: The protein found to be most downregulated was Protein S100-A7 (fold change=-1.69) followed by Beta-defensin 4A (fold change=-1.54) and Maestro heat-like repeat-containing protein family member 2B (fold change=-1.48). HPSE, AMBP, ALB, A2M, APOA2, LPO, SERPIN, S100A2 were also found to be significantly downregulated in this comparison. G1-Ctrl up: Probable phospholipid-transporting ATPase IF (fold change=1.47) was found to be most upregulated, followed by DENR (fold change=1.44) and KRT7 (fold change=1.35). SPRR1A, CASP4, S100A10, PSMB7 were also significantly upregulated in this comparison.

G2-Ctrl top3 down: The protein found to be most downregulated was SETD2 (fold change=-1.43) followed by HIVEP2 (fold change=-1.42) and HPSE (fold change=-0.56). G2-Ctrl top3 up: KRT75 (fold change=2.76) was found to be most upregulated, followed by KRT12 (fold change=1.97) and LRP1B (fold change=1.41)
G1-G2 top3 down: The protein found to be most downregulated was KRT75 (fold change=-2.0) followed by KRT12 (fold change=-1.78) and CYP1A1 (fold change=-1.62). APOA1, ALB, APOA2, S100A7, A2M, AMBP, SERPING1, APOB, C3, ITIH4, CST1, CFB, AHSG were also found to be significantly downregulated in this comparison group. G1-G2 top3 up: PKHD1 (fold change=1.42) was found to be most upregulated, followed by ATP11B (fold change=1.41) and KRT74 (fold change=1.34). S100A10 was also significantly upregulated, as observed in G1-Ctrl comparison.

Among possible comparisons, G1vsCtrl and G1vsG2 yield proteins with high fold change and low p-values. As shown in the Volcano plots (Figure 9a), the variance in G2vsCtrl is smaller than in the other two comparisons, indicating higher similarity between G2 and Ctrl subjects.

Table 15. The most prominent protein findings among the three comparisons. P-value, log2ratio and fold-change are presented, along with the pathways in which these proteins are involved. Biologically relevant findings with high statistical significance were not identified in G2vsCtrl.

protein	ttest_pvalue	l2r	ratio	fold change	biological function
G1-Ctrl					
S100A7	0.019	-0.755	0.592	-1.688	immune response
DEFB4A	0.022	-0.622	0.649	-1.539	Inflammation
A2M	0.042	-0.268	0.830	-1.204	acute phase response,coagulation
SERPINA1	0.024	-0.287	0.819	-1.220	Atherosclerosis
LPO	0.037	-0.291	0.816	-1.224	phagosome maturation
S100A10	0.030	0.226	1.170	1.170	dissolution of fibrin clot
CASP4	0.015	0.255	1.193	1.193	cell apoptosis, nephropathy
G1-G2					
S100A7	0.011	-0.501	0.706	-1.416	immune response
A2M	0.012	-0.486	0.713	-1.401	acute phase response,coagulation
C3	0.030	-0.265	0.831	-1.202	Complement
SERPING1	0.036	-0.309	0.807	-1.238	Complement
APOA1	0.015	-0.580	0.668	-1.495	LXR/FXR, atherosclerosis
SERPINA1	0.045	-0.367	0.775	-1.289	atherosclerosis, coagulation
PLG	0.033	-0.265	0.831	-1.202	Coagulation
G2-Ctrl					
SETD2	0.038	-0.516	0.698	-1.430	Enzyme
HIVEP2	0.019	-0.507	0.703	-1.421	transcription regulator
HPSE	0.029	-0.474	0.719	-1.389	Enzyme
LRP1B	0.020	0.493	1.408	1.408	Transmembrane receptor
KRT75	0.003	1.113	2.163	2.163	Other

MRM validation

We utilized Multiple Reaction Monitoring (MRM) (see Methods) in order to validate the relative quantitation obtained by the iTRAQ technology. For each group comparison, we selected the most relevant proteins, based on differential expression and clinical relevance. The proteins selected presented low p-value, high fold-change and were the most relevant to clinical pathways. In G1vsG2, 9 out of 12 proteins presented positive correlation between the iTRAQ and MRM quantitation. 9 out of 12 proteins presented positive correlation in iTRAQ and MRM quantitation in comparison G1vsCtrl as well, whereas in G2vsCtrl, 10 out of 15 presented positive correlation (Figure 9c).

Bioinformatic Analysis

IPA

For biological knowledge extraction we utilized the QIAGEN's Ingenuity® Pathway Analysis Platform (see Methods). The results are shown in Tables 16 and 17.

Table 16: Deregulated pathways identified in G1 vs G2 comparison.

Pathways	p-value	Molecules	
		Downregulated	Upregulated
Acute Phase Response Signaling	$3.16*10^{-20}$	SERPING1,C3, APOA2,C9, AHSG,AMBP,CP,FGG,PLG,IL36G,ALB,APOA1, ORM1,TF, IL1RN,ITIH4,CFB,ORM2, SERPINA1,FGB,HRG,MAP2K1,A2M	
LXR/RXR Activation	$2*10^{-15}$	APOB,C3,APOA2,C9,AHSG,AMBP,A1BG,ALB,IL36G,APOA1,TF,ORM1, IL1RN,ITIH4,ORM2,SERPINA1	GC
Atherosclerosis Signaling	$2.9*10^{-7}$	ALB,IL36G,APOB,APOA1,ORM1,IL1RN,APOA2,ORM2,SERPINA1	PRDX6
Coagulation System	$8.5*10^{-7}$	PLG,SERPINC1,SERPINA1,FGB,A2M,FGG	
Complement System	$2.5*10^{-5}$	SERPING1,C3,C9,CFB,C6	
IL-12 Signaling and Production in Macrophages	$4.4*10^{-5}$	ALB,APOB,APOA1,ORM1,APOA2,ORM2,SERPINA1,MAP2K1	
IL-10 Signaling	$4*10^{-3}$	IL36G	BLVRA, BLVRB, IL1RN
Toll-like Receptor Signaling	$5.4*10^{-3}$	IL36G	UBB,TOLLIP, IL1RN

Table 17: Deregulated pathways identified in G1 vs Ctrl comparison.

Pathways	p-value	Molecules	
		Downregulated	Upregulated
Acute Phase Response Signaling	$9.6*10^{-12}$	APOA2,AHSG,AMBP, ALB,IL36G,TF,IL1RN,IL36RN,ORM2,SERPINA1,MAP2K3,HRG,A2M,RBP4	MYD88, IL18, CP
LXR/RXR Activation	10^{-10}	IL36G,ALB,TF,IL1RN,APOA2,IL36RN,AMBP,AHSG, ORM2,SERPINA1,GC,A1BG,RBP4	IL18
Phagosome maturation	$1.3*10^{-7}$	LPO, NAPG	DYNLL1,CALR,TUBA1C,ATP6V1G1,NAPA,PRDX6, EEA1,PRDX5,PRDX1
Atherosclerosis Signaling	$1.6*10^{-6}$	ALB,IL36G, IL1RN,APOA2,IL36RN,ORM2,SERPINA1, RBP4	IL18,PRDX6
Toll-like Receptor Signaling	$2*10^{-5}$	UBB, IL1RN,IL36RN,MAP2K3	IL36G,IL18,MYD88
IL-10 Signaling	$1.2* 10^{-4}$	IL36G,IL1RN,IL36RN,MAP2K3	BLVRB,IL18

In the G1vsCtrl comparison, Acute phase response signalling, Atherosclerosis signalling and LXR/RXR- FXR/RXR activation were the top canonical pathways which were found to be activated, whereas molecules related to cardiotoxicity, hepatotoxicity and nephrotoxicity were identified in this comparison.

For the G1vsG2 comparison, similar canonical pathways were found to be deregulated. Biologically relevant findings with high statistical significance were not identified in G2vsCtrl.

The ingenuity pathway analysis software is a standard software for determining deregulated pathways connected to disease (Jimenez-Marin, Collado-Romero, Ramirez-Boo, Arce, & Garrido, 2009). The p-value reported reflects the proportion of proteins deregulated in the pathway (high proportions correspond to low p-values), and also the proportion of proteins of a pathway in the differentially expressed protein list for each comparison (high proportion of proteins that belong to a specific pathway in the whole list of differentially expressed proteins yield low p-values)

Protein-protein interactions network analysis

The three PPI networks created by the differentially expressed proteins identified in the three comparisons are shown in Figure 10. For the G1 vs Ctrl comparison 16/33 proteins form 25 PPI. In the case of the G1 vs G2 comparison 37/61 proteins form 129 PPI, whereas for the G2 vs Ctrl comparison 20/37 proteins form 31 PPI. The list of all interactions validated by experimental data is available in S12 Table.

Figure 10: PPI networks generated by STRING for the differentially expressed proteins identified in the three comparisons (a,b,c).

STRING analysis

G1 vs G2
https://string-db.org/cgi/network.pl?taskId=hsF6vmNPoBn0
G1 vs control
https://string-db.org/cgi/network.pl?taskId=ZxeEk8PwWmTK
G2 vs control
https://string-db.org/cgi/network.pl?taskId=2e0aIDIYNIXF

LINCS

We introduced in L1000CDS[2], a search engine of gene expression signatures from the LINCS L1000 dataset, the differentially expressed (up & down-regulated) proteins of Comparison G1 vs G2. Among the agents that most efficiently reversed that phenotype, the top hit was BRD-K01868942, a serotonin receptor antagonist (S9 Table).

Figure 10a: G1 vs G2

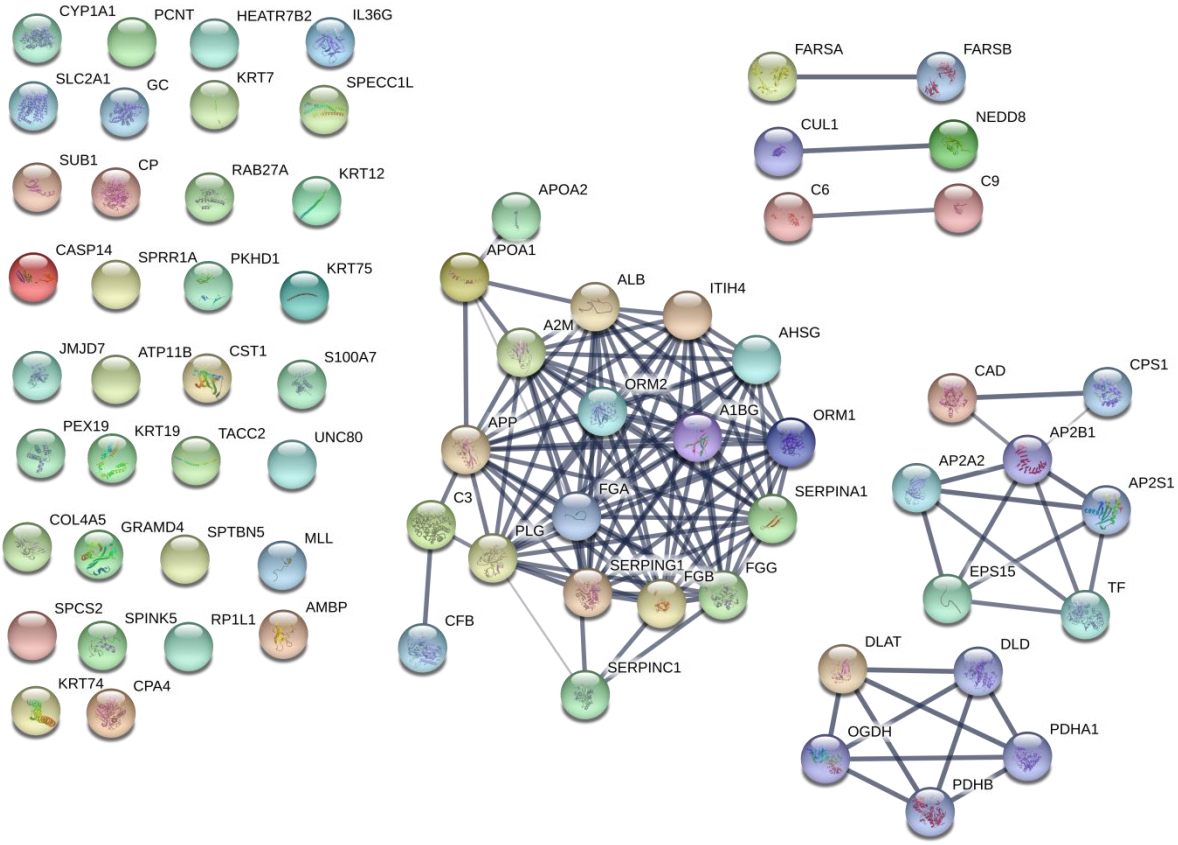


Figure 10b: G2 vs Control

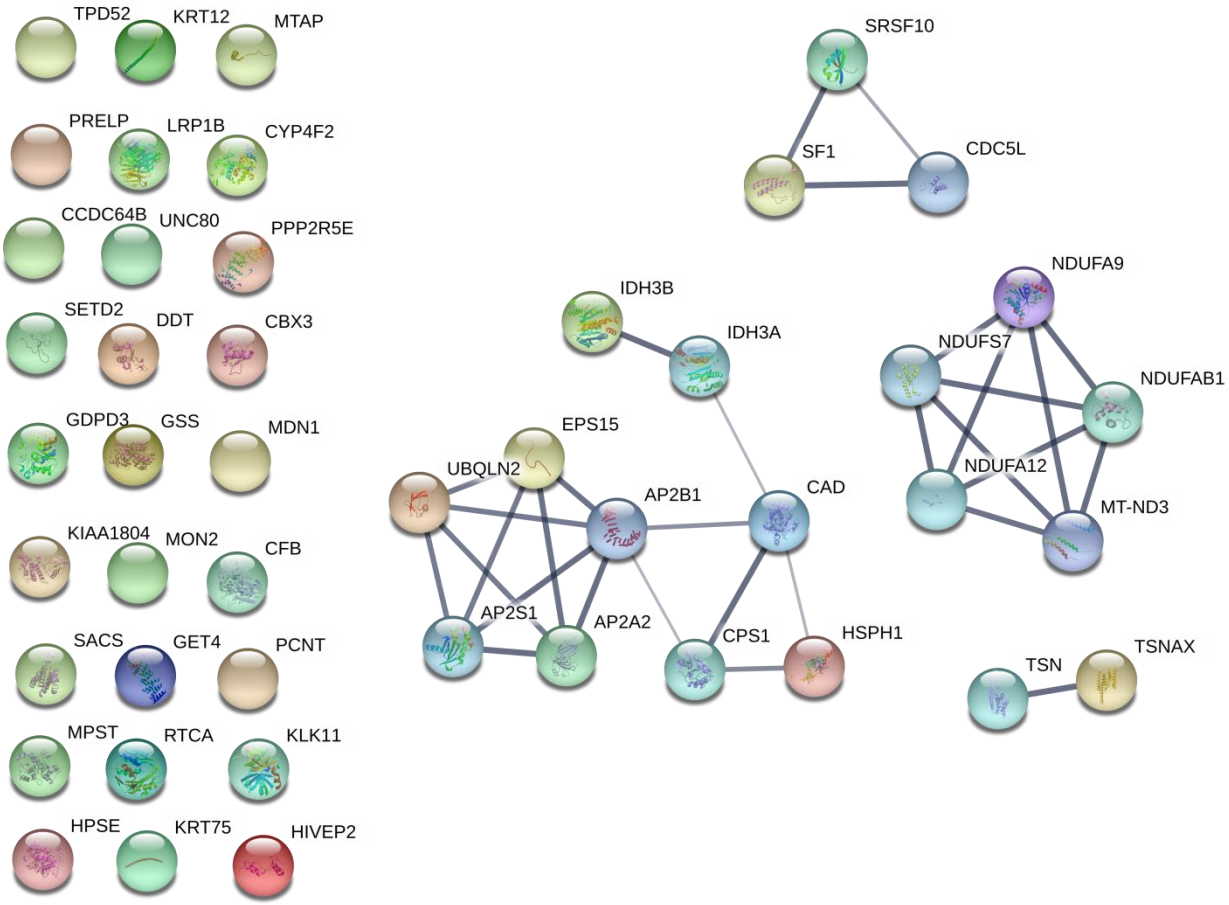
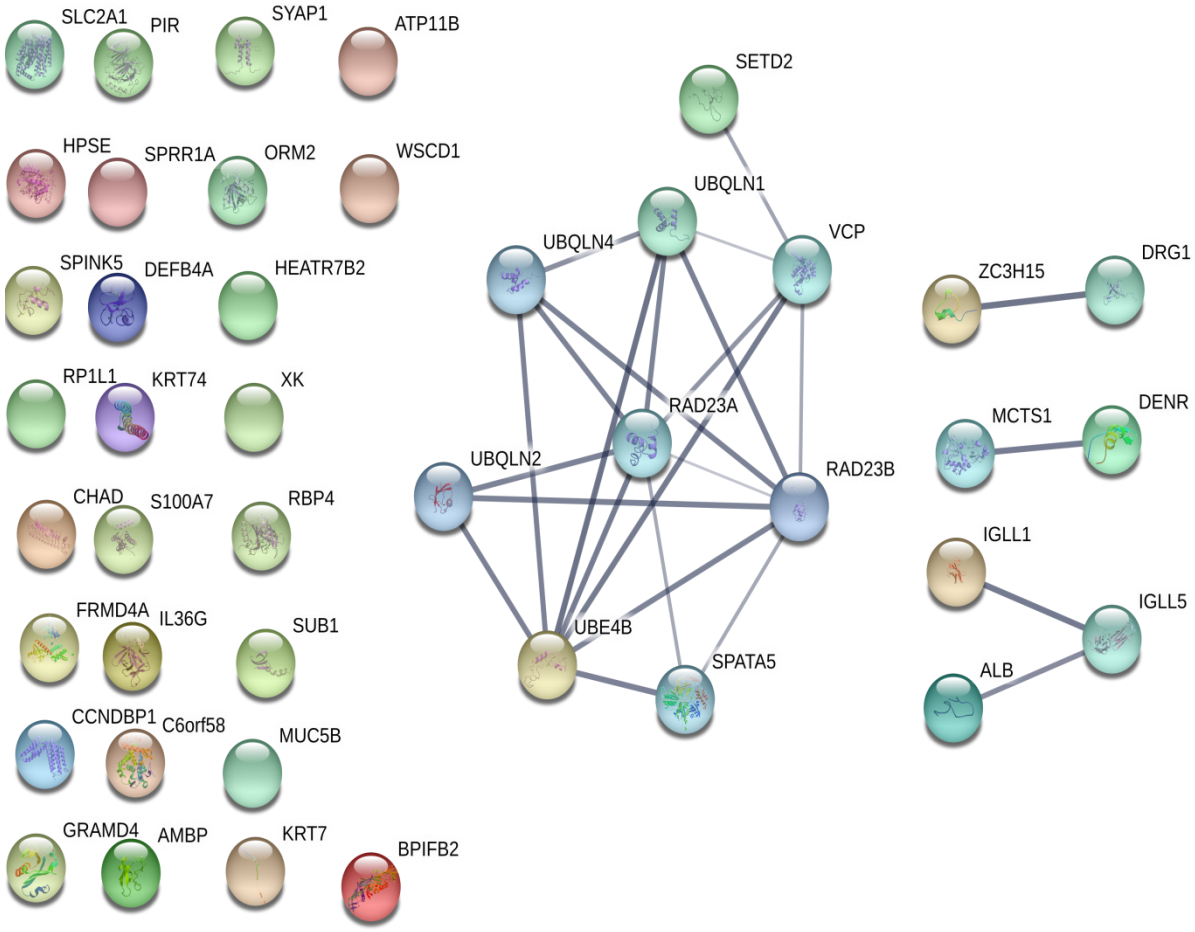


Figure 10c: G1 vs Ctrl



Discussion

Part I

Salivary characteristics and caries in Diabetes

The terms salivary hypofunction or hyposalivation and xerostomia are often used interchangeably but actually represent different clinical conditions. Hyposalivation refers to a diminished salivary flow, whereas xerostomia refers to a subjective experience of mouth dryness. (Humphrey & Williamson, 2001) This is further complicated by the fact that some patients with hyposalivation are not xerostomic and, conversely, those with xerostomia may have normal salivary flow rates. However, xerostomia is a common and primary symptom associated with salivary gland hypofunction. Usually when salivary secretion has decreased to half its normal values an individual will begin to experience xerostomia. Although there are wide individual variations, hyposalivation is usually defined by an unstimulated whole saliva flow rate of less than 0.1 mL/min, collected for 5 to 15 minutes, or chewing-stimulated whole saliva flow rate of less than 0.7 mL/min, collected for 5 minutes. (von Bultzingslowen et al., 2007)

In this study, both xerostomia and hyposalivation were recorded, in order to investigate the subjective and objective alterations in salivary flow. The results demonstrated higher xerostomia incidence and significantly decreased salivary flow in children and adolescents with poorly controlled type 1 diabetes. These data confirm the existing knowledge about salivary flow alterations in patients with diabetes, caused by hyperglycemia (additionally see review in chapter 2). (Abbate, et al., 2014; Bernardi, et al., 2007; Busato et al., 2009; Busato, et al., 2012; Edblad, et al., 2001; R. Harrison & W. H. Bowen, 1987; K. M. Karjalainen, et al., 1996; Lopez-Pintor, et al., 2016; Lopez, et al., 2003; Moore, et al., 2001; Moreira, et al., 2009; Rai, et al., 2011; Sreebny, Yu, Green, & Valdini, 1992; Zloczower, Reznick, Zouby, & Nagler, 2007) The subjective experience of dry mouth was assessed using 2 xerostomia questions (MOUTH DRY?- MOUTH MOIST?) along with 4 questions from a dental questionnaire, previously presented by Fox et al. and applied by Moore et al. that have shown to correlate with salivary dysfunction (Moore, et al., 2001) (Fox, et al., 1987). Well controlled patients presented no statistical differences in flow rate compared to healthy controls. However, the self-reported salivary impairment for the group of well controlled patients presented higher prevalence than the measured hyposalivation. This conflicting finding may be attributed to the short-term subjective feeling of dry mouth that characterize periods with elevated blood glucose concentrations, observed even in patients with an overall adequate glycemic regulation.

Salivary secretion is under the control of both the parasympathetic and sympathetic components of the autonomic nervous system. (Humphrey & Williamson, 2001) It has been suggested that neuropathy and microvascular abnormalities, with endothelial dysfunction and deterioration of microcirculation that is associated with diabetes mellitus, may play a role in disturbed saliva flow and composition. (Belazi, et al., 1998) The increased viscosity in saliva of poorly controlled patients with diabetes may, respectively, be attributed to the higher protein concentration of saliva during hyperglycemia. (Busato, et al., 2012) The alterations in flow and composition could additionally explain the lower pH values for resting saliva recorded for the poorly controlled group.

Furthermore, the results demonstrated that children and adolescents with poor regulation of chronic glycemia had significantly higher caries prevalence than the well-controlled and healthy subjects. The data of our analysis confirm the reports of Syrjala et al. (Syrjala, Niskanen, Ylostalo, & Knuuttila, 2003) and Siudikiene et al. (Siudikiene, et al., 2008), according to which, subjects with poor metabolic control had higher caries levels. Taking into account that moderate to well oral hygiene was a prerequisite for the participants in our study, the finding of higher DMFT prevalence could be related with the lower salivary flow rate observed in this group. The presence of dental caries could also be associated with higher levels of mutans streptococci and lactobacilli, as well as yeasts, which were observed in patients with poor regulation of type 1 diabetes.

Dental caries is a complex, dynamic and multifactorial process. As such, there are numerous factors to be considered when determining patient's caries risk.(D'Amario, Barone, Marzo, & Giannoni, 2006) Regarding contributing and confounding effects, the present study took into consideration various parameters in order to eliminate possible interactions. Indeed, a questionnaire analysis determined caries risk factors for all the participants in the study. Via careful selection, it eliminated potential confounding factors such as medications, systemic diseases, sugary diet/ frequent snacking, inadequate oral hygiene and low socioeconomic status for all three groups.

Interestingly, the well-controlled diabetic group presented the lowest caries prevalence, even when compared to the healthy subjects. These findings, although not statistically significant, are in agreement with the findings by Siudikiene et al. (Siudikiene, et al., 2008), and might be explained by the strict diet and close monitor of these patients.

No statistically significant differences were recorded among groups for stimulated flow rate, pH values and buffering capacity. The stimulation of salivary flow through gustatory and mechanical activity restores the pH values measured in all three groups, and offers saliva adequate buffering capacity. Saliva presents a buffering system that is able to resist pH changes and neutralize acids produced by acidogenic microorganisms. (Cho, et al., 2010) Salivary buffering capacity is promoted by carbonatebicarbonate, phosphate, and proteins systems, the most important buffering agent present in stimulated saliva. The bicarbonate system is responsible for approximately 85% of salivary buffering capacity in the pH range from 7.2 to 6.8.

(Bernardi, et al., 2007) (Singh et al., 2015)The concentration of these ions is higher in saliva collected after mechanical stimulation. As salivary flow increases, the concentration of bicarbonate ions also increases, thus explaining that buffering capacity values are not affected among three groups. The duration of the diabetes in the population studied, along with the age of the participants, could explain the lack of differences in stimulated salivary characteristics, as microcirculation and endothelial alterations may have not extensively altered the salivary glands' reaction to stimuli.

The evaluation of salivary characteristics in this study was performed with a chair-side, quick and easy to use salivary test. Such tools could be of great use for practitioners to assess caries risk in young individuals with difficulty in maintaining optimal glycemic control, especially when more complicated evaluation methods cannot be performed or need to be performed by specialized personnel. (D'Amario, et al., 2006)

Part II

Why saliva?

The issue of children's compliance in monitoring their serum glucose has shifted researchers' focus towards saliva, a non- invasive, easily collected biological fluid which presents an attractive alternative to blood samples(Lima, et al., 2010; Yeh, et al., 2010) Analysis of saliva may provide insights to biological processes for patients with diabetes and could potentially reveal early complications through biological mechanisms activated long before the appearance of clinical symptoms of the disease. It is important to note that blood collection in a pediatric population can cause poor compliance of patients, thus saliva collection for glycemic monitoring is an attractive alternative. (Kaczor-Urbanowicz, et al., 2017)

HbA1c threshold

Salivary proteomic changes of Type 1 diabetes were analysed based on the HbA1c regulation. Elevated HbA1c predicts long-term microvascular and macrovascular complications and is the only biomarker of glycemic control with strong outcome data(M. Rewers, et al., 2009). A target range of <7.5% (58mmol/mol) is recommended, following the ISPAD Consensus Guidelines, for all age-groups. Of all age-groups, adolescents show the poorest performance in achieving optimal glycemic control, an observation which is in accordance with the physiological, hormonal challenges and the increased independence in diabetes care during this period(M. Rewers, et al., 2009; Spencer, Cooper, & Milton, 2010).

Diabetes and gingival inflammation

A large number of studies suggest that diabetes is associated with an increased prevalence, extent and severity of oral inflammatory diseases such as gingivitis and periodontitis(Giuca et al., 2015; Lalla et al., 2007). Whole saliva is a combination of the secretions of the major and minor salivary glands, together with the gingival crevicular fluid (Giannobile, et al., 2009) and is the biological fluid used in the present study for proteomic analysis. Taking into account the lack of compliance in oral hygiene during adolescence, subjects with oral inflammation were excluded from the study. For that purpose, the gingival index(Loe, 1967) was recorded by a specialized dentist during a clinical examination; a score below 1 was a prerequisite for the subjects of all three groups(Loe, 1967). Thus, the proteomic analysis of saliva in our study highlights differences due to diabetic pathology and excludes the contribution of oral inflammation.

In our study, the presence of gingival inflammation was an exclusion criterion for all the participants. However, the pathway analysis indicated a deregulation of the mechanisms involved in inflammation, immune response and IL-12 signalling in poorly-controlled diabetic adolescents. Inflammation and immune response play key

role in periodontal diseases such as gingivitis and periodontitis, which are considered to be the most common complications of diabetes. (K. M. Karjalainen & Knuuttila, 1996) Additionally, the osteolytic role of the proinflammatory cytokine interleukin IL-12 was recently found to be involved in the pathogenesis of periodontal diseases. (Issaranggun Na Ayuthaya, Everts, & Pavasant, 2018) Differentially expressed proteins in these pathways, that could contribute to periodontal disease, are presented in table 15.

Subjects demographics

BMI values in control group are on the threshold between normal and overweight for 15-year-old adolescents, measured at 24kg/m^2 , a value which is not surprising taking into account the high prevalence of juvenile obesity in Greece(Krassas, Tzotzas, Tsametis, & Konstantinidis, 2001; Tzotzas et al., 2008). The aforementioned BMI differentiation in the control group did not contribute to the variance of the overall dataset at the extent of having a detectable effect.

High confidence data

For the purposes of this study, saliva from a large cohort of 36 individuals was analyzed utilizing peptide labeling & multiplexing technology (iTRAQ) for high-confidence protein identification and quantitation. In order to exceed the iTRAQ multiplexing limitation (8 samples in a single run), we devised a controlled, we devised a controlled experimental design and data normalization scheme in order to combine 6 individual iTRAQ 6-plex batches (see Methods and Figure 7-Graphical Abstract). Furthermore the protein identification was performed with the Trans Proteomic Pipeline(Pedrioli, 2010) (TPP) where the False Discovery rate was controlled both at the peptide and at the protein level, selecting for analysis only the proteins present in all the samples. Finally the expression level as calculated from the iTRAQ reporter ions for a large number of proteins was validated by MRM which is a highly sensitive and specific mass spectrometry technique. In our study, MRM was performed on three pooled samples. Pooling samples could mask individual sample variability within each group. Moreover, aberrantly high or low levels of specific proteins in individual samples could influence the concentration in the entire group. However, the results obtained by the MRM approach are in general agreement with the proteomics data derived from the individual samples. These initial findings have to be further confirmed by analysis of an independent cohort of saliva samples.

Our approach did not involve multiple testing correction of p values, which is a limitation in our study. However, a double criterion was used for the selection of differentially expressed proteins, namely t-test p-value and log2ratio p-value. Proteins were considered significant when both p-values < 0.05. This approach is equivalent to a volcano-plot based selection of differentially expressed proteins, with the added value of being systematic and not empirical since rigid statistical

criteria based on actual numerical values (both p-values < 0.05) where used as a selection threshold.

Despite the limitations, the process described above, produced a highly reliable data set which gave us the opportunity for in depth proteomic analysis of type 1 diabetes utilizing saliva, an easily and non-invasively acquired biological sample. Compared with previous proteomic studies, the present one provides a significantly higher number of reliable protein identifications (total 2031)(Rosa et al., 2012). Rao et al. has previously identified a total of 491 proteins in saliva of type 2 diabetic subjects(Rao, et al., 2009), Cabras et al. detected 120 salivary components using HPLC-ESI-MS analysis of whole human saliva of children with type 1 diabetes(Cabras, et al., 2010), while 148 proteins were detected using pooled samples per type of diabetes by Bencharit et al.(Bencharit et al., 2013) The number of proteins confidently identified in the present study is comparable to the total number of 2290 proteins that Loo et al. report by combining salivary proteomic datasets from several studies(Loo, Yan, Ramachandran, & Wong, 2010).

Studies on salivary proteome in Diabetes

It has been previously shown that salivary proteomes present alterations in type 1 and type 2 diabetic patients. Rao et al characterized the salivary proteome in subjects with pre-diabetes, type 2 diabetes and healthy controls. A total of 487 unique proteins was identified, of which 65 were found to be differentially expressed in saliva from patients with type 2 diabetes versus controls(Rao, et al., 2009). The majority of the differentially expressed proteins were associated with pathways regulating metabolism and immune response, similarly to the findings of our study (Rao, et al., 2009). Salivary proteomes also presented differences in edentulous patients with type 2 diabetes, where 96 peptides corresponding to 52 proteins were found to be differentially expressed between diabetic and non-diabetic controls(Border et al., 2012). Moreover, salivary peptidomic modifications were identified in patients with type 1 diabetes, when compared to healthy controls, indicating down-regulation of peptides involved in oral cavity host defence in these patients(Cabras, et al., 2010). Proteomic changes associated with hyperglycemia were determined by a label-free proteomic approach, showing that there is a correlation between specific proteins and HbA1c levels in patients with diabetes(Bencharit, et al., 2013). In accordance with the findings of our study, this analysis demonstrated alterations in the salivary proteomic values of various serum originating proteins including albumin, complement C3 and alpha2-macroglobulin, related to increased levels of HbA1c(Bencharit, et al., 2013).

Deregulated pathways

Based on high confidence data the bioinformatics analysis yields biologically significantly deregulated pathways. The lack of significant differences, observed in G2vsCtrl for pathways, is in accordance with the clinical data available for these two groups. Indeed, satisfactory glycemic control is the key factor for prevention of diabetic complications(Lebovitz, et al., 2006). G2 and Ctrl subjects presented similar proteomic profiles, which is in accordance with their respective health status. The similarity of the proteomic profiles of Ctrl and G2 subjects is the main reason for which when these two «healthy» groups are compared with the deregulated patients (G1), there is considerable overlap in the two lists of differentially expressed proteins. This is illustrated in Figure 3b. However, as shown in the same figure, there are also unique differentially expressed proteins. These differences are probably due to the diverging genetic background of Ctrl subjects when compared to G2- well regulated T1D patients.

Contrary to G2vsCtrl comparison, common and biologically relevant proteins are identified in the two comparisons G1vsCtrl and G1vsG2. Differential expression of proteins in the G1 group, led to activation of molecular pathways related to pathological complications, as shown in Tables 16 and 17. Acute phase response signalling, LXR/RXR activation network, atherosclerosis and coagulation pathway, immune response and toll-like receptor signalling appear to be deregulated in poorly controlled patients.

Regulation of the mechanisms controlling inflammation and synthesis of acute phase proteins is impaired by hyperglycaemia and the direct relationship between hyperglycaemia, inflammatory process and oxidative stress contributes to the development of diabetic complications (Beisswenger, 2012; Gordin et al., 2008). Additionally, functional defects of the immune system have been correlated with the metabolic control of diabetic patients and are related to increased susceptibility of these patients to infections(Moutschen, Scheen, & Lefebvre, 1992). The pathway analysis indicated a deregulation of the key mechanisms involved in inflammation and immune response in poorly-controlled diabetic adolescents.

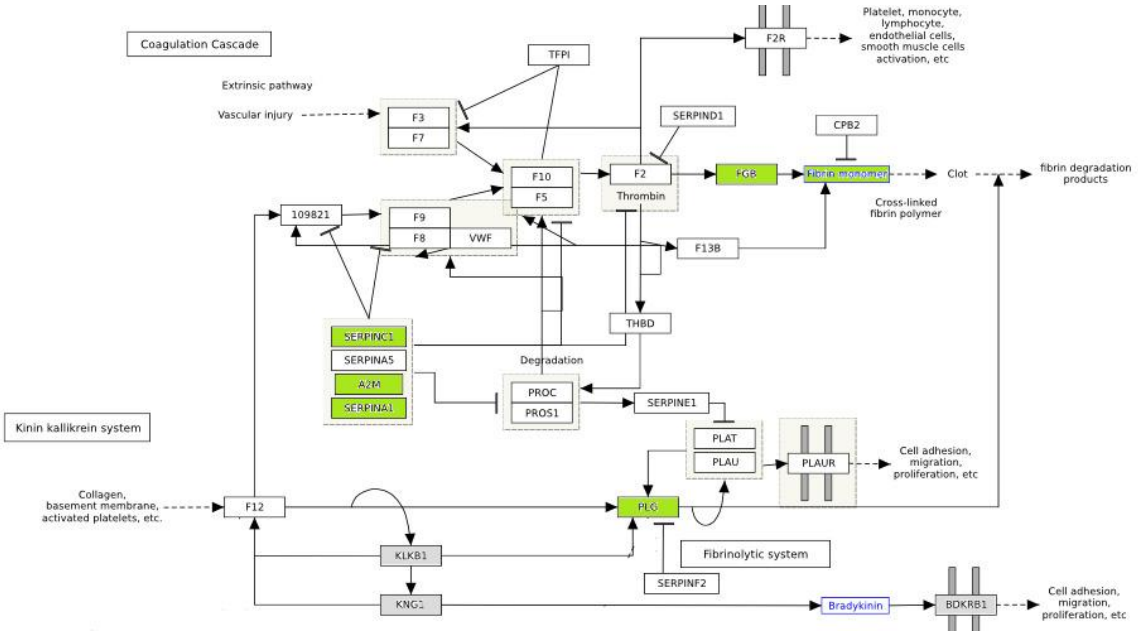
Liver X receptors (LXRs), transcription factors of a nuclear hormone receptor family, play an important role in metabolic regulation. They control cholesterol and glucose homeostasis in the body and recent studies in type 2 diabetic models have shown that LXRs regulate insulin secretion and biosynthesis via control of glucose and lipid metabolism in pancreatic b-cells(Ding et al., 2014; Efanov, Sewing, Bokvist, & Gromada, 2004). In our study, deregulation of LXR/RXR pathway in G1 could reflect the inadequate metabolic control of the disease.

Toll-like receptors (TLRs), another signalling pathway which was found to be deregulated in poorly controlled patients in our study, are proteins that play key role in the innate immune system. These immune receptors are able to recognize microbial molecules, detect infections and initiate antimicrobial host defence responses. According to new data, autoimmune diabetes is found to be triggered by

the innate immune pathways and TRLs are the mediators of this mechanism, (Zipris, 2010).

Hyperglycemia is known to play a critical role in the pathogenesis of cardiovascular disease. Numerous substances, such as growth factors, cytokines and pro-coagulant factors are related to a series of altered underlying processes that induce and promote atherogenesis(Beisswenger, 2012; Katz, et al., 2015). In our study, among differentially expressed proteins were PLG, SERPING1, SERPINC1, APOA2, FGB, A2M, which are related to endothelial dysfunction, coagulation processes and pro-atherogenic alteration mechanisms. The differentially expressed proteins involved in the coagulation pathway are illustrated in Figure 11.

Figure 11. The effect of diabetes on fibrin clot formation is presented with annotated differentially expressed proteins. In green downregulated proteins are shown. These proteins are inhibitors of fibrin clot formation. Thus, fibrin clot formation is activated in diabetes. (http://www.wikipathways.org/index.php/Pathway:WP558)



Vascular lesions are the result of an unbalance between fibrin deposition and fibrinolysis. Injury in vascular endothelial cells releases plasminogen activators and at the same time activates fibrinolysis. The role of plasminogen activators is to cleave plasminogen into plasmin, which dissolves clots. Fibrinolysis is controlled by plasminogen activator inhibitors (PAI-1) and plasmin inhibitors (a2-macroglobulin)(Beisswenger, 2012). In diabetes, premature atherosclerosis and activation of coagulation factors, combined with hypofibrinolysis all contribute to increased cardiovascular risk. Serin protease inhibitors (SERPINC1, SERPINA1) and A2 macroglobulin are downregulated in poorly control subjects (shown in green) (Figure 11), which further impairs the degradation of fibrin clots (Carr, 2001; Chung, Lin, & Kao, 2015; Pratte et al., 2009).

Blood clot formation is the last step in the atherothrombotic mechanism, and the structure of the fibrin network, among other factors, determines cardiovascular risk. Hyperglycemia induces alterations in coagulation factor plasma levels and its impact is crucial in predisposition to cardiovascular events(Katz, et al., 2015). Coagulation's deregulation appears to play an important role in glomerular hypertrophy and fibrosis of diabetic nephropathy(Sumi et al., 2011).

Moreover the differentially expressed proteins identified in the three comparisons described in our study form three distinct PPI networks as it is demonstrated in Figure 10. The fact that the majority of the proteins participate in PPI indicates that they share common functions.

In conclusion, by performing analysis at the systems biology level with rigorous statistical methodology this study provides functional insights by connecting the disease phenotype to specific biological processes (Tables 16 and 17). This functional analysis demonstrates the deregulation of biological mechanisms highly relevant to diabetic pathophysiology (inflammation, atherosclerosis signaling, coagulation, etc.) whereas the patients did not exhibit any clinical complications (retinopathy, microalbuminuria, neuropathy) associated with type 1 diabetes. Thus, our study reveals molecular features with clinical relevance that can allow physicians to assess the status of asymptomatic patients.

Potential preventive intervention

A final step to the bioinformatic analysis was the utilisation of L1000CDS^2 which is a search engine of gene expression signatures from the LINCS L1000 dataset(Vempati, et al., 2014) (see Methods). The system is a tool for identifying perturbagens whose overall effect in gene expression either mimics or reverses the gene expression pattern. When provided with the differentially expressed (up & down-regulated) proteins of Comparison G1 vs G2 and asked to return the agents that most efficiently reversed that phenotype, the top hit was BRD-K01868942 (S9 Table), a novel serotonin receptor antagonist(Lemaitre et al., 2009).

As previously demonstrated on diabetic mice, increased serotonin receptor activity induces contraction of arteries thus causing vascular dysfunction(Nelson, Harrod, & Lamping, 2012). The finding that a serotonin receptor antagonist efficiently reverses our experimental phenotype leads to the suggestion that this phenotype is at least partially induced by increased serotonin receptor activity. The above confirms our aforementioned finding of vascular dysfunction in diabetics with poor glycemic control versus well-controlled diabetics. Furthermore, this finding suggests that serotonin receptor antagonists could be potentially utilised as a preventive intervention in young patients with poor diabetic control. This possible course of intervention is further supported by the fact that serotonin antagonists improve vascular function in patients with peripheral arterial disease(Miyazaki et al., 2007). Thus, the available pharmacological data on the most prominent predicted active substance support the validity of our bioinformatics approach.

Conclusions

In the first part of this study, the salivary status and dental caries of young patients with type 1 diabetes was evaluated, in relation to the metabolic control of the disease. Xerostomia, decreased salivary flow rates and higher caries prevalence were recorded in children and adolescents with poor glycemic control, when compared to those with well control of the disease and healthy subjects. The evaluation was performed with the use of chair-side salivary tests, which provide the practitioners with an easy-to use and quick method for caries risk assessment and confirmation of clinical symptoms and signs in young patients.

The second part of this study provides the research community with a high quality proteomic resource with state-of-the-art wealth of information for a very specific patient population, which is young individuals with type-1 diabetes and poor glycemic control. In-depth analysis of data from this population indicated that differentially expressed proteins are related to acute phase response, endothelial dysfunction, inflammatory and coagulation processes in type I diabetes mellitus. Furthermore, hyperglycemia appears to be a causal link between diabetes and its complications by activating the respective molecular pathways from the early stages of the disease. Finally, a possible course of preventive intervention was revealed by molecular signatures analysis. The current work enriches the clinical landscape by providing a proof-of-concept on how proteomics and bioinformatics approaches can be applied for the elucidation of molecular pathways involved in the pathophysiology of type 1 diabetes.

References

The AACE system of intensive diabetes self-management—2002 update. (2002). *Endocrine Practice,, 8*, 40-82.

Abbate, G. M., Borghi, D., Passi, A., & Levrini, L. (2014). Correlation between unstimulated salivary flow, pH and streptococcus mutans, analysed with real time PCR, in caries-free and caries-active children. *Eur J Paediatr Dent, 15*(1), 51-54.

Abdallah, C., Dumas-Gaudot, E., Renaut, J., & Sergeant, K. (2012). Gel-based and gel-free quantitative proteomics approaches at a glance. *Int J Plant Genomics, 2012*, 494572. doi: 10.1155/2012/494572

Ai, J. Y., Smith, B., & Wong, D. T. (2012). Bioinformatics advances in saliva diagnostics. *Int J Oral Sci, 4*(2), 85-87. doi: ijos201226 [pii]
10.1038/ijos.2012.26

Al-Maskari, A. Y., Al-Maskari, M. Y., & Al-Sudairy, S. (2011). Oral Manifestations and Complications of Diabetes Mellitus: A review. *Sultan Qaboos Univ Med J, 11*(2), 179-186.

Al-Tarawneh, S. K., Border, M. B., Dibble, C. F., & Bencharit, S. (2011). Defining salivary biomarkers using mass spectrometry-based proteomics: a systematic review. *OMICS, 15*(6), 353-361. doi: 10.1089/omi.2010.0134

Al Kawas, S., Rahim, Z. H., & Ferguson, D. B. (2012). Potential uses of human salivary protein and peptide analysis in the diagnosis of disease. *Arch Oral Biol, 57*(1), 1-9. doi: S0003-9969(11)00202-0 [pii]
10.1016/j.archoralbio.2011.06.013

Albert, D. A., Ward, A., Allweiss, P., Graves, D. T., Knowler, W. C., Kunzel, C., et al. (2012). Diabetes and oral disease: implications for health professionals. *Ann N Y Acad Sci, 1255*, 1-15. doi: 10.1111/j.1749-6632.2011.06460.x

Amado, F., Lobo, M. J., Domingues, P., Duarte, J. A., & Vitorino, R. (2010). Salivary peptidomics. *Expert Rev Proteomics, 7*(5), 709-721. doi: 10.1586/epr.10.48

Amado, F. M., Ferreira, R. P., & Vitorino, R. (2013). One decade of salivary proteomics: current approaches and outstanding challenges. *Clin Biochem, 46*(6), 506-517. doi: S0009-9120(12)00579-6 [pii]
10.1016/j.clinbiochem.2012.10.024

Amerongen, A. V., & Veerman, E. C. (2002). Saliva--the defender of the oral cavity. *Oral Dis, 8*(1), 12-22.

An, Z., Chen, Y., Zhang, R., Song, Y., Sun, J., He, J., et al. (2010). Integrated ionization approach for RRLC-MS/MS-based metabonomics: finding potential biomarkers for lung cancer. *J Proteome Res, 9*(8), 4071-4081. doi: 10.1021/pr100265g

Anderson, J. E., & Thliveris, J. A. (1986). Testicular histology in streptozotocin-induced diabetes. *Anat Rec, 214*(4), 378-382. doi: 10.1002/ar.1092140407

Anderson, L. C. (1987). Parotid gland function in streptozotocin-diabetic rats. *J Dent Res, 66*(2), 425-429. doi: 10.1177/00220345870660020701

Anderson, L. C., Garrett, J. R., Thulin, A., & Proctor, G. B. (1989). Effects of streptozocin-induced diabetes on sympathetic and parasympathetic stimulation of parotid salivary gland function in rats. *Diabetes, 38*(11), 1381-1389.

Ao, S., Sun, X., Shi, X., Huang, X., Chen, F., & Zheng, S. (2017). Longitudinal investigation of salivary proteomic profiles in the development of early childhood caries. *J Dent, 61*, 21-27. doi: S0300-5712(17)30090-8 [pii]
10.1016/j.jdent.2017.04.006

Aren, G., Sepet, E., Ozdemir, D., Dinccag, N., Guvener, B., & Firatli, E. (2003). Periodontal health, salivary status, and metabolic control in children with type 1 diabetes mellitus. *J Periodontol, 74*(12), 1789-1795. doi: 10.1902/jop.2003.74.12.1789

Aro, K., Wei, F., Wong, D. T., & Tu, M. (2017). Saliva Liquid Biopsy for Point-of-Care Applications. *Front Public Health, 5*, 77. doi: 10.3389/fpubh.2017.00077

Arredouani, A., Stocchero, M., Culeddu, N., El-Sayed Moustafa, J., Tichet, J., Balkau, B., et al. (2016). Metabolomic Profile of Low Copy-Number Carriers at the Salivary Alpha-Amylase Gene Suggests a Metabolic Shift Towards Lipid-Based Energy Production. *Diabetes*. doi: db16-0315 [pii]
10.2337/db16-0315

Artigues, A., Nadeau, O. W., Rimmer, M. A., Villar, M. T., Du, X., Fenton, A. W., et al. (2016). Protein Structural Analysis via Mass Spectrometry-Based Proteomics. *Adv Exp Med Biol, 919*, 397-431. doi: 10.1007/978-3-319-41448-5_19

Aslam, B., Basit, M., Nisar, M. A., Khurshid, M., & Rasool, M. H. (2017). Proteomics: Technologies and Their Applications. *J Chromatogr Sci, 55*(2), 182-196. doi: bmw167 [pii]
10.1093/chromsci/bmw167

Atkinson, M. A., Eisenbarth, G. S., & Michels, A. W. (2014). Type 1 diabetes. *Lancet, 383*(9911), 69-82. doi: S0140-6736(13)60591-7 [pii]
10.1016/S0140-6736(13)60591-7

Bagherian, A., & Asadikaram, G. (2012). Comparison of some salivary characteristics between children with and without early childhood caries. *Indian J Dent Res, 23*(5), 628-632. doi: IndianJDentRes_2012_23_5_628_107380 [pii]
10.4103/0970-9290.107380

Bahar, G., Feinmesser, R., Shpitzer, T., Popovtzer, A., & Nagler, R. M. (2007). Salivary analysis in oral cancer patients: DNA and protein oxidation, reactive nitrogen species, and antioxidant profile. *Cancer, 109*(1), 54-59. doi: 10.1002/cncr.22386

Barbosa, E. B., Vidotto, A., Polachini, G. M., Henrique, T., Marqui, A. B., & Tajara, E. H. (2012). Proteomics: methodologies and applications to the study of human diseases. *Rev Assoc Med Bras (1992), 58*(3), 366-375. doi: S0104-42302012000300019 [pii]

Basham, R. J., Richardson, D. M., Sutcliffe, C. B., & Haas, D. W. (2009). Effect of HIV-1 infection on human DNA yield from saliva. *HIV Clin Trials, 10*(4), 282-285. doi: Y65W64K36346NK41 [pii]
10.1310/hct1004-282

Baumgartner-Parzer, S. M., Wagner, L., Pettermann, M., Grillari, J., Gessl, A., & Waldhausl, W. (1995). High-glucose--triggered apoptosis in cultured endothelial cells. *Diabetes, 44*(11), 1323-1327.

Bayes, B., Granada, M. L., Lauzurica, R., Pastor, M. C., Navarro, M., Bonet, J., et al. (2005). Effect of low doses of atorvastatin on adiponectin, glucose homeostasis, and clinical inflammatory markers in kidney transplant recipients. *Transplant Proc, 37*(9), 3808-3812. doi: S0041-1345(05)00909-7 [pii]
10.1016/j.transproceed.2005.08.064

Beisswenger, P. J. (2012). Glycation and biomarkers of vascular complications of diabetes. *Amino Acids, 42*(4), 1171-1183. doi: 10.1007/s00726-010-0784-z

Belazi, M. A., Galli-Tsinopoulou, A., Drakoulakos, D., Fleva, A., & Papanayiotou, P. H. (1998). Salivary alterations in insulin-dependent diabetes mellitus. *Int J Paediatr Dent, 8*(1), 29-33.

Belstrom, D., Constancias, F., Liu, Y., Yang, L., Drautz-Moses, D. I., Schuster, S. C., et al. (2017). Metagenomic and metatranscriptomic analysis of saliva reveals disease-associated microbiota in patients with periodontitis and dental caries. *NPJ Biofilms Microbiomes, 3*, 23. doi: 10.1038/s41522-017-0031-4
31 [pii]

Ben-Aryeh, H., Cohen, M., Kanter, Y., Szargel, R., & Laufer, D. (1988). Salivary composition in diabetic patients. *J Diabet Complications, 2*(2), 96-99.

Ben-Aryeh, H., Serouya, R., Kanter, Y., Szargel, R., & Laufer, D. (1993). Oral health and salivary composition in diabetic patients. *J Diabetes Complications, 7*(1), 57-62. doi: 1056-8727(93)90025-T [pii]

Bencharit, S., Baxter, S. S., Carlson, J., Byrd, W. C., Mayo, M. V., Border, M. B., et al. (2013). Salivary proteins associated with hyperglycemia in diabetes: a proteomic analysis. *Mol Biosyst, 9*(11), 2785-2797. doi: 10.1039/c3mb70196d

Bending, D., Zaccone, P., & Cooke, A. (2012). Inflammation and type one diabetes. *Int Immunol, 24*(6), 339-346. doi: dxs049 [pii]
10.1093/intimm/dxs049

Bernardi, M. J., Reis, A., Loguercio, A. D., Kehrig, R., Leite, M. F., & Nicolau, J. (2007). Study of the buffering capacity, pH and salivary flow rate in type 2 well-controlled and poorly controlled diabetic patients. *Oral Health Prev Dent, 5*(1), 73-78.

Boersema, P. J., Kahraman, A., & Picotti, P. (2015). Proteomics beyond large-scale protein expression analysis. *Curr Opin Biotechnol, 34*, 162-170. doi: S0958-1669(15)00014-2 [pii]
10.1016/j.copbio.2015.01.005

Boppana, S. B., Ross, S. A., Shimamura, M., Palmer, A. L., Ahmed, A., Michaels, M. G., et al. (2011). Saliva polymerase-chain-reaction assay for cytomegalovirus screening in newborns. *N Engl J Med, 364*(22), 2111-2118. doi: 10.1056/NEJMoa1006561

Border, M. B., Schwartz, S., Carlson, J., Dibble, C. F., Kohltfarber, H., Offenbacher, S., et al. (2012). Exploring salivary proteomes in edentulous patients with type 2 diabetes. *Mol Biosyst, 8*(4), 1304-1310. doi: 10.1039/c2mb05079j

Borgnakke, W. S. (2010). Salivary glucose levels are unable to predict oral candidiasis or monitor diabetes. *J Evid Based Dent Pract, 10*(4), 237-240. doi: S1532-3382(10)00188-0 [pii]
10.1016/j.jebdp.2010.09.018

Bosch, J. A., Veerman, E. C., de Geus, E. J., & Proctor, G. B. (2011). alpha-Amylase as a reliable and convenient measure of sympathetic activity: don't start salivating just yet! *Psychoneuroendocrinology, 36*(4), 449-453. doi: S0306-4530(11)00007-2 [pii]
10.1016/j.psyneuen.2010.12.019

Bozovic, D., Racic, M., & Ivkovic, N. (2013). Salivary cortisol levels as a biological marker of stress reaction. *Med Arch, 67*(5), 374-377.

Brinkmann, O., Kastratovic, D. A., Dimitrijevic, M. V., Konstantinovic, V. S., Jelovac, D. B., Antic, J., et al. (2011). Oral squamous cell carcinoma detection by salivary biomarkers in a Serbian population. *Oral Oncol, 47*(1), 51-55. doi: S1368-8375(10)00325-8 [pii]
10.1016/j.oraloncology.2010.10.009

Busato, I. M., Ignacio, S. A., Brancher, J. A., Gregio, A. M., Machado, M. A., & Azevedo-Alanis, L. R. (2009). Impact of xerostomia on the quality of life of adolescents with type 1 diabetes mellitus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 108*(3), 376-382. doi: S1079-2104(09)00292-3 [pii]
10.1016/j.tripleo.2009.05.005

Busato, I. M., Ignacio, S. A., Brancher, J. A., Moyses, S. T., & Azevedo-Alanis, L. R. (2012). Impact of clinical status and salivary conditions on xerostomia and oral health-related quality of life of adolescents with type 1 diabetes mellitus. *Community Dent Oral Epidemiol, 40*(1), 62-69. doi: 10.1111/j.1600-0528.2011.00635.x

Cabras, T., Pisano, E., Mastinu, A., Denotti, G., Pusceddu, P. P., Inzitari, R., et al. (2010). Alterations of the salivary secretory peptidome profile in children affected by type 1 diabetes. *Mol Cell Proteomics, 9*(10), 2099-2108. doi: M110.001057 [pii]
10.1074/mcp.M110.001057

Cacicedo, J. M., Yagihashi, N., Keaney, J. F., Jr., Ruderman, N. B., & Ido, Y. (2004). AMPK inhibits fatty acid-induced increases in NF-kappaB transactivation in cultured human umbilical vein endothelial cells. *Biochem Biophys Res Commun, 324*(4), 1204-1209. doi: S0006-291X(04)02245-4 [pii]
10.1016/j.bbrc.2004.09.177

Canivell, S., & Gomis, R. (2014). Diagnosis and classification of autoimmune diabetes mellitus. *Autoimmun Rev, 13*(4-5), 403-407. doi: S1568-9972(14)00032-9 [pii]
10.1016/j.autrev.2014.01.020

Capriotti, A. L., Cavaliere, C., Foglia, P., Samperi, R., & Lagana, A. (2011). Intact protein separation by chromatographic and/or electrophoretic techniques for top-down proteomics. *J Chromatogr A, 1218*(49), 8760-8776. doi: S0021-9673(11)00785-0 [pii]
10.1016/j.chroma.2011.05.094

Carleo, A., Bennett, D., & Rottoli, P. (2016). Biomarkers in sarcoidosis: the contribution of system biology. *Curr Opin Pulm Med, 22*(5), 509-514. doi: 10.1097/MCP.0000000000000306

Carr, M. E. (2001). Diabetes mellitus: a hypercoagulable state. *J Diabetes Complications, 15*(1), 44-54. doi: S1056-8727(00)00132-X [pii]

Casanova, L., Hughes, F. J., & Preshaw, P. M. (2014). Diabetes and periodontal disease: a two-way relationship. *Br Dent J, 217*(8), 433-437. doi: sj.bdj.2014.907 [pii]
10.1038/sj.bdj.2014.907

Caseiro, A., Ferreira, R., Padrao, A., Quintaneiro, C., Pereira, A., Marinheiro, R., et al. (2013). Salivary proteome and peptidome profiling in type 1 diabetes mellitus using a quantitative approach. *J Proteome Res, 12*(4), 1700-1709. doi: 10.1021/pr3010343

Caseiro, A., Vitorino, R., Barros, A. S., Ferreira, R., Calheiros-Lobo, M. J., Carvalho, D., et al. (2012). Salivary peptidome in type 1 diabetes mellitus. *Biomed Chromatogr, 26*(5), 571-582. doi: 10.1002/bmc.1677

Casqueiro, J., & Alves, C. (2012). Infections in patients with diabetes mellitus: A review of pathogenesis. *Indian J Endocrinol Metab, 16 Suppl 1*, S27-36. doi: 10.4103/2230-8210.94253
IJEM-16-27 [pii]

Castagnola, M., Cabras, T., Iavarone, F., Fanali, C., Nemolato, S., Peluso, G., et al. (2012). The human salivary proteome: a critical overview of the results obtained by different proteomic platforms. *Expert Rev Proteomics, 9*(1), 33-46. doi: 10.1586/epr.11.77

Castagnola, M., Cabras, T., Vitali, A., Sanna, M. T., & Messana, I. (2011). Biotechnological implications of the salivary proteome. *Trends Biotechnol, 29*(8), 409-418. doi: S0167-7799(11)00068-0 [pii]

10.1016/j.tibtech.2011.04.002

Chavez, E. M., Borrell, L. N., Taylor, G. W., & Ship, J. A. (2001). A longitudinal analysis of salivary flow in control subjects and older adults with type 2 diabetes. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 91*(2), 166-173. doi: S1079-2104(01)92705-2 [pii]

10.1067/moe.2001.112054

Chiappin, S., Antonelli, G., Gatti, R., & De Palo, E. F. (2007). Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta, 383*(1-2), 30-40. doi: S0009-8981(07)00213-6 [pii]

10.1016/j.cca.2007.04.011

Cho, M. A., Ko, J. Y., Kim, Y. K., & Kho, H. S. (2010). Salivary flow rate and clinical characteristics of patients with xerostomia according to its aetiology. *J Oral Rehabil, 37*(3), 185-193. doi: JOR2037 [pii]

10.1111/j.1365-2842.2009.02037.x

Choi, M. (2010). Saliva diagnostics integrate dentistry into general and preventive health care. *Int J Prosthodont, 23*(3), 189.

Chung, W. S., Lin, C. L., & Kao, C. H. (2015). Diabetes increases the risk of deep-vein thrombosis and pulmonary embolism. A population-based cohort study. *Thromb Haemost, 114*(4), 812-818. doi: 14-10-0868 [pii]

10.1160/TH14-10-0868

Cinar, A. B., Freeman, R., & Schou, L. (2018). A new complementary approach for oral health and diabetes management: health coaching. *Int Dent J, 68*(1), 54-64. doi: 10.1111/idj.12334

Collin, H. L., Uusitupa, M., Niskanen, L., Koivisto, A. M., Markkanen, H., & Meurman, J. H. (1998). Caries in patients with non-insulin-dependent diabetes mellitus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 85*(6), 680-685. doi: S1079-2104(98)90035-X [pii]

Concannon, P., Chen, W. M., Julier, C., Morahan, G., Akolkar, B., Erlich, H. A., et al. (2009). Genome-wide scan for linkage to type 1 diabetes in 2,496 multiplex families from the Type 1 Diabetes Genetics Consortium. *Diabetes, 58*(4), 1018-1022. doi: db08-1551 [pii]

10.2337/db08-1551

Concannon, P., Rich, S. S., & Nepom, G. T. (2009). Genetics of type 1A diabetes. *N Engl J Med, 360*(16), 1646-1654. doi: 360/16/1646 [pii]

10.1056/NEJMra0808284

Cook, D. G., Mendall, M. A., Whincup, P. H., Carey, I. M., Ballam, L., Morris, J. E., et al. (2000). C-reactive protein concentration in children: relationship to adiposity and other cardiovascular risk factors. *Atherosclerosis, 149*(1), 139-150. doi: S0021-9150(99)00312-3 [pii]

Cooke, C., Ahmedzai, S., & Mayberry, J. (1996). Xerostomia--a review. *Palliat Med. 1996 Oct;10(4):284-92., 10*(4), 284-292.

Costalonga, M., & Herzberg, M. C. (2014). The oral microbiome and the immunobiology of periodontal disease and caries. *Immunol Lett, 162*(2 Pt A), 22-38. doi: S0165-2478(14)00187-4 [pii]
10.1016/j.imlet.2014.08.017
Cozma, S., Dima-Cozma, L. C., Ghiciuc, C. M., Pasquali, V., Saponaro, A., & Patacchioli, F. R. (2017). Salivary cortisol and alpha-amylase: subclinical indicators of stress as cardiometabolic risk. *Braz J Med Biol Res, 50*(2), e5577. doi: S0100-879X2017000200201 [pii]
10.1590/1414-431X20165577
Craig, R., & Beavis, R. C. (2004). TANDEM: matching proteins with tandem mass spectra. *Bioinformatics, 20*(9), 1466-1467. doi: 10.1093/bioinformatics/bth092
bth092 [pii]
Crook, M. (2004). Type 2 diabetes mellitus: a disease of the innate immune system? An update. *Diabet Med, 21*(3), 203-207. doi: 1030 [pii]
Cuevas-Cordoba, B., & Santiago-Garcia, J. (2014). Saliva: a fluid of study for OMICS. *OMICS, 18*(2), 87-97. doi: 10.1089/omi.2013.0064
Cunha-Cruz, J., Scott, J., Rothen, M., Mancl, L., Lawhorn, T., Brossel, K., et al. (2013). Salivary characteristics and dental caries: evidence from general dental practices. *J Am Dent Assoc, 144*(5), e31-40. doi: S0002-8177(14)60499-2 [pii]
D'Amario, M., Barone, A., Marzo, G., & Giannoni, M. (2006). Caries-risk assessment: the role of salivary tests. *Minerva Stomatol, 55*(7-8), 449-463.
da Silva Modesto, K. B., de Godoi Simoes, J. B., de Souza, A. F., Damaceno, N., Duarte, D. A., Leite, M. F., et al. (2015). Salivary flow rate and biochemical composition analysis in stimulated whole saliva of children with cystic fibrosis. *Arch Oral Biol, 60*(11), 1650-1654. doi: S0003-9969(15)30024-8 [pii]
10.1016/j.archoralbio.2015.08.007
Dabadghao, P., Vidmar, S., & Cameron, F. J. (2001). Deteriorating diabetic control through adolescence-do the origins lie in childhood? *Diabet Med, 18*(11), 889-894. doi: 593 [pii]
Danaei G, F. M., Lu Y, Singh GM, Cowan MJ, Paciorek CJ et al. (2011). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet, 378*(9785), 31-40.
de Jong, E. P., Xie, H., Onsongo, G., Stone, M. D., Chen, X. B., Kooren, J. A., et al. (2010). Quantitative proteomics reveals myosin and actin as promising saliva biomarkers for distinguishing pre-malignant and malignant oral lesions. *PLoS One, 5*(6), e11148. doi: 10.1371/journal.pone.0011148
de Oliveira, S. A., Siqueira, M. M., Brown, D. W., Litton, P., Camacho, L. A., Castro, S. T., et al. (2000). Diagnosis of rubella infection by detecting specific immunoglobulin M antibodies in saliva samples: a clinic-based study in Niteroi, RJ, Brazil. *Rev Soc Bras Med Trop, 33*(4), 335-339.
Deepa, T., & Thirrunavukkarasu, N. (2010). Saliva as a potential diagnostic tool. *Indian J Med Sci, 64*(7), 293-306. doi: IndianJMedSci_2010_64_7_293_99854 [pii]
10.4103/0019-5359.99854
Definition, diagnosis and classification od diabetes mellitus and its complications. (1999). *(WHO/NCD/NCS/99.2).*

Demirel, F., Tepe, D., Kara, O., & Esen, I. (2013). Microvascular complications in adolescents with type 1 diabetes mellitus. *J Clin Res Pediatr Endocrinol, 5*(3), 145-149. doi: 10.4274/Jcrpe.994

Desai, G. S., & Mathews, S. T. (2014). Saliva as a non-invasive diagnostic tool for inflammation and insulin-resistance. *World J Diabetes, 5*(6), 730-738. doi: 10.4239/wjd.v5.i6.730

Deutsch, E. W. (2010). The PeptideAtlas Project. *Methods Mol Biol, 604*, 285-296.

Deutsch, E. W., Mendoza, L., Shteynberg, D., Slagel, J., Sun, Z., & Moritz, R. L. (2015). Trans-Proteomic Pipeline, a standardized data processing pipeline for large-scale reproducible proteomics informatics. *Proteomics Clin Appl, 9*(7-8), 745-754. doi: 10.1002/prca.201400164

Diabetes and oral health. Abstracts of the 19th Annual International Conference of the University Diabetes Outreach Programme of the University of the West Indies and the University of Technology, Jamaica in collaboration with the Association of Public Dental Surgeons. March 21-23, 2013. Ocho Rios, Jamaica. (2013). *West Indian Med J, 62 Suppl 1*, 20-36.

Diagnosis and classification of diabetes mellitus. (2014). *Diabetes Care, 37 Suppl 1*, S81-90. doi: 37/Supplement_1/S81 [pii]

10.2337/dc14-S081

Ding, L., Pang, S., Sun, Y., Tian, Y., Yu, L., & Dang, N. (2014). Coordinated Actions of FXR and LXR in Metabolism: From Pathogenesis to Pharmacological Targets for Type 2 Diabetes. *Int J Endocrinol, 2014*, 751859. doi: 10.1155/2014/751859

Dodds, M. W., & Dodds, A. P. (1997). Effects of glycemic control on saliva flow rates and protein composition in non-insulin-dependent diabetes mellitus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 83*(4), 465-470. doi: S1079-2104(97)90147-5 [pii]

Dodds, M. W., Johnson, D. A., & Yeh, C. K. (2005). Health benefits of saliva: a review. *J Dent, 33*(3), 223-233. doi: S0300-5712(04)00172-1 [pii]

10.1016/j.jdent.2004.10.009

Dodds, M. W., Yeh, C. K., & Johnson, D. A. (2000). Salivary alterations in type 2 (non-insulin-dependent) diabetes mellitus and hypertension. *Community Dent Oral Epidemiol, 28*(5), 373-381.

Doi, M., Sekizawa, N., Tani, Y., Tsuchiya, K., Kouyama, R., Tateno, T., et al. (2008). Late-night salivary cortisol as a screening test for the diagnosis of Cushing's syndrome in Japan. *Endocr J, 55*(1), 121-126. doi: JST.JSTAGE/endocrj/K07E-023 [pii]

Donaghue, K. C., Chiarelli, F., Trotta, D., Allgrove, J., & Dahl-Jorgensen, K. (2009). Microvascular and macrovascular complications associated with diabetes in children and adolescents. *Pediatr Diabetes, 10 Suppl 12*, 195-203. doi: PDI576 [pii]

10.1111/j.1399-5448.2009.00576.x

Donaghue, K. C., Marcovecchio, M. L., Wadwa, R. P., Chew, E. Y., Wong, T. Y., Calliari, L. E., et al. (2018). ISPAD Clinical Practice Consensus Guidelines 2018: Microvascular and macrovascular complications in children and adolescents. *Pediatr Diabetes, 19 Suppl 27*, 262-274. doi: 10.1111/pedi.12742

Dowling, P., Wormald, R., Meleady, P., Henry, M., Curran, A., & Clynes, M. (2008). Analysis of the saliva proteome from patients with head and neck squamous cell carcinoma reveals differences in abundance levels of proteins associated

with tumour progression and metastasis. *J Proteomics, 71*(2), 168-175. doi: S1874-3919(08)00066-3 [pii]
10.1016/j.jprot.2008.04.004
Duncan, B. B., & Schmidt, M. I. (2006). The epidemiology of low-grade chronic systemic inflammation and type 2 diabetes. *Diabetes Technol Ther, 8*(1), 7-17. doi: 10.1089/dia.2006.8.7
Edblad, E., Lundin, S. A., Sjodin, B., & Aman, J. (2001). Caries and salivary status in young adults with type 1 diabetes. *Swed Dent J, 25*(2), 53-60.
Efanov, A. M., Sewing, S., Bokvist, K., & Gromada, J. (2004). Liver X receptor activation stimulates insulin secretion via modulation of glucose and lipid metabolism in pancreatic beta-cells. *Diabetes, 53 Suppl 3*, S75-78. doi: 53/suppl_3/S75 [pii]
Estrada, Y. M. R. M., & Orlander, P. R. (2011). Salivary cortisol can replace free serum cortisol measurements in patients with septic shock. *Chest, 140*(5), 1216-1222. doi: S0012-3692(11)60588-6 [pii]
10.1378/chest.11-0448
Fang, X., & Zhang, W. W. (2008). Affinity separation and enrichment methods in proteomic analysis. *J Proteomics, 71*(3), 284-303. doi: S1874-3919(08)00104-8 [pii]
10.1016/j.jprot.2008.06.011
Feng, Y., & Picotti, P. (2016). Selected Reaction Monitoring to Measure Proteins of Interest in Complex Samples: A Practical Guide. *Methods Mol Biol, 1394*, 43-56. doi: 10.1007/978-1-4939-3341-9_4
Festa, A., D'Agostino, R., Jr., Howard, G., Mykkanen, L., Tracy, R. P., & Haffner, S. M. (2000). Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). *Circulation, 102*(1), 42-47.
Fine, D. H., Markowitz, K., Furgang, D., Fairlie, K., Ferrandiz, J., Nasri, C., et al. (2009). Macrophage inflammatory protein-1alpha: a salivary biomarker of bone loss in a longitudinal cohort study of children at risk for aggressive periodontal disease? *J Periodontol, 80*(1), 106-113. doi: 10.1902/jop.2009.080296
10.1902/jop.2009.080296 [pii]
Fox, P. C., Busch, K. A., & Baum, B. J. (1987). Subjective reports of xerostomia and objective measures of salivary gland performance. *J Am Dent Assoc, 115*(4), 581-584. doi: S0002-8177(87)54012-0 [pii]
Gagnum, V., Stene, L. C., Sandvik, L., Fagerland, M. W., Njolstad, P. R., Joner, G., et al. (2015). All-cause mortality in a nationwide cohort of childhood-onset diabetes in Norway 1973-2013. *Diabetologia, 58*(8), 1779-1786. doi: 10.1007/s00125-015-3623-7
Galili, D., Findler, M., & Garfunkel, A. (1994). Oral and dental complications associated with diabetes and their treatment. *Compendium. 1994 Apr;15(4):496, 498, 500-9., 15*(4), 496-509.
Ghafouri, B., Tagesson, C., & Lindahl, M. (2003). Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. *Proteomics, 3*(6), 1003-1015. doi: 10.1002/pmic.200300426
Giannobile, W. V., Beikler, T., Kinney, J. S., Ramseier, C. A., Morelli, T., & Wong, D. T. (2009). Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontol 2000, 50*, 52-64. doi: PRD288 [pii]
10.1111/j.1600-0757.2008.00288.x

Giuca, M. R., Pasini, M., Giuca, G., Caruso, S., Necozione, S., & Gatto, R. (2015). Investigation of periodontal status in type 1 diabetic adolescents. *Eur J Paediatr Dent, 16*(4), 319-323.

Giusti, L., Baldini, C., Ciregia, F., Giannaccini, G., Giacomelli, C., De Feo, F., et al. (2010). Is GRP78/BiP a potential salivary biomarker in patients with rheumatoid arthritis? *Proteomics Clin Appl, 4*(3), 315-324. doi: 10.1002/prca.200900082

Global status report on noncommunicable diseases 2010. . (2011).

Goncalves Lda, R., Soares, M. R., Nogueira, F. C., Garcia, C., Camisasca, D. R., Domont, G., et al. (2010). Comparative proteomic analysis of whole saliva from chronic periodontitis patients. *J Proteomics, 73*(7), 1334-1341. doi: S1874-3919(10)00070-9 [pii]

10.1016/j.jprot.2010.02.018

Goncalves Lda, R., Soares, M. R., Nogueira, F. C., Garcia, C. H., Camisasca, D. R., Domont, G., et al. (2011). Analysis of the salivary proteome in gingivitis patients. *J Periodontal Res, 46*(5), 599-606. doi: 10.1111/j.1600-0765.2011.01378.x

Goodson, J. M., Kantarci, A., Hartman, M. L., Denis, G. V., Stephens, D., Hasturk, H., et al. (2014). Metabolic disease risk in children by salivary biomarker analysis. *PLoS One, 9*(6), e98799. doi: 10.1371/journal.pone.0098799

PONE-D-14-07713 [pii]

Gordin, D., Forsblom, C., Ronnback, M., Parkkonen, M., Waden, J., Hietala, K., et al. (2008). Acute hyperglycaemia induces an inflammatory response in young patients with type 1 diabetes. *Ann Med, 40*(8), 627-633. doi: 793151240 [pii]

10.1080/07853890802126547

Greabu, M., Battino, M., Mohora, M., Totan, A., Didilescu, A., Spinu, T., et al. (2009). Saliva--a diagnostic window to the body, both in health and in disease. *J Med Life, 2*(2), 124-132.

Gupta, S., Nayak, M. T., Sunitha, J. D., Dawar, G., Sinha, N., & Rallan, N. S. (2017). Correlation of salivary glucose level with blood glucose level in diabetes mellitus. *J Oral Maxillofac Pathol, 21*(3), 334-339. doi: 10.4103/jomfp.JOMFP_222_15

JOMFP-21-334 [pii]

Hagger, V., Hendrieckx, C., Sturt, J., Skinner, T. C., & Speight, J. (2016). Diabetes Distress Among Adolescents with Type 1 Diabetes: a Systematic Review. *Curr Diab Rep, 16*(1), 9. doi: 10.1007/s11892-015-0694-2

10.1007/s11892-015-0694-2 [pii]

Hajishengallis, E., Forrest, C. B., & Koo, H. (2016). Early Childhood Caries: Future Perspectives in Risk Assessment. *JDR Clin Trans Res, 1*(2), 110-111. doi: 10.1177/2380084416637577

10.1177_2380084416637577 [pii]

Hajishengallis, E., Parsaei, Y., Klein, M. I., & Koo, H. (2017). Advances in the microbial etiology and pathogenesis of early childhood caries. *Mol Oral Microbiol, 32*(1), 24-34. doi: 10.1111/omi.12152

Han, X., Aslanian, A., & Yates, J. R., 3rd. (2008). Mass spectrometry for proteomics. *Curr Opin Chem Biol, 12*(5), 483-490. doi: S1367-5931(08)00117-8 [pii]

10.1016/j.cbpa.2008.07.024

Hand, A. R., & Weiss, R. E. (1984). Effects of streptozotocin-induced diabetes on the rat parotid gland. *Lab Invest, 51*(4), 429-440.

Harding, J. L., Pavkov, M. E., Magliano, D. J., Shaw, J. E., & Gregg, E. W. (2019). Global trends in diabetes complications: a review of current evidence. *Diabetologia, 62*(1), 3-16. doi: 10.1007/s00125-018-4711-2
10.1007/s00125-018-4711-2 [pii]
Harjutsalo, V., Sjoberg, L., & Tuomilehto, J. (2008). Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. *Lancet, 371*(9626), 1777-1782. doi: S0140-6736(08)60765-5 [pii]
10.1016/S0140-6736(08)60765-5
Harrison, R., & Bowen, W. (1987). Flow rate and organic constituents of whole saliva in insulin dependent diabetic children and adolescents. *Pediatric Dent*(9), 287-291.
Harrison, R., & Bowen, W. H. (1987). Flow rate and organic constituents of whole saliva in insulin-dependent diabetic children and adolescents. *Pediatr Dent, 9*(4), 287-291.
Hartman, M. L., Goodson, J. M., Barake, R., Alsmadi, O., Al-Mutawa, S., Ariga, J., et al. (2016). Salivary Biomarkers in Pediatric Metabolic Disease Research. *Pediatr Endocrinol Rev, 13*(3), 602-611.
Hassaneen, M., & Maron, J. L. (2017). Salivary Diagnostics in Pediatrics: Applicability, Translatability, and Limitations. *Front Public Health, 5*, 83. doi: 10.3389/fpubh.2017.00083
Havugimana, P. C., Hu, P., & Emili, A. (2017). Protein complexes, big data, machine learning and integrative proteomics: lessons learned over a decade of systematic analysis of protein interaction networks. *Expert Rev Proteomics, 14*(10), 845-855. doi: 10.1080/14789450.2017.1374179
Hellhammer, D. H., Wust, S., & Kudielka, B. M. (2009). Salivary cortisol as a biomarker in stress research. *Psychoneuroendocrinology, 34*(2), 163-171. doi: S0306-4530(08)00299-0 [pii]
10.1016/j.psyneuen.2008.10.026
Helmerhorst, E. J., Dawes, C., & Oppenheim, F. G. (2018). The complexity of oral physiology and its impact on salivary diagnostics. *Oral Dis, 24*(3), 363-371. doi: 10.1111/odi.12780
Hemadi, A. S., Huang, R., Zhou, Y., & Zou, J. (2017). Salivary proteins and microbiota as biomarkers for early childhood caries risk assessment. *Int J Oral Sci, 9*(11), e1. doi: ijos201735 [pii]
10.1038/ijos.2017.35
Hill, K., You, D., Inoue, M., & Oestergaard, M. Z. (2012). Child mortality estimation: accelerated progress in reducing global child mortality, 1990-2010. *PLoS Med, 9*(8), e1001303. doi: 10.1371/journal.pmed.1001303
PMEDICINE-D-11-03032 [pii]
Hirsch, I. B. (2009). Clinical review: Realistic expectations and practical use of continuous glucose monitoring for the endocrinologist. *J Clin Endocrinol Metab, 94*(7), 2232-2238. doi: jc.2008-2625 [pii]
10.1210/jc.2008-2625
Hocine, A., Belmokhtar, K., Bauley, K., Jaisson, S., Gaha, K., Oubaya, N., et al. (2015). Serum and Tissue Accumulation of Advanced Glycation End-Products Correlates with Vascular Changes. *Perit Dial Int, 35*(5), 592-594. doi: 35/5/592 [pii]
10.3747/pdi.2013.00338

Hu, S., Gao, K., Pollard, R., Arellano-Garcia, M., Zhou, H., Zhang, L., et al. (2010). Preclinical validation of salivary biomarkers for primary Sjogren's syndrome. *Arthritis Care Res (Hoboken), 62*(11), 1633-1638. doi: 10.1002/acr.20289

Humphrey, S. P., & Williamson, R. T. (2001). A review of saliva: normal composition, flow, and function. *J Prosthet Dent, 85*(2), 162-169. doi: S0022-3913(01)54032-9 [pii]

10.1067/mpr.2001.113778

Hyoty, H. (2016). Viruses in type 1 diabetes. *Pediatr Diabetes, 17 Suppl 22*, 56-64. doi: 10.1111/pedi.12370

IDF Diabetes Atlas, 8th edition. (2017).

Implications of the United Kingdom Prospective Diabetes Study. . (2003). *Diabetes Care, 26*, 28-32.

Inder, W. J., Dimeski, G., & Russell, A. (2012). Measurement of salivary cortisol in 2012 - laboratory techniques and clinical indications. *Clin Endocrinol (Oxf), 77*(5), 645-651. doi: 10.1111/j.1365-2265.2012.04508.x

Ismail, A. F., McGrath, C. P., & Yiu, C. K. Y. (2017). Oral health status of children with type 1 diabetes: a comparative study. *J Pediatr Endocrinol Metab, 30*(11), 1155-1159. doi: 10.1515/jpem-2017-0053

/j/jpem.ahead-of-print/jpem-2017-0053/jpem-2017-0053.xml [pii]

Issaranggun Na Ayuthaya, B., Everts, V., & Pavasant, P. (2018). The immunopathogenic and immunomodulatory effects of interleukin-12 in periodontal disease. *Eur J Oral Sci, 126*(2), 75-83. doi: 10.1111/eos.12405

Ivanovski, K., Naumovski, V., Kostadinova, M., Pesevska, S., Drijanska, K., & Filipce, V. (2012). Xerostomia and salivary levels of glucose and urea in patients with diabetes. *Prilozi, 33*(2), 219-229.

Iyengar, A., & Maron, J. L. (2015). Detecting infection in neonates: promises and challenges of a salivary approach. *Clin Ther, 37*(3), 523-528. doi: S0149-2918(15)00072-7 [pii]

10.1016/j.clinthera.2015.02.006

Iyengar, A., Paulus, J. K., Gerlanc, D. J., & Maron, J. L. (2014). Detection and potential utility of C-reactive protein in saliva of neonates. *Front Pediatr, 2*, 131. doi: 10.3389/fped.2014.00131

Javaid, M. A., Ahmed, A. S., Durand, R., & Tran, S. D. (2016). Saliva as a diagnostic tool for oral and systemic diseases. *J Oral Biol Craniofac Res, 6*(1), 66-75. doi: 10.1016/j.jobcr.2015.08.006

S2212-4268(15)00089-5 [pii]

Javed, F., Sundin, U., Altamash, M., Klinge, B., & Engstrom, P. E. (2009). Self-perceived oral health and salivary proteins in children with type 1 diabetes. *J Oral Rehabil, 36*(1), 39-44. doi: JOR1895 [pii]

10.1111/j.1365-2842.2008.01895.x

Jessop, D. S., & Turner-Cobb, J. M. (2008). Measurement and meaning of salivary cortisol: a focus on health and disease in children. *Stress, 11*(1), 1-14. doi: 780648151 [pii]

10.1080/10253890701365527

Jimenez-Marin, A., Collado-Romero, M., Ramirez-Boo, M., Arce, C., & Garrido, J. J. (2009). Biological pathway analysis by ArrayUnlock and Ingenuity Pathway Analysis. *BMC Proc, 3 Suppl 4*, S6. doi: 1753-6561-3-S4-S6 [pii]

10.1186/1753-6561-3-S4-S6

Juhan-Vague, I., Alessi, M. C., Mavri, A., & Morange, P. E. (2003). Plasminogen activator inhibitor-1, inflammation, obesity, insulin resistance and vascular risk. *J Thromb Haemost, 1*(7), 1575-1579. doi: 279 [pii]

K, M. P., Johnson, P., Ganesh, M., & Subhashini, A. S. (2013). Evaluation of Salivary Profile among Adult Type 2 Diabetes Mellitus Patients in South India. *J Clin Diagn Res, 7*(8), 1592-1595. doi: 10.7860/JCDR/2013/5749.3232

Kaczor-Urbanowicz, K. E., Martin Carreras-Presas, C., Aro, K., Tu, M., Garcia-Godoy, F., & Wong, D. T. (2017). Saliva diagnostics - Current views and directions. *Exp Biol Med (Maywood), 242*(5), 459-472. doi: 1535370216681550 [pii]

10.1177/1535370216681550

Kahn, H. S., Morgan, T. M., Case, L. D., Dabelea, D., Mayer-Davis, E. J., Lawrence, J. M., et al. (2009). Association of type 1 diabetes with month of birth among U.S. youth: The SEARCH for Diabetes in Youth Study. *Diabetes Care, 32*(11), 2010-2015. doi: dc09-0891 [pii]

10.2337/dc09-0891

Kanjirath, P. P., Kim, S. E., & Rohr Inglehart, M. (2011). Diabetes and oral health: the importance of oral health-related behavior. *J Dent Hyg, 85*(4), 264-272.

Karjalainen, K. (2000). Periodontal diseases, dental caries, and saliva in relation to clinical characteristics of type 1 diabetes. *Acta Univ. Oul. D*(Academic Dissertation).

Karjalainen, K. M., & Knuuttila, M. L. (1996). The onset of diabetes and poor metabolic control increases gingival bleeding in children and adolescents with insulin-dependent diabetes mellitus. *J Clin Periodontol, 23*(12), 1060-1067.

Karjalainen, K. M., Knuuttila, M. L., & Kaar, M. L. (1996). Salivary factors in children and adolescents with insulin-dependent diabetes mellitus. *Pediatr Dent, 18*(4), 306-311.

Karlik, M., Valkovic, P., Hancinova, V., Krizova, L., Tothova, L., & Celec, P. (2015). Markers of oxidative stress in plasma and saliva in patients with multiple sclerosis. *Clin Biochem, 48*(1-2), 24-28. doi: S0009-9120(14)00703-6 [pii]

10.1016/j.clinbiochem.2014.09.023

Katsareli, E. A., & Dedoussis, G. V. (2014). Biomarkers in the field of obesity and its related comorbidities. *Expert Opin Ther Targets, 18*(4), 385-401. doi: 10.1517/14728222.2014.882321

Katz, M., Giani, E., & Laffel, L. (2015). Challenges and Opportunities in the Management of Cardiovascular Risk Factors in Youth With Type 1 Diabetes: Lifestyle and Beyond. *Curr Diab Rep, 15*(12), 119. doi: 10.1007/s11892-015-0692-4

10.1007/s11892-015-0692-4 [pii]

Keane, M., Fallon, R., Riordan, A., & Shaw, B. (2015). Markedly raised levels of C-reactive protein are associated with culture-proven sepsis or necrotising enterocolitis in extremely preterm neonates. *Acta Paediatr, 104*(7), e289-293. doi: 10.1111/apa.12978

Keil, M. F. (2012). Salivary cortisol: a tool for biobehavioral research in children. *J Pediatr Nurs, 27*(3), 287-289. doi: S0882-5963(12)00050-4 [pii]

10.1016/j.pedn.2012.02.003

Kerner, W., & Bruckel, J. (2014). Definition, classification and diagnosis of diabetes mellitus. *Exp Clin Endocrinol Diabetes, 122*(7), 384-386. doi: 10.1055/s-0034-1366278

Khanna, P., Maron, J. L., & Walt, D. R. (2017). Development of a Rapid Salivary Proteomic Platform for Oral Feeding Readiness in the Preterm Newborn. *Front Pediatr, 5*, 268. doi: 10.3389/fped.2017.00268

Kinsinger, C. R., Apffel, J., Baker, M., Bian, X., Borchers, C. H., Bradshaw, R., et al. (2012). Recommendations for mass spectrometry data quality metrics for open access data (corollary to the Amsterdam principles). *Proteomics, 12*(1), 11-20. doi: 10.1002/pmic.201100562

Kofler, S., Nickel, T., & Weis, M. (2005). Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation. *Clin Sci (Lond), 108*(3), 205-213. doi: CS20040174 [pii]

10.1042/CS20040174

Kordonouri, O., Klingensmith, G., Knip, M., Holl, R. W., Aanstoot, H. J., Menon, P. S., et al. (2014). ISPAD Clinical Practice Consensus Guidelines 2014. Other complications and diabetes-associated conditions in children and adolescents. *Pediatr Diabetes, 15 Suppl 20*, 270-278. doi: 10.1111/pedi.12183

Koulmanda, M., Budo, E., Bonner-Weir, S., Qipo, A., Putheti, P., Degauque, N., et al. (2007). Modification of adverse inflammation is required to cure new-onset type 1 diabetic hosts. *Proc Natl Acad Sci U S A, 104*(32), 13074-13079. doi: 0705863104 [pii]

10.1073/pnas.0705863104

Krassas, G. E., Tzotzas, T., Tsametis, C., & Konstantinidis, T. (2001). Determinants of body mass index in Greek children and adolescents. *J Pediatr Endocrinol Metab, 14 Suppl 5*, 1327-1333; discussion 1365.

Kyriazis, I., Rekleiti, M., Beliotis, E., & Saridi, M. (2013). Diabetes in Greece- A recent approach. *Ελληνικό Περιοδικό της Νοσηλευτικής Επιστήμης, 6*(2), 60-63.

Lalla, E., Cheng, B., Lal, S., Kaplan, S., Softness, B., Greenberg, E., et al. (2007). Diabetes mellitus promotes periodontal destruction in children. *J Clin Periodontol, 34*(4), 294-298. doi: CPE1054 [pii]

10.1111/j.1600-051X.2007.01054.x

Lamster, I. B. (2012). Diabetes and oral health. What's their relationship? *Diabetes Self Manag, 29*(3), 30, 32-34.

Lebovitz, H. E., Austin, M. M., Blonde, L., Davidson, J. A., Del Prato, S., Gavin, J. R., 3rd, et al. (2006). ACE/AACE consensus conference on the implementation of outpatient management of diabetes mellitus: consensus conference recommendations. *Endocr Pract, 12 Suppl 1*, 6-12. doi: 1FHJ9E5J6JND69FJ [pii]

10.4158/EP.12.S1.6

Lee, Y. H., & Wong, D. T. (2009). Saliva: an emerging biofluid for early detection of diseases. *Am J Dent, 22*(4), 241-248.

Lemaitre, S., Lepailleur, A., Bureau, R., Butt-Gueulle, S., Lelong-Boulouard, V., Duchatelle, P., et al. (2009). Novel antagonists of serotonin-4 receptors: synthesis and biological evaluation of pyrrolothienopyrazines. *Bioorg Med Chem, 17*(6), 2607-2622. doi: S0968-0896(08)01128-0 [pii]

10.1016/j.bmc.2008.11.045

Leruez-Ville, M., Magny, J. F., Couderc, S., Pichon, C., Parodi, M., Bussieres, L., et al. (2017). Risk Factors for Congenital Cytomegalovirus Infection Following Primary and Nonprimary Maternal Infection: A Prospective Neonatal Screening Study Using Polymerase Chain Reaction in Saliva. *Clin Infect Dis, 65*(3), 398-404. doi: 3737620 [pii]

10.1093/cid/cix337
Li, L. W., Wong, H. M., Sun, L., Wen, Y. F., & McGrath, C. P. (2015). Anthropometric measurements and periodontal diseases in children and adolescents: a systematic review and meta-analysis. *Adv Nutr, 6*(6), 828-841. doi: 6/6/828 [pii]
10.3945/an.115.010017
Liatis, S., Dafoulas, G. E., Kani, C., Politi, A., Litsa, P., Sfikakis, P. P., et al. (2016). The prevalence and treatment patterns of diabetes in the Greek population based on real-world data from the nation-wide prescription database. *Diabetes Res Clin Pract, 118*, 162-167. doi: S0168-8227(16)30168-1 [pii]
10.1016/j.diabres.2016.06.018
Libby, P. (2007). Inflammatory mechanisms: the molecular basis of inflammation and disease. *Nutr Rev, 65*(12 Pt 2), S140-146.
Liljestrand, J. M., Gursoy, U. K., Hyvarinen, K., Sorsa, T., Suominen, A. L., Kononen, E., et al. (2014). Combining salivary pathogen and serum antibody levels improves their diagnostic ability in detection of periodontitis. *J Periodontol, 85*(1), 123-131. doi: 10.1902/jop.2013.130030
Lim, P. W., Garssen, J., & Sandalova, E. (2016). Potential Use of Salivary Markers for Longitudinal Monitoring of Inflammatory Immune Responses to Vaccination. *Mediators Inflamm, 2016*, 6958293. doi: 10.1155/2016/6958293
Lima, D. P., Diniz, D. G., Moimaz, S. A., Sumida, D. H., & Okamoto, A. C. (2010). Saliva: reflection of the body. *Int J Infect Dis, 14*(3), e184-188. doi: S1201-9712(09)00203-3 [pii]
10.1016/j.ijid.2009.04.022
Lippolis, R., & De Angelis, M. (2016). Proteomics and Human Diseases. *J Proteomics Bioinform, 9*, 63-74.
Loe, H. (1967). The Gingival Index, the Plaque Index and the Retention Index Systems. *J Periodontol, 38*(6), Suppl:610-616. doi: 10.1902/jop.1967.38.6.610
Loo, J. A., Yan, W., Ramachandran, P., & Wong, D. T. (2010). Comparative human salivary and plasma proteomes. *J Dent Res, 89*(10), 1016-1023. doi: 0022034510380414 [pii]
10.1177/0022034510380414
Lopez-Pintor, R. M., Casanas, E., Gonzalez-Serrano, J., Serrano, J., Ramirez, L., de Arriba, L., et al. (2016). Xerostomia, Hyposalivation, and Salivary Flow in Diabetes Patients. *J Diabetes Res, 2016*, 4372852. doi: 10.1155/2016/4372852
Lopez, M. E., Colloca, M. E., Paez, R. G., Schallmach, J. N., Koss, M. A., & Chervonagura, A. (2003). Salivary characteristics of diabetic children. *Braz Dent J, 14*(1), 26-31. doi: S0103-64402003000100005 [pii]
Malamud, D. (2011). Saliva as a diagnostic fluid. *Dent Clin North Am, 55*(1), 159-178. doi: S0011-8532(10)00081-9 [pii]
10.1016/j.cden.2010.08.004
Manadas, B., Mendes, V. M., English, J., & Dunn, M. J. (2010). Peptide fractionation in proteomics approaches. *Expert Rev Proteomics, 7*(5), 655-663. doi: 10.1586/epr.10.46
Mandel, I. D., Kutscher, A., Denning, C. R., Thompson, R. H., Jr., & Zegarelli, E. V. (1967). Salivary studies in cystic fibrosis. *Am J Dis Child, 113*(4), 431-438.
Mark, A. M. (2016). Diabetes and oral health. *J Am Dent Assoc, 147*(10), 852. doi: S0002-8177(16)30610-9 [pii]
10.1016/j.adaj.2016.07.010

Maron, J. L., Johnson, K. L., Rocke, D. M., Cohen, M. G., Liley, A. J., & Bianchi, D. W. (2010). Neonatal salivary analysis reveals global developmental gene expression changes in the premature infant. *Clin Chem, 56*(3), 409-416. doi: clinchem.2009.136234 [pii]
10.1373/clinchem.2009.136234
Martinez, K., Frazer, S. F., Dempster, M., Hamill, A., Fleming, H., & McCorry, N. K. (2018). Psychological factors associated with diabetes self-management among adolescents with Type 1 diabetes: A systematic review. *J Health Psychol, 23*(13), 1749-1765. doi: 1359105316669580 [pii]
10.1177/1359105316669580
Mattson, J., & Cerutis, D. (2001). Diabetes mellitus: a review of the literature and dental implications. *Compend Contin Educ Dent. , 22*(9), 757-764.
Meagher, R. J., & Kousvelari, E. (2018). Mobile oral heath technologies based on saliva. *Oral Dis, 24*(1-2), 194-197. doi: 10.1111/odi.12775
Mirzaii-Dizgah, I., Hejazi, S. F., Riahi, E., & Salehi, M. M. (2012). Saliva-based creatine kinase MB measurement as a potential point-of-care testing for detection of myocardial infarction. *Clin Oral Investig, 16*(3), 775-779. doi: 10.1007/s00784-011-0578-z
Mirzaii-Dizgah, I., & Riahi, E. (2013). Salivary high-sensitivity cardiac troponin T levels in patients with acute myocardial infarction. *Oral Dis, 19*(2), 180-184. doi: 10.1111/j.1601-0825.2012.01968.x
Miyazaki, M., Higashi, Y., Goto, C., Chayama, K., Yoshizumi, M., Sanada, H., et al. (2007). Sarpogrelate hydrochloride, a selective 5-HT2A antagonist, improves vascular function in patients with peripheral arterial disease. *J Cardiovasc Pharmacol, 49*(4), 221-227. doi: 10.1097/FJC.0b013e3180325af3
00005344-200704000-00006 [pii]
Monnier, V. M., Glomb, M., Elgawish, A., & Sell, D. R. (1996). The mechanism of collagen cross-linking in diabetes: a puzzle nearing resolution. *Diabetes, 45 Suppl 3*, S67-72.
Moore, P. A., Guggenheimer, J., Etzel, K. R., Weyant, R. J., & Orchard, T. (2001). Type 1 diabetes mellitus, xerostomia, and salivary flow rates. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 92*(3), 281-291. doi: S1079-2104(01)55937-5 [pii]
10.1067/moe.2001.117815
Moreira, A. R., Passos, I. A., Sampaio, F. C., Soares, M. S., & Oliveira, R. J. (2009). Flow rate, pH and calcium concentration of saliva of children and adolescents with type 1 diabetes mellitus. *Braz J Med Biol Res, 42*(8), 707-711. doi: S0100-879X2009005000006 [pii]
Morzel, M., Palicki, O., Chabanet, C., Lucchi, G., Ducoroy, P., Chambon, C., et al. (2011). Saliva electrophoretic protein profiles in infants: changes with age and impact of teeth eruption and diet transition. *Arch Oral Biol, 56*(7), 634-642. doi: S0003-9969(11)00004-5 [pii]
10.1016/j.archoralbio.2010.12.015
Moutschen, M. P., Scheen, A. J., & Lefebvre, P. J. (1992). Impaired immune responses in diabetes mellitus: analysis of the factors and mechanisms involved. Relevance to the increased susceptibility of diabetic patients to specific infections. *Diabete Metab, 18*(3), 187-201.
Murrah, V. (1985). Diabetes mellitus and associated oral manifestations: a review. *J Oral Pathol., 14*(4), 271-281.

Nagler, R. M., Hershkovich, O., Lischinsky, S., Diamond, E., & Reznick, A. Z. (2002). Saliva analysis in the clinical setting: revisiting an underused diagnostic tool. *J Investig Med, 50*(3), 214-225. doi: 10.2310/6650.2002.33436

Naidoo, T., Konkol, K., Biccard, B., Dudose, K., & McKune, A. J. (2012). Elevated salivary C-reactive protein predicted by low cardio-respiratory fitness and being overweight in African children. *Cardiovasc J Afr, 23*(9), 501-506. doi: 10.5830/CVJA-2012-058

Nasidze, I., Li, J., Quinque, D., Tang, K., & Stoneking, M. (2009). Global diversity in the human salivary microbiome. *Genome Res, 19*(4), 636-643. doi: gr.084616.108 [pii]

10.1101/gr.084616.108

Nater, U. M., & Rohleder, N. (2009). Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. *Psychoneuroendocrinology, 34*(4), 486-496. doi: S0306-4530(09)00032-8 [pii]

10.1016/j.psyneuen.2009.01.014

Nathan, D. M., Cleary, P. A., Backlund, J. Y., Genuth, S. M., Lachin, J. M., Orchard, T. J., et al. (2005). Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *N Engl J Med, 353*(25), 2643-2653. doi: 353/25/2643 [pii]

10.1056/NEJMoa052187

Naumova, E. A., Sandulescu, T., Bochnig, C., Al Khatib, P., Lee, W. K., Zimmer, S., et al. (2014). Dynamic changes in saliva after acute mental stress. *Sci Rep, 4*, 4884. doi: srep04884 [pii]

10.1038/srep04884

Nayak, S., Bhad Patil, W. A., & Doshi, U. H. (2014). The relationship between salivary insulin-like growth factor I and quantitative cervical maturational stages of skeletal maturity. *J Orthod, 41*(3), 170-174. doi: 1465313313Y.0000000091 [pii]

10.1179/1465313313Y.0000000091

Nazir, M. A., AlGhamdi, L., AlKadi, M., AlBeajan, N., AlRashoudi, L., & AlHussan, M. (2018). The burden of Diabetes, Its Oral Complications and Their Prevention and Management. *Open Access Maced J Med Sci, 6*(8), 1545-1553. doi: 10.3889/oamjms.2018.294

OAMJMS-6-1545 [pii]

Negishi, A., Ono, M., Handa, Y., Kato, H., Yamashita, K., Honda, K., et al. (2009). Large-scale quantitative clinical proteomics by label-free liquid chromatography and mass spectrometry. *Cancer Sci, 100*(3), 514-519. doi: CAS1055 [pii]

10.1111/j.1349-7006.2008.01055.x

Nelson, P. M., Harrod, J. S., & Lamping, K. G. (2012). 5HT(2A) and 5HT(2B) receptors contribute to serotonin-induced vascular dysfunction in diabetes. *Exp Diabetes Res, 2012*, 398406. doi: 10.1155/2012/398406

Newrick, P. G., Bowman, C., Green, D., O'Brien, I. A., Porter, S. R., Scully, C., et al. (1991). Parotid salivary secretion in diabetic autonomic neuropathy. *J Diabet Complications, 5*(1), 35-37.

Ngounou Wetie, A. G., Wormwood, K. L., Russell, S., Ryan, J. P., Darie, C. C., & Woods, A. G. (2015). A Pilot Proteomic Analysis of Salivary Biomarkers in Autism Spectrum Disorder. *Autism Res, 8*(3), 338-350. doi: 10.1002/aur.1450

Nokes, D. J., Enquselassie, F., Nigatu, W., Vyse, A. J., Cohen, B. J., Brown, D. W., et al. (2001). Has oral fluid the potential to replace serum for the evaluation of population immunity levels? A study of measles, rubella and hepatitis B in rural Ethiopia. *Bull World Health Organ, 79*(7), 588-595. doi: S0042-96862001000700003 [pii]

Novy, B. B. (2014). Saliva and biofilm-based diagnostics: a critical review of the literature concerning sialochemistry. *J Evid Based Dent Pract, 14 Suppl*, 27-32. doi: S1532-3382(14)00068-2 [pii]

10.1016/j.jebdp.2014.04.004

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem, 250*(10), 4007-4021.

Pappa, E., Vastardis, H., Mermelekas, G., Gerasimidi-Vazeou, A., Zoidakis, J., & Vougas, K. (2018). Saliva proteomics analysis offers insights on type 1 diabetes pathology in a pediatric population. *Frontiers in Physiology, 9, 444*. doi: 10.3389/fphys.2018.00444

Park, N. J., Li, Y., Yu, T., Brinkman, B. M., & Wong, D. T. (2006). Characterization of RNA in saliva. *Clin Chem, 52*(6), 988-994. doi: clinchem.2005.063206 [pii]

10.1373/clinchem.2005.063206

Patterson, C. C., Dahlquist, G. G., Gyurus, E., Green, A., & Soltesz, G. (2009). Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. *Lancet, 373*(9680), 2027-2033. doi: S0140-6736(09)60568-7 [pii]

10.1016/S0140-6736(09)60568-7

Pedrioli, P. G. (2010). Trans-proteomic pipeline: a pipeline for proteomic analysis. *Methods Mol Biol, 604*, 213-238. doi: 10.1007/978-1-60761-444-9_15

Pickup, J. C., & Crook, M. A. (1998). Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia, 41*(10), 1241-1248. doi: 10.1007/s001250051058

Piersigilli, F., & Bhandari, V. (2016). Biomarkers in neonatology: the new "omics" of bronchopulmonary dysplasia. *J Matern Fetal Neonatal Med, 29*(11), 1758-1764. doi: 10.3109/14767058.2015.1061495

Pitts, N. B., Zero, D. T., Marsh, P. D., Ekstrand, K., Weintraub, J. A., Ramos-Gomez, F., et al. (2017). Dental caries. *Nat Rev Dis Primers, 3*, 17030. doi: nrdp201730 [pii]

10.1038/nrdp.2017.30

Poudel, P., Griffiths, R., Wong, V. W., Arora, A., Flack, J. R., Khoo, C. L., et al. (2018). Oral health knowledge, attitudes and care practices of people with diabetes: a systematic review. *BMC Public Health, 18*(1), 577. doi: 10.1186/s12889-018-5485-7

10.1186/s12889-018-5485-7 [pii]

Pratte, K. A., Baron, A. E., Ogden, L. G., Hassell, K. L., Rewers, M., & Hokanson, J. E. (2009). Plasminogen activator inhibitor-1 is associated with coronary artery calcium in Type 1 diabetes. *J Diabetes Complications, 23*(6), 387-393. doi: S1056-8727(08)00071-8 [pii]

10.1016/j.jdiacomp.2008.07.002

Purohit, S., Sharma, A., Zhi, W., Bai, S., Hopkins, D., Steed, L., et al. (2018). Proteins of TNF-alpha and IL6 Pathways Are Elevated in Serum of Type-1 Diabetes Patients with Microalbuminuria. *Front Immunol, 9*, 154. doi: 10.3389/fimmu.2018.00154

Pussinen, P. J., & Salomaa, V. (2018). Oral health: a neglected aspect of diabetes care. *Lancet Diabetes Endocrinol, 6*(12), 917-919. doi: S2213-8587(18)30032-9 [pii]
10.1016/S2213-8587(18)30032-9
Putnam, S. K., Lopata, C., Fox, J. D., Thomeer, M. L., Rodgers, J. D., Volker, M. A., et al. (2012). Comparison of saliva collection methods in children with high-functioning autism spectrum disorders: acceptability and recovery of cortisol. *Child Psychiatry Hum Dev, 43*(4), 560-573. doi: 10.1007/s10578-012-0284-3
Qiao, Y., Wu, M., Feng, Y., Zhou, Z., Chen, L., & Chen, F. (2018). Alterations of oral microbiota distinguish children with autism spectrum disorders from healthy controls. *Sci Rep, 8*(1), 1597. doi: 10.1038/s41598-018-19982-y
10.1038/s41598-018-19982-y [pii]
Rabilloud, T., Chevallet, M., Luche, S., & Lelong, C. (2010). Two-dimensional gel electrophoresis in proteomics: Past, present and future. *J Proteomics, 73*(11), 2064-2077. doi: S1874-3919(10)00175-2 [pii]
10.1016/j.jprot.2010.05.016
Rai, K., Hegde, A. M., Kamath, A., & Shetty, S. (2011). Dental caries and salivary alterations in Type I Diabetes. *J Clin Pediatr Dent, 36*(2), 181-184.
Ranger, M., & Grunau, R. E. (2014). Early repetitive pain in preterm infants in relation to the developing brain. *Pain Manag, 4*(1), 57-67. doi: 10.2217/pmt.13.61
Rao, P. V., Reddy, A. P., Lu, X., Dasari, S., Krishnaprasad, A., Biggs, E., et al. (2009). Proteomic identification of salivary biomarkers of type-2 diabetes. *J Proteome Res, 8*(1), 239-245. doi: 10.1021/pr8003776
10.1021/pr8003776 [pii]
Report of the Expert Committee on the Diagnosis and Classification of Diabetes. (1997). *Diabetes Care* (20), 1183-1197.
Report of the Expert Committee on the Diagnosis and Classification of Diabetes. (2003). *Diabetes Care* (26), 3160-3167.
Reuterving, C. O. (1986). Pilocarpine-stimulated salivary flow rate and salivary glucose concentration in alloxan diabetic rats. Influence of severity and duration of diabetes. *Acta Physiol Scand, 126*(4), 511-515. doi: 10.1111/j.1748-1716.1986.tb07849.x
Rewers, M., Pihoker, C., Donaghue, K., Hanas, R., Swift, P., & Klingensmith, G. J. (2009). Assessment and monitoring of glycemic control in children and adolescents with diabetes. *Pediatr Diabetes, 10 Suppl 12*, 71-81. doi: PDI582 [pii]
10.1111/j.1399-5448.2009.00582.x
Rewers, M., Pihokerb, C., Donaghuec, K., Hanasd, R., P, S., & G, K. (2009). ISPAD Clinical Practice Consensus Guidelines 2009 Compendium.Assessment and monitoring of glycemic control in children and adolescents with diabetes. *Pediatric Diabetes 2009, 10*(12), 71-81.
Rich, S. S., Akolkar, B., Concannon, P., Erlich, H., Hilner, J. E., Julier, C., et al. (2009). Overview of the Type I Diabetes Genetics Consortium. *Genes Immun, 10 Suppl 1*, S1-4. doi: gene200984 [pii]
10.1038/gene.2009.84
Romano-Keeler, J., Wynn, J. L., & Maron, J. L. (2014). Great expectorations: the potential of salivary 'omic' approaches in neonatal intensive care. *J Perinatol, 34*(3), 169-173. doi: jp2013170 [pii]
10.1038/jp.2013.170

Rosa, N., Correia, M. J., Arrais, J. P., Lopes, P., Melo, J., Oliveira, J. L., et al. (2012). From the salivary proteome to the OralOme: comprehensive molecular oral biology. *Arch Oral Biol, 57*(7), 853-864. doi: S0003-9969(12)00002-7 [pii]
10.1016/j.archoralbio.2011.12.010
Rosenfeld, C. S. (2015). Microbiome Disturbances and Autism Spectrum Disorders. *Drug Metab Dispos, 43*(10), 1557-1571. doi: dmd.115.063826 [pii]
10.1124/dmd.115.063826
Rosenstock, J., Brown, A., Fischer, J., Jain, A., Littlejohn, T., Nadeau, D., et al. (1998). Efficacy and safety of acarbose in metformin-treated patients with type 2 diabetes. *Diabetes Care, 21*(12), 2050-2055.
Ryan, R., Booth, S., Spathis, A., Mollart, S., & Clow, A. (2016). Use of Salivary Diurnal Cortisol as an Outcome Measure in Randomised Controlled Trials: a Systematic Review. *Ann Behav Med, 50*(2), 210-236. doi: 10.1007/s12160-015-9753-9
10.1007/s12160-015-9753-9 [pii]
Ryu, O. H., Atkinson, J. C., Hoehn, G. T., Illei, G. G., & Hart, T. C. (2006). Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis. *Rheumatology (Oxford), 45*(9), 1077-1086. doi: kei212 [pii]
10.1093/rheumatology/kei212
Salt, I. P., Morrow, V. A., Brandie, F. M., Connell, J. M., & Petrie, J. R. (2003). High glucose inhibits insulin-stimulated nitric oxide production without reducing endothelial nitric-oxide synthase Ser1177 phosphorylation in human aortic endothelial cells. *J Biol Chem, 278*(21), 18791-18797. doi: 10.1074/jbc.M210618200
M210618200 [pii]
Samaranayake, L. (2007). Saliva as a diagnostic fluid. *Int Dent J, 57*(5), 295-299.
Saudek, C., Derr, R., & R., K. (2006). Assessing Glycemia in Diabetes Using Self-monitoring Blood Glucose and Hemoglobin A1c. *JAMA, 295*, 1688-1697.
Sauder, K. A., Stafford, J. M., Mayer-Davis, E. J., Jensen, E. T., Saydah, S., Mottl, A., et al. (2019). Co-occurrence of early diabetes-related complications in adolescents and young adults with type 1 diabetes: an observational cohort study. *Lancet Child Adolesc Health, 3*(1), 35-43. doi: S2352-4642(18)30309-2 [pii]
10.1016/S2352-4642(18)30309-2
Schafer, C. A., Schafer, J. J., Yakob, M., Lima, P., Camargo, P., & Wong, D. T. (2014). Saliva diagnostics: utilizing oral fluids to determine health status. *Monogr Oral Sci, 24*, 88-98. doi: 000358791 [pii]
10.1159/000358791
Schmidt, A. M., Yan, S. D., Brett, J., Mora, R., Nowygrod, R., & Stern, D. (1993). Regulation of human mononuclear phagocyte migration by cell surface-binding proteins for advanced glycation end products. *J Clin Invest, 91*(5), 2155-2168. doi: 10.1172/JCI116442
Serpa, J. J., Parker, C. E., Petrotchenko, E. V., Han, J., Pan, J., & Borchers, C. H. (2012). Mass spectrometry-based structural proteomics. *Eur J Mass Spectrom (Chichester), 18*(2), 251-267. doi: 10.1255/ejms.1178
Shaddox, L. M., Huang, H., Lin, T., Hou, W., Harrison, P. L., Aukhil, I., et al. (2012). Microbiological characterization in children with aggressive periodontitis. *J Dent Res, 91*(10), 927-933. doi: 0022034512456039 [pii]

10.1177/0022034512456039
Shahar, E., Pollack, S., Kedem, E., Hassoun, G., & Nagler, R. (2008). Effect of HAART on salivary composition and oxidative profile in HIV-infected patients. *Curr HIV Res, 6*(5), 447-451.
Shahbaz, S., Katti, G., Ghali, S. R., Katti, C., Diwakar, D. D., & Guduba, V. (2014). Salivary alterations in type 1 diabetes mellitus patients: Salivary glucose could be noninvasive tool for monitoring diabetes mellitus. *Indian J Dent Res, 25*(4), 420-424. doi: IndianJDentRes_2014_25_4_420_142512 [pii]
10.4103/0970-9290.142512
Sharon, A., Ben-Aryeh, H., Itzhak, B., Yoram, K., Szargel, R., & Gutman, D. (1985). Salivary composition in diabetic patients. *J Oral Med, 40*(1), 23-26.
Shirtcliff, E. A., Buck, R. L., Laughlin, M. J., Hart, T., Cole, C. R., & Slowey, P. D. (2015). Salivary cortisol results obtainable within minutes of sample collection correspond with traditional immunoassays. *Clin Ther, 37*(3), 505-514. doi: S0149-2918(15)00084-3 [pii]
10.1016/j.clinthera.2015.02.014
Simons, S. S., Cillessen, A. H., & de Weerth, C. (2017). Cortisol stress responses and children's behavioral functioning at school. *Dev Psychobiol, 59*(2), 217-224. doi: 10.1002/dev.21484
Singh, S., Sharma, A., Sood, P. B., Sood, A., Zaidi, I., & Sinha, A. (2015). Saliva as a prediction tool for dental caries: An in vivo study. *J Oral Biol Craniofac Res, 5*(2), 59-64. doi: 10.1016/j.jobcr.2015.05.001
S2212-4268(15)00043-3 [pii]
Siudikiene, J., Machiulskiene, V., Nyvad, B., Tenovuo, J., & Nedzelskiene, I. (2008). Dental caries increments and related factors in children with type 1 diabetes mellitus. *Caries Res, 42*(5), 354-362. doi: 000151582 [pii]
10.1159/000151582
Siudikiene, J., Maciulskiene, V., & Nedzelskiene, I. (2005). Dietary and oral hygiene habits in children with type I diabetes mellitus related to dental caries. *Stomatologija, 7*(2), 58-62. doi: 052-06 [pii]
Spencer, J., Cooper, H., & Milton, B. (2010). Qualitative studies of type 1 diabetes in adolescence: a systematic literature review. *Pediatr Diabetes, 11*(5), 364-375. doi: PDI603 [pii]
10.1111/j.1399-5448.2009.00603.x
Spielmann, N., & Wong, D. T. (2011). Saliva: diagnostics and therapeutic perspectives. *Oral Dis, 17*(4), 345-354. doi: 10.1111/j.1601-0825.2010.01773.x
Sreebny, L. M., Yu, A., Green, A., & Valdini, A. (1992). Xerostomia in diabetes mellitus. *Diabetes Care, 15*(7), 900-904.
Standards of medical care in diabetes--2012. (2012). *Diabetes Care, 35 Suppl 1*, S11-63. doi: 35/Supplement_1/S11 [pii]
10.2337/dc12-s011
Starzak, D. E., Konkol, K. F., & McKune, A. J. (2016). Effects of Cardiorespiratory Fitness and Obesity on Salivary Secretory IgA and Alpha-Amylase in South African Children. *Children (Basel), 3*(3). doi: children3030012 [pii]
10.3390/children3030012
Strahler, J., Skoluda, N., Kappert, M. B., & Nater, U. M. (2017). Simultaneous measurement of salivary cortisol and alpha-amylase: Application and recommendations. *Neurosci Biobehav Rev, 83*, 657-677. doi: S0149-7634(17)30026-X [pii]

10.1016/j.neubiorev.2017.08.015
Streckfus, C., Bigler, L., Dellinger, T., Dai, X., Kingman, A., & Thigpen, J. T. (2000). The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin Cancer Res, 6*(6), 2363-2370.
Streckfus, C., Bigler, L., Tucci, M., & Thigpen, J. T. (2000). A preliminary study of CA15-3, c-erbB-2, epidermal growth factor receptor, cathepsin-D, and p53 in saliva among women with breast carcinoma. *Cancer Invest, 18*(2), 101-109.
Sumi, A., Yamanaka-Hanada, N., Bai, F., Makino, T., Mizukami, H., & Ono, T. (2011). Roles of coagulation pathway and factor Xa in the progression of diabetic nephropathy in db/db mice. *Biol Pharm Bull, 34*(6), 824-830. doi: JST.JSTAGE/bpb/34.824 [pii]
Sun, C. S., & Markey, M. K. (2011). Recent advances in computational analysis of mass spectrometry for proteomic profiling. *J Mass Spectrom, 46*(5), 443-456. doi: 10.1002/jms.1909
Swaminathan, V., Prakasam, S., Puri, V., & Srinivasan, M. (2013). Role of salivary epithelial toll-like receptors 2 and 4 in modulating innate immune responses in chronic periodontitis. *J Periodontal Res, 48*(6), 757-765. doi: 10.1111/jre.12066
Syrjala, A. M., Niskanen, M. C., Ylostalo, P., & Knuuttila, M. L. (2003). Metabolic control as a modifier of the association between salivary factors and dental caries among diabetic patients. *Caries Res, 37*(2), 142-147. doi: 10.1159/000069020
69020 [pii]
Szabo, G. T., Tihanyi, R., Csulak, F., Jambor, E., Bona, A., Szabo, G., et al. (2012). Comparative salivary proteomics of cleft palate patients. *Cleft Palate Craniofac J, 49*(5), 519-523. doi: 10.1597/10-135
Team, R. C. (2016). R: A Language and Environment for Statistical Computing.
Thul, P. J., & Lindskog, C. (2018). The human protein atlas: A spatial map of the human proteome. *Protein Sci, 27*(1), 233-244. doi: 10.1002/pro.3307
Tian, C., Sun, X., Liu, X., Huang, X., Chen, F., & Zheng, S. (2017). Salivary peptidome profiling analysis for occurrence of new carious lesions in patients with severe early childhood caries. *PLoS One, 12*(8), e0182712. doi: 10.1371/journal.pone.0182712
PONE-D-17-12442 [pii]
Tishler, M., Yaron, I., Shirazi, I., & Yaron, M. (1997). Saliva: an additional diagnostic tool in Sjogren's syndrome. *Semin Arthritis Rheum, 27*(3), 173-179. doi: S0049-0172(97)80017-0 [pii]
Tu, M., Chia, D., Wei, F., & Wong, D. (2016). Liquid biopsy for detection of actionable oncogenic mutations in human cancers and electric field induced release and measurement liquid biopsy (eLB). *Analyst, 141*(2), 393-402. doi: 10.1039/c5an01863c
Twetman, S., Johansson, I., Birkhed, D., & Nederfors, T. (2002). Caries incidence in young type 1 diabetes mellitus patients in relation to metabolic control and caries-associated risk factors. *Caries Res, 36*(1), 31-35. doi: 57587 [pii]
10.1159/000057587
Tzotzas, T., Kapantais, E., Tziomalos, K., Ioannidis, I., Mortoglou, A., Bakatselos, S., et al. (2008). Epidemiological survey for the prevalence of overweight and abdominal obesity in Greek adolescents. *Obesity (Silver Spring), 16*(7), 1718-1722. doi: oby2008247 [pii]
10.1038/oby.2008.247

van Greevenbroek, M. M., Schalkwijk, C. G., & Stehouwer, C. D. (2013). Obesity-associated low-grade inflammation in type 2 diabetes mellitus: causes and consequences. *Neth J Med, 71*(4), 174-187.

Vempati, U. D., Chung, C., Mader, C., Koleti, A., Datar, N., Vidovic, D., et al. (2014). Metadata Standard and Data Exchange Specifications to Describe, Model, and Integrate Complex and Diverse High-Throughput Screening Data from the Library of Integrated Network-based Cellular Signatures (LINCS). *J Biomol Screen, 19*(5), 803-816. doi: 1087057114522514 [pii]

10.1177/1087057114522514

Vidova, V., & Spacil, Z. (2017). A review on mass spectrometry-based quantitative proteomics: Targeted and data independent acquisition. *Anal Chim Acta, 964*, 7-23. doi: S0003-2670(17)30150-2 [pii]

10.1016/j.aca.2017.01.059

Virk, S. A., Donaghue, K. C., Cho, Y. H., Benitez-Aguirre, P., Hing, S., Pryke, A., et al. (2016). Association Between HbA1c Variability and Risk of Microvascular Complications in Adolescents With Type 1 Diabetes. *J Clin Endocrinol Metab, 101*(9), 3257-3263. doi: 10.1210/jc.2015-3604

Vitorino, R., Alves, R., Barros, A., Caseiro, A., Ferreira, R., Lobo, M. C., et al. (2010). Finding new posttranslational modifications in salivary proline-rich proteins. *Proteomics, 10*(20), 3732-3742. doi: 10.1002/pmic.201000261

Vitorino, R., de Morais Guedes, S., Ferreira, R., Lobo, M. J., Duarte, J., Ferrer-Correia, A. J., et al. (2006). Two-dimensional electrophoresis study of in vitro pellicle formation and dental caries susceptibility. *Eur J Oral Sci, 114*(2), 147-153. doi: EOS328 [pii]

10.1111/j.1600-0722.2006.00328.x

Vitorino, R., Lobo, M. J., Ferrer-Correira, A. J., Dubin, J. R., Tomer, K. B., Domingues, P. M., et al. (2004). Identification of human whole saliva protein components using proteomics. *Proteomics, 4*(4), 1109-1115. doi: 10.1002/pmic.200300638

Vlassara, H. (1996). Advanced glycation end-products and atherosclerosis. *Ann Med, 28*(5), 419-426.

Vlassara, H. (1997). Recent progress in advanced glycation end products and diabetic complications. *Diabetes, 46 Suppl 2*, S19-25.

von Bültzingslowen, I., Sollecito, T., P, F., Daniels, T., Jonsson, R., & Lockhart, P. e. a. (2007). Salivary dysfunction associated with systemic diseases: systematic review and clinical management recommendations. *Oral Pathol Oral Radiol Endod 103*(1), S57.e51-S57.e15.

von Bultzingslowen, I., Sollecito, T. P., Fox, P. C., Daniels, T., Jonsson, R., Lockhart, P. B., et al. (2007). Salivary dysfunction associated with systemic diseases: systematic review and clinical management recommendations. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 103 Suppl*, S57 e51-15. doi: S1079-2104(06)00872-9 [pii]

10.1016/j.tripleo.2006.11.010

Wang, K., Wang, Y., Wang, X., Ren, Q., Han, S., Ding, L., et al. (2018). Comparative salivary proteomics analysis of children with and without dental caries using the iTRAQ/MRM approach. *J Transl Med, 16*(1), 11. doi: 10.1186/s12967-018-1388-8

10.1186/s12967-018-1388-8 [pii]

WHO. (1997). Oral Health Surveys: Basic Methods. *World Health Organization, Geneva, Switzerland*.

Wilkins, M. (2009). Proteomics data mining. *Expert Rev Proteomics, 6*(6), 599-603. doi: 10.1586/epr.09.81

Wilkins, M. R., Pasquali, C., Appel, R. D., Ou, K., Golaz, O., Sanchez, J. C., et al. (1996). From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Biotechnology (N Y), 14*(1), 61-65.

Wilkins, M. R., Sanchez, J. C., Gooley, A. A., Appel, R. D., Humphery-Smith, I., Hochstrasser, D. F., et al. (1996). Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev, 13*, 19-50.

Williamson, S., Munro, C., Pickler, R., Grap, M. J., & Elswick, R. K., Jr. (2012). Comparison of biomarkers in blood and saliva in healthy adults. *Nurs Res Pract, 2012*, 246178. doi: 10.1155/2012/246178

Wilson, A. M., Ryan, M. C., & Boyle, A. J. (2006). The novel role of C-reactive protein in cardiovascular disease: risk marker or pathogen. *Int J Cardiol, 106*(3), 291-297. doi: S0167-5273(05)00434-1 [pii]

10.1016/j.ijcard.2005.01.068

Wissel, M., Demmler, G., Gandaria. C., Cravens. S., Berg, A., Widen, A., Altrich, M., Kleiboeker, S. . (2017). Detection of Cytomegalovirus (CMV) in Saliva of Congenitally Infected Neonates. *Open Forum Infectious Diseases, 4*. doi: 10.1093/ofid/ofx163.862

Witztum, J. L., & Horkko, S. (1997). The role of oxidized LDL in atherogenesis: immunological response and anti-phospholipid antibodies. *Ann N Y Acad Sci, 811*, 88-96; discussion 96-89.

Wong, D. T. (2006). Towards a simple, saliva-based test for the detection of oral cancer 'oral fluid (saliva), which is the mirror of the body, is a perfect medium to be explored for health and disease surveillance'. *Expert Rev Mol Diagn, 6*(3), 267-272. doi: 10.1586/14737159.6.3.267

Wong, D. T. (2012). Salivary diagnostics. *Oper Dent, 37*(6), 562-570. doi: 10.2341/12-143-BL

Wong, V., Yan, T., Donald, A., & McLean, M. (2004). Saliva and bloodspot cortisol: novel sampling methods to assess hydrocortisone replacement therapy in hypoadrenal patients. *Clin Endocrinol (Oxf), 61*(1), 131-137. doi: 10.1111/j.1365-2265.2004.02062.x

CEN2062 [pii]

Wren, M. E., Shirtcliff, E. A., & Drury, S. S. (2015). Not all biofluids are created equal: chewing over salivary diagnostics and the epigenome. *Clin Ther, 37*(3), 529-539. doi: S0149-2918(15)00092-2 [pii]

10.1016/j.clinthera.2015.02.022

Wu, J. Y., Yi, C., Chung, H. R., Wang, D. J., Chang, W. C., Lee, S. Y., et al. (2010). Potential biomarkers in saliva for oral squamous cell carcinoma. *Oral Oncol, 46*(4), 226-231. doi: S1368-8375(10)00011-4 [pii]

10.1016/j.oraloncology.2010.01.007

Wu, Z. Z., Wang, J. G., & Zhang, X. L. (2009). Diagnostic model of saliva protein finger print analysis of patients with gastric cancer. *World J Gastroenterol, 15*(7), 865-870.

Xiao, H., & Wong, D. T. (2011). Proteomics and its applications for biomarker discovery in human saliva. *Bioinformation, 5*(7), 294-296.

Xiao, H., Zhang, L., Zhou, H., Lee, J. M., Garon, E. B., & Wong, D. T. (2012). Proteomic analysis of human saliva from lung cancer patients using two-

dimensional difference gel electrophoresis and mass spectrometry. *Mol Cell Proteomics, 11*(2), M111 012112. doi: M111.012112 [pii]
10.1074/mcp.M111.012112
Yang, K., Peretz-Soroka, H., Liu, Y., & Lin, F. (2016). Novel developments in mobile sensing based on the integration of microfluidic devices and smartphones. *Lab Chip, 16*(6), 943-958. doi: 10.1039/c5lc01524c
Yeh, C. K., Christodoulides, N. J., Floriano, P. N., Miller, C. S., Ebersole, J. L., Weigum, S. E., et al. (2010). Current development of saliva/oral fluid-based diagnostics. *Tex Dent J, 127*(7), 651-661.
Yeung, W. C., Rawlinson, W. D., & Craig, M. E. (2011). Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. *BMJ, 342*, d35. doi: 10.1136/bmj.d35
Zhang, A., Sun, H., Wang, P., & Wang, X. (2013). Salivary proteomics in biomedical research. *Clin Chim Acta, 415*, 261-265. doi: S0009-8981(12)00519-0 [pii]
10.1016/j.cca.2012.11.001
Zhang, J., Zhang, X., Ma, Q., Lin, J., & Chen, F. (2013). Salivary Peptidomic Analysis- The Extension of Proteomics. *J Mol Biomark Diagn, 4*(2).
Zhang, L., Xiao, H., Karlan, S., Zhou, H., Gross, J., Elashoff, D., et al. (2010). Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. *PLoS One, 5*(12), e15573. doi: 10.1371/journal.pone.0015573
Zhang, Z., Li, M., Zhang, G., Fang, P., Yao, H., Xiao, Z., et al. (2010). Identification of human gastric carcinoma biomarkers by differential protein expression analysis using 18O labeling and nanoLC-MS/MS coupled with laser capture microdissection. *Med Oncol, 27*(2), 296-303. doi: 10.1007/s12032-009-9208-x
Zhou, J., Jiang, N., Wang, S., Hu, X., Jiao, K., He, X., et al. (2016). Exploration of Human Salivary Microbiomes--Insights into the Novel Characteristics of Microbial Community Structure in Caries and Caries-Free Subjects. *PLoS One, 11*(1), e0147039. doi: 10.1371/journal.pone.0147039
PONE-D-15-31932 [pii]
Zimmermann, B. G., Park, N. J., & Wong, D. T. (2007). Genomic targets in saliva. *Ann N Y Acad Sci, 1098*, 184-191. doi: 1098/1/184 [pii]
10.1196/annals.1384.002
Zipris, D. (2010). Toll-like receptors and type 1 diabetes. *Adv Exp Med Biol, 654*, 585-610. doi: 10.1007/978-90-481-3271-3_25
Zloczower, M., Reznick, A. Z., Zouby, R. O., & Nagler, R. M. (2007). Relationship of flow rate, uric acid, peroxidase, and superoxide dismutase activity levels with complications in diabetic patients: can saliva be used to diagnose diabetes? *Antioxid Redox Signal, 9*(6), 765-773. doi: 10.1089/ars.2007.1515
Zubarev, R. A., & Makarov, A. (2013). Orbitrap mass spectrometry. *Anal Chem, 85*(11), 5288-5296. doi: 10.1021/ac4001223